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Review

Antibiotic-Resistant Gene Behavior in Constructed Wetlands Treating Sewage: A Critical Review

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Abstract: The main objective of this review is to evaluate the performance of constructed wetlands (CWs) used to reduce antibiotic-resistant genes (ARGs) during sewage treatment. To accomplish this objective, statistical and correlation analyses were performed using published data to determine the influence of operational and design parameters on ARG reduction in CWs. The effects of design and operational parameters, such as different CW configurations, seasonality, monoculture and polyculture, support medium, and hydraulic retention time (HRT), on ARG removals, were analyzed. A comparison of ARG reduction under different CW configurations showed that the hybrid configuration of surface flow (SF)–vertical subsurface flow (VSSF) achieved the highest reductions, with values of 1.55 ulog. In this case, aeration is considered an important factor to reduce ARGs in CWs, and it should be considered in future studies. However, statistical analyses showed that the ARG reductions under different CW configurations were not significant ($p > 0.05$). The same behavior was observed when the effects of operational factors on ARG reductions were analyzed ($p > 0.05$). The results of this study show that CWs are not optimal technologies to reduce ARGs in sewage. The combination of CWs with advanced wastewater technologies can be a solution for enhancing ARG reduction and reducing the spread of antibiotic resistance.

Keywords: antibiotic-resistant genes; sewage; constructed wetlands; operational factors; design factors

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1. Introduction

The emergence of antibiotic-resistant bacteria (ARB) and their dissemination into the environment through antibiotic-resistant genes (ARGs) have been recognized as one of the main concerns of the 21st century. According to the World Health Organization (WHO), it is expected that by 2050, antibiotic resistance (AR) will be the main cause of death in the world population [1]. This problem is associated with both the excessive use of antibiotics (over 100,000 tons per year) and their misuse. The misuse of antibiotics is due to ineffective prescriptions and treatments, the use of foreign or old antibiotics, and ever their use in productive sectors, such as livestock or agriculture [2,3]. In addition, it should be noted that the global SARS-CoV-2 pandemic could contribute to the development and spread of AR due to the widespread use of biocides and increased self-medication by the population [4,5].

Due to the constant consumption of antibiotics, selective pressure may occur within the gut leading to the development of AR in enteric bacteria [6]. Furthermore, taking into account that 30–70% of antibiotics are partially metabolized in the human body, both antibiotics and ARB are continuously excreted into the sewage [7]. Therefore, enteric bacteria of the genus *Escherichia*, *Salmonella*, *Enterococcus*, *Shigella*, and *Klebsiella* predominate in sewage, and it is common to detect resistant strains of each of these [8,9]. *Enterococcus* sp., for example, are naturally resistant pathogens with the ability to acquire resistance to almost all available antibiotics [9]. Due to this, the contamination of surface water with antibiotics from treated and untreated sewage is considered to be the main cause of the rapid spread of AR in the environment [3,10,11].

In general, conventional wastewater treatment plants (WWTPs) have been designed to achieve organic matter, nutrient, and pathogen removal efficiencies of 80–95%, 40–90%, and 80–99%, respectively [12,13]. These types of plants are sequentially composed of primary, secondary, and tertiary treatments where the most widely used biological technology in secondary treatment is activated sludge [14]. WWTPs receive discharges from domestic, hospital, industrial, and agricultural areas that contain a mixture of antibiotic, ARB, and ARG concentrations [15]. Many studies reveal that the dissemination of AR principally occurs during the biological treatment [16–18]. The optimal conditions of oxygen, nutrients, organic matter, and temperature in biological systems promote bacterial growth and different mechanisms for acquiring AR by microorganisms [19]. After the biological treatment, a disinfection process takes place where the bacterial load is removed. However, the reactivation of ARB and ARGs during the disinfection processes was reported, which represents a challenge for WWTPs [20]. For these reasons, conventional WWTPs do not reach optimal ARG reductions, and ARG abundance in the final effluent sometimes increases. For example, Bueno et al. [21] reported an average increase of 1.43 log copies/mL for 17 ARGs in effluent from a WWTP that used an aerobic reactor and UV disinfection [3,16,22]. Similarly, Narciso-da-Rocha et al. [23] evaluated the variations of bacterial community and ARG abundance in a conventional WWTP using activated sludge as a secondary treatment. The results of this study revealed ARG reductions close to 2 ulog with ARG abundances in the effluent close to 4 log copies/mL. Therefore, it has become a priority to find new alternatives of sewage treatment that allow ARG abundance to be reduced in an efficient, safe, and low-cost way. Advanced wastewater treatment technologies, such as advanced oxidation processes (AOPs) and membrane filtration, reported ARG reductions above 4 ulog, and they can be a suitable alternative for stopping AR dissemination into the environment [24]. However, this type of technology requires highly qualified personnel for their operation and maintenance.

Constructed wetlands (CWs) are nonconventional treatment technologies that mimic the removal processes of natural wetlands, optimizing operational and design parameters to enhance the removal of contaminants [25]. These systems have proven to be a sustainable alternative to treat sewage in developed countries, as they efficiently remove organic matter and nutrients [22,25]. According to their hydrology, CWs can be classified as surface (SF) and subsurface flow (SSF). At the same time, they can be divided according to the flow direction into horizontal (HSSF) and vertical subsurface flow (VSSF) [26]. In these systems, the combination of physical, biological, and chemical mechanisms allows the removal of contaminants. Regarding biological mechanisms, aerobic degradation and anaerobic degradation are favored by the different CW configurations. In the case of HSSF, this system promotes aerobic degradation, while in the VSSF system, pollutant removal is achieved by anaerobic degradation [3]. Mancilla et al. [27] evaluated the performance of HSSF and reported removal efficiencies between 20–80% and 10–60% for organic matter and nutrients, respectively. Regarding ARGs and ARB, different studies revealed that CWs achieved removal efficiencies of 99% and 78%, respectively. Despite these performance levels, reductions of ARGs and ARB depend on operational and design parameters of CWs. Moreover, these reductions can be influenced by physical, chemical, and biological mechanisms that reveal the complexity of removing AR elements in CWs [2,10,28–35].

Although there are reviews that have analyzed the ARG reductions achieved in CW, these are only in the form of a compilation of information, showing the new knowledge available on the subject [3,35,36]. However, in this review, an analysis is made of all the information that exists to date. This analysis revealed the contradictions among different studies when indicating the operating conditions that achieve the greatest reductions in ARGs. In this context, the objective of this review is: (1) To analyze the presence and behavior of ARGs in HC; (2) to determine the operating and design parameters that can generate greater ARG reductions; (3) to make a brief comparison between the reductions obtained by HC and advanced treatment technologies; and (4) to present future challenges to achieve a better understanding of this problem and its possible solutions. To achieve objective 2, statistical and correlation analyses were applied to ARG reduction data obtained from different available studies.

2. Antibiotic Resistance in Sewage Treated by Constructed Wetlands

2.1. Antibiotic-Resistant Genes

ARGs are units of nucleic acid information that encode proteins involved in different resistance mechanisms, such as antibiotic inactivation, target site modifications, and reduced antibiotic penetration. In the case of ARGs that are related to the tetracycline family resistance (*tet*O, *tet*B, and *tet*M), these elements encode proteins, which prevent the antibiotic from binding to the ribosome, inhibiting the antibiotic action [8,37]. This AR is a natural phenomenon used by bacteria that gives them adaptive advantages for obtaining resources in the environment compared to other competing species [38,39]. The presence of antibiotics in the environment generates a selective pressure that inhibits the growth of susceptible bacteria, favoring intrinsically resistant bacteria or those that have acquired this resistance over time [8].

Resistance acquired by bacteria can occur through the transfer of genetic material from other bacteria (of the same or a different species) or point mutations [6]. Figure 1 shows the different processes through which bacteria acquire ARGs. In this case, horizontal gene transfer (HGT) occurs through three genetic mechanisms: 1. Transformation, wherein an extracellular naked ARG is taken up by bacteria that have developed genetic competence; 2. Conjugation, wherein the genetic transfer from ARB to recipient cell occurs through cell-to-cell contact; and 3. Transduction, the mechanism through which an ARG is introduced into a cell by a virus or bacteriophage [6,40]. Vertical gene transfer is another process of AR acquisition that consists of the transfer of genetic material from parents to offspring by ARB after acquiring an AGR through one of the mechanisms mentioned above. This process allows the resistant bacteria rate in the environment to increase [6].

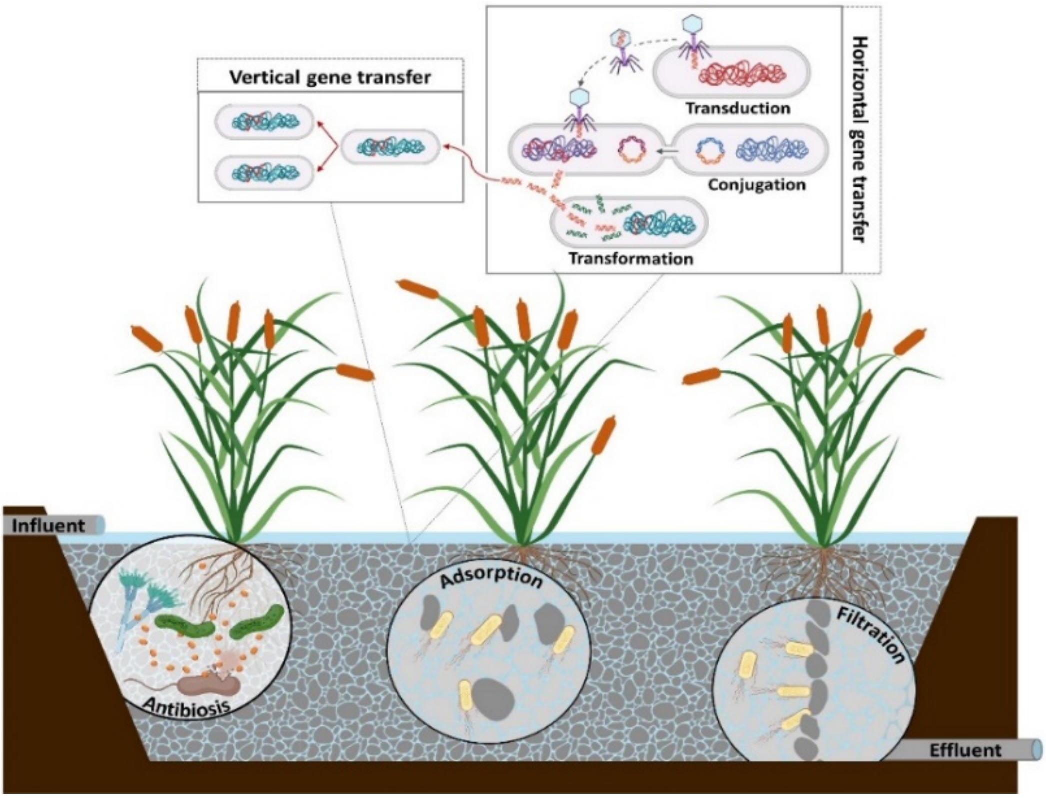

Figure 1. Processes of antibiotic-resistant gene transfer and mechanisms of antibiotic-resistant gene reduction in constructed wetlands. Note: Created with BioRender.com (accessed on 1 April 2022).

As seen in Figure 1, the gene transfer mechanisms mentioned above occur simultaneously within a CW system [3], showing that operational and design conditions are important for reducing ARGs. Therefore, understanding the behavior of ARGs and ARB under different conditions will allow CWs to be optimized, avoiding an increase in ARG abundances in the final effluents.

2.2. Mechanisms of Antibiotic-Resistant Gene Reduction in Constructed Wetlands

As mentioned above, the effectiveness of ARG reduction in CWs depends on the involved conditions and mechanisms. Different studies have shown a positive correlation between the absolute abundance of ARGs and the rRNA 16S gene, a microbial marker [2,3]. This information could indicate that ARGs are transported by fecal microorganisms in sewage and that the reduction of these microorganisms is related to ARG reductions in the effluents [2,3,35]. The principal mechanisms of ARG reduction in CWs are shown in Figure 1. The antibiosis mechanism is related to the production of low molecular weight antibiotics by a group of bacteria or fungi. They can inhibit the growth of ARB in CWs and therefore decrease ARG abundance [41]. Similarly, it has been reported that plant roots are capable of releasing antibiotic compounds. Shirdashtzadeh et al. [42] and Chandrasena et al. [43] found an efficient inhibition of *E. coli* growth due to antibiotic release by the plant *Malaleuca ericifolia*. Likewise, Li et al. [44] reported that extracts from macrophytes, such as *Phragmites communis*, *Typha latifolia*, *Arundo donax*, *Polygonum hydropiper*, and *Polygonum orientale*, achieved reductions close to 100% for coliphages T4 and f2.

Other mechanisms of ARB and ARG reduction in CWs are associated with physical processes, such as filtration and adsorption. In both mechanisms, the support medium and rooting capacity of macrophytes play a fundamental role [3,22]. Liu et al. [28] reported reductions of 50% for the *tet* gene, while Dires et al. [45] achieved an ARB reduction of 77.5%. Both studies suggested that the principal mechanism responsible for achieving these reductions was filtration by zeolite and gravel, respectively. In the case of adsorption, this mechanism is related to the interactions between the contaminants and the support medium or plant roots due to the sorption properties and ionic composition of the medium [46]. Du et al. [2] studied ARG reduction in CWs that used zeolite as a support medium. They determined values of 95.3% for the *sul* and *tet* genes. These results can be explained by the porous morphology and larger surface area of zeolite.

2.3. Antibiotic-Resistant Gene Reductions in Constructed Wetlands

Different types of ARGs can be detected in sewage [10,35,47]. Table 1 shows the absolute abundances and reductions of different ARGs in CWs treating sewage. For rural 0′and urban areas, these values in the influents fluctuated between 1.43×10^6–1.25×10^8 copies/mL and 1.05×10^4–1.58×10^8 copies/mL, respectively. In both types of sewage, the average abundances of ARGs are in the order of 10^7 copies/mL. However, rural areas are characterized by low populations and scattered households, such that the discharge of untreated sewage into the environment is common, especially in underdeveloped countries [48]. This situation poses a significant risk for antibiotic dissemination into the environment.

Table 1. Absolute abundances and reductions of different antibiotic-resistant genes in constructed wetlands treating sewage.

Sewage	Flow Configuration	Macrophyte Type	Support Medium	HRT	ARGs	Absolute Abundance (Copies/mL)		Reduction (Ulog)	Range	Ref
						Influent (×10^6)	Effluent (×10^6)			
Urban	HSSF, VSSF, SF, VSSF–HSSF	*P. australis*, *T. angustifolia*, *T. dealbata*, *C. alterfolius*, *I. tectorum*	tuff, gravel, sand, zeolite	0.18–6	*sul*1	8.18	7.10	0.33	−0.49–1.01	[10,11,29,30,33,34,49]
					*sul*2	8.95	7.48	0.35	0.04–0.9	
					*sul*3	6.92	6.04	0.13	−0.27–0.75	
					*erm*B	4.25	1.56	0.21	0.08–0.82	
					*erm*C	4.43	1.62	0.42	0.30–0.67	
					*tet*M	0.91	0.25	0.36	0.34–0.63	
					*tet*O	0.01	0.04	0.34	0.32–0.90	
					*tet*X	0.04	0.005	0.60	0.31–0.69	
					*flo*R	0.00007	0.00004	0.75	−0.02–0.88	
					*cml*A	2.64	0.07	0.36	0.29–0.74	
Rural	SF–VSSF, SF	*P. australis*, *T. dealbata*, *T. orientalis*, *P. cordata*, *M. vercillatum*, *I. tectorum*	chaff, soil	0.25–1.5	*sul*1	1.14	0.05	0.70	0.42–1.55	[31,50]
					*sul*2	0.01	0.001	0.65	0.41–1.34	
					*sul*3	1.47	0.003	0.78	0.22–0.78	
					*tet*M	1.02	0.01	0.66	0.30–2.69	
					*tet*O	0.42	0.002	0.73	0.51–1.69	
					*erm*B	0.83	0.007	2.03	0.12–2.03	
					*erm*C	0.02	0.01	0.24	0.14–0.27	

Note: HSSF: horizontal subsurface flow; VSSF: vertical subsurface flow; SF: surface flow; VSSF–HSSF: hybrid vertical subsurface flow–horizontal subsurface flow; SF–VSSF: hybrid superficial flow–vertical subsurface flow; *P. australis*: *Phragmites australis; T. angustifolia: Typha angustifolia; T. dealbata: Thalia dealbata; C. alterfolius: Cyperus alterfolius; I. tectorum: Iris tectorum;* HRT: hydraulic retention time; ARGs: antibiotic-resistant genes.

The occurrence of ARGs in influent with an average abundance of 2.0×10^7 copies/mL shows the risks associated with the occurrence of HTG and ARB proliferation. These processes can trigger the dissemination of AR into the environment [3,11]. Moreover, concentrations of antibiotics, pesticides, disinfectants, and heavy metals in sewage can increase this risk [6,49].

In addition, Table 1 shows the operating and design parameters of the CWs used by the evaluated studies to reduce ARG abundances in sewage. These parameters include conventional flow configurations, such as SF, HSSF, VSSF, and also hybrid configurations. The type of macrophytes used, support medium, and hydraulic retention time (HRT) can also be visualized.

Regarding the performance of CWs at reducing ARG abundances in sewage, the ARG reductions were above 0.3 ulog except for *erm*B and *tet*M. However, these systems achieved variable reductions that can be observed in the wide ranges reported. These ranges fluctuated from negative values (−0.49 to −0.02 ulog) to values above the average of 0.3 ulog. For urban sewage, negative reductions were reported for *sul*1, *sul*2, and *sul*3, with values of −0.49, −0.27, and −0.02 ulog, respectively. These negative reductions indicate an increase in ARG abundance in the effluent. This behavior can be related to the presence of antibiotic and coselective agents in CWs. In the case of rural sewage, higher reductions were reported for *tet*M and *erm*B, with values of 2.69 and 2.03 ulog, respectively. Regarding the wide ranges of reductions reported in this study (−0.49 to 2.69 ulog), this result indicates that the ARG reductions in CWs depend on operational and design parameters, a topic that will be discussed in later sections of this study.

3. Statistical and Correlation Analyses to Determine the Effects of Operational and Design Parameters on Antibiotic-Resistant Gene Reductions in Constructed Wetlands

To understand the behavior of ARGs in CWs used to treat sewage, an exhaustive bibliographic analysis was performed using different databases, such as Web of Science and Scopus.

3.1. Statistical Analyses to Determine the Effect of Flow Configuration on Antibiotic-Resistant Genes

First, a bibliographic study was performed to determine the effect of CW flow configurations on ARG reductions. For this analysis, the absolute abundance of ARGs (*sul*1, *sul*2, *sul*3, *erm*B, *erm*C, *tet*O, *tet*X, *tet*G, *tet*M, *flo*R, and *cml*A) in influent and effluent from SF, HSSF, VSSF, and hybrid configurations published in different studies was considered and selected as input parameters. With these data, the ARG reduction was calculated and compared. It is important to mention that the number of publications used for this study was $n = 15$. Then, statistical analyses of the selected data were carried out using Rstudio version 1.2.1335, with a significance level of $p = 0.05$. Shapiro–Wilk and Fligner–Killen tests were applied to analyze normality and the homogeneity of variance, respectively. Then, an ANOVA test was carried out for data with a normal distribution, while Kruskal–Wallis was applied for data without a normal distribution.

3.2. Correlation Analyses to Determine the Effects of Operational Parameters on Antibiotic-Resistant Gene Reductions

Statistical and correlation analyses were carried out to evaluate the effects of seasonality (warm and cold seasons), plantation pattern (monoculture and polyculture), support medium (zeolite and gravel), and HRT (0.5–2 h, 6–24 h, and 48–96 h) on ARG reductions (*sul*1, *sul*2, *erm*B, and *tet*M). A correlation analysis was conducted with RStudio version 1.2.1335, using the "corrplot" package. A correlation factor >0.7 was used for this analysis. For statistical analyses, Shapiro–Wilk and Fligner–Killen tests were applied to analyze normality and homogeneity of variance, respectively. Student's *t*-test was performed for data with a normal distribution, while the Wilcoxon–Mann–Whitney test was applied for data without a normal distribution. The significance level used in these analyses was $p = 0.05$.

4. Antibiotic-Resistant Gene Reduction Performance of Constructed Wetlands

4.1. Effects of Flow Configuration on Antibiotic-Resistant Gene Reduction in Constructed Wetlands

ARG behaviors depend on the biotic and abiotic characteristics of the environment [15]. For this reason, the hydrology of CWs is an important factor. CWs are classified depending on their flow, such as SF and SSF CWs. SSF can in turn be divided into VSSF and HSSF. Moreover, different CW configurations are combined in sequence to improve their pollutant removal performance by forming hybrid CWs [33].

In SF, sewage is exposed to the atmosphere [51]. Sedimentation, filtration, oxidation, reduction, adsorption, and precipitation processes take place due to the slow movement of sewage through the wetland [52]. In VSSF, sewage is fed intermittently onto the bed surface, flooding the entire surface; then, via gravity, it percolates and drains through the support medium. This system increases oxygen availability, promoting aerobic degradation [53]. In HSSF, sewage flows horizontally through the support medium and roots. In this case, ARGs present in sewage make contact with aerobic, anaerobic, and anoxic zones. In aerobic zones close to the surface, processes, such as oxidation and hydrolysis, take place, while in the anaerobic zones, chemical and biological degradation are favored in the support medium [52].

Figure 2 shows ARG reductions in different CW flow configurations. It is important to mention that these results were obtained from published data on ARG abundances in different studies. The ARG reductions in CW flow configurations present in the following order: SF–VSSF > VSSF > SF > HSSF > SF–SF > VSSF–HSSF, with average values of 1.55, 0.67, 0.52, 0.42, 0.41, and 0.25 ulog, respectively. The highest ARG reductions were reported for SF–VSSF hybrid configurations, with average values of 2.60 ulog. This result may be related to the aeration conditions of this CW combination. Studies indicate that greater aeration toward the rhizosphere promotes biodegradation processes. This condition favors the growth and biological activity of microbial communities and increases chemical degradation by redox reactions [2,33,49]. Chen et al. [33] reported that continuous artificial aeration with a rate of 10 m^3/h improved ARG reductions, achieving ARG removal efficiencies of 87.8–99.1%.

Intermittent aeration in CWs has also been shown to be an effective method of improving the reduction of nitrogenous contaminants, such as ARGs. Hou et al. [54] indicated that intermittent aeration of 0.8–1.2 mg/L, with a pattern of 20 min of aeration and 100 min without aeration, presented ammonium and total nitrogen removal efficiencies of 94.6% and 82.6%, respectively.

VSSF presented ARG reductions 0.15 ulog greater than those obtained by SF. The same tendency was observed by Huang et al. [55], who found that VSSF systems achieved ARG removal efficiencies above 62%. The authors suggested that these greater removal efficiencies in VSSF systems were related to the filtration capacity of the support medium. Likewise, Chen et al. [30] compared ARG reductions using different CW flow configurations and plant species. They concluded that HSSF and VSSF systems obtained higher ARG reductions than the SF system, with 0.90 and 0.77 ulog compared to 0.62 ulog, respectively. These results are associated with an adsorption mechanism that takes place by support medium. This result could not be visualized in Figure 2, as SF obtained reductions 0.1 ulog greater than HSSF.

Finally, the VSSF–HSSF hybrid system presented lower ARG reductions, with values close to 0.02 ulog. Likewise, increases in *erm*B, *tet*G, and *flo*R abundances were observed, with values of 4.18×10^4, 1.02×10^6, and 6.75×10^4 copies/mL, respectively. This result may be due to the saturation characteristics of HSSFs that promote anoxic/anaerobic zones close to the support medium. Avila et al. [10] studied how the conditions of saturation and unsaturation influenced the reduction of ARGs. They found that CWs with saturated conditions favor the accumulation of ARGs in the support medium, generating the AR dissemination mediated by HGT.

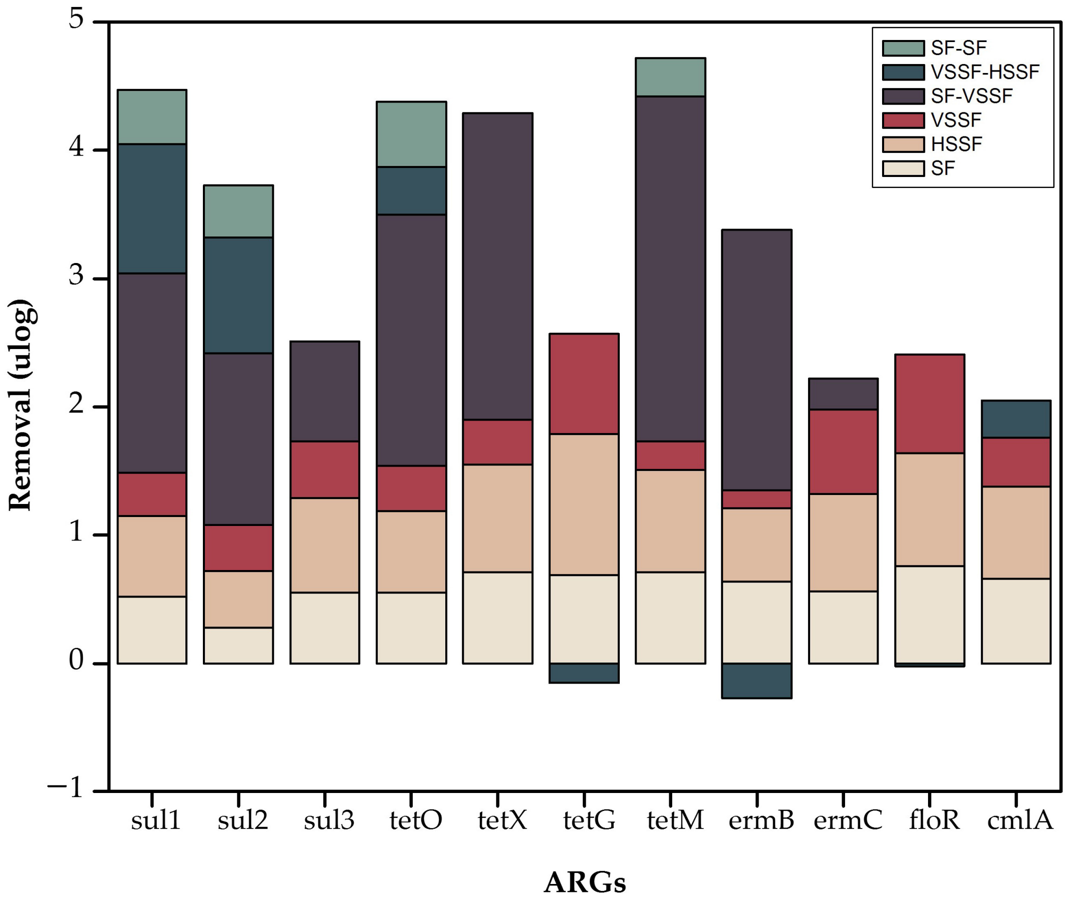

Figure 2. ARG reductions in different constructed wetland flow configurations. SF: surface flow; HSSF: horizontal subsurface flow; VSSF: vertical subsurface flow; SF–VSSF: hybrid surface flow–vertical subsurface flow; VSSF–HSSF: hybrid vertical subsurface flow–horizontal subsurface flow; SF–SF: hybrid surface flow–surface flow.

Although these results indicated that the hybrid SF–VSSF configuration is the most optimal to reduce ARG, statistical analyses indicated that there is no significant difference between different CW flow configurations ($p > 0.05$). Table 2 shows statistical analyses for CW configurations.

Table 2. Statistical analysis to evaluate the significance level of flow configurations in the reduction of antibiotic-resistant genes in constructed wetlands.

Parameter	ARGs	*p*-Value	Significant Difference
CW configuration: HSSF, VSSF, SF, VSSF–HSSF, SF–SF, SF–VSSF	*sul*1	2.85	No
	*sul*2	4.05	No
	*sul*3	0.52	No
	*erm*B	5.56	No
	*erm*C	0.89	No
	*tet*O	0.66	No
	*tet*X	0.52	No
	*tet*G	0.51	No
	*tet*M	0.55	No
	*flo*R	0.52	No
	*cml*A	0.89	No

Note: CW: constructed wetland; HSSF: horizontal subsurface flow; VSSF: vertical subsurface flow; SF: surface flow; VSSF–HSSF: hybrid vertical subsurface flow–horizontal subsurface flow; SF–VSSF: hybrid superficial flow–vertical subsurface flow; SF–SF: hybrid superficial flow–superficial flow; ARGs: antibiotic-resistant genes. ($p < 0.05$).

These results can be explained by the methodology, as the selected data have considerable variation. In accordance with studies by Chen et al. [30], ARG behavior depends on the biotic and abiotic characteristics of the environment. Therefore, differences among countries, drug handling habits, and CW operating conditions may be the reason why the ARG reductions in the different flow configurations are not significant ($p > 0.05$). In addition, this result may indicate that flow configuration is not a determining factor in ARG reduction. Other factors must be taken into account to maximize the filtration, adsorption, and biodegradation mechanisms [11,30,34,49]. However, it is important to consider aeration as a relevant parameter when optimizing a CW to reduce ARGs in future studies.

4.2. Effects of Operational and Design Parameters on Antibiotic-Resistant Gene Reductions in Constructed Wetlands

Figure 3 shows a correlation analysis to determine the influence of the main operational and design parameters on ARG reductions. These parameters include seasonality, monoculture and polyculture, support media, and HRT.

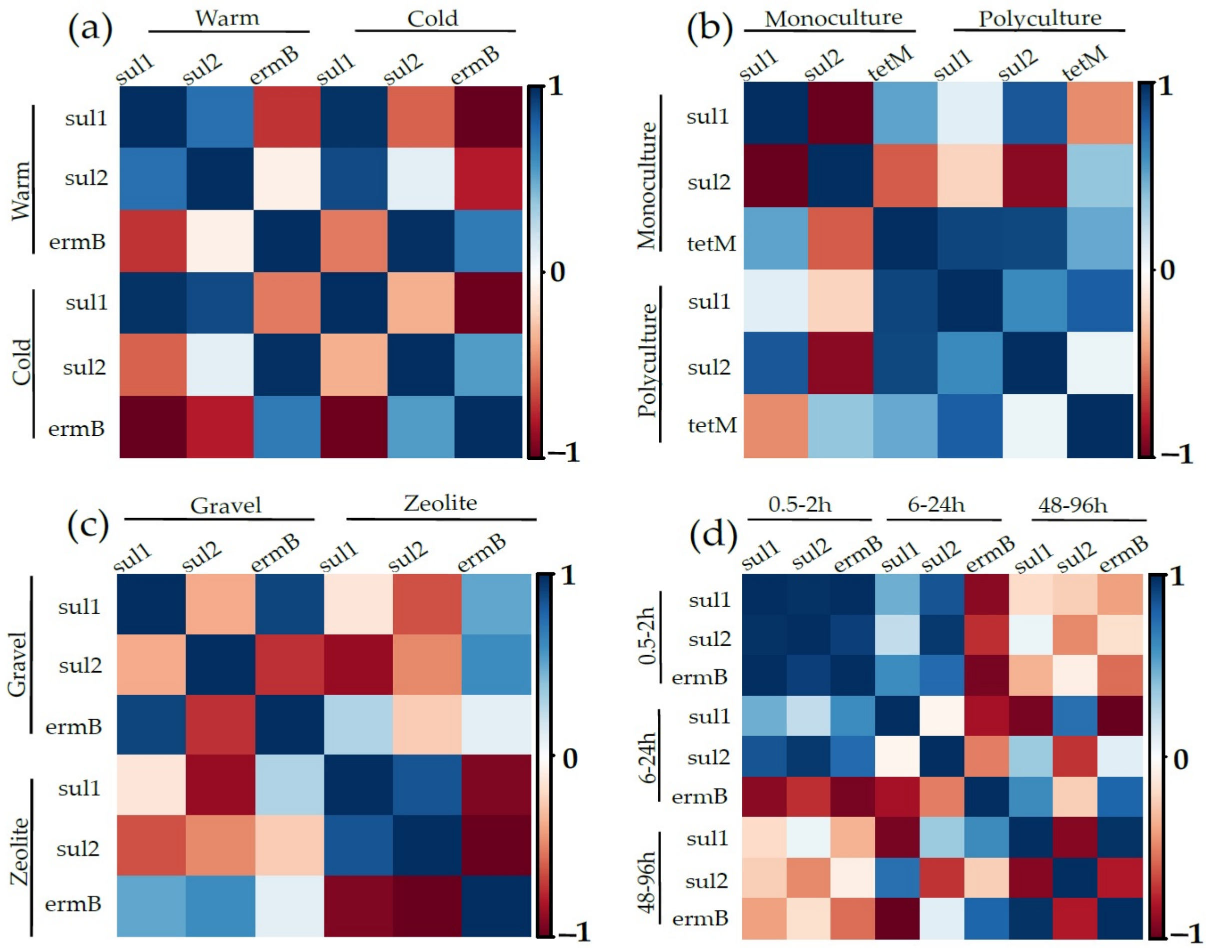

Figure 3. Correlation analysis to determine the influence on antibiotic-resistant gene reductions of (**a**) seasonality, (**b**) monoculture and polyculture, (**c**) support medium, and (**d**) hydraulic retention time.

4.2.1. Effects of Seasonality

Temperature variations generate various changes in the behavior of the microbial communities in the CW system [3]. Therefore, the effect of warm and cold seasons on the reduction of *sul*1, *sul*2, and *erm*B was evaluated by a correlation analysis (Figure 3a). In this analysis, it was noted that *sul*1 reduction in warm seasons correlates positively (0.99) with *sul*1 reduction in cold seasons. This behavior suggests that seasonality is not a determining factor for sul1 reduction for the data used in this review, while for the other ARGs, the information is not conclusive. However, these results differ from studies, such as Fang et al. [31], who found an 18% greater reduction in ARG during winter compared to summer. By contrast, Li et al. [56] indicated a 16% higher ARG reduction in summer compared to those obtained during winter. Likewise, Sabri et al. [11] evidenced negative correlations between temperature and ARG abundances. Abou-kandil et al. [34] attributed these results to the fact that during summer, the metabolic activity and biomass responsible for ARG biodegradation intensified.

4.2.2. Effects of Monoculture and Polyculture

The presence of vegetation allows pollutant removal through direct or indirect degradation [26,57]. At the same time, macrophyte plants can contribute to AR transmission and proliferation because they retain ARGs and ARB in their roots and the support medium [30,31]. To evaluate the effect of plantation pattern—monoculture or polyculture—on the reduction of *sul*1, *sul*2, and *tet*M, correlation analyses were also performed (Figure 3b). The figure shows a negative correlation (−0.89) between *sul*2 reduction in monoculture and *sul*2 reduction in polyculture. This indicates that under the evaluated conditions, plantation pattern is an influential factor in achieving higher *sul*2 reductions; the results were not conclusive for the other ARGs. These results are similar to those reported by Abou-kandil et al. [34] and Rajan et al. [58], who determined 4.5×10^4 copies/mL greater reductions of *E. coli* in polyculture systems, which may result in a more effective reduction of ARB containing *sul*1 and *sul*2. This may be because the roots of polyculture generate a matrix that allows better filtration. This behavior differs from that found in studies by Leiva et al. [59], who report that there are no significant differences ($p > 0.05$) in bacterial removal between monoculture and polyculture systems. Likewise, Cardinal et al. [60] indicated that the presence of macrophytes in monoculture or polyculture cultivation did not produce a considerable change in the reduction of ARB. Contrary to these studies, Licata et al. [61] reported a 4% higher removal of *E. coli* in a monoculture compared to polyculture. Meanwhile, Christofilopoulos et al. [62] indicated that ARG reductions depend on operational conditions and the bacteria community present in the CW system. They reported increases in abundance of *sul*1 and *qnr*A in *E. coli* in planted systems, with values of 3.18×10^9 and 3.8×10^9 gene/μg, respectively, while in *Enterococcus* sp., *sul*1 abundance increased by 2.0×10^4 gene/μg. These results confirm that ARG behaviors depend on both environmental characteristics and the physiological characteristics of ARB. Therefore, these factors should be taken into account when analyzing ARG reductions in future studies.

4.2.3. Effects of the Support Medium

"Support medium" refers to the filter media that cover the CW bed. This system supports living organisms and plants, influences water movement, and allows the adsorption and filtration of contaminants [63]. In an examination of the effect of gravel and zeolite on the reduction of *sul*1, *sul*2, and *erm*B by correlation analysis (Figure 3c), no significant correlations were observed (correlation factor <0.7). Thus, this study reveals that the support medium has no effect on ARG reductions in the operating conditions evaluated. This result was not expected, because authors, such as Du et al. [2], demonstrated a high adsorption of antibiotics and ARGs on zeolite, reaching removal efficiencies of 95%. Similarly, Abou-kandil et al. [34] and Chen et al. [29] indicated that zeolite is the best support medium for reducing ARGs due to its larger surface area and micropores that allow a better sorption capacity. However, this sorption capacity may be the cause of the increased abundance of ARB in effluents. The support medium can retain ARB, increasing bacterial proliferation and HGT mechanisms [34,60]. Likewise, studies by Song et al. [32] using sand at different heights in a CW indicated that the lower layer contains higher relative abundances of *tet*A than the surface layers, with abundances of 0.057 and 0.006 genes/16S rRNA, respectively. Therefore, ARG adsorption in the lower layers could generate increases in ARG abundance and cause the CW to act as a reservoir for AR dissemination.

4.2.4. Effects of Hydraulic Retention Time

The HRT is the time in which the sewage and system components are in contact [64]. This parameter depends on the occurrence of pollutants, degradation rates, and the treatment objective [52]. In this study, the effects of different HRT ranges (0.5–2 h, 6–24 h, and 48–96 h) on the reduction of *sul*1, *sul*2, and *erm*B in CWs were evaluated via a correlation analysis (Figure 3d). The reductions of *sul*1 and *sul*2 in the 6–24 h range are negatively correlated (−0.95, −0.72) with *sul*1 and *sul*2 reductions in the 48–96 h range. Likewise, reductions of *erm*B in the 0.5–2 h range were negatively correlated the with *erm*B reduction in the 6–24 h range (−0.95). Chen et al. [29] reported 36% higher ARG removal efficiencies by increasing the HRT of the system from 9.6 h to 28.8 h. The removal efficiencies for the *qnr*, *cml*, and *tet* genes stood out, with 95, 96, and 90%, respectively [29]. Liu et al. 2021 [65] studied the abundances of ARG in sediments, indicating higher abundances of *tet*M and *tet*W operating at an HRT of 24 h compared to 96 h and 240 h. Based on these two studies, it can be inferred that an HRT range of 6–24 h could achieve more efficient reductions of *sul*1, *sul*2, *erm*B, and *tet*M. The results obtained in this section demonstrate that ARG behaviors depend on the environmental conditions and the analyzed ARG type. The statistical analyses shown in Table 3 show that the differences observed were not significant ($p > 0.05$) when the CWs were operated with different seasonality, support medium, plantation pattern, and HRT. This result, as mentioned in the previous section, may be due to the methodology used. At the same time, it could be related to the difficulty of optimizing these technologies to reduce certain ARG types without generating increases in other ARGs. This tendency indicates that the CWs are not optimal technologies for treating biological pollutants, since the mechanisms involved in their reduction can also lead to an increase in ARGs, causing these systems to act as biological reactors.

Table 3. Statistical analyses to evaluate the significance level of different operating parameters on the reduction of antibiotic-resistant genes in constructed wetlands.

Operating Parameter	Comparison	*p*-Value ARG Reduction				Significant Difference
		*sul*1	*sul*2	*erm*B	*tet*M	
Seasonality	Warm–Cold	0.99	0.57	0.33	*	No
Support medium	Gravel–Zeolite	0.19	0.18	0.60	*	No
Plantation pattern	Monoculture–Polyculture	0.97	0.97	*	0.21	No
HRT	(0.5–2 h), (6–24 h), and (48–96 h)	5.14	4.36	4.23	*	No

Note: HRT: hydraulic retention time; ARG: antibiotic-resistant gene; *: data not available. ($p < 0.05$)

Similar results were obtained by Ávila et al. [10], who found no significant differences when they evaluated the effect of operational and design parameters on ARG reductions ($p > 0.05$). Likewise, Sabri et al. [11] did not observe significant changes in ARG ($p > 0.05$) in the water column, while the difference in ARG abundance could be seen only in the support medium. Abou-kandil et al. [34] indicated that ARGs persisted in the biofilm formed on the support medium. This tendency is consistent with the results reported by Chen et al. [30], who indicated that adsorption processes on the support medium and biological processes in macrophytes can cause AR transmission and proliferation. In addition, He et al. [49] reported 70% increases in the abundances of all evaluated ARGs, with the exception of *erm*B. The same result was obtained by Liu et al. [28], Nõlvak et al. [66], Huang et al. [67], and Berglund et al. [68]. These studies indicated that the use of CWs did not generate a risk of them acting as ARG reservoirs as long as there was a low level of antibiotic exposure (100–2000 ng/L) and the bacteria present had a low metabolic rate. However, Helt et al. [69] established that a single antibiotic at subinhibitory concentrations generated significant increases in resistance, not only to the antibiotic but also to others.

Although a decrease in ARG abundance in effluent compared to influent was observed, this phenomenon takes place due to the formation of the biofilm between the support medium and the plant roots. Similarly, studies reported that ARG abundances can be 0.22 genes/16S rRNA gene higher in the support medium compared to those in the water column [31]. In this case, the ARB present tend to migrate quite rapidly to the biofilms [60]. This means that over time, the ARG proportion in the support medium may exceed the levels present in the water column, producing a reservoir of resistance that may eventually migrate into the effluent [3].

5. Use of Advanced Sewage Treatment Technologies to Enhance Antibiotic-Resistant-Gene Reduction in Constructed Wetlands

Although CWs are easy-to-implement treatment technologies with low operating costs, the scientific evidence in this review indicates that they are not suitable for efficient reduction of ARGs. Table 4 shows advanced sewage treatment and ARG reductions achieved using different treatment processes. AOPs use reactive oxygen species with high redox potential to degrade several organic agents, such as nucleic acids and cell membranes [70]. Among AOPs, processes such as UV irradiation, ozonation, Fenton, and Fenton/UV have been the most studied for the deactivation of microorganisms because they cause damage on the cell membrane and nucleic acids [22].

Table 4. Advanced sewage technologies for reducing antibiotic-resistant genes.

Technology	Process	ARG	Reduction (ulog)	Range	References
AOPs	UV–Fenton, ozonation, UV-activated persulfate, and UV-C/H_2O_2	*sul*1	1.23 ± 1.00	0.38–2.44	[70–74]
		*sul*2	1.45 ± 1.16	0.24–2.57	
		*qnr*S	2.21 ± 2.22	0.44–3.79	
		*tet*O	1.92 ± 2.37	0.25–3.60	
		tetW	1.71 ± 1.62	0.57–2.86	
		*cml*A	2.16 ± 0.93	1.50–2.82	
		bla_{OXA}	1.38 ± 1.12	0.58–2.17	
		bla_{TEM}	0.90 ± 0.88	0.27–1.53	
Membrane filtration	Microfiltration, ultrafiltration, nanofiltration, reverse osmosis, microfiltration, membrane bioreactor, and ultrafiltration	*sul*1	3.26 ± 1.03	2.65–4.45	[75–78]
		*sul*2	3.71 ± 0.40	1.50–4.00	
		*tet*W	3.42 ± 3.76	0.76–6.09	
		*tet*M	3.98 ± 3.58	1.45–6.51	
		*tet*A	4.88 ± 1.58	3.76–6.00	
		*erm*B	0.94 ± 1.00	0.23–1.65	
		*qnr*A	0.91 ± 0.70	0.41–1.40	

Note: AOP: Advanced oxidation process.

Likewise, membrane filtration technologies allow particle and solute retention by molecular weight and hydrodynamic radius [56]. Membrane filtration technologies include microfiltration, ultrafiltration, nanofiltration, and reverse osmosis [75]. Both technologies (AOP and membrane filtration) achieved higher ARG reductions than those obtained by the CW systems, with reduction values of 3.8, 6.5, and 1.1 ulog, respectively. While these advanced technologies achieved suitable reductions, their implementation presents disadvantages. In the case of AOPs, transformation products more toxic than the original compounds can be generated [70,73]. Moreover, studies showed that UV and ozone treatment can generate regrowth of inactivated microorganisms [71,73]. Meanwhile, membrane processes can result in high energy consumption and maintenance costs due to biofouling [22].

Several studies reported improvements in removal efficiency for various contaminants by combining CWs with advanced treatment technologies. Kong et al. [79] studied a VSSF integrated with a membrane bioreactor system as a pretreatment to treat synthetic sewage. They found higher removals of chemical oxygen demand (COD) and turbidity, with values of 62% and 49%, respectively. Azaizeh et al. [80] studied the reduction of *E. coli* in sewage in CWs integrated with a UV disinfection system, finding reductions of 1–2 ulog using CW alone. With the addition of UV technology, the inactivation of *E. coli* was almost total. This same result was reported by Russo et al. [81] for the treatment of secondary effluent from a WWTP with 3 HSSFs integrated with a UV unit. Their data indicated improvements in the reduction of *E. coli*, CT, *enterococcus* sp., coliphages, and *C. perfringen* spores through the disinfection process, which achieved complete elimination of *E. coli*. Thus, although there are currently no specific studies of ARGs in these integrated systems, Table 4 predicts increases in ARG reduction efficiency.

Figure 4 summarizes the main results obtained in this study. Regardless of the operating and design parameters used, CWs are not optimal technologies to achieve efficient ARG reduction. Therefore, in order to improve the performance of ARG removal by CWs, it is suggested to perform CW studies combined with advanced technologies. The plantation pattern of macrophytes evaluated, support medium, seasonality, and HRT are also schematized.

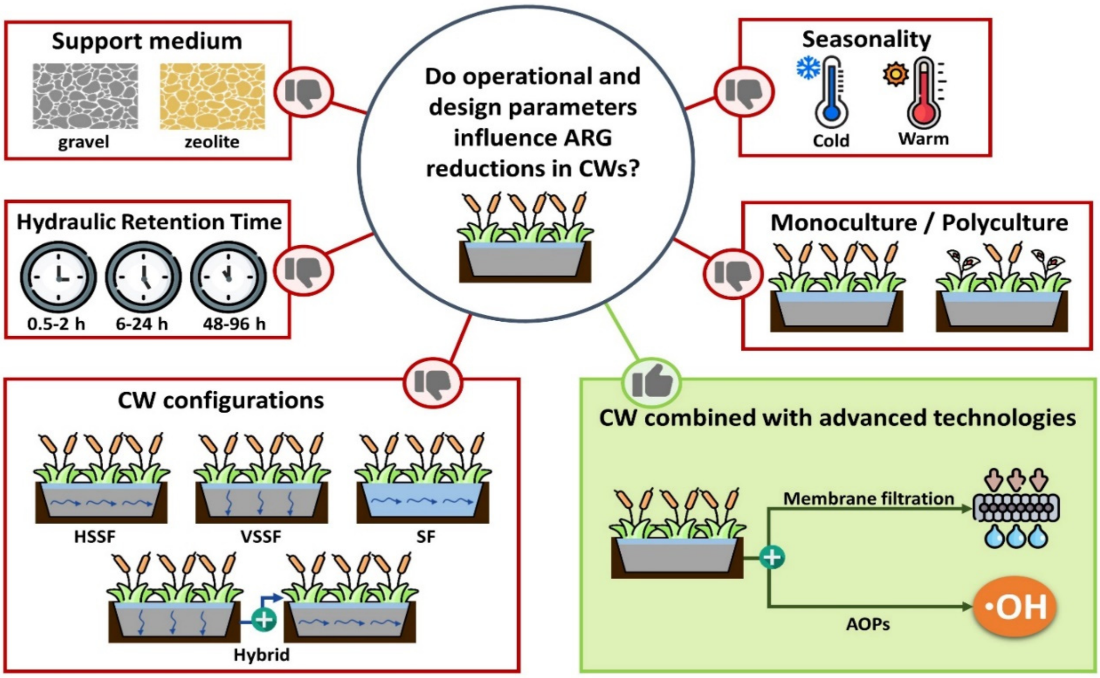

Figure 4. Graphic description of principal results obtained in this study. CW: constructed wetlands; AOPs: advanced oxidation processes.

6. Conclusions

Due to the occurrence of ARG, ARB, and antibiotics in sewage, the search for new alternative treatment technologies is imperative. CWs seem to be a good alternative for removing AR elements. However, the influence of different operational and design parameters is still defined. Contrary to other published reviews, this study performed statistical and correlation analyses using published data for determining the influence of operational and design parameters on ARG reductions in CWs.

Regarding the effects of the flow configuration on ARG reductions, the results of this reviewed indicated that the SF–VSSF hybrid flow configuration allows a higher reduction of ARG, with an average of 1.55 ulog. However, the statistical analysis showed that these differences were not significant ($p > 0.05$). Aeration seems to be an important parameter to take into account to decrease ARG abundances in effluents and should be considered in future studies. Statistical analysis of operating parameters, such as seasonality, monoculture or polyculture, support medium, and HRT, showed a nonsignificant reduction in ARGs ($p > 0.05$). This same behavior was observed in the correlation analyses, which showed no positive or negative results for the different operating and design parameters evaluated.

From the results obtained in this review, it is concluded that the technologies that use bacterial processes for the reduction of biological pollutants, such as ARGs, are not efficient. Due to the environment generated in CWs, processes such as HGT and ARB proliferation may be favored. To take advantage of the operating costs of using CWs to treat sewage, it is suggested that these systems be integrated with advanced treatment technologies to improve the ARG reduction performance of CWs.

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