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Fates of Intracellular and Extracellular Antibiotic Resistance Genes in Full Scale Waste Stabilization Pond Systems

Immanuela Karina*, Iftita Rahmatika, Cindy Rianti Priadi
Universitas Indonesia, Indonesia
e-mail: Immanuela.karina@ui.ac.id

KEYWORDS:
antibiotic resistance genes; intracellular and extracellular; wastewater; full-scale waste stabilization; horizontal gene transfer

ABSTRACT
Waste stabilization ponds (WSP) are conventional wastewater treatment technologies that utilize biological processes and have become key hotspots for the proliferation of antibiotic resistance genes (ARGs). During the wastewater treatment process, bacterial inactivation may release ARGs into the environment, with two primary phases of presence: intracellular (*iARGs*) and extracellular (*eARGs*). The objective of this study was to assess the abundance and removal efficiency of ARGs during the WSP process, and to explore the correlation between ARGs, total bacterial populations, and *integron 1* (*intl1*) as a marker for horizontal gene transfer. The results showed that raw wastewater had *iARGs* abundance ranging from 4.69×10^5 to 5.99×10^6 gene copies/ml, and *eARGs* from 5.91×10^3 to 1.04×10^5 gene copies/ml. After treatment through the WSP, intracellular ARGs, *intl1*, and 16S rRNA genes were reduced by 0.20 to 1.73 logs, while extracellular ARGs were removed by 0.05 to 0.79 logs. Interestingly, *intl1* and 16S rRNA genes showed amplification at the outlet of the wastewater treatment plant by -0.21 and -0.87 logs, respectively. The study revealed that the relative abundance of ARGs was higher in the extracellular phase, with *iARGs* and *eARGs* showing values from 7.75×10^-5 to 4.52×10^-2 and 1.33×10^-4 to 1.58×10^-1, respectively. A significant positive correlation was observed between ARGs and the total bacterial population, as well as with *intl1*, indicating the potential for co-occurrence and horizontal gene transfer in the microbial community within wastewater. This research highlights the environmental implications of ARGs persistence and proliferation in wastewater treatment processes, offering insights into the management of ARGs in wastewater treatment systems.

INTRODUCTION

Antimicrobial resistance (AMR) is a condition where microorganisms, namely bacteria, viruses, protozoa and fungi no longer respond to the effects of antibiotics which are ideally active against infections of these microorganisms. (Mancuso, Midiri, Gerace, & Biondo, 2021). In 2019, WHO designated AMR as one of the 10 global public health threats.(WHO, 2023). AMR makes infections more difficult to treat, increasing the risk of spreading infectious diseases and death. The AMR phenomenon in bacteria is estimated to contribute to 4.95 million global deaths, of which 1.27 million are directly caused by AMR.(Antimicrobial Resistance Collaborators, 2022). In addition, AMR causes economic losses in developed and developing countries due to increased costs of disease care and duration of hospital stays. Globally, disease care costs will increase by $300 billion to $1 trillion per year by 2050 due to AMR.(Ahmad & Khan, 2019).OECD member countries are expected to experience cumulative losses of $20-35 trillion by 2050 (Pulingam, et al., 2022).

Resistance ability is facilitated by various antibiotic resistance genes (ARGs) which are formed due to excessive, inappropriate use of antibiotics, and not following usage guidelines for animals and humans.(Prestinaci, Pezzotti, & Pantosti, 2015). Due to continuous exposure to antibiotics, bacteria are able to adapt to environmental stressors. Bacterial genes mutate and form new traits that encode resistance to antibiotics. Antibiotic resistance traits can also be transferred between bacteria that are not related by parent-offspring through a process called horizontal gene transfer.(Cecchini, Langer, & Slawomirski, 2015).The problem of ARGs

proliferation faces a more significant challenge because ARGs are divided into 2 types, namely intracellular and extracellular ARGs which have different fate and transport mechanisms. After the bacterial host is inactivated and lysed due to disinfection, intracellular ARGs can transform into extracellular ARGs (eARGs). Extracellular ARGs can associate and interact with solids, organic molecules, and biofilms which will increase their persistence.(Lin, et al., 2024). However, studies on the distribution of ARGs in the environment have focused more on the iARGs phase and ignored eARGs.(Zhang, et al.).

Wastewater and wastewater treatment plants (WWTP) act as reservoirs and main sources of AMR entry into other environmental matrices.(Kunhikannan, et al., 2021).Wastewater treatment generally utilizes biological processes, where biological reactors contain an abundance of microorganisms and high nutrients so that they can function as incubators for ARGs and antibiotic resistant bacteria (ARB).(Yu, Li, Zhang, & He, 2023). In addition, IPAL contains stressors such as antibiotic residues, pharmaceuticals, biocides and heavy metals that support the occurrence of horizontal gene transfer processes which will expand the prevalence of antibiotic resistance genes.(Karkman, Do, Walsh, & Virta, Antibiotic-Resistance Genes in Waste Water, 2018). ARGs and genes encoding resistance to other stressors are usually present together on mobile genetic elements or have similar regulatory mechanisms.(Zhao, Yu, & Zhang, 2023).

Conventional wastewater treatment plants are designed to remove traditional pollutants such as suspended solids, nutrients, and coliforms, but do not focus on removing antibiotics and antibiotic resistance genes, which are emerging contaminants.(Qin, et al., 2020). Moreover, there is no regulation that controls the dissemination of ARGs in the environment. Several studies have found that ARGs are still detected in wastewater treatment plant effluent after going through physical, chemical, and biological treatment stages.(Drane, Sheehan, Whelan, Ariel, & Kinobe, 2024). IPAL is able to inactivate and destroy bacterial cell components, but extracellular DNA carrying eARGs can persist in the aquatic environment for a long period of time.(Sivalingam, Poté, & Prabakar, 2020).

Appropriate wastewater treatment is the key to controlling the distribution of ARGs in the environment and human population. Ineffective wastewater treatment will expand the dissemination of ARGs to other environmental matrices, such as surface water, groundwater, and soil through effluent discharge. Therefore, this study aims to (1) Analyze the prevalence of intracellular and extracellular ARGs in conventional wastewater treatment plants; (2) Evaluate the performance of conventional wastewater treatment plants in removing intracellular and extracellular ARGs; and (3) Analyze the correlations between ARGs, integrons, and total bacterial population.

METHOD

The IPAL processing flow and sampling points can be seen in

Figure 2. **Comparison of Absolute Abundance of iARGs and eARGs at Each Sampling Point in WWTP: a)**. Wastewater sampling was conducted at a centralized IPAL X in West Java Province, Indonesia, which processes domestic waste. IPAL X has a maximum capacity of 80,000 m3/day with a utilized capacity of 40%. IPAL uses conventional processing technology with the main process being a biological process. From the collection channel, the

wastewater processing flow in IPAL X is divided into two series of waste stabilization ponds called line A and line B. Each series has 3 anaerobic pond units, 2 facultative pond units, and 2 maturation pond units arranged in series. Before entering the biological pond, there is a physical pre-treatment process in the form of waste filtering by manual bar screen and mechanical fine screen, and sand sedimentation by grit chamber. The effluent of IPAL X will be discharged into the Citarum River.

Sampling was carried out using the grab sampling method, namely on January 16, 2025, using sterile glass bottles. Sampling was carried out at 4 points, namely the inlet or after passing through the bar screen (INF), the 3rd anaerobic pond outlet (AN.3), the 2nd facultative pond outlet (F.2), and the point before discharge represented by the 2nd maturation pond outlet (M.2). Samples from Line A and Line B were composited and homogenized by shaking vertically. The samples were put into an icebox and the samples were preserved by cooling at 4°C for transport to the laboratory.

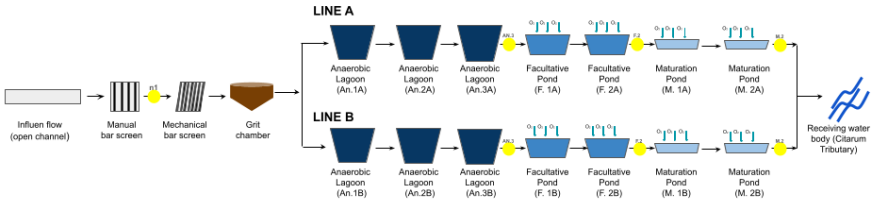


Figure 1. Treatment processes of the Full-scale Domestic WWTP. The solid-lined black arrows show the wastewater flows; and the yellow dots indicate the sampling points

DNA Extraction

Wastewater samples were filtered with a sterile 0.22-µm diameter mixed cellulose esters (MCE) membrane (Millipore, USA) using a vacuum pump. The sample volume used was 100 ml for the inlet sample (INF) and 150 ml for the samples from anaerobic (AN.2), facultative (F.2), and maturation (M.2) ponds. At this stage, intracellular DNA and extracellular DNA were separated. Intracellular DNA is a DNA molecule measuring > 0.22-µm and is retained on the membrane filter. Meanwhile, extracellular DNA is a DNA molecule measuring <0.22-µm so that it passes through the 0.22-µm filter and is contained in the sample filtrate. The complete stages of separation and extraction of iDNA and eDNA can be seen in the **Kesalahan! Sumber referensi tidak ditemukan.**The iDNA concentrate on the surface of the MCE membrane was extracted using the DNEasy Powerwater kit (QIAGEN, Netherlands) according to the manual instructions.

Meanwhile, extracellular DNA was extracted using the isopropanol precipitation method following the guidelines developed by Kirtane & Deiner (2024) and Zhao et al. (2020) with some modifications (Kirtane & Deiner, 2024);(Zhao, et al., 2020). The first stage in the isopropanol precipitation method is the addition of salt. The addition of salt will encourage precipitation, because salt increases the ionic strength of the solution and reduces the electrostatic repulsion force between DNA molecules so that nucleic acids will aggregate (Kirtane & Deiner, 2024). The type of cation used in this study is 3 M sodium acetate (Merck, Germany). The volume of sodium acetate added is 0.1 of the sample volume. Since the sample volume used is 100 – 150 ml, the volume of sodium acetate used is 10 – 15 ml. After that, a precipitant in the form of pure isopropanol (Merck, Germany) is added. Based on Li et al. (2020), the recommended isopropanol/sample ratio is 0.5; 0.75; or 1(Li, et al., 2020). In this

study, the author added 100-150 ml of isopropanol to achieve an isopropanol/sample volume ratio of 1. The sample was then incubated in a freezer for 24 hours at a temperature of -20℃. The third stage of the alcohol precipitation method is centrifugation to form nucleic acid pellets. The sample was inserted into a centrifuge tube and centrifugation machine (.Gyrozen 1248R) at a relative centrifugal force (RCF) of 3.134 x g for 30 min. Finally, the supernatant was discarded using a micropipette. The pellet was cleaned with 70% ethanol (Merck, Germany) twice to remove impurities, then dried naturally. The pellet was eluted with 200 µl of 10x TE buffer solution (Servicebio, Hubei, China) for further quantification.

Real Time PCR for ARGs Quantification

There are 2 types of ARGs analyzed, namely tetA and sul1, 1 type of integrase genes intl1, and housekeeping gene 16S-rRNA. Real-time PCR was performed with Type 7500 Fast (Applied Biosystem, USA) using qPCR kit type THUNDERBIRDTM Next SYBR® qPCR Mix (Toyobo, Japan). The amplification reaction includes initial denaturation of DNA at a temperature of 95° C for 5 minutes, followed by 45 cycles of 95° C for 10 seconds, annealing at 55° C for 20 seconds, and extension at 72° C for 20 seconds. Melting curve analysis was performed by gradually increasing the temperature from 65° C to 95° C to check for non-specific amplification (Kasuga, Nagasawa, Suzuki, Kurisu, & Furumai, 2022). Primers used in analysis qPCRcan be seen on **Kesalahan! Sumber referensi tidak ditemukan.. Kesalahan! Sumber referensi tidak ditemukan.**

Number of gene copies in the detected sample volume is obtained using the standard curve method. In the standard curve method, the standard solution of plasmid extract containing the five target sequences is diluted with a dilution series of 10^3 - 10^8 copies/µl. Then, CT-value analysis of the series of standard solutions was performed. The standard curve with $R^2 \geq 0.994$ was acceptable. The results of the standard curve creation can be seen in the Kesalahan! Sumber referensi tidak ditemukan. Gene quantity was analyzed in absolute abundance (gene copies/ml) and relative abundance of ARGs and integration genes to the 16S-rRNA gene (gene copies/16S-rRNA copies).

Log-removal of ARGs, MGEs, and 16S-rRNA

Absolute abundance and describes the total concentration of target genes in wastewater samples. Absolute abundance is obtained from the results of reading the CT value of the qPCR system which is inputted into the standard curve equation. Meanwhile, relative abundance is defined as the absolute abundance of ARGs and intl1 normalized by the absolute abundance of the 16S-rRNA gene. The 16S-rRNA gene represents the bacterial biomass in wastewater that has the potential to be a host for ARGs(Li, Qiu, Li, Liang, & Huang, 2019). Relative abundance is calculated using equation (1):

$$Relative\ abundance = {C_j^i}\Big/{C_j^{16s}} \quad (1)$$

i = Target genes type
j = Sampling point
C_j^i = Concentration of genes type i at sampling point j (gene copies/ml)

C_j^{16S}= Concentration of 16S-rRNA gene at sampling point j (gene copies/ml)

The fluctuation and removal rate of ARGs, MGE, and 16S-rRNA by the wastewater treatment system can be calculated using equation (2):

$$Log\ removal = log\left(C_0/C_f\right) \quad (2)$$

Log removal = Removal of target genes by the WWTP treatment unit, relative to the inlet
C_0= Absolute abundance of target genes in WWTP (gene copies/ml)
C_f= Absolute abundance of target genes after each stage of treatment (gene copies/ml)

Statistical Analysis

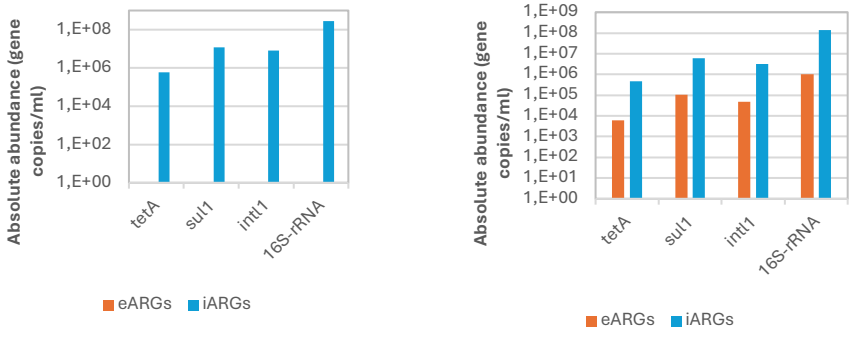
Statistical analysis was performed with Jamovi 2.6.26 and Microsoft Excel. One-way ANOVA and Spearman's correlation test were performed with p-value <0.05 indicating a significant correlation. Two-tailed independent t-test analysis was used to assess the significance of differences in relative abundance of ARGs at each sampling point and removal of ARGs relative to the inlet. The calculation of Spearman's correlation coefficient aims to analyze the effect of MGE intl1 and bacterial abundance (represented by 16S-rRNA gene) on ARG abundance. Interpretation of Spearman's correlation coefficient refers to (Schober, Boer, & Schwarte, 2018). The |r value| between 0 - 0.1 indicates a negligible correlation, |r value| between 0.1 and 0.39 indicates a weak correlation, |r value | between 0.4 and 0.69 indicates a moderate correlation, |r value | between 0.7 and 0.89 indicates a strong correlation; and |r value | between 0.9 and 1.0 indicates a very strong correlation.

RESULTS AND DISCUSSION

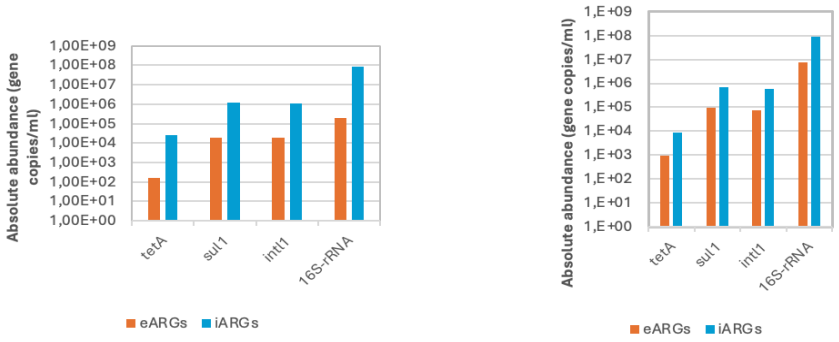
Absolute Abundance

The four target genes (tetA, sul1, intl1, and 16S-rRNA) were detected in the intracellular and extracellular phases at the influent (INF), facultative pond (F.2) and maturation pond 2 (M.2). However, in the anaerobic lagoon (AN.3), the four target genes were only detected in the intracellular phase. Except in the anaerobic lagoon, the absolute abundance of iARGs was 1-2 orders of magnitude higher than that of eARGs (

Figure 2. **Comparison of Absolute Abundance of iARGs and eARGs at Each Sampling Point in WWTP: a))**



(a) (b)



(c) (d)

Figure 2. Comparison of Absolute Abundance of iARGs and eARGs at Each Sampling Point in WWTP: a)Influent; b) Anaerobic Pond; (c) Facultative Pond; (d) Outlet/Maturation Pond

The abundance of iARGs tetA and sul1 were in the range of 8.64×10^3 – 1.21×10^7 gene copies/ml. Whereas the abundance of intl1 and 16S-rRNA in the intracellular phase were in the range 6.04×10^5 – $8.15\times 10^6$and 8.78×10^7 – 2.72×10^8 gene copies/ml, respectively **(Figure 3a)**. The waste stabilization pond (WSP) was able to remove the four intracellular genes significantly ($p < 0.05$) with a removal rate of 0.20 - 1.73 log ($p < 0.05$). However, there was significant amplification in the anaerobic pond (AN.3). TetA, sul1, and 16S-rRNA genes experienced a significant increase ($p < 0.05$) of -0.10 log, -0.30 log, and -0.28 log relative to the inlet, respectively. Meanwhile, intl1 also experienced an insignificant increase ($p > 0.05$) of -0.40 log. Removal of iARGs in each treatment step of WWTP X can be seen in **Figure 3c.**

The removal eficiency of anaerobic – facultative – maturation system at WWTP X was lower than conventional WWTP in a research by Yuan et al. (2009) which utilized the biological reactor Cyclic Activated Sludge Technology (CAST) and Modified Sequential Batch Reactor (MSBR) (Yuan, et al., 2019). Based on the study, the biological reactor system – secondary clarifier was able to remove intracellular sul1 and tetA by 2.6 logs and 2.5 logs, respectively. The removal rate of iARGs in the WSP system of WWTP X was also lower than the removal rate of the anaerobic – anoxic – oxic (AAO) reactor in the study by Zhang et al. (2018), where the sul1 gene was removed by 1.98 – 2.24 logs and tetC (comparable to tetA genes in this study) by 2.27 – 2.92 logs) (Zhang, et al., 2018). Meanwhile, Huang et al. (2019) found that the AAO-MBR biological system of the intracellular gene sul1 was 1.54 - 5.95 logs and intl1 was 1.56 - 5.97 logs, which is much higher than the waste stabilization pond WWTP X (Li, Qiu, Li, Liang, & Huang, 2019). The intracellular tetA, sul1, and intl1 genes were also still detected in M.2, which is the outlet of the WWTP X with the concentration of 8.64×10^3 – 7.09×10^5 gene copies/ml. This value was smaller than the research by (Li, Zhang, Liu, Wen, & Wang, 2025) who found that iARGs tetA and sul1 is still persisted in WWTP effluent even after passing through the tertiary treatment stages of Ultrafiltration, UV, and chlorination, namely 0.64×10^5 and 1.3×10^5gene copies/ml.

The absolute abundance of eARGs tetA and sul1 were in the range of ND – 1.04×10^5 gene copies/ml. At the influent sampling point, the absolute abundance of tetA, sul1, intl1, and 16S-rRNA were 5.71×10^3, 1.04×10^5, 4.79×10^4, and 1.03×10^6gene copies/ml, respectively **(Figure 3b)**. In the anaerobic pond (AN.3), the four target genes were completely

eliminated to below detection limit. The concentration four target genes in facultative pond (F.2) is higher that the anaerobic lagoon (AN.3), but still lower that the influent (INF) by 0.39 – 1.55 log. In the maturation pond (M.2), eARGs tetA and sul1 were eliminated by 0.80 log and 0.05 log, respectively ($p > 0.05$). Meanwhile, intl1 was amplified by -0.21 log ($p > 0.05$), and 16S-rRNA was significantly amplified ($p < 0.05$) by -0.87 log. The absolute abundance of extracellular tetA and sul1 genes in M.2 was 5.67 and 4.80 fold higher than in F.2. Removal of eARGs in each treatment step of WWTP X can be seen in **Figure 3d.**

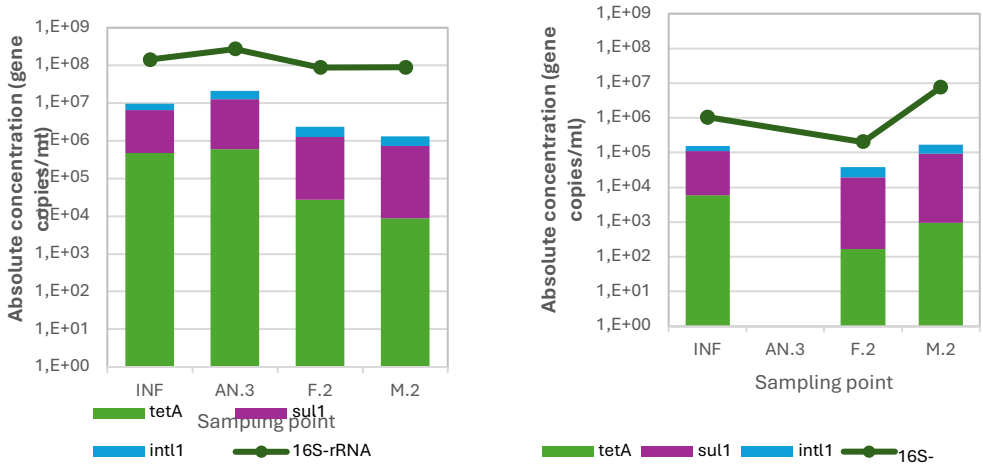
In the second maturation pond 2 (M.2) which is the outlet of WWTP X, the absolute abundance of 16S-rRNA gene was 7.64×10^6 gene copies/ml. Extracellular tetA, sul1, and intl1 genes were also still detected in maturation pond 2, with the concentration of 9.49×10^2- 9.32×10^4 gene copies/ml. This value is lower than the research by Wang et al. (2020) who found that free eARGs were detected at 10^7- 0.39×10^8 gene copies/ml in the effluent of five WWTPs using membrane bioreactor (MBR) technology, including the intl1 gene and several tetracycline resistance genes (Wang, et al., 2020). Amplification of extracellular ARGs after biological treatment also occurred in the study by Yuan, et al. (2019). Based on the research, the biological reactor system – secondary clarifier is able to remove absorbed eARGs sul1 and tetA by 0.95 log and 0.9 log respectively (Yuan, et al., 2019). However, sul1 and tetA in the free eARGs phase are amplified by -0.3 log and -0.25 log. Research by Liang, et al. (2022) also found an amplification of extracellular intl1 after anoxic-oxic-anoxic-oxic (A^2O^2) system, which was -0.3 to -0.8 log (Liang, et al., 2022).

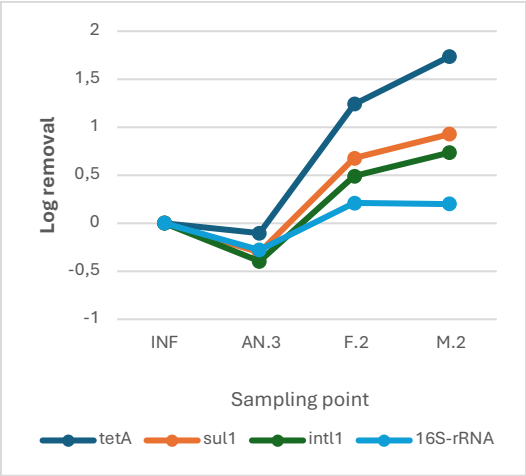
Maturation ponds ideally aim to inactivate coliforms and pathogens by utilizing light penetration (Verbyla, Sperling, & Maiga, 2017). Solar radiation will destroy the DNA molecules of bacteria, viruses, and other microorganisms through photolysis reactions (Wang, Han, Li, Liu, & Yan, 2022). However, in this study, the amplification of extracellular ARGs and intl1 in the maturation pond of WWTP X occurred because light penetration was only able to inactivate bacteria through cell wall degradation and enzymatic reactions, but was not yet able to degrade the bacterial DNA chain (ie molecular lesion (Ghosh, Chen, & Hu, 2022). As a result, intracellular DNA is released into the environment as eARGs after bacterial lysis (Drane, Sheehan, Whelan, Ariel, & Kinobe, 2024). This is reflected in the contrasting distribution pattern of eARGs and iARGs in the maturation pool. In the maturation pool, the removal rate of intracellular tetA, sul1, and intl1 genes was higher than other units, in contrast to eARGs which were amplified. In line with this phenomenon, Dunn & Silverman (2021) also found that the rate of degradation of intracellular DNA containing tetA and sul2 sequences was faster than extracellular DNA after 16 hours of experiment (Dunn & Silverman, 2021).

There are several other factors causing differences in the distribution and removal patterns of intracellular and extracellular ARGs in WWTPs. The first factor is the tendency of eARGs to be adsorbed by sediment, organic matter, or other larger molecules (Deng, et al., 2023) (Mardalisa, et al., 2025). This factor has the potential to occur in eARGs in anaerobic ponds that experience removal until below the detection limit. As a result, the adsorbed eARGs do not pass the 0.22 um membrane filtration and are retained on the membrane surface with iARGs. This possibility could occur because visually, anaerobic ponds have the highest turbidity compared to other sampling points. Similar findings also occurred in the study of Yuan et al. (2019) who found that activated sludge has a high suspended solids content so that eARGs are freely adsorbed on particles (Yuan, et al., 2019). Dissolved solids or dissolved organic matter (DOM) also affect the level of ARGs removal in wastewater. Dissolved organic matter (DOM) is able to absorb sunlight and produce highly reactive hydroxyl radical (HO) molecules

(Ballmer, McNeill, & Deiner, 2024). HO molecules will induce DNA chain damage, especially in extracellular DNA. High eARGs removal in anaerobic ponds can occur because hypothetically, the DOM content at this point is higher than in facultative ponds and maturation ponds.

The second factor is the difference in physical-chemical parameters in the inlet channel, anaerobic pond, facultative pond, and maturation pond, one of which is the availability of nutrients. Intracellular ARGs dominate in nutrient-rich environments (Zarei-Baygi & Smith, 2021). Intracellular ARGs increase in aquatic ecosystems that have high carbon and nitrogen content.(Liu, et al., 2024). Environments with high nutrient availability support the survival and replication capabilities of bacteria hosting ARGs (Liu, et al., 2024). Extracellular ARGs were not detected in the anaerobic pond because the four ARGs were more in the intracellular phase. This is evident from the results of iARGs concentration in the anaerobic pond is in contrast with eARGs. Extracellular ARGs were removed until below the detection limit, whereas iARGs in the anaerobic pond were amplified and had the highest concentration compared to other sampling points. However, further analysis is needed on nutrient concentrations at each sampling point in the WWTP X. Another physical-chemical factor that also affects the distribution pattern of ARGs is oxygen content. The activity of microorganisms in anaerobic conditions is lower, including cell secretions that also release DNA sequences containing ARGs into the environment (Barancheshme & Munir, 2018). Anaerobic conditions prevent the propagation of eARGs through horizontal gene transfer (Qin, et al., 2020).

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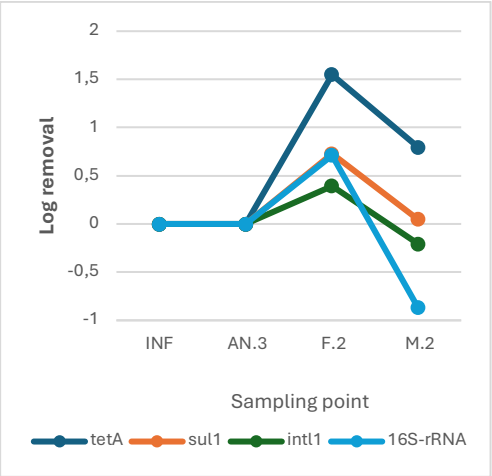
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Sampling point
tetA
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intl1
16S-rRNA

(d)

Figure 3.Composition of Antibiotic Resistance Genes in Each Sampling Point of WWTP: (a) iARGs and (b) eARGs in each stage of WWTP treatment. Removal of Antibiotic Resistance Genes in Each Treatment Step: (c) iARGs; (d) eARGs

Relative Abundance

The relative abundance of iARGs and intl1 at the inlet point was in the range of 3.04×10^{-3} - 4.25×10^{-2}. Meanwhile, the relative abundance of iARGs and intl1 in the WSP unit was 7.75×10^{-5} - 4.52×10^{-2}. The relative abundance of iARGs in this WWTP was lower than the average relative abundance of 18 WWTPs that utilize AAO and oxidation ditch technology, which is 1.1×10^{-3} – 1.00 for sul1 and 7.84 - 6.08 for intl1 (Zhang, et al., 2019). The relative abundance of iAGs in WSP is similar to the relative abundance of sul1, tetW, and intl1 genes in the effluent of wastewater treatment plants utilizing oxidation ditch technology and natural wetlands, which is 7.75×10^{-5} - 4.52×10^{-2} (Sabri, et al., 2020). The fluctuation of relative abundance of iARGs in WWTP was similar to the absolute abundance, except for the tetA gene. The relative abundance of intracellular tetA continued to decrease throughout the sampling points ($p<0.05$). The sul1 and intl1 genes increased in the anaerobic pond (AN.3) ($p>0.05$) and decreased in units F.2 and M.2 (**Figure 3**)

The relative abundance of eARGs at the inlet point was 1.09×10^{-2}– 1.58×10^{-1}, 3.57 – 3.71 fold higher than iARGs. This trend was different from the absolute abundance, where the absolute abundance of iARGs is higher than eARGs, similar to a study by (Xin, et al., 2023). The relative abundance of extracelullar sul1, tetA, and intl1 decreased along the INF-F.2 point ($p>0.05$) similar to the absolute abundance trend. The eARGs and intl1 genes are also decreased along the F.2 – M.2 point ($P>0.05$). This is different from the absolute abundance trend where the three extracellular genes in maturation pond 2 (M.2) increased compared to the facultative pond (F.2). The relative abundance of eARGs in the waste stabilization pond unit was 1.33×10^{-4}– 9.62×10^{-2}. Research by Zhou, et al. (2019) found the relative abundance of sul1 in the IPAL utilizing AAO-MBR technology of 7.6×10^{-4}-9.0×10^{-4} (Zhou, Zhu, Yan, Wang, & Wang, 2019), lower than the abundance of sul1 in the biological pond of the WWTP X. Fluctuations in the relative abundance of eARGs can be seen in **Figure 3**

Relative abundance is highly dependent on the microbial composition in wastewater. The 16S-rRNA gene represents the total abundance of bacteria in wastewater, which reflects the number of potential hosts and influences the frequency of horizontal transmission of ARGs (Wang, Han, Li, Liu, & Yan, 2022). Bacteria that commonly live in wastewater are coliform and enterococcus (Manoharan, Ishaque, & Ahn, 2022). Meanwhile, based on research Yang et al. (2020) the bacteria with the highest abundance in the Anoxic – aerobic (A/O) tank on a pig farm are proteobacteria which are also

potential hosts of ARGs (Yang, et al., 2020),. Zhang, et.al (2015) research in WWTP using AAO-MBR technology also found Proteobacteria as the most dominant bacterial group (69.1%) at the WWTP influent, while the dominant bacterial group at the WWTP efluent was Firmicutes (Zhang, et al., 2015). Wang, et.al (2022) found a significant positive correlation between ARGs and microbial communities, such as *Patescibacteria, Bacteroidota*, and *Actinobacteriota*, which shows that these bacterial phyla have the potential to carry ARGs (Wang, et al., 2022). The ability of bacterial hosts to survive depends on species selection based on ideal growth conditions (Mardalisa, et al., 2025), including the availability and optimum oxygen requirements of bacteria. Fluctuations in the relative abundance of anaerobic, facultative, and maturation ponds at WWTP X occurred because the three ponds had different physicochemical characteristics, including differences in oxygen content. Theoretically, the lowest dissolved oxygen content was in the anaerobic pond because it had the greatest depth (depth = 4 m), followed by the facultative pond (depth = 2 m), and the maturation pond had the highest dissolved oxygen content (depth = 1.5 m). This condition occurred in the study by Yang, et.al (2020), which found that denitrifying bacteria were more abundant in anoxic tanks which had lower DO content compared to aerobic tanks(Yang, et al., 2020). Fluctuations in the relative and absolute abundances of ARGs and intl1 were similar. This suggests that bacteria and ARGs are eliminated at comparable rates (McConnell, et al., 2018).

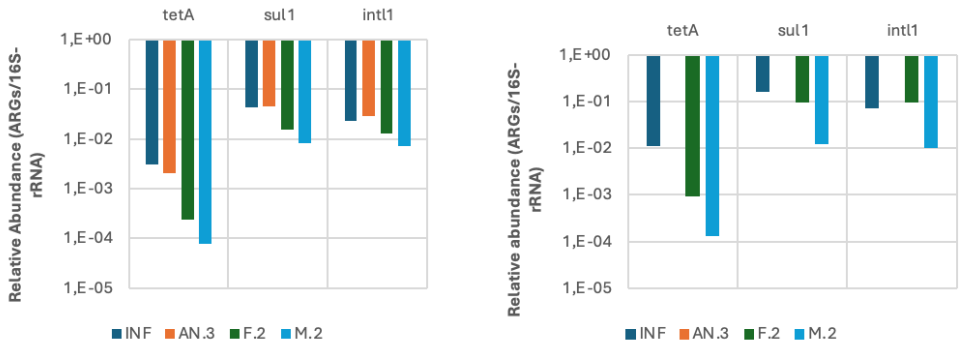

Figure 4. Fluctuations in the Relative Abundance of Antibiotic Resistance Genes in Domestic Wastewater Treatment Plants

(a) iARGs; (b) eARGs

Correlation of ARGs, intl1 and 16S-rRNA

Integrons and 16S-rRNA are two factors that influence the level of ARGs dissemination. Intl1 is an indicator of anthropogenic antibiotic consumption that describes the level of horizontal gene transfer in the environment.(Johnson, et al., 2016);(Conco, et al., 2022). Meanwhile, the

abundance of 16S-rRNA genes also illustrates the potential for HGT in wastewater, because the higher the density of the bacterial community, the greater the potential for HGT (Alexander, Bollmann, Seitz, & Schwartz, 2015). Based on the results of the Spearman test on **Table 1.** Correlation **between ARGs, intl1, and 16S-rRNA in intracellular phase Table 2. Correlation between ARGs, intl1, and 16S-rRNA in intracellular phase**absolute abundance (gene copies/ml) of tetA and sul1, both in the intracellular and extracellular phases, had a strong positive correlation with the 16S-rRNA gene $R > 0.7$) $p < 0.05$). This result is consistent with the findings of Du et al. (2015) who found that the removal of sul1 in the anaerobic - anoxic - aerobic (A/A/0) system and membrane bioreactor was strongly and significantly correlated with the removal of the 16S-rDNA gene. ($R = 0.908$ - 0.915, $p < 0.01$)ρ(Du, et al., 2015). This means that the removal of ARGs occurs due to the process of removing bacteria in the wastewater treatment plant (eg sedimentation of biomass in sludge). 16S-rRNA also has a strong and significant correlation with intl1 for intracellular intl1 ($R = 0.755$, $p < 0.05$) and extracellular intl1 ($R = 0.851$ $p < 0.05$) . The correlation between 16S-rRNA and intl1 indicates the potential for horizontal gene transfer in the bacterial community mediated by intl1 (Wen, et al., 2024).

Intracellular and extracellular ARGs also had a strong and significant correlation ($R> 0.7$, $p < 0.05$) with intl1. The correlation between tetA and intl1 was stronger in the intracellular phase, while the correlation between sul1 and intl1 was stronger in the extracellular phase. Xin et al. (2023) also found a strong positive correlation between intl1 and iARGs, including sul1 and several tetracycline resistance genes of tetO, tetX, tetW, tetM, tetL, and tetQ, but did not find a strong correlation between intl1 and eARGs (Xin, et al., 2023). Yang et al. (2020) research in conventional WWTP using anoxic-oxic (A/O) technology also found a very strong correlation between intl1 and sul1, as well as a moderate-strong correlation between intl1 and tetA.(Yang, et al., 2020). According to research at Zhengzhou City WWTP using anaerobic-anoxic-aerobic (A/A/O) technology, intl1 has a strong and significant correlation with tetA and sul1 genes, both in the intracellular and extracellular phases. However, the correlation between intl1 and ARGs is weaker in the extracellular phase, indicating that ARGs carried by intl1 can be degraded by nucleases after being released from bacterial cells.(Deng, et al., 2023). The strong correlation between ARGs sul1 and tetA (Spearman's > 0.7, $p < 0.05$) in both intracellular and extracellular phases indicates that both ARGs are co-located in the same bacterial host or MGE (Amarasekara, et al., 2023);(Zhao, et al., 2023).

Table 1. Correlation between ARGs, intl1, and 16S-rRNA in intracellular phase

	tetA	sul1	intl1	16S-rRNA
tetA	-			

sul1	0.732*	-		
intl1	0.811*	0.963**	-	
16S-rRNA	0.881**	0.760*	0.755*	-

* Indicates significant correlation (p-value < 0.05).
** Indicates remarkably significant correlation (p < 0.001).

Table 2. Correlation between ARGs, intl1, and 16S-rRNA in intracellular phase

	tetA	**sul1**	**intl1**	**16S-rRNA**
tetA	-			
sul1	0.766*	-		
intl1	0.709*	0.972**	-	
16S-rRNA	0.773*	0.809*	0.851**	-

* Indicates significant correlation (p-value < 0.05).
** Indicates remarkably significant correlation (p < 0.001).

CONCLUSION

This study found that Waste Stabilization Ponds (WSPs) are effective at reducing intracellular antibiotic resistance genes (ARGs) like *tetA*, *sul1*, and *intl1*, but extracellular ARGs can increase, especially in the maturation pond. A strong positive correlation between ARGs, *intl1*, and 16S-rRNA gene abundance suggests a high potential for horizontal gene transfer among bacteria in wastewater. While some ARGs are removed, the persistence of others in the treated effluent raises concerns about the long-term effectiveness of WSPs in controlling antibiotic resistance. The findings highlight the need for improved treatment methods that target both intracellular and extracellular ARGs, and suggest future research should compare different technologies, investigate microbial community roles, assess environmental factors influencing ARG dynamics, and evaluate the long-term environmental and public health impacts of residual ARGs in treated wastewater.

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