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Biofiltration: A breeding ground for antibiotic resistance genes

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Abstract

Wastewater treatment plants (WWTPs) play a central role in limiting the environmental dissemination of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs), yet their performance can fluctuate following operational disturbances. This study investigated ARB and ARG dynamics over three months (March to May 2024) following the restart of the biofiltration stage of a municipal WWTP post-flood. Chromogenic extended-spectrum beta-lactamase (ESBL)- and carbapenemase (CARBA)-resistant CHROMagar ESBL and CHROMagar mSuperCarba were used to cultivate the bacteria plates from the influent, secondary clarifier, biofiltration and effluent samples. Phenotypic profiling of isolates combined with colony PCR of key ESBL genes, namely, *bla-TEM, bla-CTX-M,* and*bla-SHV,* and CARBA genes, namely, *NDM* and *Oxa-48* was performed. Along with the relative abundance of the genes co-occurrence patterns of multi-gene profiles of ESBL isolates was also investigated. Removal of gram-negative bacteria stabilized within three months, however, ESBL- and CARBA-resistant bacteria did not. The relative abundance of ESBL-resistant bacteria increased 3.7-fold and CARBA-resistant bacteria 6.7-fold from influent to effluent in May, with additional proliferation in the biofiltration stage from the secondary clarifier (2.3-fold ESBL; 5.1-fold CARBA). Genotypic screening of the isolates revealed that the most prevalent ESBL genes, *bla-SHV*, *bla-CTX-M*and *bla-TEM,* frequently co-occur, with a particularly strong co-association between *bla-SHV* and *bla-TEM*and striking persistence of the *NDM gene*, which was detected in up to 87% of the effluent isolates in May. Spatially resolved sampling further enabled the identification of resistance hotspots, pin-pointing biofiltration as a key zone of gene exchange and ARG persistence. Together, these findings highlight the critical need for targeted monitoring strategies in engineered water environments.

1. Introduction

Antimicrobial resistance (AMR) has become a global threat in recent years, causing more than half a million deaths associated with AMR and 133,000 deaths directly due to AMR in Europe in 2019 [1]. Globally, this number is even greater (1.27 million) [2]. This threat is not limited to only humans and can also cause food security and safety issues due to drug-resistant infections in animals and plants. The recent increase in resistance in gram-negative bacteria has attracted particular interest from clinicians. A report published in 2017 discussed how gram-negative bacteria, in particular, harbor special abilities against antibiotics, increasing their resistance to a broad range of antibiotics and causing increased infections worldwide. The report also highlights how, in Europe, resistance toward third-generation cephalosporins in *E. coli* and *K. pneumoniae* has increased significantly, including a high percentage of carbapenem resistance in *K. pneumoniae*. In Germany in particular, *E. coli* has been found to cause the most nosocomial infections [3]. Gram-negative bacteria have also been reported as an increasing risk by other researchers [4, 5].

Wastewater treatment plants (WWTPs) are believed to be mixing pots for antibiotic-resistant bacteria owing to their regular influx of bacteria from households, hospitals and industries. Several studies have reported that WWTPs release large quantities of antibiotic resistance genes (ARGs) into the environment

due to ineffective treatment processes at ARGs despite a reduction in bacterial concentrations [6, 7, 8]. Several other factors, such as residual antibiotics, selective pressure and high cell density, can trigger the exchange of genes. Microbial biofilms and activated sludge within WWTPs act as major reservoirs for ARB and ARGs and facilitate horizontal gene transfer (HGT), enabling the enrichment and persistence of resistant populations. Although activated sludge and biofilms remove organic pollutants and nutrients from wastewater, they also facilitate HGT by harboring virulence factor genes and ARGs [9, 10], which can enter the natural environment through effluent discharge.

Surveillance of ARGs in WWTPs is highly important, but it is not enough to consider only the inflow and outflow of WWTPs. It is also important to study how the stage of treatment acts as a critical control point where co-occurrence or even resistance can be amplified and become more persistent. Studying the co-occurrence of ESBL bacteria is also important given that the multigene ESBL profile can often indicate the emergence of high-risk clones or integron-mediated gene cassettes. There are limited data on gene co-occurrence or multistage studies of WWTPs, which focus on the impact on the enrichment or reduction of multigene bacteria.

This study was designed to investigate how antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) persist, accumulate and disseminate within a municipal WWTP, with particular emphasis on the biofiltration stage. To capture both spatial and temporal variation, samples were collected across treatment stages and over three months. Gram-negative ESBL- and CARBA-resistant isolates were genotypically screened for key resistance determinants, including *bla-TEM, bla-SHV, bla-CTX-M, NDM* and *oxa-48*. The integrase gene *IntI1* was also assessed to evaluate the contribution of mobile gene elements to gene transfer within the system. Through this approach, the study aims to answer the following research questions:

1. Does the removal efficiency of WWTPs change with respect to antibiotic-resistant bacteria (ARB)?
2. What role do wastewater treatment plants play in the enrichment or reduction of ARB within the treatment process?
3. Is there an accumulation or amplification of resistance within the treatment stage?
4. Do certain genes exhibit greater persistence or stability across treatment stages than others do?
5. Are there identifiable co-selection patterns among ESBL genes within the resistant isolates recovered from WWTPs?

By integrating phenotypic resistance profiles with genotypic screening of key ESBL and CARBA genes, as well as the integrase gene, this study provides a comprehensive assessment of how resistance is maintained, transferred and potentially disseminated through the wastewater treatment process.

2. Materials and Methods

2.1. Sampling locations:

The samples were collected from a municipal WWTP located in Celle, Lower Saxony, Germany. The analyzed WWTP has a separate sewer system. Celle municipal WWTP has a treatment capacity of approximately 120,000 people. In addition to conventional treatment steps, the facility operates a biofiltration stage containing clay pellets with a biofilm around them. The effluent from the secondary clarifier is passed through this biofiltration unit, where the biofilm reduces the remaining nutrients before final discharge. Prior to the sampling campaign, the biofiltration stage had been shut down for two months (28.12.2023–28.02.2024) because of exceptionally high-water volumes during a regional flood event. Sampling was conducted three weeks following the restart of the biofiltration unit, once a month for a period of three months thereafter, encompassing the months of March, April and May in 2024. During normal operation, the biofilm on the clay pellet is washed off once a day via high-volume water agitation. The resulting biomass-rich water is returned back to the influent stream for re-treatment, potentially redistributing biofilm-associated microorganisms and resistance determinants throughout the system.

Samples were collected from four different stages of WWTPs in the morning between 9 and 10 AM. Samples from the biofiltration stage were collected before the washing of the clay pellets.

1. A) Water samples from influent
2. B) Water samples after secondary clarifier
3. C) Clay pellets from the biofiltration stage
4. D) Water sample from effluent

All the water samples were collected via a qualified sampling technique, and the clay pellets from biofiltration were collected as grab samples [11]. The samples were transported to Biosafety Lab II at Leibniz University Hannover at 4°C. The samples were then processed for further experiments.

2.2 Sample processing

A volume of 50 mL clay pellets was mixed with 50 mL autoclaved normal saline (0.9%) at a 1:1 V/V ratio and mixed by shaking the closed bottles vigorously for five minutes. The normal saline-containing biofilm from the clay pellet was then transferred to a clean bottle. This process was repeated three times, and the samples were pooled in the same bottle. The biofilm containing normal saline was then used for plating. Wastewater samples from the WWTP and normal saline containing biofilms from clay pellets were used to make serial dilutions for cultivation.

2.3 Plating of samples:

This study focused on gram-negative bacteria that are resistant to ESBL and CARBA. To cultivate the ARB, two different antibiotic resistance plates were used: CHROMagar ESBL and CHROMagar mSuperCarba (Mast Diagnostica GmbH, Reinfeld, Germany). MacConkey agar plates were prepared from the granulated MacConkey agar medium according to the manufacturer's instructions (Carl Roth GmbH, Karlsruhe, Germany). These plates were used as controls for the growth of all gram-negative bacteria (including resistant and sensitive bacteria). Using serial dilution, 100 µL of each diluted sample was

inoculated on plates via the spread plate method. For antibiotic resistance plates, influent and biofilm-containing samples (biofiltration) were diluted to 10^{-1}; for effluent and secondary clarifiers, 100 µL and 1000 µL undiluted samples were plated. For MacConkey, agar influent and biofilm-containing samples (biofiltration) were diluted to 10^{-3} dilution, and for effluent and secondary clarifiers, 100 µL and 1000 µL undiluted samples were plated. The plates were then incubated at 37 ± 2°C for 24 hrs.

2.4 Phenotypic identification of antibiotic-resistant bacteria:

After cultivation, the colonies were identified according to the manufacturer's instructions according to the color of the colonies. The number of colony-forming units was calculated via Eq. 1.

CFUs/mL = $\frac{N*DF}{V}$ ……. Eq. 1

where *N* represents the number of colonies, *DF* represents the dilution factor and *V* represents the volume plated.

The identified colonies were then picked via a sterilized toothpick and transferred into a sterilized 1.5 mL tube containing 50 µL of normal saline (0.9% NaCl). The colonies were mixed in normal saline and stored at -80°C. Up to ten colonies of each bacterium per plate were collected for subsequent analysis, provided that sufficient colony growth was present.

2.5 Genotypic characterization of colonies via qPCR

The colonies were further characterized via colony PCR for the presence of various antibiotic genes. The primers used in the study are listed in Table 1 and were purchased from Biomers (Biomers.net GmbH, Germany) and Eurofins (Eurofins Genomics, Germany). For qPCR, a 400 nM concentration of primers and a 200 nM concentration of probe were utilized. A template of 2 µL of a mixture of colonies mixed in normal saline was used.

Table 1
Primers and probes used in the study.

Primers	Sequence	Reference
bla-CTX-M	CGATGTGCAGTACCAGTAA	[12]
	TTAGTGACCAGAATCAGCGG	
bla-TEM	GCATCTTACGGATGGCATGA	[13]
	GTCCTCCGATCGTTGTCAGAA	
bla-SHV	TCCCATGATGAGCACCTTTAAA	[13]
	TCCTGCTGGCGATAGTGGAT	
bla-NDM	GCCACACCAGTGACAATATC	[14]
	GTGCTCAGTGTCGGCAT	
Oxa-48	GGCACGTATGAGCAAGATGC	[15]
	GTTTGACAATACGCTGGCTGC	
Intl1	GCCTTGATGTTACCCGAGAG	[16]
	GATCGGTCGAATGCGTGT	
	(Cy5) CGCCACTCATTCGCCACCCA (BHQ3)	

The 4X CAPITAL qPCR probe master mix (Biotech Rabbit, Germany) and PCR-grade water (Jena Bioscience GmbH, Germany) were utilized for qPCR. qPCR was performed using Bio-Rad CFX-96 via the following program: 98°C for 2 min 30 s, 40 cycles of 95°C for 5 s, 59°C for 15 s and 68°C for 15 s. Primers that were not used with probes were supplemented with

EVA green dye at a 500 nM concentration (Jena Bioscience GmbH, Germany), and a melt curve was run from 50°C to 99°C in 0.5°C increments with a 5 s dwell per step. The relative abundance of positive colonies was calculated via Eq. 2.

Relative abundance (%) = $\left(\frac{Positive\ colonies}{Total\ colonies\ tested}\right) \times 100$ ..... Eq. 2

2.6 Data analysis

The data were analyzed and visualized via Excel and R.

3. Results and Discussion

3.1 Detection and removal efficiency of gram-negative bacteria in influent and effluent

Figure 1 illustrates the colony-forming units (CFUs) per millilitre of gram-negative bacteria in both the influent and effluent. Despite consistently elevated bacterial concentrations in influent samples throughout the sampling months, WWTPs demonstrated a strong capacity for microbial reduction. The removal efficiency reached 99.45%, lowering the effluent concentration to 8.6×10^2 CFU/mL, as shown in Fig. 1. Notably, German wastewater regulations do not prescribe microbiological limits for treated wastewater discharged into bathing waters; instead, regulatory compliance is based primarily on chemical parameters such as BOD_5, COD, and other physicochemical indicators (Abwasserverordnung [AbwV], 2024).

Figure 2 (ESBL) illustrates a gradual increase in ESBL-resistant bacterial colonies across the sampling months, indicating a dynamic environment in which selective pressures may drive the development or persistence of resistance. From March to May, the number of ESBL-resistant bacteria in the influent samples increased by approximately 6.9-fold, whereas that in the effluent samples increased 4.9-fold, reaching 4.5×10^2 CFU/mL in May. In contrast, CARBA-resistant bacteria in the influent samples from March to May remained relatively stable, with an average concentration of 7.5×10^3 CFU/mL. The effluent samples, however, exhibited a more than 5-fold reduction, resulting in 2.7×10^2 CFU/mL in May (Fig. 2 CARBA). Sampling began after the flood and continued for three months. During this period, the removal efficiency for gram-negative bacteria gradually stabilized.

A consistently high removal efficiency was observed for ESBL-resistant bacteria, as reflected by up to 98% reductions from influent to effluent across sampling months. Conversely, CARBA-resistant bacteria presented greater variability, with only 80.7% removal efficiency recorded in March, which increased to 96.3% in May, suggesting month- and resistance-specific differences in treatment performance (Fig. 2).

While previous studies have concentrated on *E. coli* and *K. pneumoniae*, it is equally important to include other clinically relevant pathogens, such as *P. aeruginosa* or *A. baumannii*, to obtain a more comprehensive overview [17, 18, 19]. Our study revealed that WWTPs need constant monitoring and may require improvements in their treatment steps. This approach is essential for reducing overall microbial levels and limiting the spread of antibiotic-resistant bacteria. This section also shows that the removal efficiency of WWTPs can vary depending on the type of resistant bacteria, as illustrated in Figs. 1 and 2.

3.2 Relative abundance of antibiotic-resistant bacteria (ARB) in influent and effluent

Figure 3 shows the relative abundance of antibioticresistant bacteria (ARB) in the influent and effluent samples. The relative abundance of ESBL-resistant bacteria in effluent increased from 1.2% to 54.4% from March to May. However, for CARBAresistance, the relative abundance increased from 19.7% to 31.9%. Notably, the proportion of ESBLproducing bacteria in the effluent increased by 2.0 to 3.7fold compared with that in the influent. For CARBAresistant bacteria, the increase in the relative abundance of ARB from influent and effluent was even more pronounced, ranging from 1.6fold to 12.9fold, with the highest enrichment observed in March (Figure 3).

Several factors may contribute to this trend, such as selective pressure in WWTPs, horizontal gene transfer, and treatment technology limitations [1, 20, 21, 22, 23]. Our results revealed that the relative abundance of antibiotic-resistant bacteria increased during the wastewater treatment process, leading to a relatively high relative abundance of ESBL- and CARBA-resistant bacteria in the effluent. This highlights a critical limitation of current wastewater treatment: despite achieving high overall removal efficiencies for Gram-negative bacteria (as shown in Fig. 1 and Fig. 2), the system remains ineffective at reducing and may even enrich clinically relevant ARB. A comparative study investigating factors influencing ABR reported similar findings, with treated wastewater containing more resistant bacteria than influent [24].

To identify the specific stage acting as the reservoir, samples from two other stages of the WWTP were analyzed, and their abundances are compared in Fig. 4, with the results discussed in the following section.

3.3 Relative ARB abundance across four WWTP stages: biofiltration as a turning point

Figure 4 shows the relative ARB abundance across four treatment stages, namely, influent, secondary clarifier, biofiltration and effluent, to identify potential reservoirs of ARB within the WWTP. We first examined how the relative abundance of ARB changed from the influent to the secondary clarifier. For ESBL-resistant bacteria, the increase ranged from 1.3-fold to 2-fold between March and May, whereas CARBA-resistant bacteria showed a more modest increase, increasing from 1.4-fold to 1.6-fold over the same period. As shown in the previous section, the increase in effluent relative abundance was further confirmed by examining the fold change from influent to effluent. For ESBL-resistant bacteria, the percentage of ESBL-resistant bacteria increased from 2-fold to 3.7-fold, resulting in 52.4% of ESBL-resistant bacteria being discharged into the receiving river. In contrast, CARBA-resistant bacteria displayed a different trend, with fold changes decreasing from 12.9-fold in March to 6.7-fold in May and reaching the lowest value of 1.6-fold in April (Fig. 4).

The increase in relative abundance from the secondary clarifier to the effluent indicated that the conditions between these two stages altered the resistant community. The elevated levels of ARB observed after the biofiltration step suggest that this may act as a reservoir and amplification zone, promoting the persistence and proliferation of resistant strains. Biofilm-rich environments, such as clay–pellet biofilters, provide favorable conditions for bacterial persistence, protection from environmental stress, and increased opportunities for horizontal gene transfer. This accumulation is likely driven by multiple interacting factors. The diverse microbial community present on clay pellets offers a broad genetic reservoir that facilitates ARG dissemination and the emergence of multidrug-resistant (MDR) strains. Additionally, the biofiltration process promotes structured biofilms that further increase gene exchange and stabilize resistant populations [10, 25].

Treatment stage-wise analysis revealed that ARB abundance did not decline uniformly throughout the treatment process. However, both the influent and the secondary clarifier showed a steady increase in

ARB abundance over time in later sampling months. This section answers the question that arose at the beginning of this manuscript: the biofiltration stage not only serves as a reservoir of antibiotic-resistant bacteria but also actively enriches the treated wastewater with resistant strains as it passes through this treatment step.

To further characterize the ARG distribution across WWTP stages, colony PCR was performed to target ESBL- and CARBA-associated genes, along with the mobile genetic element *IntI1*. The stage-specific patterns of these genes are discussed in the following section.

3.4 Genotypic confirmation and distribution of ESBL- and CARBA resistance genes

Figure 5 shows the relative abundance of ESBL- and CARBA resistance genes along with the mobile genetic element *IntI1*. ESBL resistance genes were dominant in different sampling months. In the influent and secondary clarifier, *bla-CTX-M* had the highest relative abundance in March (50.0% & 23.1% relative abundance), which is consistent with previous reports linking *CTX-M* prevalence to colder conditions and increased rainfall [26]. In contrast, April and May were dominated by *bla-TEM* (88.5% & 73.7%) and *bla-SHV* (100% & 93.0%), respectively, indicating a shift in ESBL gene prevalence later in the sampling period. Compared with those in the other sampling months, the relative abundances of *bla-SHV* and *bla-CTX-M* in the biofiltration and effluent stages were relatively greater in May than in the other sampling months, with up to 63% of the bacteria with the *bla-SHV* gene in the receiving water (Fig. 5).

CARBA resistance genes presented more distinct temporal and stage-specific differences. The *NDM* abundance increased notably in May from the influent (72.0%) to the secondary clarifier (100%), and 87.2% of the isolates in the effluent samples carried the NDM gene. WWTP seems effective in eliminating *oxa-48*, which remained relatively low in influent throughout the sampling period. The treatment stage-specific patterns revealed a 4-fold increase in the relative abundance of NDM within the secondary clarifier and a 3-fold increase in the biofiltration stage *for NDM* from April to May (Fig. 5). These findings suggest that *NDM* is not only more viable but also more resilient. This gene has shown a consistent trend with its role as a broad-spectrum CARBA resistance gene capable of persisting in WWTPs [1, 26].

The mobile genetic element *IntI1* showed a temporal pattern similar to that of the other ARGs, with a low relative abundance in March (31.3%) and substantially higher levels in April (89.8%) and May (94%) across all WWTP stages. This indicates the widespread presence of class 1 integrons during the later sampling months. There was a clear trend toward a high relative abundance of *IntI1* in the secondary clarifier and in biofiltration, reaching the highest abundance of 94% in April in biofiltration. Notably, up to 29% of the isolates in the effluent remained *IntI1* positive, demonstrating limited removal of integron-carrying bacteria through the treatment process (Fig. 5). The strong and persistent detection of *IntI1* across samples suggests constant integron-mediated dissemination of resistance genes within the WWTP.

Analysis of the relative gene-abundance map revealed clear differences in the persistence and distribution of ARGs across the WWTP stages and sampling months. Among the ESBL determinants, *bla-TEM* and *bla-SHV* consistently presented the highest overall abundance, which is consistent with their known environmental stability and plasmid-mediated mobility [27].

Compared with the OXA-48 gene, the *NDM* gene also demonstrated high resilience, appearing in a greater number of isolates. This finding is particularly striking because *NDM* is generally considered a rare antibiotic resistance determinant in wastewater. Its unexpectedly high prevalence suggests that certain conditions in the system may favor the persistence or even the enrichment of *NDM*-carrying bacteria [28]. A study of the German clinical urban system and rural system (without clinical interference) reported high *NDM* gene presence in the clinical urban system but not in the rural system [29]. This finding indicates that a high number of isolates carrying *NDM* genes in this study could be introduced by clinical wastewater entering the municipal WWTP. Our study indicates that both biofiltration and secondary clarifiers act as reservoirs and ARGs. We also observed that *NDM* and *bla-SHV* were more persistent in later sampling months, suggesting their growing stability and potential enrichment within the treatment system.

To further investigate the co-occurrence of ESBL genes in isolates with and without *intI1*, a gene map analysis was generated, which is discussed in the following section.

3.5 Resistance gene profiles and co-occurrence patterns in ESBL-resistant colonies

Given the complex fluctuation pattern and the presence of multiple ESBL-associated genes in the isolates, a gene map was constructed. As shown in Fig. 6, this map illustrates that many ESBL-resistant isolates carry more than one resistance gene. The genes are further categorized on the basis of whether they occur with or without the *IntI1* integron, highlighting the role of mobile genetic elements in shaping resistance profiles. Across all months, the influent consistently contained the highest proportion of isolates carrying all three genes, while in the downstream stages, isolates with *bla-SHV* and *bla-TEM* combinations increasingly accumulated (Fig. 6).

Without *IntI1*, the influent presented the highest proportion of triple-positive isolates (16.7%), followed by isolates harboring the *SHV & CTX-M* combination (10%) in March. In April and May, the same pattern was followed, with the triple-positive isolates (40% and 24%, respectively) having the highest percentage, but the *bla-SHV and bla-TEM* dual-positive isolates (25 and 24%, respectively) having the second highest percentage. As water progressed through the treatment process, these patterns shifted to biofiltration, and the dominant combination became *bla-SHV* and *bla-TEM* (6.7%) in March. In April and May, secondary clarifier and biofiltration both resulted in high concentrations of dual- and triple-positive isolates. However, effluent isolates always had more triple-positive genes, indicating that isolates carrying 3 genes were more resilient throughout the treatment process (Fig. 6). Colony PCR revealed clear temporal and stage-dependent patterns in the co-occurrence of ESBL genes within resistant isolates. In March, only 6% of the effluent isolates carried at least one ESBL gene, but by May, this

number had increased to 71%, representing a more than 10-fold increase. Cooccurrence increased even more dramatically, increasing from 0% in March isolates to 50% in May isolates, indicating a 50-fold increase in multigene ESBL profiles (Fig. 6). These findings demonstrate that the effluent, which represents the final stage of treatment, accumulates a greater proportion of isolates with complex resistance profiles over time.

When the presence of *IntI1* was considered, it became evident that integrons were not consistently linked to multigene ESBL profiles. Although the *IntI1* gene is abundant across isolates at all treatment stages (Fig. 5), several isolates carrying two or three ESBL genes lack this integron, suggesting that other mobile genetic elements, such as plasmids or transposons, likely facilitate gene exchange within the system (Fig. 6) [27]. While additional ESBL genes may contribute to resistance, the three genes examined here represent the most commonly reported ESBL determinants globally and therefore serve as strong indicators of resistance dynamics in wastewater environments [8, 12, 23, 26]. The substantial increase in multigene ESBL profiles over time reflects increasing resistome complexity and suggests intensified gene exchange within the treatment system, particularly in the biofiltration stage. This finding shows that the presence of *IntI1* has little influence on whether isolates carry multiple ESBL genes. However, *bla-TEM* and *bla-SHV* frequently occur together, and the effluent displays the highest occurrence of all three ESBL resistance genes, indicating strong co-selection and persistence of multigene resistance profiles during the treatment process.

4. Conclusions

This study demonstrates that different stages of the investigated wastewater treatment plant harbor distinct and highly persistent, ARB and ARGs including ESBL and CARBA genes of major clinical relevance. Our findings provide evidence that WWTPs enrich and disseminate ARGs. The presence of recently emerging clinical resistance genes particularly the high presence of *NDM* in isolates from the final effluent indicates genetic exchange between clinically associated and environmental bacteria. The detection of multiple ARGs in effluent isolates confirms their release in environment waters, enabling further dissemination among environmental microbial communities. The results also show that the biological treatment processes currently in operations are not sufficient to reduce the diversity or prevalence of ARGs detectable in the final effluent. Instead, the biofiltration stage acts as a reservoir that amplifies resistant populations and facilitates co-occurrence of multiple ESBL genes, underscoring its role as a hotspot for ARG persistence. Genotypic analysis showed shifting distribution of ESBL genes across treatment stages and frequent co-occurrence of *bla-SHV*, *bla-CTX-M* and *bla-TEM*, within single isolates. The increased detection of *IntI1* suggests active mobilization of ARGs within biofiltration environment, although co-occurrence patterns indicate plasmid or transposons as likely drivers of gene transfer.

Since our analysis focused on isolates cultivated on selective media, the Resistome described here represents only a portion of the total ARG pool. Future work should incorporate whole-community

metagenomic approaches to capture the full spectrum of resistance determinants circulating within WWTPs.

Overall, these findings highlight the dual role of WWTPs as both mitigation points and amplifying hubs for antibiotic resistance with biofiltration stage as key zones of persistence. Targeted monitoring and optimization of biological treatment processes are needed to limit environmental dissemination of antibiotic resistance.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authorship contribution

K.S. conceptualized the manuscript, conducted the investigation, created the visualizations, performed the formal analysis and wrote the original draft. J.L. assisted with the investigation and edited the manuscript. U.D. edited the manuscript. D.S. helped with the investigation and also edited the manuscript. M.W. reviewed the manuscript and was responsible for project administration and funding acquisition. R.N. reviewed and edited the manuscript and was responsible for supervision and funding acquisition. All authors have read and approved the final manuscript.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the authors used Microsoft copilot and deepL to improve the language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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Author Contribution

K.S. conceptualised the manuscript, conducted the investigation, created the visualisations, performed the formal analysis and wrote the original draft. J.L. assisted with the investigation and edited the manuscript. U.D. edited the manuscript. D.S. helped with the investigation and also edited the manuscript. M.W. reviewed the manuscript and was responsible for project administration and funding acquisition. R.N. reviewed and edited the manuscript and was responsible for supervision and funding acquisition. All authors have read and approved the final manuscript.

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Data Availability

The datasets analyzed during the current study are available from the corresponding author on request.

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Figures

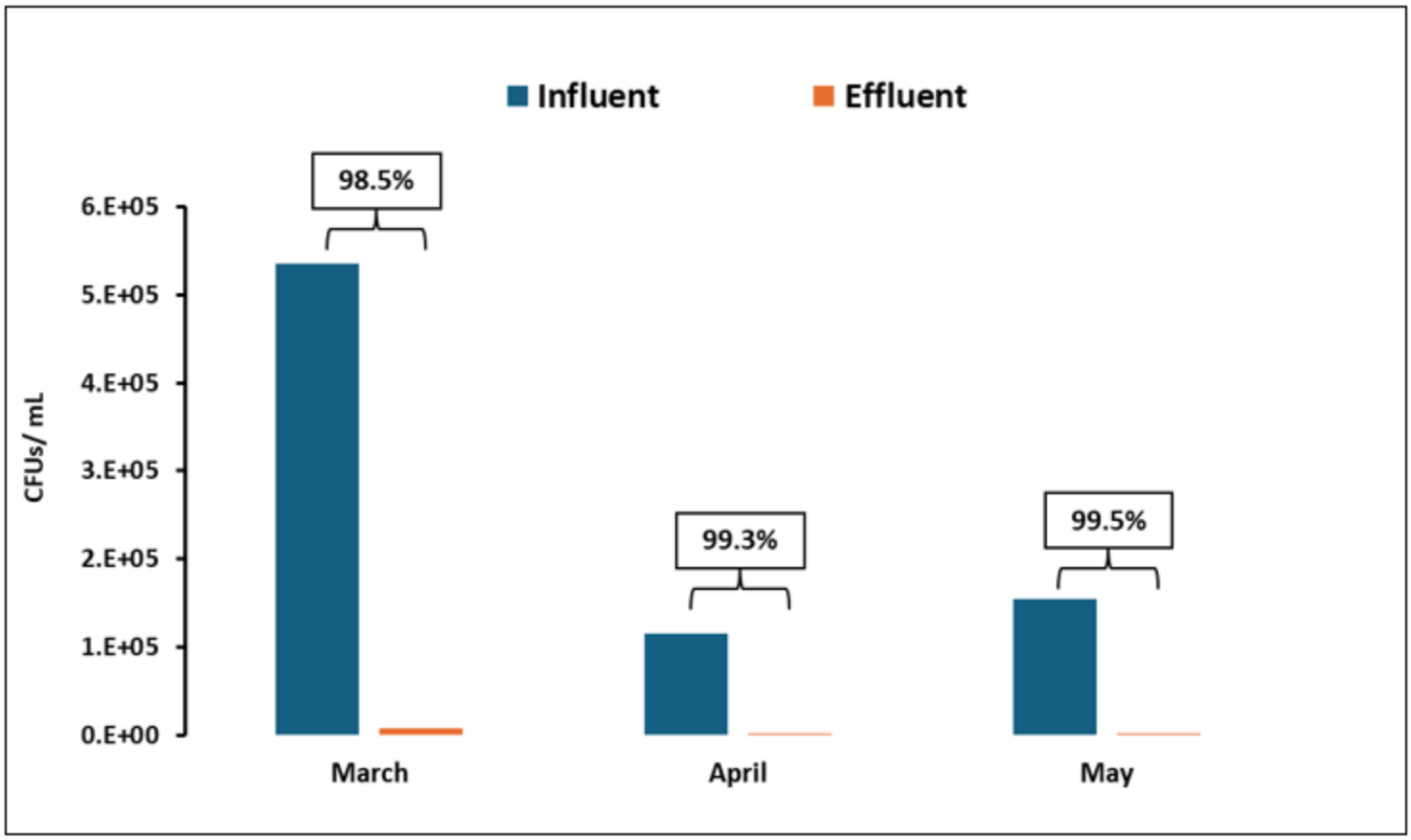


Figure 1

Figure 1: Concentration of Gram-negative bacteria (CFUs/mL) in influent and effluent samples of the WWTP, along with the corresponding removal efficiency (%). Bacterial counts were determined using MacConkey agar.

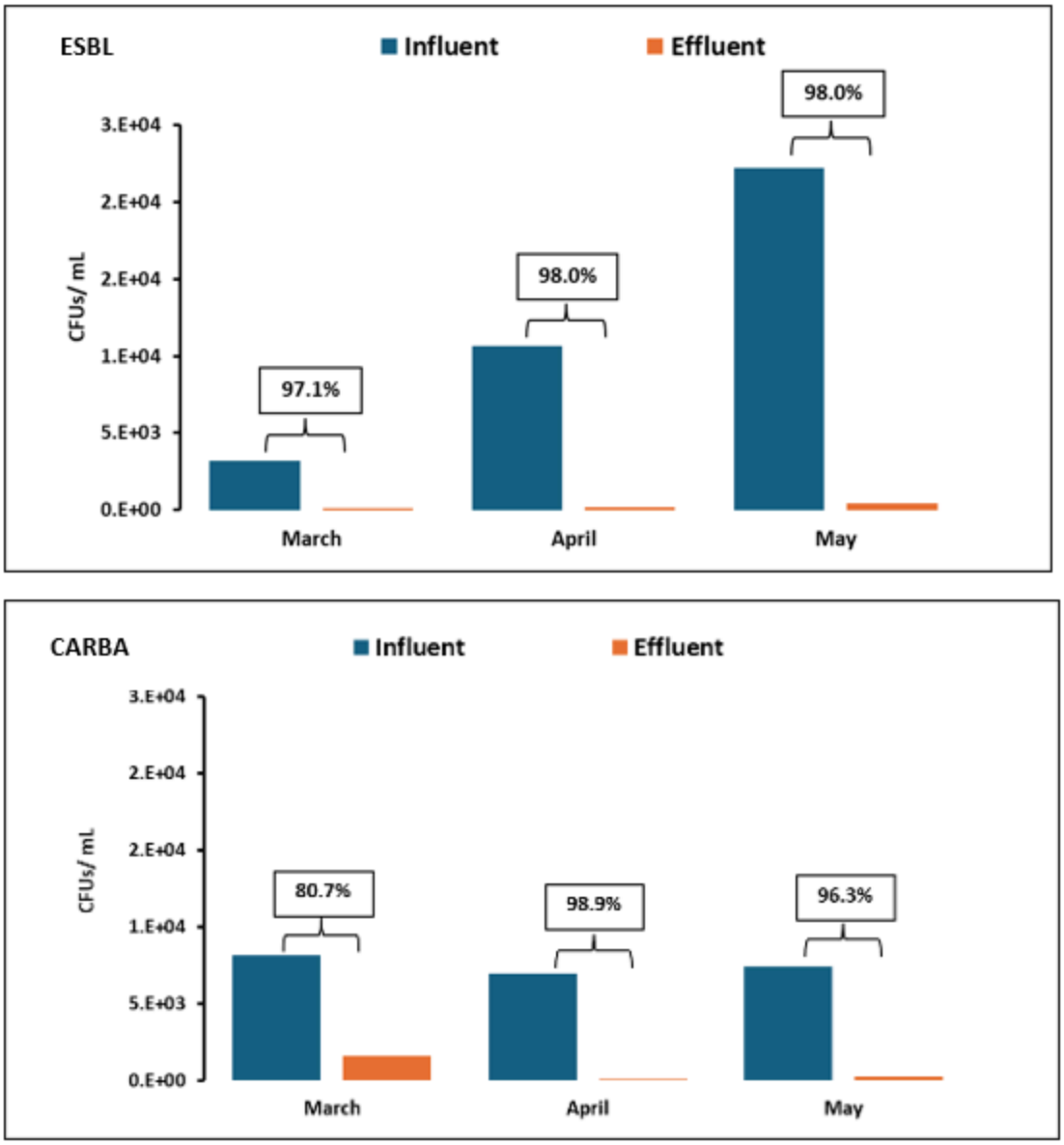


Figure 2

Figure 2 Antibiotic-resistant bacteria (CFUs/mL) in the influent and effluent of WWTPs and their removal efficiency (%). Bacteria were cultivated on CHROMagar ESBL and CHROMagar mSuperCarba

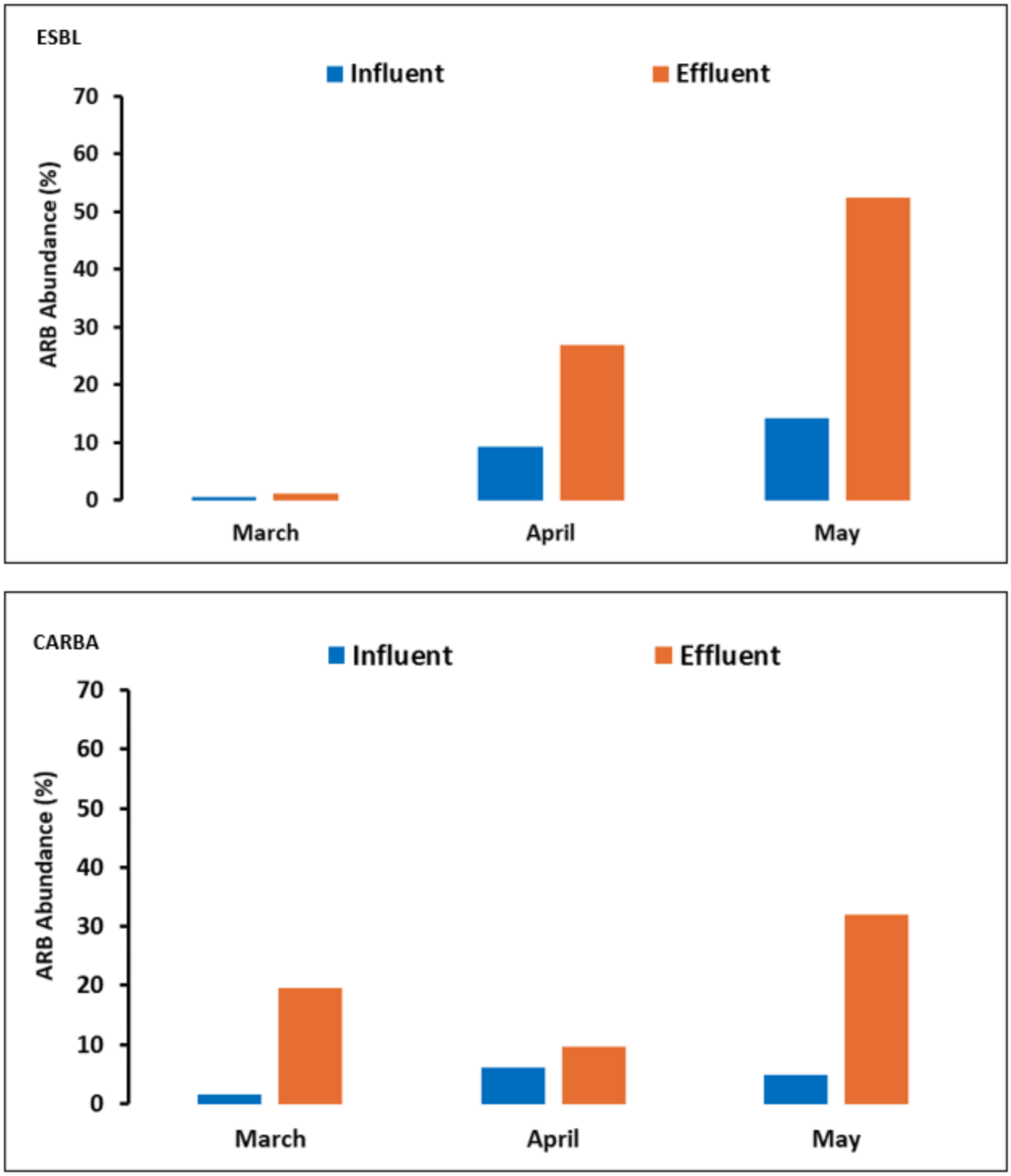


Figure 3

Figure 3 Relative abundance of phenotypically resistant bacteria (%) in influent and effluent samples of WWTP. The abundance is calculated as the ratio of CFUs on MacConkey plates with ESBL and CARBA plates, respectively.

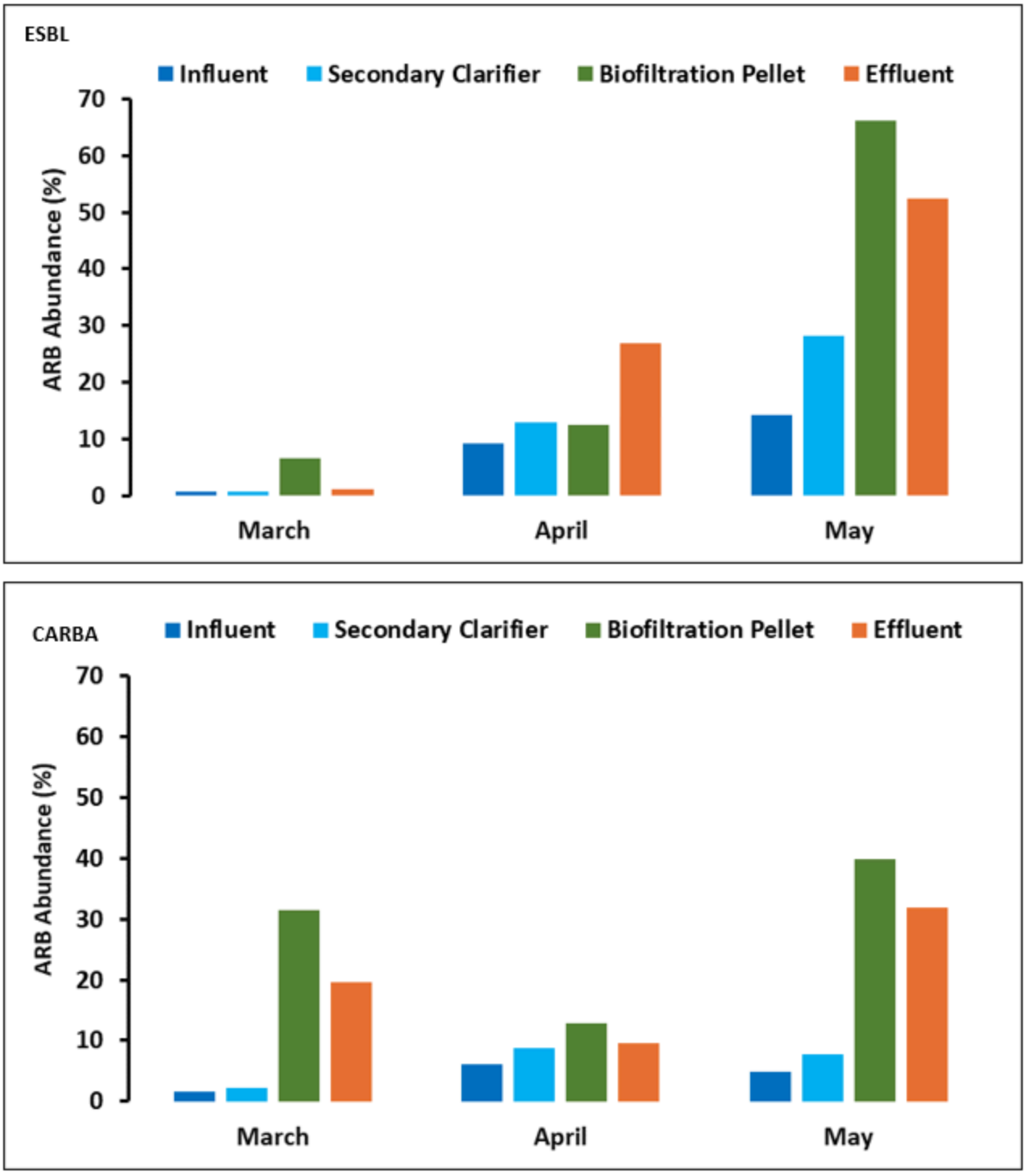


Figure 4

Figure 4 Relative abundance of phenotypically resistant bacteria (%) in four different stages of the WWTP. The abundance was calculated as the ratio of CFUs on MacConkey plates with ESBL and CARBA plates.



Figure 5

Figure 5 Relative abundance (%) of phenotypically resistant isolates harboring at least one ARG in samples collected from various stages of the WWTP during March–May 2024. ARG presence was confirmed using qPCR and the abundance was calculated using eq. 2. IN= influent, SC= secondary clarifier, BF= biofiltration, EF= effluent, MGE= mobile genetic element.

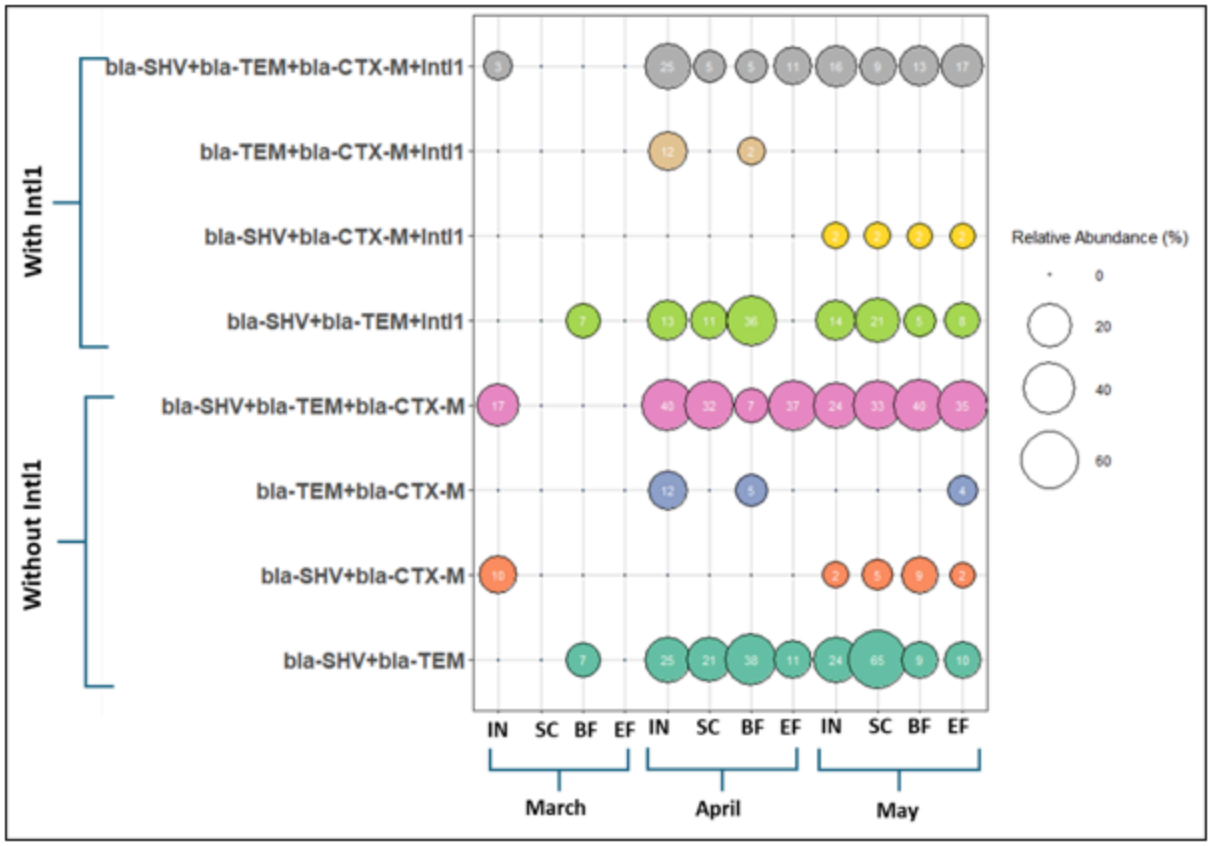


Figure 6

Figure 6: Relative abundance (%) of colonies containing more than one ESBL gene with and without Intl1 gene from samples collected from various parts of wastewater treatment plant. Samples collected in March, April and May 2024. The genes were confirmed using qPCR and abundance was calculated using eq. 2. IN= influent, SC= secondary clarifier, BF= biofiltration, EF= effluent, MGE= mobile genetic element.