



Removing Antibiotic-Resistant Bacteria (ARB) Carrying Genes (ARGs): Challenges and Future Trends

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Abstract

Developed control of chemical disinfection techniques is beginning to be progressively significant in order to equilibrate under-treatment (minimal pathogen demobilization) and over-treatment (immoderate consumption of disinfectant and disinfection by-products generation) that way giving great ecological and economic advantages. This work reviews the most recent and pertinent researches in this field of eliminating Antibiotic-resistant Bacteria (ARB) carrying genes (ARGs) during wastewater treatment especially disinfection. Traditional disinfection techniques may not be efficient in demobilizing ARB and the simultaneous liberation of ARB and antibiotics at sub-lethal concentrations into municipal wastewater treatment plant effluent may promote the development of resistance among bacteria in receiving water. The pathway of the influences of diverse disinfection techniques in water and wastewater (chlorination, UV irradiation, Fenton reaction, ozonation, and photocatalytic oxidation) deserves more attention. The impacts of constructed wetlands and nanotechnology on ARB and ARG have to be more explored. As the best available technology, membranes processes should be widely adopted through the world for removing ARB and ARGs from the perspective of reusing treated wastewater as drinking water. These safe barriers against pollutants diffusion in nature merit more technical and economic expansion for their larger industrial application especially in developing countries.

Subject Areas

Bioengineering, Biotechnology, Microbiology

Keywords

Antibiotic-Resistant Bacteria (ARB), Antibiotic Resistance Genes (ARGs), Wastewater Treatment, Disinfection, Oxidation, *Escherichia coli*

1. Introduction

The excess of antibiotic resistance is a worldwide problem for human and animal health. This is due to the fact that this resistance decreases the performance of antibiotics and the treatability of infectious diseases [1] [2]. Antibiotics may choose antibiotic-resistant bacteria (ARB) carrying genes (ARGs) responsible for antibiotic-resistance routes [3] [4] [5]. Employing antibiotics may unavoidably stimulate ARB; however, the misusing or overusing antibiotics is linked to the prevalence of ARB in clinical and animal agriculture domains [3] [6]. There has been mounting worry concerning the existence of ARB and their resistance genes in aquatic mediums, since antibiotic resistance may be spread through splitting ARGs between bacterial communities [7] [8]. Mobile genetic elements, like plasmids, integrons, and transposons, are implicated in ARG splitting via horizontal gene transfer (HGT) phenomena, like conjugation (cell-to-cell contact), transduction (virus-mediated), and transformation (the uptake of exogenous genetic materials) [3]. Taking into account the HGT routes, in both free form (extracellular) and within host cells (intracellular), ARGs are seen to be pollutants of worry in natural and engineered water systems [7] [8] [9].

Urban wastewaters are established as one of the main origins of ARB and ARGs in aquatic mediums [10] [11]. Traditional biological wastewater treatments are unable to completely remove the ARB and ARGs [11] [12], and can even conduct selective elevations of multi-resistant bacterial species [13]. Disinfecting wastewater effluent using chlorine or UV irradiation has been largely exercised to save the microbiological quality of potable water sources and sensitive receiving waters [14] [15] [16] [17] [18]. Improved wastewater treatment employing ozone or UV/H₂O₂ has as well obtained elevating interest in several developed nations and has been applied in numerous nations to remove diverse trace organic pollutants [19]-[25] exercising opposite environmental influences, like hormones and pharmaceuticals [26]. Under such conditions, there has been rising attention in the performance of employing traditional and advanced wastewater disinfection techniques as obstacles versus antibiotic-resistance spreading via diminishing the quantities of ARB and ARGs [1] [27]-[33].

Disinfection (oxidation) techniques, like chlorine, ozone, UV, and UV/H₂O₂, have been tested to demobilize ARB and ARGs in wastewater effluent matrixes in laboratory, pilot, and full-scale investigations [9] [12] [34]-[44]. Generally, wastewater disinfection techniques below usual treatment circumstances may importantly diminish global ARB amounts (frequently by more than several logs); however, at the same time conducting to ARB selection (*i.e.*, an augmenta-

tion of the relative proportions of ARB amongst the surviving bacterial cells) [34] [38] [45] [46]. This makes more difficult the evaluation of disinfection's performance in diminishing the capacity of antibiotic-resistance spreading [47] [48] [49] [50]. Diminishing ARG quantities, which was assessed by the quantitative polymerase chain reaction (qPCR) technique, was frequently much less important contrasted to that of ARB, showing the more resistant quality of DNA versus bacterial cells themselves. In general, wastewater disinfection techniques have to be accurately estimated and then regulated to attain enough degrees of ARG demobilization [1].

The demobilization performance of ARGs throughout wastewater disinfection is a function of diverse parameters [51]. Primarily, the kind of ARGs is fundamental. If ARGs are existing within extracellular or intracellular DNA (from now on designated as e-ARG and i-ARG), they can influence the efficiency of ARG deterioration by oxidants or UV [9]. The influence of ARG kind on its demobilization performance by diverse disinfection techniques is deficiently established. Secondly, varying levels of ARG demobilization may be quantified following which analytical techniques are employed. Lately, qPCR-based techniques have been largely used to measure ARG deterioration. Most former investigations employed the qPCR technique optimized for short amplicons (e.g., 100 - 200 bp). Nevertheless, the short amplicon-based qPCR techniques may undervalue ARG demolition and related absence of ARG biological role [39] [52] [53]. Thirdly, the running circumstances of disinfection technologies (such as oxidant or UV injection) and water characteristics (like dissolved organic matter [54]-[60]) may greatly touch ARG demobilization degrees. Most past researches were performed below the particular situations formed by disinfection technologies and wastewater matrixes, which shortens possibilities for inter-comparison and generalization of the finds. In this regard, it is advised to define principle-based kinetic parameters for ARG demobilization in clean water matrixes (such as no oxidant demand). At most, few investigations have accurately defined commonly viable rate constants for ARG demobilization, like for ozonation [35] and UV disinfection [39] [52]. To conclude, regardless of huge modern research advance, it remains hard to foresee the demobilization degrees of varying sorts of ARGs throughout wastewater disinfection or oxidation at a changing running technique or water matrix situations [1].

This work reviews the most recent and pertinent researches in this field of eliminating ARB and ARG through wastewater treatment.

2. Demobilization of Plasmid-Encoded Antibiotic Resistance Genes Using Chlorine, UV, and UV/H₂O₂

Yoon *et al.* [1] evaluated the demobilization efficacy of plasmid-encoded antibiotic resistance genes (ARGs) both in extracellular form (e-ARG) and existing inside *Escherichia coli* (*E. coli*) (intracellular form, i-ARG) throughout water treatment [61] [62] [63] [64] [65] using chlorine, UV (254 nm), and UV/H₂O₂.

They employed a quantitative real-time PCR (qPCR) technique to measure the ARG demolition to *amp^R* (850 bp) and *kan^R* (806 bp) amplicons, both of which are located in the pUC4K plasmid. The plate count and flow cytometry techniques were as well utilized to control the bacterial demobilization factors, like cultivability and membrane deterioration [66], respectively. They measured the kinetics of *E. coli* [67] demobilization and ARG demolition in phosphate-buffered solutions. The ARG demolition took place much more tardily than *E. coli* demobilization in all situations. To attain 4-log lowering of ARG concentration at pH 7, the needed chlorine subjection and UV fluence were 33 - 72 (mg × min)/L for chlorine and 50 - 130 mJ/cm² for UV and UV/H₂O₂. After augmenting pH from 7 to 8, the averages of ARG demolition diminished for chlorine; however, they did not change for UV and UV/H₂O₂. The i-ARGs generally depicted lower averages of deterioration contrasted to the e-ARGs because of the preservative functions of cellular components versus oxidants and UV. The participation of •OH radicals [68] [69] to i-ARG demolition was modest in UV/H₂O₂ because of important •OH radical scavenging via cellular components. In all situations, the ARG demolition averages were identical for *amp^R* against *kan^R*, except for the chlorination of e-ARGs, in which the deterioration to *amp^R* took place faster than that to *kan^R*. Chlorine and UV dose-dependent ARG demobilization degrees evaluated in a wastewater effluent matrix could be rationally interpreted using the kinetic data gained from the phosphate-buffered solutions and the predictable oxidant (chlorine and •OH radicals) demands by water matrix components. These findings may be helpful in regulating chlorine and UV-based disinfection setups to obtain ARG demobilization [1].

Rizzo *et al.* [70] compared the impact of UV radiation on antibiotic-resistant *E. coli* strains with that of chlorination method. Below the tried circumstances, UV disinfection technique conducted to a completed emobilization following 60min of irradiation (1.25×10^4 μWs/cm²) contrasted to 120 min chlorine residence period (initial chlorine dose of 2 mg/L). Further, no modification in *E. coli* strains' resistance to amoxicillin (AMX) (minimum inhibiting concentration (MIC) > 256 mg/L) and sulfamethoxazole (SMZ) (MIC > 1024 mg/L) could be found following UV application. At the same time, the treatment touched resistance of the lower resistance strain to ciprofloxacin (CPX) (MIC diminished by 33% and 50% following 60 and 120 min, respectively). Contrarily, chlorination method did not influence antibiotic resistance of the tested *E. coli* strains. Finally, the impact of UV radiation on the mixture of three antibiotics was also examined and photodegradation data fit quite well pseudo first order kinetic models with $t_{1/2}$ values of 14, 20 and 25 min for CPX, AMX and SMZ, respectively. Following these findings, classical disinfection techniques may not be efficient in demobilizing ARB, and the simultaneous liberation of ARB and antibiotics at sub-lethal concentrations into municipal WWTP effluent may promote the development of resistance among bacteria in receiving water.

3. Contribution of Full-Scale Wastewater Treatment Plants on Antibiotic Resistant Bacteria Environmental Spreading

Turolla *et al.* [71] studied the existence of antibiotic-resistant bacteria (ARB) in wastewater and focused on the contribution of the wastewater treatment plants (WWTPs) in raising or restricting antibiotic resistance. They controlled *E. coli* and total heterotrophic bacteria (THB) resistance to ampicillin, chloramphenicol, and tetracycline in three WWTPs situated in Milan (Italy), varying between them for the running factors of biological technology, for the disinfection techniques (founded on sodium hypochlorite, UV radiation, peracetic acid (PAA) [72]) and for the discharge limits to be satisfied. They gathered wastewater from three sampling points over the treatment succession (WWTP influent, effluent from sand filtration, WWTP effluent). Antibiotic resistance to ampicillin was detected both for *E. coli* and for THB. Ampicillin resistant bacteria in the WWTP influents were 20% - 47% of *E. coli* and 16% - 25% of THB counts [71]. Restricted resistance to chloramphenicol was found only for *E. coli*; however, neither for *E. coli* nor for THB tetracycline resistance was noted. The biological treatment and sand filtration conducted to a diminution in the maximum percentage of ampicillin-resistant bacteria (20% - 29% for *E. coli*, 11% - 21% for THB). Nevertheless, the usually followed factors did not appear convenient to boost an explication of WWTP contribution in ARB diffusion. PAA was efficient in selectively performing on antibiotic-resistant THB, in contrast to UV radiation and sodium hypochlorite [71].

PAA is an organic chemical employed efficaciously as a disinfectant in wastewater treatments. So far, at minimum injections it may provoke selection; therefore, Turolla *et al.* [73] assessed the impact of minimum injections of PAA on *Enterococcus faecium* as a proxy of human-related microbial waste (Figure 1). They treated bacteria using elevating injections of PAA (from 0 to 25 mg/L min) and incubated in regrowth tests below non-growing, restricting circumstances and below growing, appropriate situations. The modifications in bacterial plenty, in bacterial phenotype (number and composition of small cell clusters), and in the plenty of an ARG were estimated. The trials established that the chosen injections of PAA efficaciously eliminated enterococci, and generated a long-lasting influence following PAA demobilization. The comparative plenty of small clusters augmented throughout the test if contrasted with that of the inoculum. Further, below growing suitable situations, the comparative plenty of small clusters diminished and the number of cells per cluster augmented with elevating PAA injections. Robust constancy of the evaluated ARG was observed, not depicting any influence throughout the full test. The findings established the likelihood of small injections of PAA to demobilize bacteria. Nevertheless, the stress formed by PAA disinfection encouraged a bacterial adaptation, even if potentially without touching the plenty of the ARG.

Manoli *et al.* [74] offered the development, verification, and pilot-scale validation of a new CT-based real-time disinfection control procedure, derived from

first principles and implemented to PAA disinfection of domestic secondary effluent wastewater. They performed validation tests employing a 3-m³ pilot contact basin of which the hydraulic efficacy was first characterized by means of tracer trials and then mathematically modeled employing the well-established theoretical framework of continuous stirred-tank reactors in series (Figure 2). The analytical model defining hydraulic efficacy was then expanded to consider disinfectant demand/decay and microbial demobilization kinetics. The integrated model was successfully employed to foresee, and control, residual PAA as well as microbial concentration in the pilot effluent. Validation investigations once and for all backed that the new CT-based control procedure was superior in keeping constant disinfection efficiency, wanted microbial counts, and low residual disinfectant under variable flow and wastewater quality. If compared with flow pacing, the CT-based control needed two times less the quantity of chemical for the identical treatment target (<100 CFU/100mL). Outstandingly, the

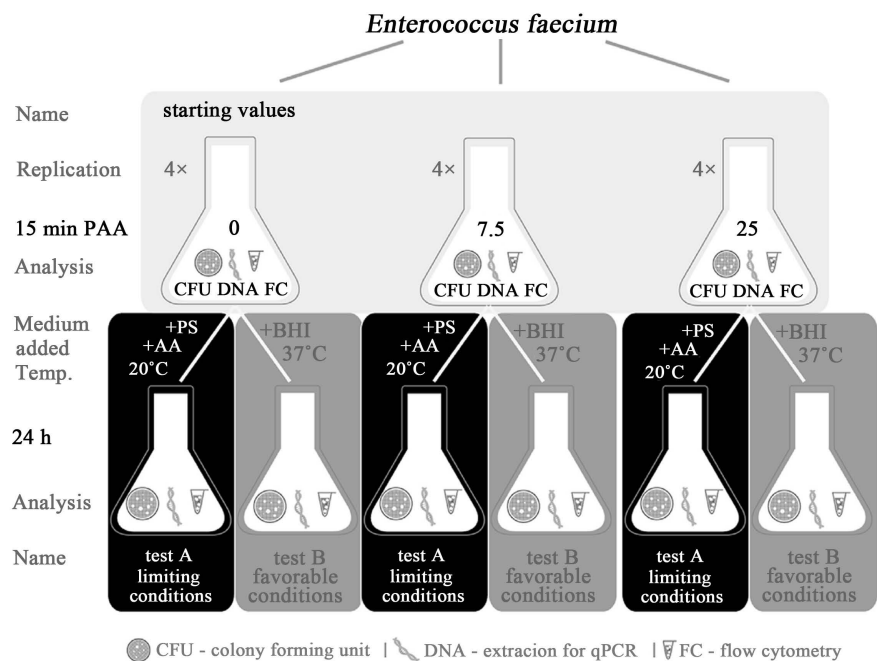


Figure 1. Graphical depiction of the experimental design (disinfection treatments and regrowth tests under limiting (test A) and favorable conditions (test B)). Abbreviations: BHI, brain-heart infusion broth; CFU, colony forming units; DNA, extraction for qPCR; FC, flow cytometry; PAA, Peracetic Acid; PS, physiological solution [73].

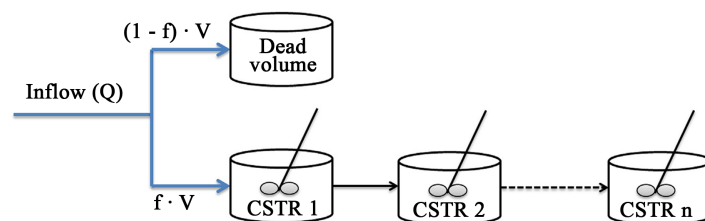
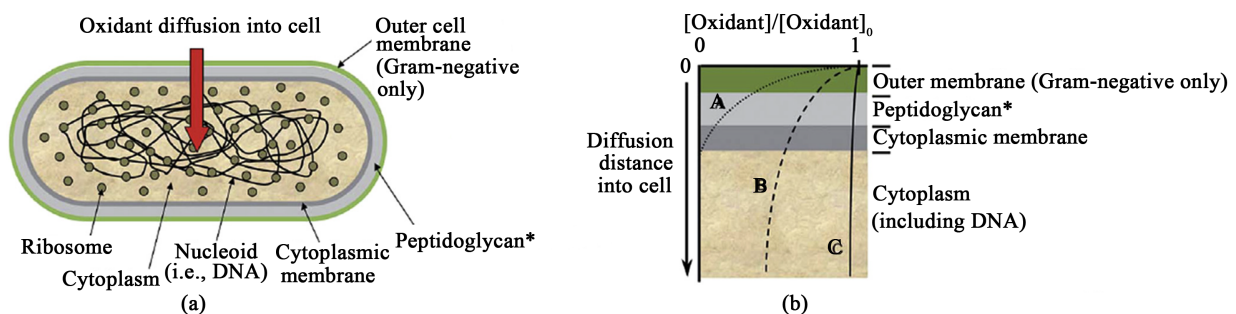


Figure 2. Conceptual representation of the n-CSTRs model modified by the addition of an effective volume factor (f) [74].

CT-based control procedure may be widespread to diverse chemical disinfection techniques like chlorination and ozonation, solo or in integration with physical treatment methods like membranes [75] [76] [77] [78] and UV irradiation.

Researchers [79] presented an overview of the present understanding about demobilization of ARB and ARG (Figure 3). They also focused on the pathway of the influences of diverse disinfection techniques in water and wastewater (chlorination, UV irradiation, Fenton reaction, ozonation, and photocatalytic oxidation) (Figure 4). They also discussed the impacts of constructed wetlands (CWs) and nanotechnology [80] on ARB and ARG (Figure 5).

McConnell *et al.* [82] tried to answer the question how single treatment stages in two tertiary WWTPs influenced the elimination (copies/mL) and relative plenty of ARGs (copies/copies 16S rRNA genes). Nine ARG markers, exemplifying resistance to frequently employed antibiotics, as well as one integron gene (*int1*) to evaluate ARG mobility capacity, were measured utilizing quantitative real-time PCR (qPCR). Both WWTPs satisfied provincial effluent regulations for the elimination of carbonaceous oxygen demand (CBOD₅) and total suspended solids. Eight of the ten ARG markers (*int1*, *su1*, *su2*, *tet(O)*, *ermB*, *bla_{CTX-M}*, *bla_{TEM}*, *qnrS*) were found in all samples. At the opposite, *mecA* was observed intermittently and *vanA* stayed under the detection limit in all samples. The total ARG marker plenty diminished by log 1.77 ($p < 0.05$) in the WWTP employing an aerated lagoon (AL), and by 2.69 logs ($p < 0.05$) via treatment in the plant utilizing a biological nutrient removal (BNR) system. The BNR and secondary clarifier stages in both WWTPs afforded the most elimination of ARGs. The comparative plenty of ARGs stayed unaltered at the AL plant and depicted a diminishing tendency at the BNR plant (Figure 6). Degrees of CBOD₅, nitrate and the human *Bacteroides* fecal marker matched with ARG concentrations, proposing such variables may be helpful in divining ARG reduction. Finally, the effluent coming from the WWTPs comprised eight of the followed ARG markers in concentrations varying from 0.01 to 3.6 log copies/mL, illustrating their liberation into nature; nevertheless, the comparative plenty of ARGs was not reinforced throughout treatment in the two WWTPs.



*Gram-positive cells have a thick peptidoglycan layer, while Gram-negative cells have a thin peptidoglycan layer surrounded by protein-rich periplasm.

Figure 3. Overview of (a) a generic vegetative bacterial cell, and (b) variations in concentrations of several hypothetical oxidants with increasing diffusion distance into the cell (where “A” represents an oxidant with high reactivity toward cell envelope constituents, “B” represents an oxidant with moderate reactivity toward cell envelope constituents and DNA, and “C” represents an oxidant with low reactivity toward all cell constituents) [9] [79].

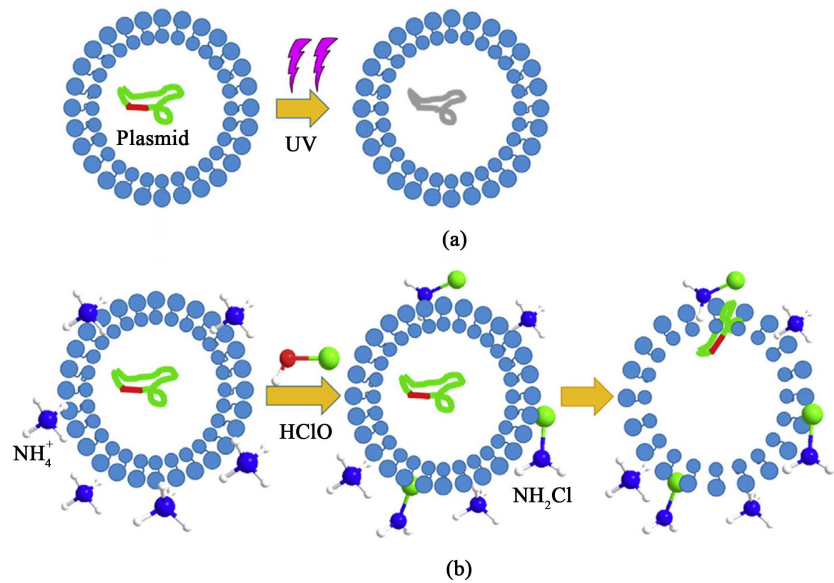


Figure 4. Comparison of the mechanisms of UV disinfection (a) and chlorination (b) affecting the ARGs conjugation transfer [79] [81].

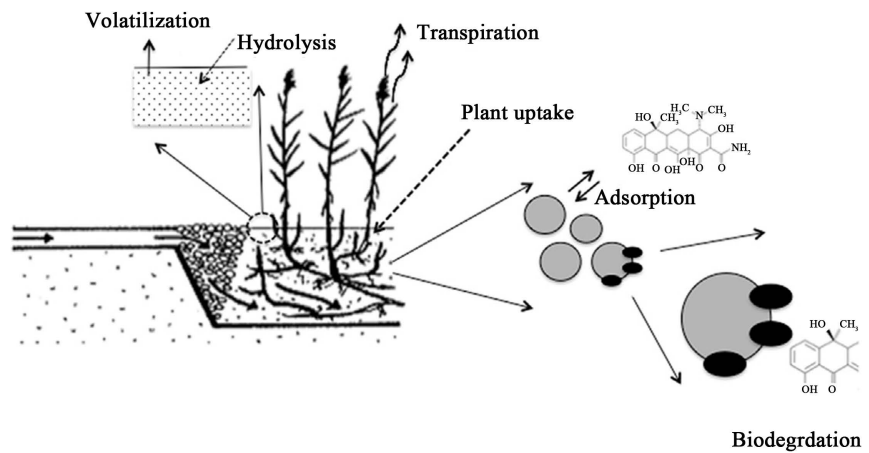


Figure 5. Major mechanisms of pharmaceutical removal in a constructed wetland (CW) [79].

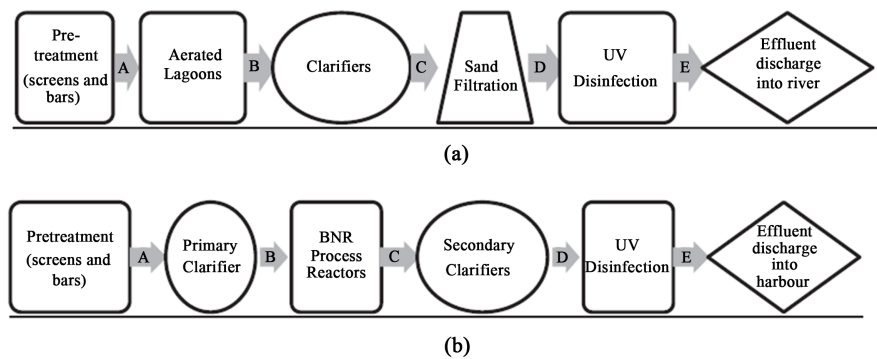


Figure 6. Schematic layout of the treatment trains of (a) the aerated lagoon (AL) plant and (b) the biological nutrient removal (BNR) plant with sampling sites indicated by letters (A - E) [82]. (a) AL plant; (b) BNR plant.

4. Conclusions

The main points drawn from this work may be given as:

1) Numerous investigations have illustrated the spread of ARB and ARG in WWTP effluents, sludge, biosolids, urban solid waste leachates, soils, rivers, lakes, and surface water of livestock farms of diverse areas of the world. The discovery of ARG comprises sulfonamide, tetracycline, beta-lactam, and fluoroquinolone resistance genes. There is a shortage of details regarding the plenty and behavior of ARB and ARG, mostly in WWTPs. Moreover, a more explained comprehension of resistance pathways and their diffusion would help in conveniently evaluating the hazard related to ARB and ARG to public health and ecosystems [79].

2) Researches on the demobilization of ARG via disinfection techniques in real drinking water and WWTPs are so restricted. Chemical disinfectants comprising chlorine, ozone, and Fenton reagent, have been shown efficient in demobilizing ARB and ARG. Various log units of demobilization performance were attained, which changed with the injections of the oxidants. The demobilization degrees of chosen ARG were 1.65 - 2.2, 0.60, and 2.42 - 3.38 log units for chlorination, ozonation, and Fenton oxidation, respectively. Nevertheless, much more investigation is necessitated to enhance the comprehension of the removal of ARG from treated water employing chemical disinfection techniques, especially chlorination which is largely employed all over the world. Restricted research on the usage of UV irradiation to demobilize ARB and ARG has shown its performance; however, a small UV injection was not able in diminishing the frequency of conjugative transfer. More important, an elevated UV injection was hardly fit to diminish the frequency of conjugative transfer. Photocatalytical methods employing TiO_2 have depicted performance in demobilizing ARB and ARG; however, the necessitated treatment has to be for a prolonged residence time (hundreds of min). Improving photocatalysts below visible light may ameliorate the performance of photocatalytical remediation [79].

3) Thanks to the environmentally-friendly type of the CWs, several types of research are dedicated to exploring their aptness for eliminating antibiotics from wastewater. Physicochemical routes implied in eliminating antibiotics flowing in a CW are in fact well authenticated. Nevertheless, it has not been deeply studied what microorganisms are in charge of the antibiotic elimination in a CW. Furthermore, the manner in which ARGs are grown or decreased in a CW has not been enough examined. In fact, the running circumstances below which ARGs are grown or decreased should be more examined. Especially, the connection among the flow scheme of a CW and the plenty of ARB or ARG remains to be investigated. Nanoparticles are coming into the wastewater effluent and sludge; however, their impact on the ARB and ARG remains widely obscure. Next investigations have to involve the role of nanomaterials in the route of diffusion of ARG across genera to estimate the viability of ARB and residual copy numbers of ARG [79].

4) Water reusing is aligned to broader, international R & D trends [83] [84]. Indeed, water reusing is applied through all the industrialized countries and several developing nations [85] [86] [87] [88]. Water reusing is the best solution to overcome the pollutants diffusion through nature [89] [90] [91] [92] [93]. Indeed, WWTPs have to be upgraded to treat wastewater at the highest level to reuse it as drinking potable [94] [95] [96].

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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