β-Lactam Resistance Genes in Wastewater Treatment Plants

Subjects: Microbiology

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Antimicrobial resistance (AMR) is one of the largest global concerns due to its influence in multiple areas, which is consistent with One Health's concept of close interconnections between people, animals, plants, and their shared environments. Antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) circulate constantly in various niches, sediments, water sources, soil, and wastes of the animal and plant sectors, and is linked to human activities. Sewage of different origins gets to the wastewater treatment plants (WWTPs), where ARB and ARG removal efficiency is still insufficient, leading to their transmission to discharge points and further dissemination. Thus, WWTPs are believed to be reservoirs of ARGs and the source of spreading AMR. According to a World Health Organization report, the most critical pathogens for public health include Gram-negative bacteria resistant to third-generation cephalosporins and carbapenems (last-choice drugs), which represent β-lactams, the most widely used antibiotics. Therefore, this research presents the available research data for ARGs in WWTPs that confer resistance to β-lactam antibiotics, with a particular emphasis on clinically important life-threatening mechanisms of resistance, including extended-spectrum β-lactamases (ESBLs) and carbapenemases (KPC, NDM).

antibiotic resistance genes | wastewater treatment plant | β-lactamase | extended-spectrum β-lactamase | carbapenemases

1. Introduction

Antibiotics are widely used to prevent and treat infections in humans, animals, and plants, but their high and incorrect consumption have made them increasingly ineffective due to antimicrobial-resistant microorganisms emerging and spreading globally. Thus, antimicrobial resistance (AMR) was announced by the World Health Organization (WHO) as one of the top global public health threats facing humanity [1]. Some Gram-negative bacteria, such as carbapenem-resistant *Pseudomonas aeruginosa*, *Acinetobacter baumannii,* and *Enterobacterales* resistant to third-generation cephalosporins and carbapenems are considered to be of particular importance, and the WHO and Centers for Disease Control and Prevention (CDC) included them in the group of critical pathogens due to the fact that they are a major cause of nosocomial infections with high morbidity and mortality [2][3]. Systematic analysis estimated 4.95 million deaths associated with bacterial AMR in 2019 and indicated β-lactam-resistant (mainly to third generation cephalosporins and carbapenem) bacteria as the major cause of death [4]. Tremendously dangerous microorganisms accumulate various AMR mechanisms that leads to their multi-drug resistance (MDR), extensive-drug resistance (XDR), or even pan-drug resistance (PDR), leaving

few, one, or no therapeutic options left, respectively. Consequently, infections caused by such bacteria carry an extremely high risk of death [5][6].

AMR is ubiquitous, associated with agriculture and livestock, medical, and veterinary settings, but it is also observed in many aquatic environments, which is in line with One Health's concept (available online: https://www.cdc.gov/onehealth/index.html accessed on 23 May 2022) of close interconnections between people, animals, plants, and their shared environments (**Figure 1**) [7].

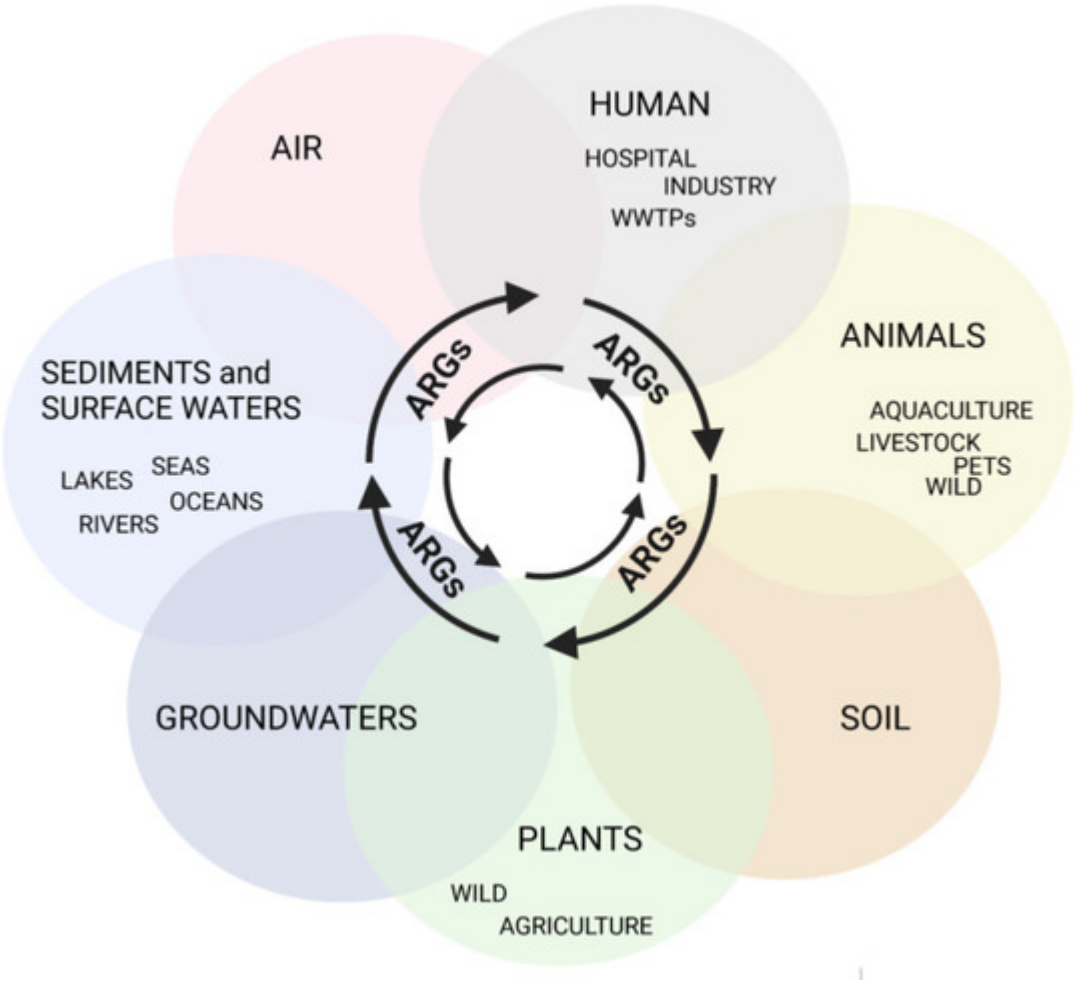


Figure 1. Routes of ARG transmission in the total environment, created with BioRender (available online: https://biorender.com, accessed on 23 May 2022).

Many aspects related to geographic location, socio-economic level, climate, antibiotic consumption, and the technology of the treatment process affect the abundance of antimicrobial resistance genes (ARGs), the bacteria carrying them (antibiotic-resistant bacteria, ARB), and their dissemination in the environment [8][9][10][11][12][13][14][15][16].

The genetic background of the AMR transmission process is of great importance. Resistance mechanisms are genetically based and linked with many genes localized on a bacterial chromosome or, what is more dangerous, on

mobile genetic elements (MGEs) [17][18]. Bacteria interacting with each other and exchanging genes by horizontal gene transfer (HGT) may lead to situations wherein previously sensitive and nonpathogenic strains may get resistance determinants and become virulent or reservoirs of ARGs for further transmission. These microorganisms, as well as resistance genes, may be discharged from WWTP systems into natural water bodies like lakes, rivers, and seas [19][20][21][22][23][24] which plays an important role in their further dissemination into human, animal and plant populations [25][26][27]. Therefore, it is believed that the WWTPs are reservoirs of ARGs, so-called “hotspots”, and one of the sources of spreading AMR, especially clinically relevant ARGs [28][29][30][31].

A great effort has been made to fight AMR and many global strategies have been taken, including developing new drugs and vaccines, improving the diagnostics of resistance mechanisms, the rational use of antibiotics, infection prevention and control, and developing new technological methods for the treatment and disinfection of wastewaters [32][33][34][35] as well as the monitoring of AMR and identifying the migration routes of bacteria with important mechanisms of resistance in the environment [16][30][31][34][36][37][38][39].

Zhuang et al. analyzed PubMed publications from the last 30 years (1990–2020) concerning reports of ARGs in the environment and showed that, on all continents, the highest frequency was related to genes encoding β-lactamases, enzymes that inactivate β-lactams, the most-used group of antibiotics [40]. Therefore, this research presents available research data on the identification of β-lactamase genes in WWTPs.

For this manual research of articles from the last decade, studies of β-lactamase genes in wastewater samples and from bacteria isolated from these type of samples were analyzed, including direct WWTP (i.e., influent, sewage sludge, effluent) and WWTP-related samples (i.e., air near bioreactors, discharge points).

2. Clinically Significant β-Lactam Resistance Genes in Wastewater Treatment Plants—The Occurrence and Distribution

According to the β-lactamase database (available online: BLDB; http://bldb.eu/ accessed on 23 May 2022), these enzymes constitute a very heterogeneous group with more than 7000 genetic variants identified. Within each of the four classes (A, B, C, and D), β-lactamases of particular clinical importance can be distinguished. These are detected consistently in environmental niches, including WWTPs (**Figure 2**) [40].

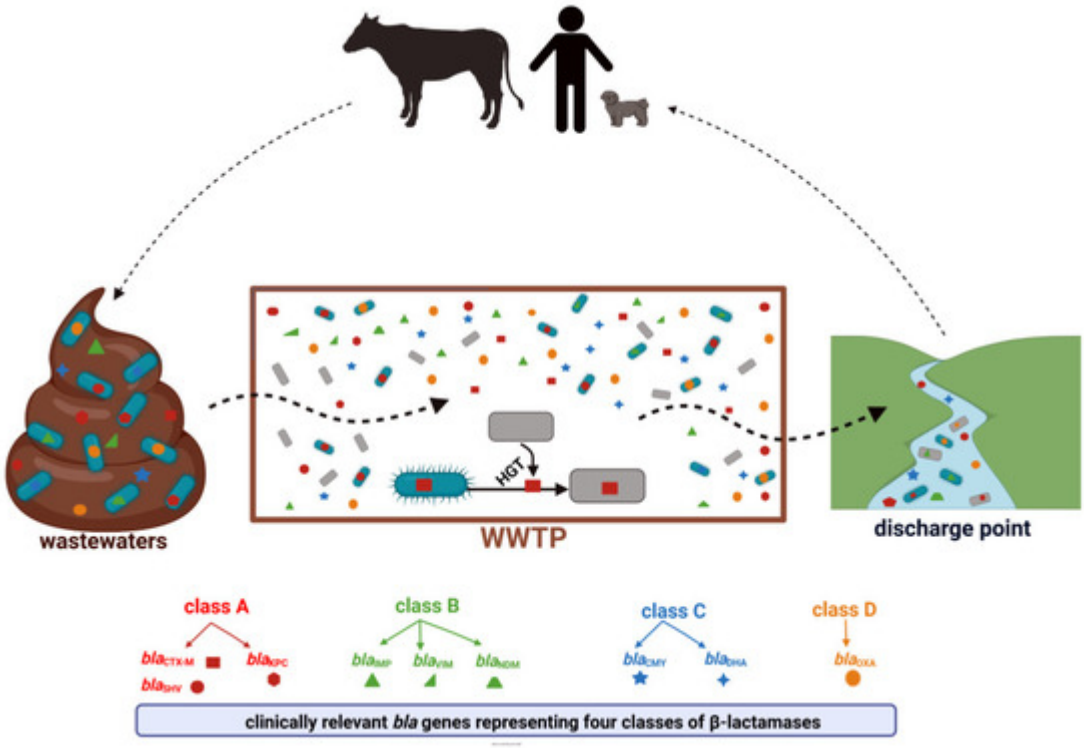


Figure 2. WWTP as a hotspot for the transmission of clinically relevant β-lactam resistance genes, created with BioRender available online: https://biorender.com, accessed on 23 May 2022).

2.1. Class A β-Lactamases

Class A β-lactamases are serine proteases that hydrolyze, on various levels, penicillins, monobactams, cephalosporins, and carbapenems and may be inhibited by β-lactamase inhibitors (e.g., clavulanic acid, sulbactam, tazobactam). It is the most diverse group, consisting of the enzymes with various spectra of hydrolysis, generally divided into: (i) a group with a narrow spectrum, e.g., carbenicillin-hydrolyzing β-lactamase (CARB) and *Pseudomonas aeruginosa* β-lactamase (PSE); (ii) a group with extended spectrum (ESBL) enzymes that originated from the first group but modified due to point mutations within the genes encoding them, which results in broadening their spectrum of hydrolyzing, e.g., cefotaximase-München-lactamase (CTX-M), Temoniera-lactamase (TEM), and sulfhydryl variable-lactamase (SHV); and (iii) a group with extremely extended spectrum including carbapenems—antibiotics of the last resort, e.g., Guiana extended-spectrum (GES), Klebsiella pneumoniae carbapenemase (KPC), Serratia marcescens enzyme (SME), and Serratia fonticola carbapenemase A (SFC-1) [41]. Among all, ESBL- and carbapenemase KPC-producing bacteria attracted the largest amount of clinical concern [42] [43] [44][45] [46][47] [48] [49][50][51].

2.1.1. Class A β-Lactamases—Occurrence and Variability in WWTPs-Linked Samples

Due to the global spread of class A β-lactamases, it is a commonly, or even predominantly, detected group in WWTPs. In a multi-national study of WWTPs from Denmark, Spain, and the United Kingdom (UK) with high-throughput qPCR used, these β-lactamases was leading, accounting for 70% of all detected *bla* genes [52]. Among them, the most relevant were two groups linked with ESBL and KPC enzymes. It is noteworthy that, among the

ESBL group, the most common in clinical settings and in various wastewater sources is CTX-M encoded by *bla*$_{CTX-M}$, carried mainly by *Enterobacterales* [53]. In this review, *bla*$_{CTX-M}$ was detected in the majority of included studies and, in many, had the highest prevalence [54][55][56][57][58][59][60][61][62][63][64][65][66][67][68]. However, *bla*$_{SHV}$ and/or *bla*$_{TEM}$ were found frequently as well [15][69][70][71][72]. In some studies, *bla*$_{TEM}$ was predominant, e.g., in an Irish study [73], as well as in Colombia [74], Poland and Portugal [9][75], Belgium [76], the US [77], and Africa [78]. Another significant group representing the KPC family encoded by *bla*$_{KPC}$ genes was detected in numerous WWTPs from European [52][56][76][79][80][81][82][83][84][85][86][87][88], as well as from American [77][89][90][91], African [59][78][92], and Asian countries [93][94]. Moreover, analysis of reviewed articles, especially those using developed techniques as high-throughput qPCR, whole-genome sequencing, or metagenomics, shows a high variety of detected genes of the discussed β-lactamases, not only representing *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$, and *bla*$_{KPC}$ families, but also others less frequently associated with public health, i.e., BEL, cfxA, GES, PER, SME, VEB, and others [52][79][83][89][94][95][96][97][98].

Environmental studies based on the analysis of bacterial strains during the treatment process most often concern the most critical pathogens posing the greatest threat, mainly *Enterobacterales*. In the reviewed literature, the predominantly tested and detected species among this bacteria family were *Escherichia coli* and *K. pneumoniae* [54][55][58][59][61][64][65][66][68][70][72][86][99][100][101][102][103][104][105][106][107][108][109][110][111][112]; however, different species of *Citrobacter* spp., *Enterobacter* spp., *Pseudomonas* spp., *Aeromonas* spp., or others were noted as well [58][63][67][74][79][89][91][96][97][108][113][114][115].

It is noteworthy that antibiotic susceptibility testing of the studied ESBL-producing strains isolated from the WWTPs confirms a high percentage of multi-drug resistance. It was also noted that these bacteria may survive the treatment process and that the WWTPs were unable to eradicate them completely. Generally, the number of MDR isolates decreased during the treatment, but for some, their proportion was still significant in effluents, in some even higher than in influent samples [57][58][60][73][75][79][81][84][86][105][106][110][112][116][117]. Moreover, analyzing downstream river or marine samples where final effluents are released, MDR isolates carrying ESBL enzymes were commonly detected [20][66][75][79][117].

Molecular typing concerning bacteria isolated from WWTPs confirmed high genetic relatedness between bacteria from WWTPs and human- and animal-associated sources, as well as the presence of clinically important lineages such as pandemic ST131 *E. coli* in WWTPs-related samples. Liedhegner et al. compared *E. coli* isolated from samples of various environmental compartments from one geographic area (clinical samples, hospital wastewater, and WWTP). The data including antibiotic resistance, virulence, and ESBL gene profiles confirmed high phenotypic and genotypic similarity across strains of these different origins and demonstrated potential health risks related to ESBL transmission [112]. An interesting study conducted by Raven et al. showed genetic relatedness between *E. coli* isolated from 20 WWTPs in the UK, livestock farms, retail meat, and isolates responsible for human blood infections. The genomic analysis of i.e., ESBL-producing isolates revealed that the three most common sequence types (STs) associated with bloodstream infections (ST131, ST73, and ST95) and the specific and most common for livestock (ST10) were found in wastewater samples [107]. In many other studies, human-associated, multidrug-resistant, and highly virulent clone ST131 *E. coli* was detected in WWTP samples as well [62][74][100][118][119][120][121].

2.1.2. Class A β-Lactamases—Removal during the Treatment Process

Concerning the removal of class A β-lactam ARGs, there is no universal target panel in qPCR studies; however, it has been noted that, although the WWTPs could effectively eliminate examined genes, their abundance was still reported in effluents and receiving water bodies. For example, in the study of Schages et al., strains harboring *bla*$_{CTX-M}$ were isolated from the effluent [110], as well as in a Japanese study wherein strains possessing ARGs belonging to the *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-9}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ families survived even after sterilization [111]. Other studies reported similar results of the ARGs' presence in effluent samples [95][122][123][124][125]. In Polish research from Kozieglowy, it was noticed that the wastewater treatment process leads to a significant increase in the relative abundance of *bla*$_{TEM}$ and *bla*$_{GES}$ genes, while the abundance of *bla*$_{KPC}$ decreased. Finally, the removal efficiency of ARGs was the least for *bla*$_{GES}$ (94.8%) and *bla*$_{CTX-M}$ (95.3%), while for other genes, it was >98% [56]. In another study, the presence of *bla*$_{KPC}$ was completely eliminated even after the first mechanical procedure [80]. In a Chinese survey comparing bacteria carrying *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$, isolated from influent and effluent, higher prevalence was noted in influent samples, except for *bla*$_{CTX-M}$, which was more frequently detected in effluent samples [116]. Significant differences between influent and effluent were described in a Romanian investigation and concerned *bla*$_{SHV-100, -145}$, which were decreased during treatment [72]. Interestingly, Neudorf et al. analyzed 3 WWTPs in Arctic Canada and noted a decrease of *bla*$_{TEM}$ abundance in two sites with a passive system and no significant changes for a third WWTP with a mechanical system. Moreover, no differences were found for *bla*$_{CTX-M}$ in all treatment plants [126]. A Spanish study by Rodriguez-Mozaz et al. demonstrated an increased frequency of *bla*$_{TEM}$ during the treatment process [127], while in a study of three WWTPs from Finland and Estonia, no significant changes were noted for *bla*$_{CTX-M-32}$, unlike *bla*$_{SHV-34}$, of which the relative concentration was increased in effluent samples but only in one tested WWTP [128]. Comparable data with similar *bla*$_{CTX-M}$ and *bla*$_{TEM}$ concentrations in influent and effluent samples were obtained in a study of five WWTPs in Tunisia; however, the abundance of the genes was higher in the effluent in a WWTP receiving additional hospital wastewater [129]. The occurrence of class A β-lactamases ARGs was also detected in downstream river samples whence final effluents were discharged, e.g., in a multi-national study including sixteen WWTPs from ten European countries [88], in a study conducted by Zieliński et al., wherein the predominant *bla*$_{TEM}$ was noted in receiving river water samples [15], and in a study performed by Osińska et al., wherein the presence of *bla*$_{SHV}$ and *bla*$_{TEM}$ in receiving river samples was confirmed [71].

WWTPs pose a health risk, not only because treated wastewater containing AMR genes or MDR bacteria are transferred into surface water bodies, but also because these pollutants are discharged into the air surrounding WWTPs through bioaerosol generated from bioreactors [15][55]. The study of the carriage of ESBL-producing *Enterobacterales* in WWTP workers and surrounding residents shows that these groups are much more like to acquire bacteria harboring the ESBL mechanism [25], thus confirming the direct influence of WWTPs on spreading ARGs into air. The contribution of WWTPs' bioaerosols in ARGs and ARB propagation into air and different environments is commonly investigated [130][131][132][133]. For example, Gaviria-Figueroa et al. studied bioaerosol samples collected downwind from sludge aeration tanks and showed a significant presence of clinically relevant class A β-lactamases, along with other classes of these enzymes and different antibiotic groups [134].

2.2. Class B β-Lactamases

Class B β-lactamases consist of a wide variety of metallo-β-lactamases (MBLs), enzymes able to hydrolyze almost all β-lactams: penicillins, cephalosporins, clinically available β-lactamase inhibitors, and carbapenems, except monobactams. They use zinc ions for activity, hence the name “metallo-” and susceptibility to metallic ion chelators like EDTA. Numerous variants are distinguished and grouped into three subclasses, among which the most widespread MBLs are imipenem-resistant *Pseudomonas* (IMP), Verona integron-encoded metallo-β-lactamase (VIM), and New Delhi metallo-β-lactamase (NDM), all representing subclass B1 [41][135][136][137]. MBLs initially detected in *P. aeruginosa* are frequently found nowadays in *K. pneumoniae* and other *Enterobacterales* [49][50][51][138][139][140] [141][142][143][144][145]. Although there is substantial geographic variability in the prevalence of MBL enzymes, they are noted worldwide and the speed of their dissemination is alarming, especially NDM enzymes [41][146][147][148][149].

2.2.1. IMP and VIM β-Lactamases in WWTPs-Linked Samples

As with the previously discussed ARGs, the environment plays a role in the transmission of bla_{IMP} and bla_{VIM} encoding MBLs enzymes with clinical importance, IMP and VIM, respectively. Although the majority of reports focus on hospital wastewater, these genes were detected also in samples of wastewater treatment plants from the US [69][89][90][134], Canada [91], China [57][69], and Singapore [94] as well as from many European countries, such as Sweden [83][96], Switzerland [86], the UK [115], Germany [87][110][123][150], Poland [56][79][80][151], Slovakia [102], and Romania [81]. A multi-national study concerning urban WWTPs in Denmark, Spain, and the UK showed the permanent presence of bla_{VIM} during the treatment process even in downstream river samples, in contrast to other tested genes, which were reduced under a detectable level [52]. Interesting results were presented by Khan et al., who compared Klebsiella oxytoca strains isolated from clinical sources (hospital wastewater) and the river receiving effluents from WWTP in Örebro, Sweden. Results obtained for two selected strains—the same antibiotic susceptibility patterns, antibiotic resistance gene profiles (i.e., bla_{VIM-1}, bla_{OXA-10}, bla_{ACC-1}), MLST type, furthermore phylogenetic relationship based on core genome single nucleotide polymorphism (SNP) analysis, and core genome MLST—suggest the transfer of K. oxytoca-producing carbapenemases from the hospital setting to the aquatic environment, which may pose a threat to the community [152].

2.2.2. New Delhi Metallo-β-Lactamase (NDM) in WWTPs-Linked Samples

According to epidemiological data, NDMs seem to pose the greatest threat among class B β-lactamases. Genes encoding them were noted in many aquatic environments, including animal production wastewaters, industrial, domestic sewage, tap water, surface water, and groundwater. However, hospital wastewater is considered to be a major source of bla_{NDM} variants [153][154][155]. As the geographical origin of NDM-producing bacteria is India, multiple publications detecting bla_{NDM}, especially in hospital sewage, come from India [156][157][158], together with other Asian [95][159][160][161] and African countries [92][162]. Interesting results were reported by Marathe et al., who studied hospital wastewater from Mumbai, India. Shotgun metagenomics revealed the presence of β-lactamase genes encoding clinically important MBLs, such as NDM, VIM, and IMP with bla_{NDM} as the most common carbapenemase-encoding gene. Additionally, 27 unique MBL genes not known yet were detected, which showed

the huge potential of the metagenomic approach [163]. However, NDM-lactamases in Asian countries were not only detected in hospital sewage samples. Analysis of rivers and sewage treatment plants in five Indian states also showed an abundance of *bla*$_{NDM}$ [64]. Similarly obtained data from southwest China showed a wide distribution of *bla*$_{NDM}$ in hospital sewage, WWTP effluent, and river samples. Interestingly, the gene was found in many different bacterial species belonging to *Enterobacterales*, genus Acinetobacter, and *Pseudomonas* [164]. The data from northern China [165][166] and Saudi Arabia [167] also confirm the presence of *bla*$_{NDM}$ in WWTP samples. *bla*$_{NDM}$ has spread globally, and several variants were noted not only in India and China but in many other countries in various water samples, including those from WWTPs and the surface waters of WWTP discharge points in the UK [115], Belgium [76], Switzerland [86], Germany [87], Poland [56][151], the Czech Republic [168], Romania [72][81], Spain [85], Africa [78][92], and the US [77][89][90]. Interesting results concern the Irish study conducted by Mahon et al. They examined the genetic relationship between NDM-possessing *E. coli* and *K. pneumoniae* (separately) cultivated from three locally linked sources: sewage samples from the collection system, freshwater streams, and clinical isolates. *E. coli* were considered indistinguishable, and *K. pneumoniae* were very closely related. These results confirm that water sewage plays an important role in the resistance transfer process [169]. Another analysis by Walsh et al. concerning public tap water and seepage water from sites around New Delhi also indicates that the environment has an undeniable influence on the propagation of NDM resistance [170].

Data regarding the wastewater treatment process show a different level of the transmission of bacteria with the NDM mechanism during the treatment process and the effectiveness of *bla*$_{NDM}$ reduction. In a Polish urban WWTP from Kozieglowy, Makowska et al. studied β-lactamase genes in the genomes of ESBL-producing and carbapenem-resistant coliforms isolated from each stage of the treatment process. They found that *bla*$_{NDM}$ and *bla*$_{VIM}$ were present in all stages and that the highest frequency was recorded in isolates from effluent compared to raw sewage, which indicates that the treatment process in the mechanical–biological treatment plant is insufficient in eliminating *bla*$_{NDM}$ and the organisms carrying them [56]. Similarly, data from two WWTPs in north China show the persistent and prevailing presence of *bla*$_{NDM}$ even after disinfection [165] and the propagation of *bla*$_{NDM}$ from a WWTP into its receiving river [166]. Other studies measuring absolute (copies/mL) and relative (copies/16S) abundance of *bla*$_{NDM}$ in influent and effluent also confirm deficient reduction [85][171]. However, Divyashree et al., who studied treated and untreated effluents from hospital samples in Mangalore, South India, showed the absence of *bla*$_{NDM}$ in treated effluents [172]. A Polish study also showed a complete reduction of *bla*$_{NDM}$ in the treatment process, even after the initial treatment stage [80], similar to a multi-center study from Denmark, Spain, and the UK [52].

2.3. Class C β-Lactamases

β-lactamases belonging to class C (AmpC) confer resistance to broad-spectrum β-lactams including penicillins, monobactams, and, most of all, cephalosporins (except fourth and fifth generations). Three mechanisms of resistance are noted: (i) chromosomal resistance induced by β–lactams; (ii) derepression due to mutations in AmpC regulatory genes, which results in overexpression and the production of the enzyme at a very high level; and (iii) the presence of plasmid-mediated AmpC genes (pAmpC) that are easily transmissible, even between different species, thus posing the highest health risk among class C β-lactamases. Several families of plasmid-encoded

AmpC variants were reported within the next decade, i.e., ACC, CIT (variants CMY, LAT, BIL), DHA, EBC (variants ACT, MIR), FOX, and MOX, differing in bacterial species of origin. The most commonly found among the strains responsible for human infections are ACC, CMY, and DHA enzymes encoded by *bla*$_{ACC}$, *bla*$_{CMY}$, and *bla*$_{DHA}$ genes, respectively. Clinically relevant bacteria producing pAmpC enzymes are mainly *Citrobacter* spp., *Salmonella* spp., and *Shigella* spp., but they were also found in other *Enterobacterales*, including *K. pneumoniae*, *Enterobacter aerogenes*, *Proteus mirabilis*, *Morganella morganii*, and *K. oxytoca* [146][173][174][175].

2.3.1. Class C β-Lactamases in WWTPs-Linked Samples

Similar to the clinical surveillance of pAmpC, environmental studies concerning wastewaters and WWTPs report the predominance of genes encoding CMY and DHA enzymes. Kwak et al. conducted an antimicrobial resistance analysis of *E. coli* in urban and hospital wastewaters. They noticed that, among β-lactam-resistant ARB, almost all (97%) were confirmed to possess ESBL or pAmpC, and among pAmpC, all were detected as carrying the *bla*$_{CMY-2}$ variant [103]. This variant, as well as others representing the CMY and DHA families, were detected in many other European studies of WWTPs from Germany [110][122][123], Romania [72], Sweden [83][96], Portugal [75][97], Poland [79][80], Slovakia [102], and Spain [176], as well as in studies conducted in Africa [114], North America [67][77][89][134][177], South America [74], and Asia [64][94]. Interestingly, Yim et al. investigated samples for plasmid-mediated quinolone resistance genes from a WWTP in Canada and detected the presence of qnrB4-AmpC (*bla*$_{DHA-1}$) genes in plasmids among *Citrobacter freundii* isolates. These were almost identical to those found in pathogenic *Klebsiella* isolates. Results of SNP analysis may suggest their dissemination from WWTP strains into clinical strains, which supports that WWTPs are a source of AMR spread [177].

In the reviewed studies, AmpC genes were detected in different stages of the treatment process, as well as in surface waters related to WWTPs. Alexander et al. conducted research on 20 critical points in aquatic systems, including WWTPs, and showed that, although the abundance at individual points and sampling periods over 2 years was variable, the presence of the AmpC genes was found in all sampling sites [150]. In another study, Su et al. analyzed the AmpC genes in *Escherichia coli* from two municipal WWTPs in China and noted that AmpC was detected in all treatment stages [178]. In s multi-national study, Yang et al. used shotgun metagenomics on activated sludge samples of 15 WWTPs from China, Singapore, the US, and Canada and detected the highest abundance of AmpC genes among all tested β-lactam resistance genes. They also found very high genetic diversity of AmpC genes [69]. Generally, metagenomic studies or studies using high throughput PCR are very useful in detecting multiple variants of genes encoding AmpC and representing different families, including, i.e., FOX, MOX, MIR, ACT, and ACC [52][80][83][89][91][94][96][110][134].

Although *bla*$_{CMY}$ and *bla*$_{DHA}$ are the most often detected and prevalent pAmpC genes, in some studies, other variants are predominant. For example, Amador et al. showed that, among the AmpC-producing *Enterobacterales* isolated from Portuguese WWTP samples, the dominant was *bla*$_{EBC}$, followed by *bla*$_{FOX}$ and *bla*$_{CIT}$ [75]; Piotrowska et al. analyzed *Aeromonas* spp. strains isolated from urban WWTPs in Warsaw, Poland, and found *bla*$_{FOX}$ to be the most abundant, followed by *bla*$_{MOX}$ and *bla*$_{ACC}$ [84]. For comparison, Fadare and Okoh studied *Enterobacterales*

isolated from the effluents of two WWTPs in South Africa and reported that the most predominant were *bla*$_{CIT}$ and *bla*$_{ACC}$, whereas *bla*$_{FOX}$ was detected in only one isolate [59].

Due to the lower frequency and speed of spread compared to other β-lactam resistance mechanisms, AmpC enzymes do not represent such a high risk. However, they are present in WWTP samples including effluents, and as a result of plasmid-localized and HGT present during the treatment process, this group may still pose a health risk and needs to be monitored.

2.4. Class D β-Lactamases

According to the BLDB, class D β-lactamases, known as oxacillinases, include more than 1,000 enzymes divided into 19 groups, among which the OXA group is the most numerous and clinically relevant. Among these, carbapenem-hydrolyzing class D enzymes (CHDLs) pose the greatest risk [173]. The substrate spectrum of the variants and level of hydrolyzing may significantly differ; however, all class D β-lactamases are not inhibited by β-lactam inhibitors, and they confer resistance to the amino-, carboxy-, and ureidopenicillins [179]. Although not classical ESBLs, as defined by inhibition by clavulanate, several of the OXA-type β-lactamase variants, such as OXA-11 and OXA-14 to OXA-20, are associated with an ESBL phenotype in that they confer resistance to some of the late-generation cephalosporins [180]. Within the OXA family, only a small fraction has a functional role as a carbapenemase. Among these are OXA-23, OXA-40, and the increasingly prevalent OXA-48, with its related variants, OXA-162, OXA-181, and OXA-232 [181]. The major enterobacterial class D carbapenemase, OXA-48, was first reported in a Turkish *K. pneumoniae* isolate in 2001 [182]. Thereafter, OXA-48 and related variants have been found in almost all *Enterobacterales*, mainly in *K. pneumoniae* and *E. coli*, that spread globally, causing endemic states in the Middle East, North Africa, India, and some European countries [49][50][51].

2.4.1. OXA Family β-Lactamases Carried in ARB

The reviewed approaches concerning class D β-lactamases are focused on bacterial strains carrying *bla*$_{OXA}$ isolated from WWTP samples. The majority of these studies confirm a *bla*$_{OXA}$ presence in isolates from both untreated and treated samples, and the prevalent variants are *bla*$_{OXA-1}$ and *bla*$_{OXA-48}$. Multiple examples come from European countries: a Czech study reported ESBL-producing *Enterobacterales* carrying *bla*$_{OXA-1}$ and isolated from effluent; globally spread MDR clones of *E. coli* ST131 and *K. pneumoniae* ST321 and ST323 harboring large FIIK plasmids with multiple antibiotic-resistance genes were found among tested strains [100]; a Spanish study detected *bla*$_{OXA-1}$ in strains isolated from effluents of two out of 21 tested WWTPs [63]; two German studies reported the presence of *bla*$_{OXA-51}$ and *bla*$_{OXA-48}$ in carbapenemase-producing bacteria [87] and *bla*$_{OXA-58}$, *bla*$_{OXA-48}$ and *bla*$_{OXA-23}$ in bacterial strains isolated from influent, activated sludge and effluent [110]; four Polish studies identified *bla*$_{OXA}$ genes among ceftazidime- or meropenem-resistant bacterial strains [79], *Aeromonas* spp. strains isolated from raw sewage, activated sludge, and effluent [84], ESBL-producing *Enterobacterales* [55] and *Acinetobacter* spp. isolates [151]; an Austrian study of carbapenemase-producing *Enterobacterales* from activated sludge confirmed harboring *bla*$_{OXA-48}$ [82]; and a study concerning the WWTP in Basel, Switzerland, where carbapenemase-resistant *Enterobacterales* and other Gram-negative bacteria isolated from municipal and hospital

wastewater and WWTP receiving this sewage were compared, and identical isolates from the WWTP and wastewater samples were detected, including OXA-48-producing *E. coli* ST38 and *Citrobacter* spp. [86]. Similarly, a molecular epidemiology approach was conducted in a Romanian study. Surleac et al. detected variants of bla_{OXA} in *K. pneumoniae* isolated from samples of three WWTPs [72], while Teban-Man et al. compared carbapenemase-producing *K. pneumoniae* isolated from the influent and effluent of two WWTPs with and without hospital input and found that bla_{OXA-48} was carried by strains isolated from raw and treated samples of WWTPs collecting hospital wastewater. In the second WWTP, the gene was observed only in strains from influent. Moreover, isolates harboring bla_{OXA-48} were genetically typed, which showed they belonged to sequence types of high-risk clones (ST258, ST101, ST147, ST2502). These clones were associated with clinical settings and reported to be multi-drug resistant [81]. In a study of a Swedish WWTP, Gram-negative bacteria harboring bla_{OXA} were noted in influent, effluent, and recipient waters of the river and lake [96]. However, in a Portuguese study conducted by Araujo et al., bla_{OXA} was detected only in strains isolated from raw sewage samples [97]. Another Portuguese investigation of ampicillin-resistant *Enterobacterales* isolated from influent and effluent showed different results; bla_{OXA} was the most prevalent gene among tested ESBL-producing strains [75]. There are significantly fewer studies detecting bla_{OXA} in the African region and they cover Algeria, where bla_{OXA-1} was detected [61]; Durban, South Africa, where cefotaxime-resistant *E. coli* were studied and bla_{OXA-1} was found as well [78]; Eastern Cape Province, South Africa, where $bla_{OXA-1-like}$ and $bla_{OXA-48-like}$ variants harbored by *Enterobacterales* isolated from effluents of WWTPs were noted [59]; and Tunisia, where *C. freundii* isolate carrying $bla_{OXA-204}$ [108] and *Enterobacterales* strains possessing bla_{OXA-1} [114] were detected. American studies concerning WWTPs also confirm bla_{OXA} presence in bacteria isolated from WWTP samples [67][77][89][91][106][112][120].

2.4.2. OXA Family β-Lactamases in Direct WWTP Samples—Occurrence and Removal

Multiple studies report the presence of bla_{OXA} in direct WWTP samples and determine the concentration and relative abundance of selected gene variants to define the efficiency of the treatment process. Comparable to previously discussed β-lactamases, bacteria producing OXA enzymes, as well as bla_{OXA}, can be detected after the treatment process. For example, the study of two WWTPs in the Brussels region determined the relative abundance of bla_{OXA-48} in different stages of the treatment, as well as in samples of the river as the discharge point for the WWTP effluents. In that study, Proia et al. showed a significant increase of bla_{OXA-48} from influent to effluent and from upstream to downstream river samples [76]. Similarly, in Kozieglowy, a Polish WWTP, it was reported that the wastewater treatment process leads to a significant increase in the relative abundance of bla_{OXA-48} genes in the effluent [56], whereas in research from the Baltic Sea area, the relative abundance of bla_{OXA-58} was decreased in the effluent; however it was weakly significant and found only in one of the three studied WWTPs [128]. In the German study, the absolute abundance of selected bla_{OXA} genes was determined, and when comparing raw and treated samples from WWTP, a significant decrease was reported regarding bla_{OXA-58} and bla_{OXA-48} but not bla_{OXA-23} [110]. Similar results were obtained in a multi-national study of WWTPs from ten European countries, where qPCR and absolute abundance were performed for selected bla_{OXA} genes. It was noticeable that, among all tested β-lactamase genes, bla_{OXA-58} was found in all tested samples, had the highest absolute abundance, and was significantly reduced during treatment [88]. In three Swedish municipal sludge treatment plants, a metagenomics approach was conducted, and many variants of bla_{OXA} were detected at all stages of the treatment process. Some

of them, like *bla*$_{OXA-48}$, were consistently enriched in treated sludge compared to primary sludge [83]. Other metagenomic approaches or using qPCR provide similar results—the presence of multiple *bla*$_{OXA}$ gene variants, including effluent samples [80][96][123][134], while others detected only single or a few variants [52][56][76][88][94][95][98][128][151]. Interestingly, in a Polish study, where *bla*$_{OXA}$ was detected as one of the prevalent tested genes in influent and effluent samples, comparative metagenomic analysis of DNA from WWTP samples and employees' swabs revealed the presence of similar ARGs in both types of samples with significantly higher concentrations than in control samples [15]. Other studies that report the presence of *bla*$_{OXA}$ genes at different stages of the treatment process include the research of Yang et al., wherein activated sewage sludge from 15 WWTPs was tested, and three variants (*bla*$_{OXA-1}$, *bla*$_{OXA-2}$ and *bla*$_{OXA-10}$) were detected [69], while in WWTP active sludge in South Carolina, in the US, a higher variability among *bla*$_{OXA}$ (seven variants) was noted [134]. Interesting results concerning the seasonal increase of *bla*$_{OXA}$ concentration between the summer and winter seasons were reported in the study of four small-scale domestic WWTPs. Furthermore, *bla*$_{OXA}$ in winter was prevalent among tested ARGs in raw sewage, as well as in effluent samples; additionally, the gene was detected in receiving river samples, in both the winter and summer seasons [71]. Results of a multi-national study, analyzing samples from Denmark, Spain, and the UK, indicated a country-specific presence for *bla*$_{OXA-10}$ detected only in WWTPs from the UK [52].

The above data, showing the presence of *bla*$_{OXA}$ genes and bacteria harboring them in WWTPs and related samples, confirms that WWTPs are a hotspot for antibiotic-resistant gene transmission into not only the aquatic compartments of the environment but also to the atmospheric air, creating an additional health risk for the workers of WWTPs.

3. Conclusions

AMR is a serious and urgent problem, and it is clear that the environment plays a key role in the process of transmission and propagation of ARGs and ARB with life-threatening clinical consequences. The multitude of publications confirms that β-lactamases genes encoding especially ESBLs (TEM, SHV, CTX-M) and KPC, NDM, and OXA carbapenemases, which pose one of the greatest health risks, are widely found in WWTPs and disseminated to further portions of the environment. Molecular analysis shows repeatedly high genetic relatedness between environmental and clinical isolates, e.g., ST131 *E. coli*. Generally, different kinds of sewage treatment processes do not eliminate these ARGs completely. Furthermore, some data indicate an increased level of β-lactam ARGs in effluent or even the presence of the genes and bacteria harboring them in samples after additional disinfection treatments.

Due to β-lactam ARGs' potential to transfer via mobile genetic elements through horizontal gene transfer, their abundance in water samples discharged from WWTPs into natural aquatic sources used by humans or animals suggests a potential risk of transmission resistance determinants into pathogenic and non-pathogenic bacteria and acquiring multidrug resistance as well as the participation of WWTPs in AMR transmission route and distribution into surrounding ecosystems and clinical settings. The growing problem of AMR and the spread of clinically relevant ARGs related to, i.e., β-lactams in the environment, indicate the need to improve and evaluate the

procedures of wastewater treatment and disinfection; thus, ARB, ARGs, and factors influencing their selection and co-selection during the treatment process would be completely removed.

The development and improvement of techniques used in testing wastewater for antibiotic resistance has been very significant in recent years. There are more and more publications indicating the use of modern metagenomic assays, which enables broadening the knowledge of the complexity and structural and functional biodiversity of microbial communities—i.e., analysis of resistance genes; taxonomic assignment; functional genes characterization; the identification of the HGT mechanism and mobile elements involved in the gene transmission; and exploring relationships between pathogenic and non-pathogenic species and susceptible and resistant bacteria. Therefore metagenomic analysis seems to be a very useful tool to understand the process of AMR transmission. However, the clinical surveillance of resistant strains responsible for life-threatening infections and nosocomial outbreaks caused by β-lactam-resistant strains also involve molecular techniques, but still the gold standard are culture-based methods detecting the expression of genes and the resistance mechanism. Therefore, according to the One Health's concept, collaborative approaches concerning AMR in the environment and clinical setting are indispensable and should combine new technology with standard microbiological methods. As WWTPs are the crucial points on the routes of ARB and ARGs' spread, they should be deeply explored, which would help to understand the process and make it possible to introduce procedures to stop, or at least slow down, the spreading of antibiotic resistance.

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