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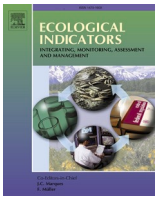

Differentiation of environment and wastewater treatment plants by core antibiotic resistance genes and *aadA2* as indicators in South Korea

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ABSTRACT

Antibiotic resistance is reportedly disseminated from wastewater treatment plants (WWTPs) into aquatic environments. In this study, it was hypothesized that discharge of effluent from WWTPs may influence resistome of the adjacent aquatic environment. Thus, we aimed at investigation of resistome in WWTPs and receiving waters, to observe the dynamics of resistome and to determine the indicators for consistent comparison of contamination level. In this study, twenty-seven samples of upstreams (n = 9), downstreams (n = 9), and effluents (n = 9) samples from nine WWTPs located in the Nakdong and Yeongsan Rivers, South Korea, were collected for four years, and a total of 343 antibiotic resistance genes (ARGs) were quantitatively amplified using SmartChip analysis. The five core ARGs (*aadA*, *aadA2*, *aadA5*, *strB*, and *sul1*) were commonly detected in all samples. Three (*aadA*, *aadA2*, *aadA5*) of the five core ARGs were significantly more abundant in the effluent than in the aquatic environment. Interestingly, the water sources of the aquatic environments and effluents could be differentiated based on the abundance of the five core ARGs. The decision-making tree model supported the classification of the two water sources: aquatic environments and effluents. Taken together, considering the ubiquitous presence of the five ARGs with significantly different abundance between water sources, core ARGs including *aadA2* are recommended as indicator genes to monitor ARGs contamination by employing quantitative analysis in South Korea. Determination of indicators would help consistently evaluate contamination level by ARGs.

1. Introduction

Wastewater treatment plants (WWTPs) have been widely investigated for their role in antibiotic resistance (AR) since they are known to be favourable environments for the proliferation of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Bouki et al., 2013). In influent samples of WWTPs, a mixture of contaminants, such as antibiotics, heavy metals, and other chemicals, are in circulation, promoting the evolution of ARB into multidrug-resistant (MDR) bacteria (Rizzo et al., 2013). WWTPs receive sewage containing feces or urine of patients (Rafraf et al., 2016; Zhang et al., 2020). They are considered as one of the most important sources of AR (Shin et al., 2021), which can reach the receiving water environment (Czekalski et al., 2014). WWTPs are therefore regarded as playing a key role in interchanges of ARGs and ARB between human society and environmental settings (Shin et al., 2022), possibly causing AR to spread into the environment (Martinez, 2009). In WWTPs, enriched ARGs and ARB affect their abundance even if there is a reduction in AR after the treatment process (Buri et al., 2021). In South Korea, WWTPs and aquatic environments were examined for ARB and ARGs, and metagenomic analysis revealed alterations in the diversity and abundance of ARGs (Raza et al., 2021). The genes *sul1* and *APH(3")-lb* were significantly more abundant in effluents than influents (Raza et al., 2021). In another study it was demonstrated that the abundance of ARGs increased from upstream to downstream in the Han River, which is considered to result from anthropogenic activity (Lee et al., 2020).

Surveillance is critical in the evaluation of ARB and ARG contamination levels. However, comprehensive analysis is 1) time and cost-consuming, 2) laborious and 3) difficult to consistently compare due to the different established methodologies. Thus, consistent framework needs to be founded to reliably compare global datasets of AR contamination. The determinants of AR, such as classes of aminoglycosides, β-lactams, sulfonamides, and tetracyclines, have been proposed as possible indicators for evaluating ARG contamination levels (Berendonk et al., 2015). Rapid monitoring of such determinants is therefore an important tool to establish a direct strategy against AR contamination. Indicator ARGs have been quantitatively analysed in aquatic environments (Narciso-da-Rocha et al., 2014; Yu et al., 2018). Recently, the

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abundance of *sul1* and *intI1* genes, proposed as indicator genes, were evaluated in marine environments, and the authors suggested other indicator genes, such as *tetM* and bla_{CTX-M}, should be also investigated (Bourdonnais et al., 2022). However, potential indicator ARGs have not been proposed in South Korea. Determination and evaluation of indicator ARGs were needed because it is unclear that previously reported genes can act as indicators in South Korea. For assessment of potential indicators in South Korea. we conducted a comprehensive analysis of the resistome and core ARGs of the nine WWTPs and adjacent rivers located in the major cities of two provinces. It was expected that the abundance of indicator ARGs would correlate with contamination levels of the sources. Additionally, candidate indicators should be associated with mobile genetic elements to allow risk assessment in aquatic environments. Thus, assuming that the effluents are contaminated by anthropogenic activity, we hypothesized that the resistome in the effluents of the WWTPs would be significantly different from that in the aquatic environments and that core ARGs could allow differentiation of water sources according to the ARGs' abundance.

2. Materials and methods

2.1. Collection and processing of samples

The sampling was performed in Nakdong and Yeongsan River streams, and nine WWTPs four and five of which are located on Nakdong and Yeongsan River streams, respectively (Fig. 1). The Nakdong and Yeongsan River streams are two of the biggest ones in South Korea. One and five liter of surface water of adjacent upstream/downstream of the river streams and effluent of each WWTP were collected using sterile bottles, respectively. The upstream and downstream are located within about 500 m from the discharge point of effluent of WWTPs. The sampling was carried out in every June from 2016 to 2019. Samples were shipped immediately to the laboratory under cool condition (4 °C). One liter of upstream and downstream samples and five litres of effluent samples were pre-filtered through a 10 μm pore size membrane filter and thereafter through a 0.22 μm pore size membrane filter (Advantec, Tokyo, Japan). DNA was extracted from the membranes using a MoBio PowerWater DNA isolation kit (MoBio Laboratories Inc., CA, USA), according to the manufacturer's instructions. Extracted DNA was stored at −20 °C for further analysis.

2.2. High-throughput quantification of ARGs with SmartChip analysis

Quantitative polymerase chain reaction (qPCR) was performed using a SmartChip Real-time PCR system (WaferGen Inc., USA) targeting 343 ARGs, and in this study almost all classes of ARGs were investigated. qPCR was performed in 100 nL containing 1 × LightCycler 480 SYBR® Green I Master Mix (Roche Inc., USA), ~ 5 ng/μL DNA template, 500 nM each of forward and reverse primer, and nuclease-free PCR-grade water. The reaction was performed under the following conditions: initial denaturation at 95 °C for 10 min; 40 cycles of denaturation at 95 °C for 30 s; and annealing at 60 °C for 30 s. Melting curve analysis was autogenerated by the program. Amplification was conducted in triplicate, and the housekeeping genes, such as *rpoB*, *mdh*, and *gapA,* were amplified as positive controls. The results were analysed using SmartChip qPCR software (version 2.7.0.1). Amplification efficiency beyond the range of 1.8 to 2.2 was discarded and a threshold cycle (Ct) of 27 was used as the detection limit (Zhu et al., 2013). Only samples successfully amplified in triplicate were analysed. The gene copy number was calculated according to a previous study (Ouyang et al., 2015). The copy number of ARGs was calculated using a previously described equation (Looft et al., 2012) and normalized to the copy number of 16S rDNA for determining relative abundance.

2.3. Data analyses

The study data was collectively analysed after four-year study. Further data analysis was done using R studio version 1.1.463 (http://www.rstudio.com/) and FunRich. The data analysis flow is 1) the calculation of relative abundance of ARGs, 2) statistical analysis for evaluation of significant difference among the samples and 3) determination of factors to discriminate the sample types by linear discriminant analysis (LDA) and decision-making tree. First, relative abundance of ARGs was calculated by copy number of ARGs divided by that of 16S rDNA. Then, shapiro test was applied using the relative abundance of ARGs for normality test. Statistical comparison of the relative abundance of ARGs i) among upstream, downstream, and effluents, and ii) between the aquatic environments (upstream and downstream) and WWTP effluents was performed using Analysis of Variance analysis (ANOVA). Last, LDA was performed with installation of "klaR," "psych," "MASS," "ggord," and "devtools" to classify the water source of the samples (upstream, downstream, and effluent) based on the abundance

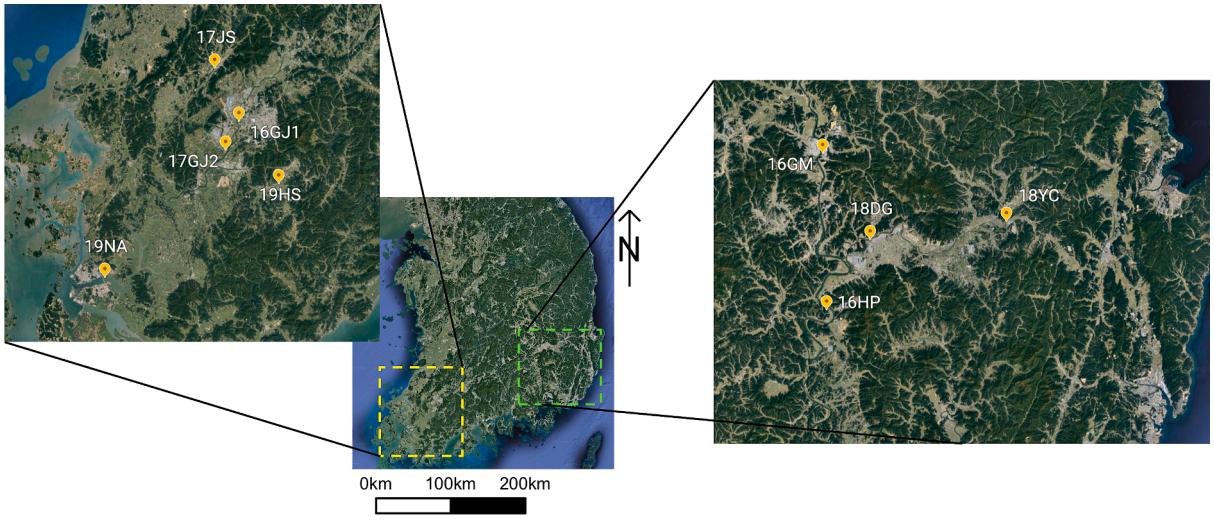

Fig. 1. Nine wastewater treatment plants marked as yellow points for sampling sites in Yeongsan and Nakdong River streams of South Korea. The five and four points on left and right maps represent the sites in Yeongsan and Nakdong River streams, respectively. The name of each wastewater treatment plant is written in white color. GJ1, GJ2 and DG are big cities, and the others are medium-size regions in South Korea. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of ARGs. The decision-making tree model was constructed with installation of "party," "caret," tidyselect", and "rpart".

3. Results

3.1. 1 Comparison of the ARG abundance

The relative abundance of ARG classes (defined as all the resistance genes to each antibiotic) is calculated as the copy number of ARGs normalized by that of 16S rDNA. The relative abundance of ARG was specifically compared within the sampling points of upstream, downstream, and effluent for each WWTP (Fig. 2). Although a variation in the abundance of ARGs was observed, aminoglycoside resistance genes were the most abundant, followed by miscellaneous ARGs, sulfonamide, and tetracycline while fluoroquinolone and glycopeptide resistance genes were rarely observed in any of the samples. No specific distribution pattern of the ARG classes was found in the upstream, downstream, and effluent. The combined relative abundance of all ARGs per each sample type was not significantly different among the upstream, downstream, and effluent samples (data not shown). Specifically, no significant difference in the relative abundance of aminoglycoside, β-lactam, sulfonamide, and tetracycline resistance genes was observed among the three types of samples (data not shown).

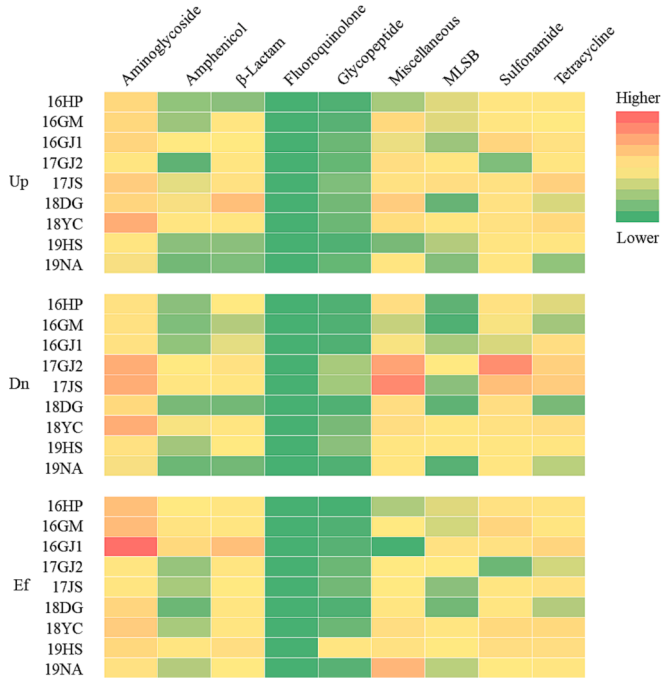

Fig. 2. Comparison of the relative abundance of antibiotic resistance gene (ARG) classes in each sampling point. The relative abundance of ARG classes is the copy number of ARGs divided by that of 16S rDNA. Red and green colors indicate higher and lower abundance of ARG classes, respectively. For example, the abundance of aminoglycoside resistance genes was highest in the effluent of 16GJ1 while it was lowest in upstream of 19NA. 16HP: Hyeonpung wastewater treatment plant (WWTP) in 2016, 16GM: Gumi WWTP in 2016, 16GJ1: Gwangju 1st WWTP in 2016, 17GJ2: Gwangju 2nd WWTP in 2017, 17JS: Jangseong WWTP in 2017, 18DG: Daegu WWTP in 2018, 18YC: Yeongcheon WWTP in 2018, 19HS: Hwansun WWTP in 2019, 19NA: Namak WWTP in 2019. Up: upstream, Dn: downstream, Ef: effluent. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Identification of core ARGs in upstream, downstream, and effluent of WWTPs

Core ARGs were defined as ARGs that were constantly detected in all samples of each type (upstream, downstream, and effluent). The core ARGs per sample type are shown in the Venn diagram (Fig. 3). The five main core ARGs common in all sample types (represented by the center of the Venn diagram) were sulfonamide *sul1* and aminoglycoside *aadA2*, *aadA*, *aadA5* and *strB*. In the upstream samples, core ARGs of aminoglycoside (*aadA*, *aadA1*, *aadA2*, *aadA5*, *aphA1*, *aac(6′)-Ib*, *aac(6′)-II,* and *strB*), β-lactam (bla_{OXA-1}), chloramphenicol (*floR*), macrolide-lincosamide-streptogramin B (*ermB*, *ereA*, *matA/mel,* and *vatE*), sulfonamide (*sul1*), and tetracycline (*tetC*, and *tetG*) were detected. In downstream, core ARGs of aminoglycoside (*aadA*, *aadA2*, *aadA5*, *aphA1,* and *strB*), β-lactam (bla_{SFO}), chloramphenicol (*floR*), macrolide-lincosamide-streptogramin B (*vatE*), and sulfonamide (*sul1*) were detected. In the effluent samples, core ARGs of aminoglycoside (*aadA*, *aadA1*, *aadA2*, *aadA5*, *and strB*), β-lactam (bla_{OXA-10}, and bla_{GES}), chloramphenicol (*catB3,* and *catB8*), and sulfonamide (*sul1*) were present. Three genes, bla_{OXA-10}, bla_{GES}, *and catB8,* were found exclusively in the effluents.

3.3. Comparison of the relative abundance of core ARGs

The relative abundances of the five main core ARGs between the upstream and downstream regions, and effluents, were compared, and between the aquatic environments (upstream and downstream) and effluents (Fig. 4). The relative abundance of the *strB* and *sul1* genes was not significantly different among the upstream, downstream, and effluent samples, and between aquatic environments and effluents. The relative abundance of *the aadA5* gene was significantly different ($p < 0.05$) among upstream, downstream, and effluent, whilst a significant difference ($p < 0.05$) of the *aadA* and *aadA2* genes was observed only between upstream and effluent. Comparing aquatic environments and effluents, the *strB* and *sul1* genes were also not different; however, the relative abundance of *aadA*, *aadA2*, and *aadA5* was significantly different ($p < 0.05$).

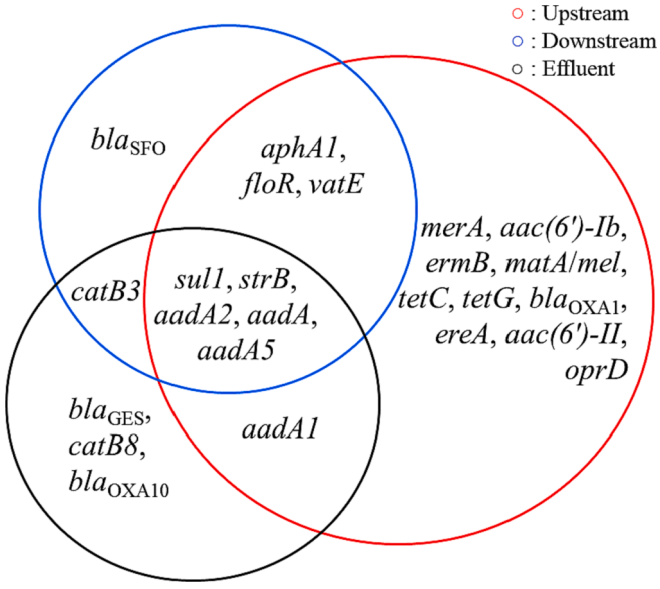

Fig. 3. Determination of core antibiotic resistance genes (ARGs) in upstream, downstream, and effluent of wastewater treatment plants. The size of Venn diagram depends on the number of ARGs.

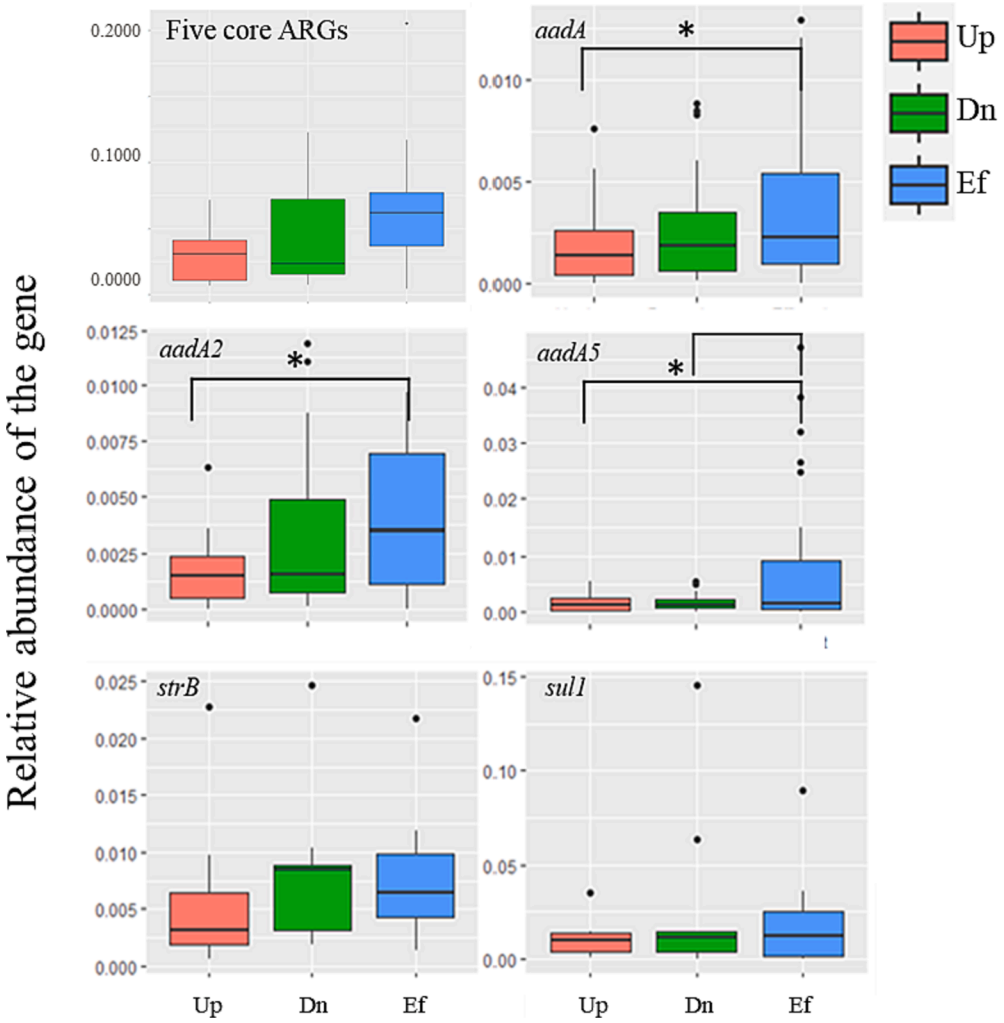

Fig. 4. Comparison of the abundance of three core ARGs (*aadA*, *aadA2*, *aadA5*, *strB*, and *sul1*) among upstream, downstreams, and effluents. The relative abundance of ARG classes is the copy number of ARGs divided by that of 16S rDNA. An asterisk (*) denotes a statistically significant difference ($p < 0.05$) among upstream, downstream, and effluent.

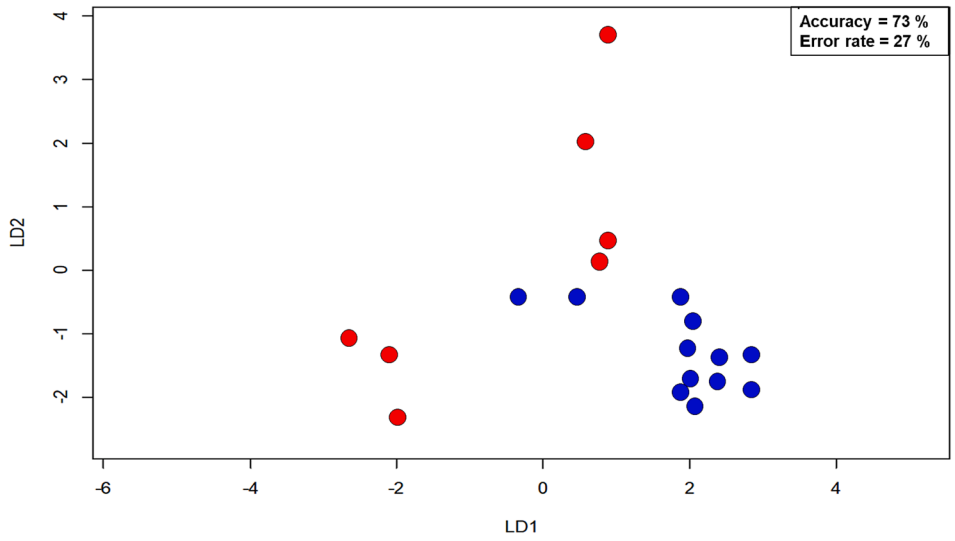

Fig. 5. Linear discriminant analysis for the five core ARGs (*aadA*, *aadA2*, *aadA5*, *strB*, and *sul1*) between the aquatic environments (red point) and effluents (blue point) of WWTPs. LDA plot classified the groups of the aquatic environment (red point) and the effluent (blue point) with the 73 % accuracy of classification and 27 % or error rate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Characterization of core ARGs in the aquatic environments and effluents of WWTPs

Linear discriminant analysis (LDA) showed that the relative abundance of the core ARGs could not be used to discern among upstream, downstream, and effluent water sources (data not shown) yet could classify the groups of water sources of the aquatic environments and effluents (Fig. 5). These different characteristics of the core resistome in aquatic environments versus effluents are supported by the decision-making tree model (Fig. 6). The relative abundances of the five core ARGs and *aadA2* gene was selected for the construction of the decision-making tree. For the differentiation of the water sources of the aquatic environments and effluents, two nodes were generated by the abundance of the *aadA2* gene (either $<$ or $\geq$ 0.0031 copy number of the gene; that of 16S rDNA). In the left branch, the relative abundance of core ARGs divided the groups of aquatic environments and effluents based on either $<$ or $\geq$ 0.054 copy number of the ARG per 16S rDNA. Using this decision-making tree, the characteristics of the core resistome in aquatic environments and effluents were determined to be different.

4. Discussion

4.1. 1. Influence of effluent discharge on receiving water bodies

Effluent discharges containing contaminants, such as residues of antibiotics, heavy metals, ARGs and ARB, reportedly promote the proliferation of ARGs and ARB by selective pressures (Buri et al., 2021; Lorenzo et al., 2018; Xu et al., 2015). However, in this study, the relative abundances of 343 ARGs did not significantly differ among the upstream, downstream, and effluent samples. This suggests that ARGs have been widely disseminated and continuously circulating in aquatic environments and WWTPs for some time. ARGs are reportedly ubiquitous in natural environments (Berglund, 2015) and have been a natural phenomenon for 30,000 years (Costa et al., 2011). In natural environments, horizontal gene transfer (HGT) events also contribute to the proliferation and dissemination of ARGs among different bacterial strains (Blahna et al., 2006). A previous study showed that the abundance of ARGs is significantly lower in pristine areas than in urban areas (Ouyang et al., 2015). However, considering that the upstream of the WWTPs might have been affected by various unexpected factors such as 1) another WWTP located upstream, 2) other factors such as run-off from various non-point sources, 3) the scale of WWTPs, and 4) the flow rate of streams, the lack of a significant difference in the relative abundance of 343 ARGs between upstream and downstream is valid.

4.2. Core resistome in the aquatic environment and the effluent of the WWTPs

We determined core ARGs constantly present in all samples of each type, and found five core genes ubiquitous in all types of the samples. The presence of plasmid-mediated *sul1* in all samples indicates that the *sul1* gene has been transferred between bacterial strains by horizontal gene transfer (HGT) regardless of water compartments (Wu et al., 2010). In addition, *sul1* has been found in a broad range of hosts because of its linkage to integron integrases (He et al., 2014). It has also been proposed as a potential indicator gene, together with *tetW* and *fexA* genes from chicken feedlots (He et al., 2014) and marker genes, for tracking anthropogenic sources (Pruden et al., 2012; Storteboom et al., 2010). The detection of the two aminoglycoside resistance genes (*strB* and *aadA*) in all samples may have linkages to clinical settings, since reportedly these genes have been found in all hospital sewages along with several other ARGs, including β-lactams (bla_{GES}, bla_{OXA}, and bla_{VEB}) (Pärnänen et al., 2022). It was showed that $>$ 90 % of these genes persisted after treatment processes that the analysed samples had undergone, which might explain their widespread distribution in aquatic environments. The presence of bla_{OXA-10}, bla_{GES}, *and catB8*, suggests that these genes were possibly derived from anthropogenic activity. As mentioned above, the presence of bla_{OXA} and bla_{GES} may mirror the clinical resistome (Pärnänen et al., 2022). It should be noted that the chloramphenicol resistance gene *catB8* was frequently detected in the integron gene cassette as *aacA4-catB8-aadA1* from human pathogenic *Acinetobacter baumannii* (Chen et al., 2015; Xia et al., 2016), even though it was not detected in the sites analysed for this study.

4.3. Determination of indicator genes

We found that some indicator genes, suggested in this study, are overlapped with previous studies. In China, the genes *aadA* and *sul1* were reportedly the main ARGs in 16 sites, including hospitals, WWTPs, sediments along rivers, animal farms, and agricultural soils (Yuan et al., 2022). The *sul1* gene is known to be highly conserved in the region of the class 1 integron, which is used as a proxy for anthropogenic pollution in the river system (Chaturvedi et al., 2021). The colocalization of the *sul1* gene in class 1 integrons contributes to its persistence and dissemination in various environmental settings (Jiang et al., 2019). The gene *aadA* is also known to be localized to class integrons in soil environments (Binh et al., 2009). The aminoglycoside resistance genes *aadA2*, *aadA*, *aadA5*, and *strB*, which are associated with healthcare infections, have been mainly investigated in clinical settings (Kishk R et al., 2021). According to another study, the *aadA* was detected in large quantities in influents, effluents, downstreams, and even upstreams (Khan et al., 2019). Likewise, the five core ARGs in this study have been well documented as

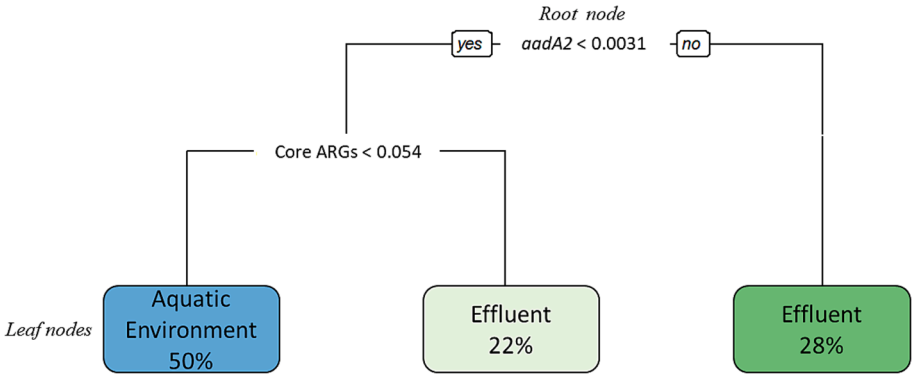

Fig. 6. Decision-making tree model using the abundance of core ARGs (*aadA*, *aadA2*, *aadA5*, *strB*, and *sul1*) and *aadA2* gene for classification of the groups of the aquatic environments (upstream and downstream) and effluent of WWTPs. First, two nodes were generated by the relative abundance (copy number of *aadA2* gene per 16S rDNA. In turn, the groups of aquatic environments and effluents were differentiated based on 0.054 copy number of the ARG per 16S rDNA.

being ubiquitous in natural environments and disseminated by class 1 integrons. This study proposes the potential indicator ARGs being overlapped in those of other countries. We suggests those indicators for global comparison of contamination level by ARGs.

4.4. Discrimination of water sources by core ARGs and aadA2 gene

The properties of the core ARGs differ between aquatic environments and effluents, based on the abundance of the five main core ARGs. Interestingly, the effluents carried the higher abundance of the five core ARGs and *aadA2* genes than the aquatic environments, which suggests that there are still a few significant ARGs for differentiating between the aquatic environments and effluents. In addition, this indicates that the effluents are more contaminated by ARGs than the aquatic environments, suggesting that the core ARGs are indeed indicators between the aquatic environments and effluents. It has been well documented that anthropogenic activities are contamination sources for ARGs and WWTPs act as a interface between the human community and the aquatic environments (Osińska et al., 2020; Rodriguez-Mozaz et al., 2020); hence, it is acceptable to imply that the core ARGs could be potential indicators to evaluate the AR contamination level. Genetic determinants have been documented for indicator ARGs including *int1*, *sul1*, *sul2*, $bla_{\text{CTX-M}}$, bla_{TEM}, $bla_{\text{NDM-1}}$, bla_{VIM}, bla_{KPC}, *qnrS*, *aac-(6´)-Ib-cr*, *vanA*, *mecA*, *ermB*, *ermF*, *tetM*, and *aph* genes (Berendonk et al., 2015); the authors described that standard core indicators (like ARGs in this study) are a requirement for the swift and easy comparability between studies worldwide (Berendonk et al., 2015). Among the indicators, only *sul1* overlapped, which was possibly due to geographical differences. Thus, in South Korea, these genes can be used for the assessment of contamination by AR in aquatic environments. Based on our findings, analyses of the core resistome could provide a means for easy comparison amongst global studies aimed at evaluating the contamination level by AR on the resistome in various environmental settings.

4.5. Limitation of this study

The limitations of this study include that samples were collected only once a year (June) from the various sites of WWTPs and adjacent rivers. Also, although randomly selected sites might reduce the statistical significance, we believe the sites we selected nonetheless cover the comprehensive geographical distribution of ARGs. Another consideration is the absence of other factors that might contribute to the distribution of ARGs in the studied sites. For example, environmental variables, such as total nitrogen, total phosphate, temperature, and pH, can influence the resistome and microbial community. Further studies are required to complement the knowledge gaps in this study to verify the utility of these core ARGs.

5. Conclusions

The current study was conducted over four years and investigated the abundance of a total of 343 ARGs from effluent of nine WWTPs and adjacent upstream and downstream. We found that the abundance of three core ARGs was significantly higher in the effluents than in the aquatic environments. Notably, the water sources were differentiated based on the abundance of the core ARGs, indicating that those genes can be potential indicators to assess AR contamination level in the aquatic environments and effluents. With numerous studies on contamination indicators in aquatic environments, we believe that quantitative analysis of the five core ARGs and *aadA2* would be appropriate indicators for consistent global surveillance. Application and quantitative analysis of core ARGs and *aadA2* gene would allow rapid and easy evaluation of the contamination status by AR in the aquatic environment as well as other environmental settings including lake, WWTPs and fish farms.

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CRediT authorship contribution statement

Hanseob Shin: Conceptualization, Software, Data curation, Writing – original draft. **Yongjin Kim:** Methodology, Visualization. **Hor-Gil Hur:** Conceptualization, Project administration, Writing – review & editing.

Declaration of 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.

Data availability

Data will be made available on request.

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