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From Wetlands to Worries: A Study of the Constructed Wetlands Model in Antibiotic Resistance Alteration

Ridwan Muhamad Rifai[1]*, Anie Yulistyorini[1], Daffa Rizalif Lahardo[2], Bakhrul Mukhid Fadilah Ramadani[2], Jenvia Rista Pratiwi[1]

[1] *Study Programme of Environmental Engineering, Faculty of Engineering,*
The State University of Malang, Malang, 65145, INDONESIA

[2] *Study Programme of Civil Engineering, Faculty of Engineering,*
The State University of Malang, Malang, 65145, INDONESIA

*Corresponding Author: ridwan.muhamadrifai.ft@um.ac.id

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Abstract

Antibiotic resistance in wastewater is an emerging health concern, as resistant coliform bacteria complicate treatment processes and pose significant risks to public health. Constructed wetlands (CWs) offer a promising and sustainable solution for wastewater treatment, although their effectiveness in reducing coliforms and mitigating antibiotic resistance varies. This study aimed to evaluate the effectiveness of a single CW system employing *Typha* sp. as the phytoremediation agent planted in Lightweight Expanded Clay Aggregate (LECA) on antibiotic resistance alteration. Samples were collected from both the inlet and outlet of the system after a 48-hour treatment. Total coliform enumeration and single colony isolation were performed to assess the abundance of coliform bacteria and antibiotic resistance. The minimum inhibitory concentration (MIC) of penicillin-G was tested using the diffusion disk method. Results showed a significant reduction in total coliform abundance (0.52 log reduction, 69.53%, *p*-val = 0.001). However, antibiotic resistance was increased, with both inlet and outlet samples exhibiting a MIC of 800μg/ml and diameter inhibition zones of 7.8±1.8 mm and 2.7±0.9 mm at 33 units of Penicillin-G, respectively. These findings suggest that CWs may promote antibiotic resistance in certain circumstances, potentially due to treatment efficiency, microbial dynamics, and horizontal gene transfer following selective pressures.

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1. Introduction

Wastewater is one of the most critical hotspots for antibiotic resistance bacteria (ARB) and genes (ARGs), which are currently considered emerging environmental contaminants in the aquatic environment [1], [2]. These two elements have been linked to the spread of antibiotic resistance in aquatic ecosystems, posing significant risks to human health. The highlight is that ARB belongs to faecal coliforms, such as *Escherichia coli*, which signifies the faecal contamination from domestic activities [3]. The persistence of ARB and ARGs in wastewater may lead to waterborne disease incidence, which is harder to treat.

In Indonesia, antibiotic resistance has been commonly identified in several spots of wastewater. For instance, wastewater from 122 dairy farms in East Java contained 36.26% of multi-drug-resistant *E. coli* to tetracycline-streptomycin-trimethoprim [4]. Another study involving dairy farms showed that 69.3% of all samples contained E. coli, where antibiotic resistance and multi-resistance incidence were 99.17% and 84.25%, respectively [5]. In another case, 32% of *E. coli* isolates from hospital wastewater were identified to possess an extended spectrum β-lactamase, raising environmental health concerns [6].

The β-lactam antibiotics, e.g., penicillin, remain the most consumed antibiotics in outpatients in Indonesia [7]. On the other hand, according to a 2019 survey of all populations in Indonesia, safe sanitation was available for only 7.5% of them, whereas 7.6% practised open defecation [8]. This combination increases the probability of water-borne disease caused by resistant bacteria from faecal contamination. Even more, one study conducted in East Lombok, Indonesia, showed that 100% of isolated *Escherichia coli* from water sources near waste disposal were resistant to penicillin [9]. Therefore, it is essential to monitor and countermeasure antibiotic resistance in a comprehensive manner.

One effort to combat antimicrobial resistance in the environment is by treating wastewater before discharging it. Urban wastewater treatment plants (WWTPs) are crucial in determining antibiotic resistance management since these systems act as the recipient and supplier [10]. Full-scale municipal WWTPs have been recognised to remove antibiotic residues, ARB, and ARGs of varying magnitude, ranging from 53% to 99% [11]. Nonetheless, it is more challenging for conventional WWTPs to reduce ARGs than ARB [12]. To reduce ARB and ARGs, conventional WWTPs using activated sludge, for instance, do not perform effectively. It needs to be coupled with advanced post-treatment [13].

Not only improving the quality of wastewater by reducing organic pollutants, but constructed wetlands (CWs) have also been widely known to alter the antibiotic resistance state [14], [15]. The plant and rhizosphere microbial communities in CWs act in the process of biodegradation, cell uptake, and surface adsorption, altering the fraction of ARB and ARGs in the wastewater feed [15]. A study involving *Phragmites australis* in a CW showed high removal percentages of antibiotic residues and antibiotic resistance [16]. In addition, CWs with a hydraulic retention time of 1 to 3 days could reduce ARB up to 1.5 log [17]. The decrease in bacterial abundance in CW effluents may indicate the release of a lower amount of ARGs into receiving environments. Even though CWs are promising, it is also known that WWTPs may act as a silent source of antibiotic resistance in the environment, as they elevate the number of antibiotic resistance genes in their effluent [18], [19].

In light of these concerns, this study aims to investigate the antibiotic resistance state of domestic wastewater treated by CWs on a local scale. Specifically, we will examine the minimum inhibitory concentration (MIC) of the most widely used antibiotic for both inlet and outlet samples and the number of faecal coliforms present. The research involved a novel combination of local domestic wastewater, LECA as substrate, and *Typha* sp. as the phytoremediation agent. By understanding the antibiotic resistance state of wastewater treated by CWs, we hope to provide valuable insights for improving wastewater treatment practices and mitigating the spread of antibiotic resistance in the environment.

2. Materials and Methods

2.1 System Design and Operation

The wastewater treatment plant was assembled by attaching three modules in parallel, i.e., the equalisation tank, double-chamber septic tank, and the horizontal subsurface flow constructed wetlands (HSFCWs) module in series alignment. *Typha* sp. was used in the CWs as the primary phytoremediation agent, planted on lightweight expanded clay aggregate (LECA). Domestic wastewater was collected from a local public facility in Malang, Indonesia. The following parameters in Table 1 were obtained from a preceded optimisation stage.

Table 1 *Operation parameters for the constructed wetlands model*

Parameters	Values
Dimension ($l \times w \times d$)	(69.2 × 17.3 ×26.0) cm
Feed flow rate (Q_f)	5.9 ml/min
Aeration flow rate (Q_a)	2 x (1.5 l/min)
Hydraulic loading rate	7 $cm^3/cm^2/d$
Hydraulic retention time (θ_d)	48 h

The system was run for six weeks before sampling, which was taken from the inlet of the equalisation tank and the outlet of the CWs module. The illustration can be seen in Fig. 1.

2.2 Coliform Enumeration and Isolation

Water samples were cultivated on Eosin Methylene Blue (EMB) agar using the spread method and incubated for 22±2h at 35±0.5°C [20]. Colonies showing green metallic, dark purple complex, and brown-pink colouration were counted. One blue-green colour with a dark purple complex colony was isolated from each agar plate and streaked on EMB agar to purify the colony. The plates were incubated for 22±2h at 35±0.5°C. The purification step was done three times sequentially. Purified colonies were then cultivated in Mueller-Hinton broth media as a working culture. Gram staining was performed to confirm the coliform isolates. In addition, the wastewaters were assessed for NH_3, COD, BOD, and Total Suspended Solid (TSS) to depict the domestic wastewater quality standards [21].

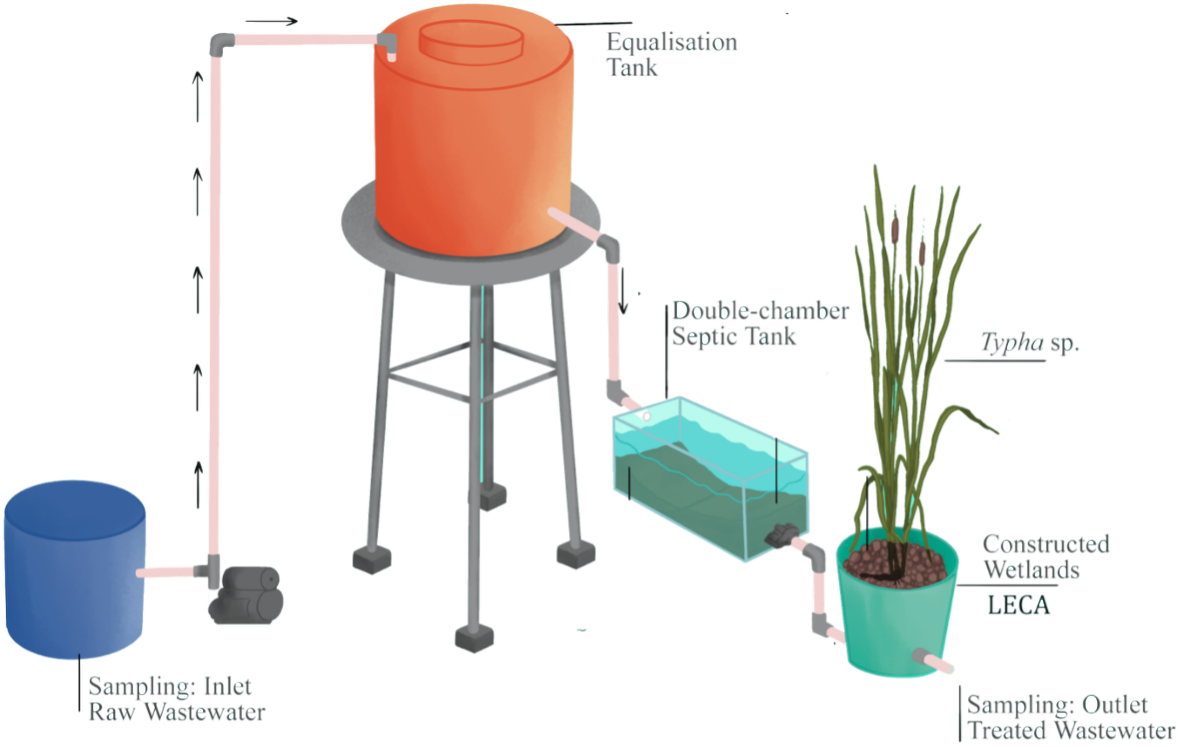

Fig. 1 *A wastewater treatment system set up in this research involved an equalisation tank, double-chamber septic tank, and constructed wetlands module*

2.3 Antibiotic Resistance Assessment

Determination of antibiotic resistance included the Clinical and Laboratory Standards Institute methods, where Penicillin-G was used in antibiotic tests. As much as 2 g pure penicillin-G, equivalent to 2,000,000 units, was diluted in sterile deionised water to produce a concentration gradient from 0 to 4,000 µg/ml. Disks were prepared with Whatman No. 44 paper with a diameter of 5.00 mm and then immersed in the antibiotic solutions. Purified colonies were transferred to Mueller-Hinton broth and incubated for 22±2h at 35±0.5°C until a turbidity equivalent of McFarland 0.5 standard was shown. The culture was smudged on Mueller-Hinton agar using a sterile swab. Disks containing antibiotics were then set on agar plates and incubated upside down for 22±2h at 37±0.5°C [22]. The resistance of each isolated culture was determined by measuring the inhibition zone using a calliper and compared to the antibiotic sensitivity standard [23].

2.4 Data Analysis

Removal percentage was estimated based on the viable coliform in CFU/ml of wastewater as formulated in Eq. (1). On the other hand, resistance magnitude was estimated from the inhibition zone diameter as formulated in Eq. (2).

$$\text{Removel }(\%) = 1 - \left[\left(\frac{CFU}{ml} \right) \text{in outlet} \Big/ \left(\frac{CFU}{ml} \right) \text{in inlet} \right] \tag{1}$$

$$\text{Resistance} = \frac{\varnothing \text{ inhibition zone in outlet}}{\varnothing \text{ inhibition zone in inlet}} \times 100\% \tag{2}$$

The number of viable coliforms between the inlet and outlet was compared and analysed for reduction and difference using the t-test. The same analyses were performed to compare inhibition zones between coliform isolates from both the inlet and the outlet. Data analysis was done using Microsoft Excel 2045 and IBM SPSS Statistics 27.

3. Results

3.1 Coliform Removal

As shown in Fig. 2, all domestic wastewater parameters in the CWs outlet were decreased compared to the raw wastewater. The percentage reduction of each parameter was 85.00%, 58.25%, 24.17%, and 80.00% for NH_3, COD, BOD, and TSS, respectively. All parameters in treated wastewater met the legal limit of domestic sewage. This condition shows that the system could reduce the pollutants contained in the wastewater while the remediation process was working. The result indicated the effectiveness of the CWs in improving water quality and was expected to be positively correlated with ARB and ARGs removal.

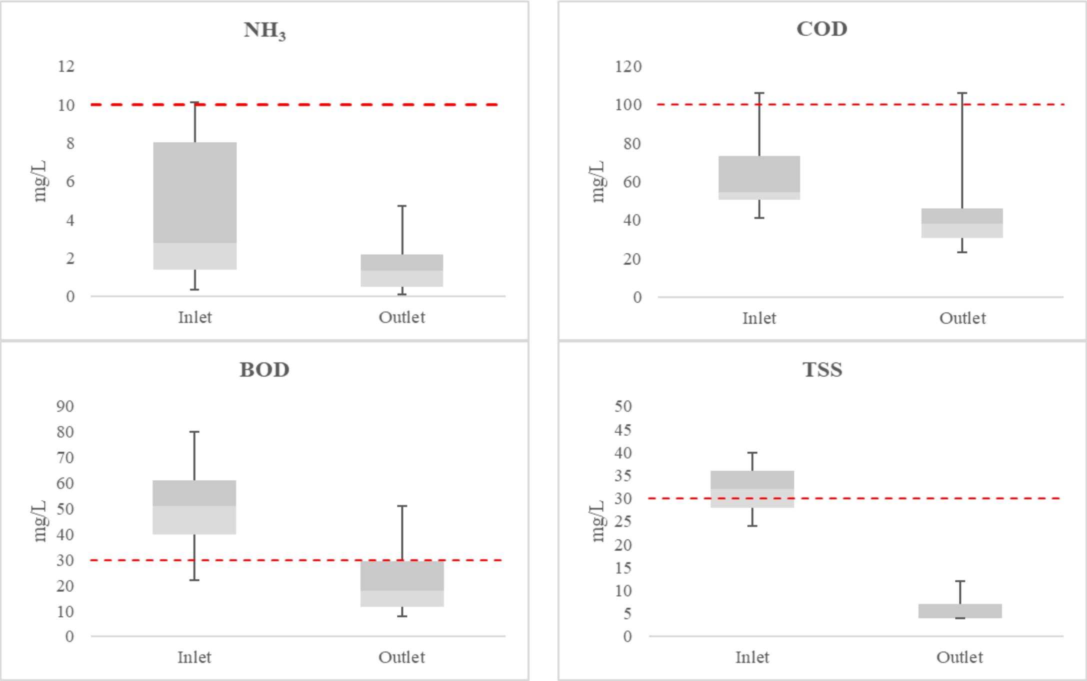

Fig. 2 *Wastewater quality parameters change in the inlet and outlet of the CWs system. The red line denotes the legal limit of the Ministry of Environment Regulation No. P.68/2016*

A reduction was also observed in the faecal coliform parameter. As shown in Fig. 3, the number of viable colonies of faecal coliform decreased with a percentage removal of 69.53%, dropping from an average of 1.20E+03 CFU/ml to 3.7E+02 CFU/ml. This reduction corresponds to a 0.516 log reduction. Statistical analysis at α = 0.05 showed that the faecal coliform abundance in the treated wastewater was significantly lower than in the raw wastewater (*p-val* = 0.001). The observed reduction highlights the effectiveness of the treatment process in significantly decreasing faecal coliform levels. This is crucial for public health, as faecal coliform bacteria are indicators of faecal contamination and can pose serious health risks if not adequately removed from wastewater.

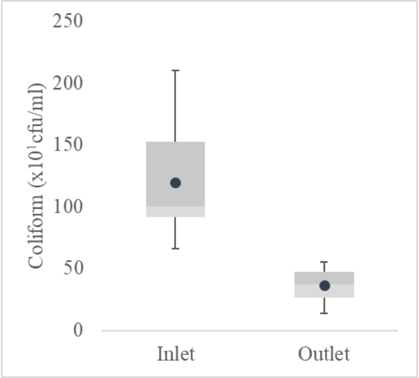

Fig. 3 *Faecal coliform abundance in the inlet (raw wastewater) and outlet (treated wastewater) of the CWs system*

3.2 Resistance Magnitude

Two isolates of faecal coliform were obtained from both raw and treated wastewater. The morphological features of the isolates were blue-green-coloured round colonies with a dark purple tint in the centre. Gram staining confirmed the isolates were faecal coliform as the bacteria appeared to be Gram-negative coccoid. Fig. 4 provides the visual representation of bacteria isolates.

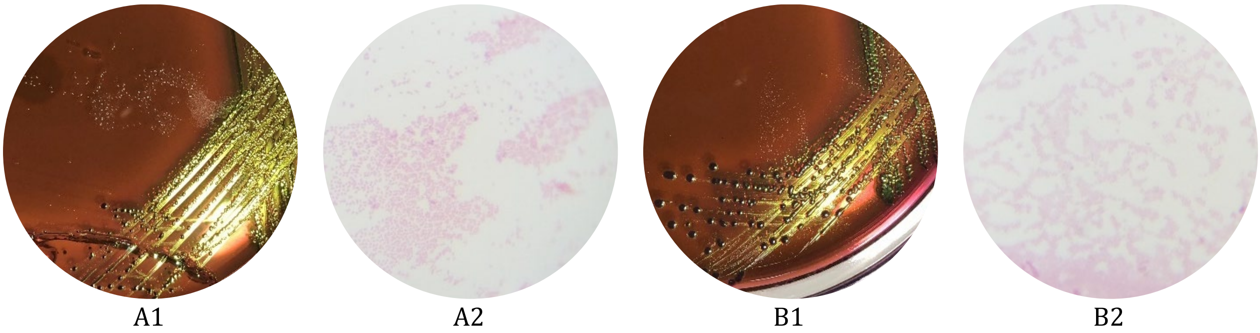

Fig. 4 *The macro- and microscopic visual representation of faecal coliform isolates from raw wastewater (A1-A2) and treated wastewater (B1-B2)*

Isolates were tested against Penicillin-G solution with concentrations ranging between 50 to 4,000 μg/ml or 0.4 to 33 μg Penicillin-G. The inhibition zone was not identified until concentrations reached 400 μg/ml and started to appear at 800 μg/ml. The condition applied to both isolates from the inlet and outlet of the system. This indicates that the MIC reached 800 μg/ml or 6.6 μg of Penicillin. The standard MIC breakpoint for 1 unit (1 μg) of Penicillin-G was >0.25 mg/l and <0.12 mg/l for resistant and sensitive isolates, respectively. Therefore, both isolates from the inlet and outlet of the CW system were considered resistant to Penicillin-G. The details of MIC and inhibition zone diameter as shown in Table 2.

Table 2 *MIC and inhibition zone diameter of inlet and outlet isolates against Penicillin-G*

Penicillin-G		Inhibition Zone Ø (mm)	
Conc. (μg/ml)	Unit (μg)	Inlet Isolate	Outlet Isolate
50	0.4	0	0
100	0.8	0	0
200	1.6	0	0
400	3.3	0	0
800	6.6	2.6±1.3	1.9±0.8
1,000	8.2	2.2±0.8	1.7±0.7
2,000	16.5	4.0±0.8	2.5±0.8
4,000	33.0	7.8±1.8	2.7±0.9

Table 2 shows that even though both inlet and outlet isolates had the MIC of 800 μg/ml, the inhibition zone diameters were smaller in the outlet isolate. This indicates that the outlet isolate had a higher resistance feature to Penicillin-G, or in other words, the antibiotic resistance was probably promoted after the treatment of the CWs system. The inhibition zone diameter graph, as seen in Fig. 5, confirms that Penicillin-G alters the growth of faecal coliform isolate, which came from the inlet, less intensely. Nonetheless, statistical analysis at $\alpha = 0.05$ did not conclude that the inhibition zone diameter of the inlet isolate was significantly larger than the outlet isolate (*p-val* = 0.090). Overall, the resistance in treated wastewater was calculated as 1.89 times higher than the resistance in raw wastewater.

4. Discussion

CWs are known for efficiently removing pollutants such as organic matter, bacteria, and antibiotic residues [15]. The efficiency of pollutant removal in our experiment, which ranged from 24.17% for BOD to 85% for NH_3, is yet to be considered a satisfactory result. Even though the treated wastewater met the legal limit, the reduction did not attain a 1 log reduction. Using LECA might also contribute to the result obtained since the best substrate to remove pollutants in CWs was suggested as zeolite [24]. The system used in this study, HSFCWs, was less efficient in reducing organic matter than the vertical subsurface flow constructed wetlands (VSFCWs) [15]. Nonetheless, both HSFCWs and VSFCWs can remove bacteria with satisfactory results. In line with the topic of this research, one of the advantages of HSFCWs is the better performance in removing ARGs. One study discovered that the removal of ARGs in HSFCW is 50% higher than in VSFCW [25]. In addition, continuous aeration, as in the system employed in this research, enhances the degradation of chemical pollution [26].

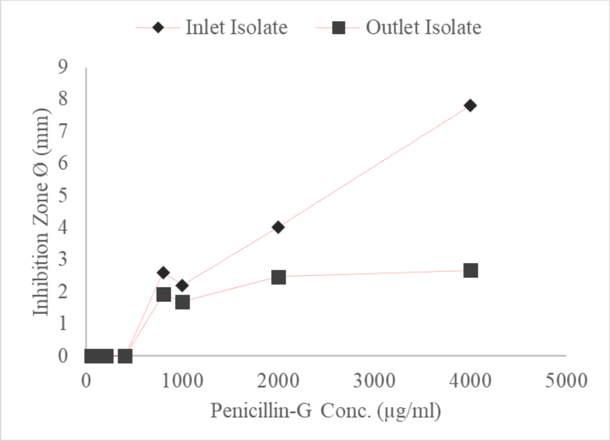

Fig. 5 *Comparison of inhibition zone diameter of inlet and outlet isolates on different antibiotic concentrations*

Even though bacteria removal occurred in the HSFCWs system during the experiment at 0.456 log reduction, making the treated wastewater meet the legal regulation, the antibiotic resistance state seems to have improved after the treatment. According to general opinion, the decrease of organic matter and NH_3 in the treated wastewater should have a positive correlation with ARG removal [17]. Further, the reduction of ARGs should be associated with the number of viable bacteria, including those that possess the antibiotic resistance feature [16]. This condition suggests that there was a mechanism altering the ARB and ARGs during the treatment of wastewater in the CWs. The increase in antibiotic resistance was also observed in several previous studies. For example, treatment of hospital wastewater indicated a 1.55 log reduction or 97.2% removal of E. coli, but increased antibiotic resistance from 3.8 to 20%, 53.8 to 60%, and 56.3 to 80% for meropenem, ciprofloxacin, and cefixime, respectively [27].

WWTP is considered a high-stress environment that shapes the surviving communities of bacteria due to the presence of antibiotic residues [28]. The bacteria with high tolerance and defensive response are likely to survive the conditions, which means the bacteria population possessing ARGs has the advantage. This can promote horizontal gene transfer by encoding the resistance feature between bacteria and increasing the resistance after treatment [19], [25]. The microbial dynamics are another reason for the increase in antibiotic resistance after treatment. Following selective pressures in the system, bacteria with a high prevalence of resistance to penicillin-G increase in population, making the ARGs more persistent in WWTP's microbiome [18], [29]. In addition, continuous aeration in our system, while reducing the pollutants, might lead to oxidative stress and induce antibiotic resistance mechanisms [30].

However, the efficiency of the treatment process itself significantly influences the microbial dynamics and antibiotic resistance prevalence [31]. This is supported by a study where multiple CWs were integrated into a more robust WWTP and successfully reduced more than 99% of ARGs in the treated effluent [32]. In contrast, the efficiency of the HSFCW in this research was less than that reported in previous studies. This lower efficiency may contribute to promoting antibiotic resistance rather than reducing it. Therefore, inefficient treatment systems pose a potential risk by increasing the abundance of ARB and ARGs. It is crucial to design and implement efficient treatment systems to ensure that the treatment process addresses the problem of antibiotic resistance effectively rather than exacerbating it. Practical design considerations include optimising the hydraulic loading rate, selecting appropriate plant species, and incorporating multiple stages of treatment to enhance overall efficiency. By ensuring high efficiency in the treatment process, we can mitigate the risks associated with the spread of antibiotic resistance in treated effluent, thereby protecting public health and the environment.

5. Conclusion

This study assessed the effectiveness of a horizontal subsurface flow constructed wetland (HSFCW) system using *Typha* sp. and Lightweight Expanded Clay Aggregate (LECA) in treating domestic wastewater and altering antibiotic resistance. The system achieved significant reductions in NH_3 (85.00%), COD (58.25%), BOD (24.17%), and TSS (80.00%), with all treated parameters meeting legal limits. Faecal coliform levels also decreased by 69.53%, corresponding to a 0.516 log reduction.

Despite these improvements, the study found increased antibiotic resistance in treated wastewater. Penicillin-G's minimum inhibitory concentration (MIC) for faecal coliform isolates was 800 µg/ml for both inlet and outlet samples, indicating resistance. Treated wastewater showed a 1.89 times higher resistance than raw wastewater, suggesting the treatment process may have promoted antibiotic resistance. These findings highlight the need for more efficient treatment systems to remove pollutants and mitigate antibiotic resistance effectively. Optimising hydraulic loading rates, selecting appropriate plant species, and incorporating multiple treatment stages can enhance system efficiency and better manage antibiotic resistance, thereby protecting public health and the environment.

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Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of the paper.

Author Contribution

The authors confirm contribution to the paper as follows: ***Study conception and design:*** *Ridwan Muhamad Rifai, Anie Yulistyorini;* ***Data collection:*** *Daffa Rizalif Lahardo, Bakhrul Mukhid Fadilah Ramadani;* ***Analysis and interpretation of results:*** *Ridwan Muhamad Rifai, Daffa Rizalif Lahardo, Jenvia Rista Pratiwi;* ***Draft manuscript preparation:*** *Ridwan Muhamad Rifai, Bakhrul Mukhid Fadilah Ramadani. All authors reviewed the results and approved the final version of the manuscript.*

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