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Original Research

Distinct ARG profiles associated with class 1 integrons in municipal and industrial wastewater treatment plants

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ABSTRACT

Class 1 integrons facilitate horizontal gene transfer, significantly influencing antibiotic resistance gene (ARG) dissemination within microbial communities. Wastewater treatment plants (WWTPs) are critical reservoirs of ARGs and integrons, yet the integron-mediated dynamics of ARG transfer across different WWTP types remain poorly understood. Here we show distinct ARG profiles associated with class 1 integrons in municipal and industrial WWTPs using a novel approach combining nested-like high-throughput qPCR and PacBio sequencing. Although industrial WWTPs contained higher absolute integron abundances, their relative ARG content was lower (1.27×10^7–9.59×10^7 copies per ng integron) compared to municipal WWTPs (3.72×10^7–1.98×10^8 copies per ng integron). Of the 132,084 coding sequences detected from integrons, 56.8% encoded antibiotic resistance, with industrial plants showing lower ARG proportions, reduced ARG array diversity, and greater incorporation of non-ARG sequences. These findings suggest industrial WWTP integrons integrate a broader array of exogenous genes, reflecting adaptation to complex wastewater compositions. This work enhances our understanding of integron-driven ARG dynamics in wastewater and offers a robust strategy for environmental integron analysis.

1. Introduction

Antibiotics are powerful therapeutic agents crucial in treating bacterial infections and safeguarding human health. However, the rapid emergence and spread of antibiotic resistance in both clinical settings and the environment poses a significant threat to effective antibiotic therapy [1]. Pathogens continuously challenge the efficacy of therapy by developing multidrug resistance, while the development of new antibiotics progresses relatively slowly [2]. The spread of antibiotic-resistant genes (ARGs) and the development of multidrug-resistant pathogens raise serious societal concerns. Wastewater treatment plants (WWTPs) serve as both barriers and hotspots for the dissemination of ARGs [3]. Uncovering the presence, distribution, and dispersal characteristics of resistome in WWTPs is therefore critical work that is widely underway but remains incomplete.

Integrons are versatile gene acquisition systems present in prokaryotes. They facilitate the incorporation of exogenous resistance genes through the action of integrase, making them vital mediators in acquiring and evolving multiple antibiotic resistance in microbes. Among the different classes of integrons, class 1 integrons are recognized for harboring the greatest diversity of antibiotic resistance gene cassettes, thereby playing a prominent role in integron-mediated multidrug resistance [4,5]. Multiple antibiotic resistance gene cassettes carried by class 1 integrons have frequently been detected in clinical settings [4,6] and pose a serious threat to patient health. The challenge of class 1 integron-mediated multidrug resistance requires significant attention, and

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necessary efforts should be made to mitigate its spread in the environment. As hotspots for ARGs, the characteristics of ARGs associated with class 1 integrons in WWTPs, particularly the arrays of multiple antibiotic resistance gene cassettes, warrant focused investigation.

In WWTPs, a significant positive correlation has been recognized between the class 1 integron-integrase gene, *intI1*, and the overall abundance of ARGs [7]. Increased *intI1* has also been proposed as a proxy for anthropogenic pollution due to its association with genes that confer resistance to antibiotics, disinfectants, and heavy metals [8]. Class 1 integrons are believed to be a crucial genetic element carrying ARGs in WWTPs. However, most previous studies have relied on the quantitative polymerase chain reaction (qPCR) method [9,10], which evaluates the abundance of *intI1* and ARGs but does not provide information on the types, abundance, or arrangements of ARGs carried by integrons. Only a few studies have investigated class 1 integron-associated ARGs in WWTPs and reported the abundance of ARGs on class 1 integrons in these systems [11–13].

Previous studies of class 1 integrons in WWTPs have two notable deficiencies. First, these studies have primarily focused on municipal WWTPs, while the characteristics of ARGs associated with class 1 integrons in industrial WWTPs remain unexplored. The type of wastewater significantly affects the abundance of ARGs and *intI1*, with higher levels commonly found in industrial WWTPs than in municipal WWTPs [14,15]. However, whether industrial WWTPs harbor more ARGs associated with class 1 integrons remains unclear.

Furthermore, the effect of wastewater type on microbial community structure is considered an important reason for the variation of ARGs in WWTPs [16]. Notably, gene cassette arrays on integrons can dynamically change under the action of integrase; thus, the genes on class 1 integrons do not fully align with the microbial community. Due to the complex conditions of industrial wastewater, microorganisms in these settings may experience heightened selective pressures compared to those in municipal WWTPs. Therefore, we hypothesize that class 1 integrons in industrial WWTPs may incorporate a broader range of exogenous gene cassettes. This leads to a lower overall abundance of ARGs associated with class 1 integrons in these facilities. To validate this hypothesis, it was necessary to undertake a comprehensive analysis of ARGs on class 1 integrons in industrial WWTPs and systematically compare the gene cassette characteristics between industrial and municipal WWTPs.

The second deficiency lies in the methodology employed to analyze class 1 integron-associated ARGs. Previous studies have utilized clone libraries and Illumina sequencing for this purpose [11,13,17,18]. However, the low throughput of clone libraries limits the analysis of gene cassette arrays on integrons, while the short-read length of Illumina sequencing is insufficient for capturing the full length of class 1 integrons. The complexity of integrons, which feature repeated integration sites and random arrangements of gene cassettes on integrons, also complicates the accurate assembly of shotgun sequencing reads. Consequently, the analysis of gene cassette arrays on class 1 integrons has proven challenging, and quantitative assessments of ARGs on these integrons have also been limited.

To address these limitations, this study proposes a novel methodology that combines nested-like qPCR and PacBio sequencing to analyze ARGs and gene cassette arrays associated with class 1 integrons. Initially, conventional PCR was employed to amplify class 1 integrons, followed by a quantitative analysis of ARGs on the integrons using high-throughput qPCR with the amplicons as the deoxyribonucleic acid (DNA) template. This approach enabled an evaluation of the relative abundance of ARGs on class 1 integrons. The main amplification bands of class 1 integrons, retrieved from electrophoresis gel, were then subjected to PacBio sequencing. The long-read length and high throughput of PacBio sequencing allow for accurate analysis of the gene cassette arrays on class 1 integrons.

Utilizing the above strategy, this study characterized the types and relative abundance of ARGs carried by class 1 integrons and elucidated the genetic arrangement of gene cassette arrays in nine WWTPs: five industrial and four municipal. A comprehensive comparison of the characteristics of ARGs on class 1 integrons between industrial and municipal WWTPs was conducted to evaluate the hypothesis that class 1 integrons in industrial WWTPs may incorporate a wider array of exogenous gene cassettes than those in municipal WWTPs. Activated sludge, consisting of abundant microorganisms, serves as the foundation of WWTPs and is the source of ARGs exported from WWTPs through effluent and excess sludge discharges. For this reason, activated sludge samples were collected for the analysis of class 1 integrons in the industrial and municipal WWTPs. This study not only enhances our understanding of class 1 integron-associated ARGs in WWTPs but also introduces a novel strategy for investigating class 1 integrons in environmental samples.

2. Materials and methods

2.1. Sample collection and DNA extraction

Activated sludge samples were collected from the aerobic units of nine WWTPs in April 2021 (Supplementary Table S1). XC, XB, SF, and SYM are four WWTPs treating municipal wastewater. SYI is a WWTP treating integrated wastewater from an industrial park containing mainly fine chemicals and dyes production factories. GB and XHC are WWTPs in two pharmaceutical factories; the GB factory mainly produces quinolone and macrolide antibiotics, and XHC mainly produces vitamin A, vitamin E, and astaxanthin. YT and YN are WWTPs in two pesticide factories; YT produces various herbicides, fungicides, and insecticides, and YN produces mainly glufosinate and some other herbicides. The samples were centrifuged at 10,000×*g* for 10 min and stored at −20 °C after the supernatant was discarded. DNA extraction was performed using the PowerSoil DNA Isolation Kit (Mobio, the United States).

2.2. Amplification and extraction of class 1 integrons

The gene cassette-containing region of class 1 integrons was amplified with a primer set of 5'CS (5'-GGCATCCAAGCAGCAAG-3') and 3'CS (5'-AAGCAGACTTGACCTGA-3') [19], which are complementary to the 5' and 3' conserved segments of class 1 integrons, respectively. The thermal program was 95 °C for 10 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min 30 s; then 72 °C for 10 min. The amplification was performed with a 25 μL reaction mixture consisting of 12.5 μL 2 × TransStart FastPfu PCR SuperMix (TransGen, Beijing, China), 1 μL forward primer, 1 μL reverse primer, 1 μL DNA template, and 9.5 μL double-distilled water (ddH_2O).

Electrophoresis was performed after PCR. The gel comprised 1% agarose in 1 × TAE buffer with SYBR Safe DNA Gel Stain (Thermo Fisher, the United States) incorporated. The PCR products were electrolyzed within the gel for 30 min at an applied voltage of 150 V. As the PCR products of empty integrons with no gene cassettes are 153 bp in size, consisting of 97 bp of the 5' conserved segment and 56 bp of the 3' conserved segment [20], the bands in the gel at about 153 bp were not extracted. The amplification products with at least one gene cassette were extracted with a SanPrep Column DNA Gel Extraction Kit (Sangon Biotech,

Shanghai, China) by cutting the gel in the range of 200–4500 bp. The recovered amplification products were quantified with a Qubit 4.0 and stored at −20 °C for further analysis.

2.3. Quantification of intI1 and class 1 integron-associated ARGs

The *intI1* gene abundance was quantified with primers of IntI1-F (5'- CCTCCCGCACGATGATC-3') and IntI1-R (5'- TCCACGCATCGTCAGGC -3') [21] on a QuantStudio 3 real-time PCR system (ABI, the United States). The qPCR was conducted with a 20 μL reaction mixture consisting of 10 μL SYBR Green qPCR Mastermixes (QIAGEN, Germany), 1 μL forward primer, 1 μL reverse primer, 1 μL DNA template, and 7 μL ddH_2O. The thermal program was 94 °C for 9 min, followed by 40 cycles of 94 °C for 30 s, 57.5 °C for 30 s, and 72 °C for 60 s. A standard curve was constructed using a standard plasmid containing the targeted sequence.

Using a WaferGen SmartChip real-time PCR system (WaferGen Inc., Fremont, the United States), high-throughput qPCR (HT-qPCR) was adopted to analyze the ARGs on class 1 integrons, with the integron amplification products extracted from agarose gel used as the DNA template. One microliter of purified 16S rRNA gene amplified from *Escherichia coli* DH5α with primers of 27F and 1492R was added into the DNA template as the reference gene. Each qPCR assay contained 215 primer sets for ARG analysis and 1 primer set for 16S rRNA gene measurement (Supplementary Table S2). Each PCR reaction was conducted in a 100 nL mixture consisting of 1 × LightCycler 480 SYBR Green I Master (Roche Applied Sciences, IN, the United States), 1 mg mL^{-1} bovine serum albumin (New England Biolaboratories, MA, the United States), 500 nM primers, and 2 ng μL^{-1} DNA template. The thermal program for HT-qPCR was 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. The ARGs on class 1 integrons were analyzed for their relative abundance, defined as copies per ng integron. All assays were performed in triplicate.

2.4. PacBio sequencing of class 1 integrons and bioinformatic analysis

For PacBio sequencing, class 1 integrons were amplified using barcode-marked 5'CS and 3'CS primers. The PCR reactions were conducted in triplicate within a 20 μL mixture containing 4 μL 5 × FastPfu Buffer, 2 μL 2.5 mM dNTPs, 0.8 μL 5 μM each primer, 0.4 μL FastPfu Polymerase, and 10 ng DNA template. The thermal program was 95 °C for 5 min, 27 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; then 72 °C for 10 min. PCR products were analyzed using agarose electrophoresis, and some specific integron bands on the gel were retrieved using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, CA, the United States). SMRTbell libraries were prepared from the retrieved integron bands using blunt-ligation according to the manufacturer's protocol (Pacific Biosciences). The purified SMRTbell libraries were then subjected to sequencing on dedicated PacBio Sequel II 8M cells using a sequencing kit 2.0. PacBio raw reads were processed using SMRT Link Analysis software version 9.0 to obtain demultiplexed circular consensus sequence reads with the following settings: minimum number of passes = 3 and minimum predicted accuracy = 0.99. Raw reads were processed through the SMRT Portal to filter low-quality sequences. The remaining sequences were further filtered by removing barcodes, primer sequences, chirmas, and sequences that contained 10 consecutive identical bases.

The filtered data were subjected to coding sequence (CDS) prediction and functional annotation using PROKKA (v1.13) with default parameters. CDSs were searched against the SARG database (v2.2) using BLASTP (v2.6.0) with a threshold *e*-value of 10^{-5}. A CDS was annotated as a potential ARG with a cutoff value of sequence identity of ≥80%, query coverage of ≥70%, and an alignment length corresponding to ≥25 amino acids. Gene subtype abundance was normalized by the total number of reads for each sample according to a previous study [11]. The risk level of all the ARG hits was evaluated using the arg_ranker v2.0 [22]. The raw data were deposited into the National Center for Biotechnology Information Sequence Read Archive database (accession number: PRJNA1111236).

2.5. Data analysis

Principal component analysis (PCA) and nonmetric multidimensional scaling (NMDS) ordinations were conducted in the R environment using the 'vegan' package. The Procustes analysis was also performed using vegan, based on the results of PCA. The diversity of shared ARGs among different samples was visualized using the 'UpSetR' package. The arrangement of ARGs on integron sequences was visualized using the 'genoPlotR' package. The statistical significance of the difference in gene abundance between municipal and industrial samples was analyzed using the independent *t*-test with SPSS Statistics after logarithmic transformation of the gene abundance values.

3. Results and discussion

3.1. IntI1 abundance and electrophoresis analysis of class 1 integrons

The absolute abundance of *intI1* was 2.33 × 10^7–1.66 × 10^9 copies per g sludge, with a relative abundance of 0.002–0.111 copies per 16S rRNA copy in the activated sludge (Fig. 1). Consistent with previous studies [7,9], the high abundance of *intI1* indicated a significant presence of class 1 integrons in WWTPs. Both the absolute abundance ($P < 0.01$) and relative abundance ($P < 0.05$) of *intI1* were significantly higher in industrial WWTP samples (SYI, GB, XHC, YT, and YN) than in the municipal samples (XC, XB, SF, and SYM) (Fig. 1), indicating a higher abundance of

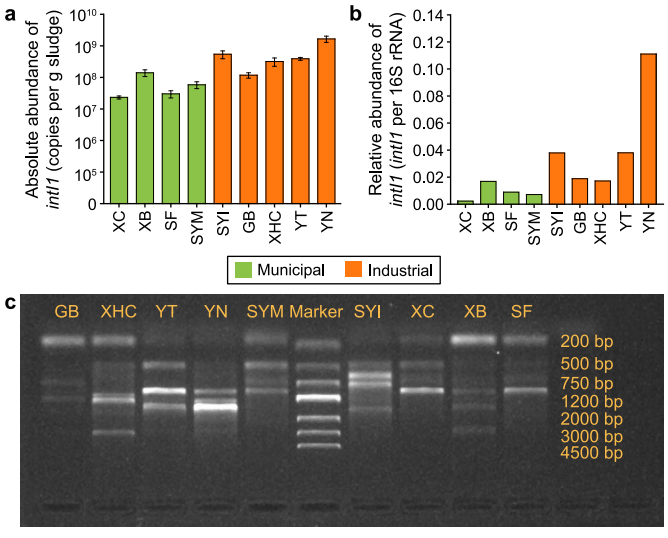

Fig. 1. *IntI1* gene abundance and the electrophoretogram of class 1 integron polymerase chain reaction (PCR) products. **a**, Absolute abundance of *intI1* gene. **b**, Relative abundance of *intI1* gene. **c**, Electrophoretogram of class 1 integron PCR products. Error bars represent standard errors of triplicate tests. XC, XB, SF, SYM, SYI, GB, XHC, YT, and YN are the wastewater treatment plants from which activated sludge samples were collected for analysis.

class 1 integrons in the WWTPs treating industrial wastewater. This finding is consistent with some previous studies suggesting that industrial WWTPs tend to harbor higher levels of class 1 integrons than municipal WWTPs [14,23].

The industrial WWTPs possessed a higher absolute abundance ($P < 0.01$) of total ARGs in the sludge than the municipal WWTPs (Supplementary Fig. S1). The PCA indicated the differences in both ARG compositions and microbial communities between industrial and municipal WWTPs (Supplementary Fig. S1). Furthermore, the Procrustes analysis (Supplementary Fig. S1) confirmed the significant influence of microbial communities on ARG characteristics in WWTPs ($P < 0.05$). However, whether the high abundance of class 1 integrons correlates with a higher prevalence of ARGs carried by class 1 integrons in industrial WWTPs has not previously been determined. The ARGs carried by class 1 integrons should be further analyzed with more targeted approaches.

Electrophoresis of class 1 integron PCR products showed diverse bands for each sample. The shortest band was the empty integrons, which had no gene cassettes with a size of 153 bp [20]. The longer bands indicated the integrons carrying gene cassettes. Generally, the bands of the integrons showed relatively strong brightness in the majority of industrial samples—YT, YN, SYI, and XHC (Fig. 1c)—which was consistent with the high *intI1* abundance in these industrial samples revealed by qPCR (Fig. 1a).

3.2. Types and relative abundance of ARGs on class 1 integrons

A total of 48–64 ARG subtypes were detected on class 1 integrons in each sludge sample using nested-like HT-qPCR, with 57–62 subtypes detected in municipal WWTPs and 48–64 subtypes detected in industrial WWTPs. These ARGs are associated with resistance to a wide range of antibiotic classes (Fig. 2a). Antibiotic deactivation was the dominant resistance mechanism of the ARGs on class 1 integrons, accounting for 42.1–47.9% of all detected ARG subtypes (Fig. 2b). Cellular protection and efflux pumps were present in similar proportions but to a lesser extent than antibiotic deactivation. The relative abundance of total detected ARGs on class 1 integrons was generally lower in industrial WWTPs (1.27×10^7–9.59×10^7 copies per ng integron) than in municipal WWTPs (3.72×10^7–1.98×10^8 copies per ng integron) (Fig. 2c), which contrasted with the higher abundance of *intI1* and total ARGs in the industrial WWTPs than in the municipal ones. However, the difference between the industrial and municipal samples was insignificant ($P = 0.088$), because GB, as an industrial WWTP treating antibiotic wastewater, harbored a relatively high abundance of ARGs on class 1 integrons compared to other industrial samples. The overall abundance of aminoglycoside ARGs was the highest ARG type, representing 48.2–96.3% of all detected ARGs on the class 1 integrons in eight out of the nine sludge samples. This is consistent with previous findings [11,13] that aminoglycoside resistance genes are the most abundant type of ARGs on class 1 integrons in activated sludge samples. The relatively low abundance of aminoglycoside ARGs in industrial WWTPs (1.38×10^6–7.49×10^7 copies per ng integron) compared to municipal WWTPs (2.93×10^7–1.90×10^8 copies per ng integron) was a contributor to the lower abundance of ARGs on class 1 integrons in industrial WWTPs.

In addition to aminoglycoside, β-lactamase is another abundant ARG type on class 1 integrons found in municipal WWTPs [11,13]. In investigations utilizing the clone library method, aminoglycoside and β-lactamase ARGs, such as *aadA*, *aacA*, and bla_{OXA}, have been found as to be the major gene cassettes on class 1 integrons in hospital effluent and the WWTP impacted by hospital effluent [20]. Diverse aminoglycoside and β-lactamase resistance gene cassette sequences have been deposited in GenBank and have become the most important proportion of gene cassette sequences in the database [24]. Illumina sequencing of gene cassettes across 12 urban WWTPs in Europe revealed that more than 50% of all detected gene cassettes contained ARGs in most facilities, with aminoglycoside and β-lactamase ARGs dominating in five and six WWTPs, respectively. [12]. In this study, β-lactamase ARGs were also relatively abundant on class 1 integrons in the municipal WWTPs (1.46×10^6–2.11×10^6 copies per ng integron), but exhibited low abundance on class 1 integrons in the industrial WWTPs (1.24×10^3–3.44×10^4 copies per ng integron) (Supplementary Fig. S2). The lower relative abundance of β-lactamase ARGs on the class 1 integrons in industrial WWTPs ($P < 0.01$) was another contributor to the lower relative abundance of ARGs on class 1 integrons in industrial WWTPs than in municipal ones.

The PCA showed that the composition of ARGs on class 1 integrons in three of the four municipal WWTPs (SYM, SF, and XC) was relatively similar, while that in XB was quite different (Fig. 2d). The composition of ARGs on class 1 integrons in the industrial WWTPs exhibited heterogeneity, both between themselves and in comparison to the municipal WWTPs. The Procrustes analysis of similarities between the class 1 integron-associated ARGs and the microbial community, as well as between the class 1 integron-

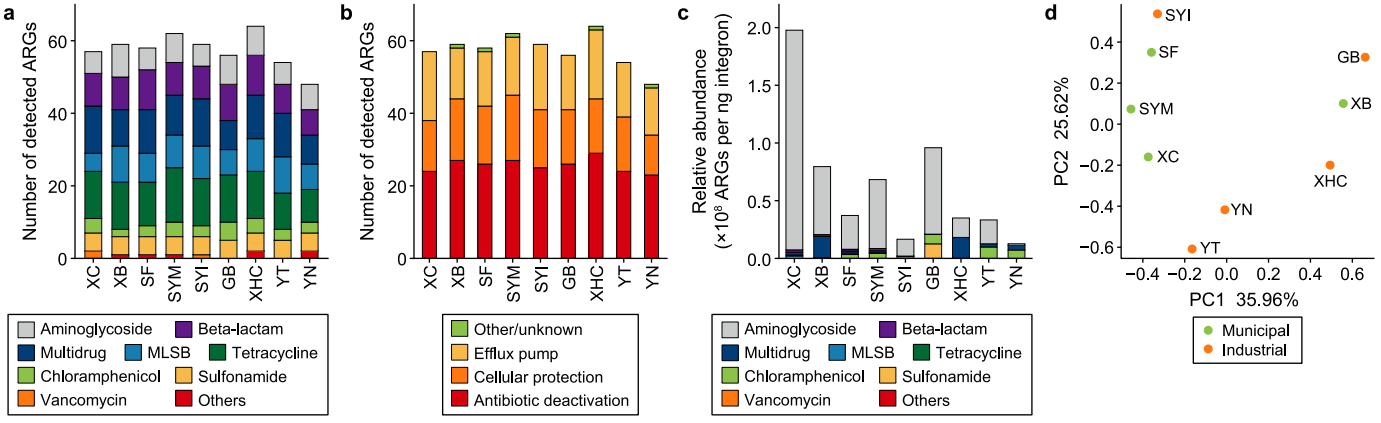

Fig. 2. Types and abundance of detected antibiotic resistance genes (ARGs) on the class 1 integrons and principal component analysis (PCA). **a**, Detected ARGs classified by antibiotic classes. **b**, Detected ARGs classified by resistance mechanisms. **c**, Relative abundance of ARGs on class 1 integrons. **d**, PCA of the relative abundance of ARGs on class 1 integrons. MLSB: macrolide-lincosamide-streptogramin B.

associated ARGs and the total ARGs, yielded statistically insignificant results ($P > 0.05$) (Supplementary Fig. S3). The Pearson correlation analysis also showed a paucity of statistically significant correlations between class 1 integron-associated ARG types and the microbial taxa at the class level (Supplementary Table S3). These findings suggest that ARGs on class 1 integrons are heterogeneous compared to the microbial community and total ARGs, likely due to the recombination of gene cassette arrays on integrons mediated by integrase [4]. Nevertheless, Planctomycetes, Saccharimonadia, and Thermoleophilia demonstrated positive correlations with β_lactamase and macrolide-lincosamide-streptogramin B (MLSB) ARGs on the class 1 integrons, while Dadabacteriia exhibited positive correlations with Sulfonamide ARGs. Furthermore, Actinobacteria and Thermoleophilia positively correlated with the total abundance of ARGs on class 1 integrons (Supplementary Table S3). These microorganisms may serve as probable hosts for class 1 integron ARGs. Class 1 integrons have been identified in various microorganisms [13,17], including *Aeromonas*, *Pseudoxanthomonas*, *Comamonas*, *Escherichia*, *Klebsiella*, *Pseudomonas*, *Salmonella*, *Acinetobacter*, and *Enterobacter*, suggesting a widespread distribution of class 1 integrons in microbial communities.

In previous studies, many ARGs in samples, such as *tetA*, *tetC*, *tetO*, *qnrA*, *ermA*, *ermB*, *mphA*, *ereB*, *vatB*, *blaPSE-1*, *sulI*, *sulII*, and *vanA*, have shown positive correlations with *intI1* using the conventional qPCR method [25–27]. However, in this study, the above-mentioned genes were either not detected or detected at very low levels on class 1 integrons. This suggests that inferring the presence or abundance of genes on integrons based solely on correlation might not be effective. Utilizing integron PCR products as the template for qPCR analysis, the absolute abundance of ARGs on class 1 integrons could not be quantified, potentially leading to an amplification of disparities in gene abundance during the initial amplification step. However, the nested-like HT-qPCR strategy enabled the comparison of the abundance of different genes on class 1 integrons. The reliability of the nested-like qPCR method was validated using control samples (Supplementary Text S1). This method was first used to indicate the notable differences in class 1 integron-associated ARG abundance between the industrial and municipal WWTPs. In contrast to the higher prevalence of class 1 integrons in the industrial WWTPs, the relative abundance of ARGs carried by class 1 integrons in these facilities was generally lower than that in municipal WWTPs. Based on this method, the relative abundance of ARGs on class 1 integrons was revealed as copies per ng integron. Given the variability in the length of class 1 integrons, an accurate calculation of the absolute abundance of class 1 integrons carrying ARGs in the sludge is not possible. However, estimations were conducted according to the length range of class 1 integrons revealed by electrophoresis analysis. The estimations indicated that the proportion of ARGs carried by class 1 integrons in the total ARGs of sludge was approximately 10^{-4}–10^{-3} (Supplementary Text S2), reflecting the important but not major contribution of class 1 integrons to the overall ARGs of sludge.

3.3. Gene cassette array analysis of class 1 integrons

PacPio sequencing was utilized to complement the arrangement information of the gene cassette arrays on the class 1 integrons. Given that the majority of the samples exhibited relatively bright bands around 1000 bp for class 1 integrons, and to eliminate the potential influence of gene cassette array length differences on the comparison of their arrangement characteristics, the approximately 1000 bp bands were extracted from the electrophoresis gel and sequenced on the PacBio platform. Because the bands of GB and XB were faint, they were not sequenced. A total of 94,051 clean reads were obtained from the seven samples, with a range of 9621 to 15,579 reads per sample (Supplementary Table S4). The mean length of these clean reads ranged from 825 to 1205 in the samples. A total of 132,084 coding sequences (CDSs) were inferred with putative functions, and 56.8% of these CDSs in all the samples (ranging from 12.7% to 87.7%) were predicted to encode antibiotic resistance (Fig. 3a). The predicted ARGs accounted for a high proportion of the detected CDSs in the three municipal WWTP samples, ranging from 67.8% to 87.7%. In the industrial WWTP samples, the proportions of ARGs in YN and YT were also relatively high, at 62.1% and 85.7%, respectively. However, the proportion of ARGs in the detected CDSs was only 17.9% and 12.7% in SYI and XHC, respectively. The results demonstrated that the percentage of ARGs on class 1 integrons of SYI and XHC was significantly lower than that of the municipal samples, while the percentage of ARGs on class 1 integrons of YN and YT was slightly lower than that of the municipal samples.

The nested-like HT-qPCR (Fig. 2c) revealed that the relative abundance of ARGs on class 1 integrons in industrial samples, including YN and YT, was lower than that in municipal samples. PacBio sequencing showed a relatively high proportion of ARGs on the class 1 integrons of YN and YT. This discrepancy might be due to the sequencing targeting class 1 integrons of around 1000 bp, thereby not capturing all band lengths. The sequencing data

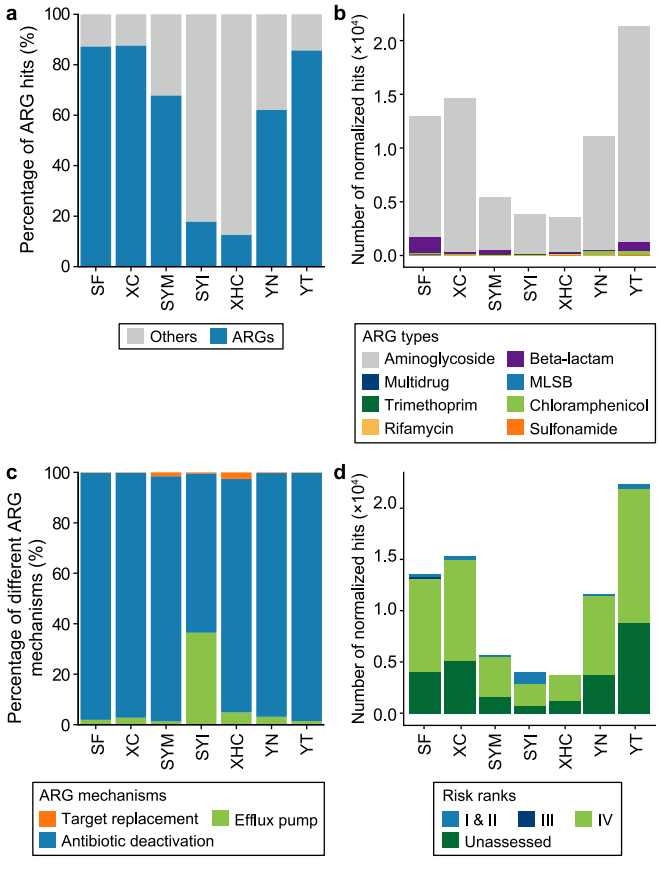

Fig. 3. Composition of class 1 integron-associated antibiotic resistance genes (ARGs) based on PacBio sequencing data. **a**, The percentage of ARG hits in different samples. **b**, The number of normalized hits for different ARG types (normalized by the average number of total reads for each sample). **c**, The percentage of different ARG mechanisms. **d**, The number of normalized hits for ARGs with different risk ranks. Rank I: mobile ARGs already present in pathogens. Rank II: mobile ARGs not yet present in pathogens. Rank III: non-mobile ARGs. Rank IV: not human-associated ARGs. MLSB: macrolide-lincosamide-streptogramin B.

(Fig. 3a) suggest that class 1 integrons around 1000 bp may contain a higher proportion of ARGs than integrons of other lengths in YN and YT. Nonetheless, the sequencing results still indicated an overall lower proportion of ARGs in the class 1 integrons of industrial samples than municipal samples, consistent with the findings of the nested-like HT-qPCR analysis.

The number of normalized ARG hits ranged from 3713.3 (XHC) to 21,831.5 (YT) (Fig. 3b). These ARGs conferred resistance to a range of antibiotics, including aminoglycoside, beta-lactam, chloramphenicol, trimethoprim, multidrug, MLSB, rifamycin, and sulfonamide. Among them, aminoglycoside resistance genes accounted for 93.4% of ARG hits across all the samples, and *aadA*, which confers resistance to aminoglycoside, was the most frequently integrated ARG subtype. The predominant resistance mechanism of ARGs was identified as antibiotic deactivation, accounting for 63.1%–98.4% of the samples (Fig. 3c). Efflux pump was another dominant resistance mechanism in the SYI sample (36.4%). Of all the ARGs on the integrons, 13 unique ARG subtypes were identified as the most significant threat to public health, including three aminoglycoside (*aac(6')-I*, *ant(2'')-I*, and *aadA*), four beta-lactam (*GES-11*, *OXA-1*, *OXA-10*, and *OXA-4*), two chloramphenicol (*catB* and *cmlA*), and four trimethoprim resistance genes (*dfrA5*, *dfrA12*, *dfrA14*, and *dfrB1*). These ARGs in the top two ranks (I and II), as prioritized by the arg_ranker, might be transmissible between environments due to their prevalence in mobile genetic elements of human pathogens and phylogenetically diverse bacterial hosts, thus posing great risks to human health. The normalized hits of these high-risk ARGs ranged from 59.3 (XHC) to 1157.2 (SYI) (Fig. 3d).

NMDS analysis showed that the composition of class 1 integron-associated ARGs was significantly different between municipal and industrial WWTPs (Fig. 4a). Specifically, the composition of ARGs in the industrial WWTPs displayed greater variability, which might be owing to the more diversified functional requirements under the complex selection pressures from industrial wastewaters, leading to the integration of a wider range of exogenous gene cassettes.

The number of detected ARG subtypes was also generally higher in municipal WWTPs (ranging from 42 to 55) than in industrial WWTPs (ranging from 13 to 22) (Fig. 4b). Many ARG subtypes were shared or unique among the three municipal WWTPs. The number of integron sequences carrying ARGs in the SYI and XHC samples was lower than that observed in three municipal WWTPs (Fig. 4c). The number of array arrangements containing at least two ARGs was found to be lower in all the industrial WWTPs than in the municipal WWTPs, indicating that the diversity of ARG-associated arrays (≥2 ARGs) in industrial WWTPs was generally lower than that in municipal WWTPs.

In contrast, the number of class 1 integron sequences not carrying ARGs, as well as the number of non-ARG CDSs carried by class 1 integrons in industrial WWTPs, was generally higher than that in municipal WWTPs (Fig. 5a). Since only class 1 integrons of around 1000 bp in length were sequenced, there might be a bias in using these results to infer the overall class 1 integron gene cassette arrays of the samples. However, nested-like HT-qPCR analysis indicated that the relative abundance of ARGs on class 1 integrons from industrial samples was lower than that in municipal samples (Fig. 2c). This suggests that industrial samples might contain more empty integrons lacking gene cassette arrays or more integrons with a higher proportion of non-ARG gene cassettes. Furthermore, the electropherogram of class 1 integrons showed less prominent empty integron bands in industrial samples from SYI, YN, and YT (Fig. 1c), further supporting the idea that these integrons carry a larger proportion of non-ARG gene cassettes. These obtained non-ARG CDSs were mainly associated with

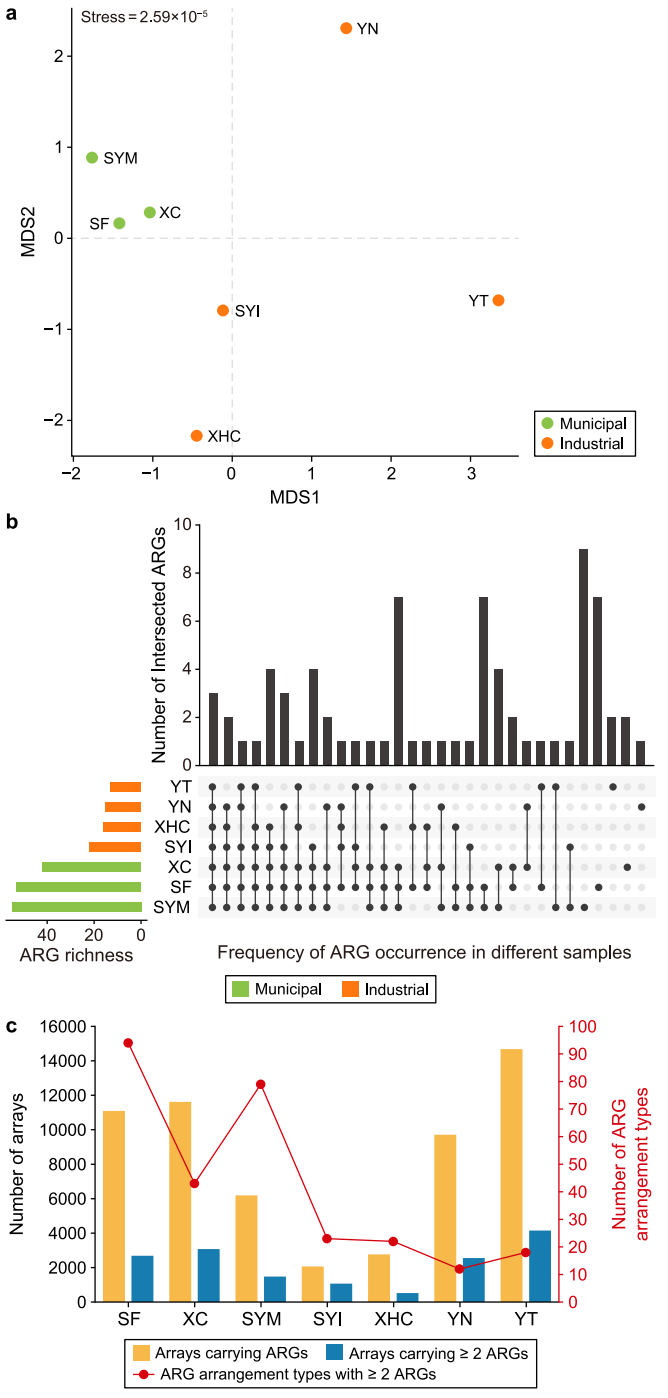

Fig. 4. Compositional difference of class 1 integron-associated antibiotic resistance genes (ARGs) between municipal and industrial wastewater treatment plants. **a**, Non-metric multidimensional scaling (NMDS) analysis of ARGs composition based on Bray-Curtis distance. A stress value less than 0.05 indicates an effective dimensionality reduction. **b**, Upset plot of ARGs identified in different samples. The horizontal bars on the left represent the number of ARGs identified in each sample. Dots and lines represent subsets of samples. The vertical histogram represents the number of ARGs shared by all samples within each subset. **c**, The number of gene cassette arrays carrying ARGs and number of ARG arrangement types.

hypothetical proteins, transport, stress resistance, and biosynthesis functions (Fig. 5b). This confirms that class 1 integrons in the industrial WWTPs mediated the transfer of more diverse functional genes, likely to help microorganisms adapt to harsh environments.

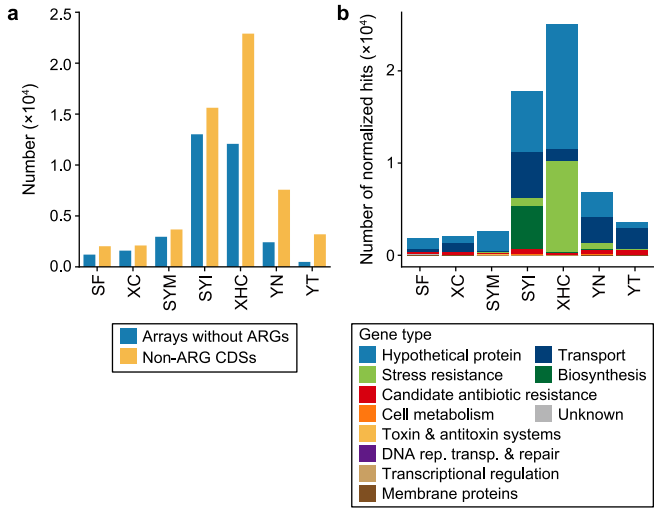

Fig. 5. Composition of other class 1 integron-associated genes based on PacBio sequencing data. **a**, The number of gene cassette arrays without antibiotic resistance genes (ARGs) and non-ARG coding sequences (CDSs). **b**, The number of normalized hits for different gene types. DNA rep. transp. & repair: DNA replication, transcription, and repair; Candidate antibiotic resistance: these genes have no annotation information in the SARG database, but have been annotated by Pokka as being associated with antibiotic resistance.

There were 58,129 integron sequences carrying at least one ARG hit identified in all samples, and 26.8% of them carried at least two ARG hits. A total of 180 distinct integron-associated ARG arrangements (≥2 ARGs) were identified in all the samples (Supplementary Table S5). Among them, |*aadA*|*aadA*| was the most prevalent ARG arrangement in all the samples, followed by |*aadA*|*aadA*|*aadA*|. Noteworthy variations in the prevalence of specific ARG arrangements were observed across different samples. For instance, the number of |*aac(6')*|*aadA*| arrangements was highest in the SYI sample, while the |*aac(6')*|*catB*| arrangement was highest in the YT sample.

A total of 19 multi-resistant integron sequences carrying three or more ARG subtypes were selected to visualize the ARGs' pairwise co-occurrence (Fig. 6a), with most of them identified in the municipal WWTP samples (ten sequences in SF, five sequences in SYM). Importantly, the 13 high-risk ARGs (top two ranks) frequently co-occurred on the integron sequences, further underscoring the high risk of integron-borne ARGs in the municipal WWTPs. Some identified ARG arrangements, including |*aadA*|*catB*|, |*aadA*|*cmlA*|, and |*aadA*|*OXA-2*|, were reported in a previous study [13], indicating certain similarities of gene cassette arrays among WWTPs.

The high-risk ARG arrangements identified in this study, such as |*aadA*|*aadA*|, |*aadA*|*dfrA17*|, and |*aadA*|*dfrA1*|, were similar to the ARG composition of the detected gene cassette arrays in *Enterobacteriaceae* isolated from patients in hospitals and community clinics [28]. The integron gene cassettes in *E. coli* from healthy individuals and animals were also found to be rich in the arrangements of *dfr* and *aad*, such as |*dfrA1*|*aadA1*| [29], |*dfrA17*|*aadA5*| [29], |*dfrA1*|*aadA2*| [30], and |*dfrA17*|*aadA5*| [30]. This indicates that the ARG arrangements on class 1 integrons in WWTPs had certain similarities with clinic samples, probably due to the exchange of antibiotic resistomes between environmental bacteria and human pathogens [31]. However, the identification of more unique ARG arrangements in this and previous studies highlights the diversity of gene cassette arrays on class 1 integrons in WWTP environments [11,13]. Unique ARG arrangements, such as |*qacG*|*qacG*| in biofilm reactors [17] and |*aadA1*|*qacEΔ1*|*sul1*| and |*catB8*|*aadA1*|*qacEΔ1*|*sul1*| in WWTP effluent-impacted river sediment [32], have also been reported. This might be attributed to the dynamic nature of gene cassette arrays on integrons under the action of integrases.

The comparison between municipal and industrial WWTPs revealed that municipal WWTPs exhibited a greater diversity of ARG-associated gene cassette arrays on class 1 integrons than industrial WWTPs. Class 1 integrons in municipal WWTPs also carried a larger number of ARGs than those in industrial WWTPs. In contrast, class 1 integrons in industrial WWTPs integrated a greater variety of non-ARG gene cassettes, probably due to the more complex wastewater quality and the fact that microorganisms could be subjected to a wider variety of selective pressures (Fig. 5). The number of class 1 integrons carrying gene cassette arrays with three or more non-ARG CDSs was also generally higher in industrial WWTPs (Fig. 6b). In the XHC sample in particular, there were nine integron sequences carrying at least six non-ARG CDSs (Fig. 6c). Most of these CDSs do not have accurate information on functional annotations. This suggests that the role of class 1 integrons in the microbial ecology of industrial WWTPs requires further study.

3.4. Environmental implications

To address the limitations of previous methodologies for analyzing class 1 integron-associated ARGs, a novel approach that combined nested-like qPCR and PacBio sequencing was employed in this study to gain insights into the differences in the characteristics of ARGs and gene cassette arrays on class 1 integrons between municipal and industrial WWTPs. Although nested-like qPCR did not permit the direct quantification of ARG-carrying integrons, it enabled a comparison of the relative abundance of ARGs on integrons between different samples. PacBio sequencing allowed the high-throughput analysis of integron sequences and the acquisition of substantial information regarding gene cassette arrays.

Compared to previous studies employing clone libraries and Illumina sequencing, the present strategy is capable of obtaining more data regarding the prevalence of ARGs on class 1 integrons, as well as a more comprehensive insight into gene cassette arrays. By employing this strategy, this study uncovered the distinctive characteristics of ARGs on class 1 integrons in industrial and municipal WWTPs. The relative abundance of ARGs and the diversity of ARG gene cassette arrays on class 1 integrons were both found to be lower in industrial WWTPs than in municipal WWTPs. In line with these findings, the abundance of non-ARG CDSs and non-ARG CDS gene cassette arrays was observed to be higher in industrial WWTPs. These results support the hypothesis that class 1 integrons in industrial WWTPs may integrate a greater diversity of exogenous genes beyond ARGs to help microorganisms in response to more complex water quality conditions and diverse selective pressures.

The results of this study indicate the presence of class 1 integrons in all samples from both municipal and industrial WWTPs, along with the abundant ARGs on the class 1 integrons. This finding indicates the high prevalence of class 1 integrons and their associated ARGs in environmental samples. Furthermore, the results showed that class 1 integron-associated ARGs were an important but non-dominant component of the overall ARGs of sludge. However, the gene cassette arrays with multiple ARGs on the class 1 integrons increase the risk of multidrug resistance proliferation. Consequently, it is argued that the significance attributed to class 1 integron-associated ARGs is not contingent on their contribution to the abundance of ARGs in environments but on their pivotal role in the development and propagation of

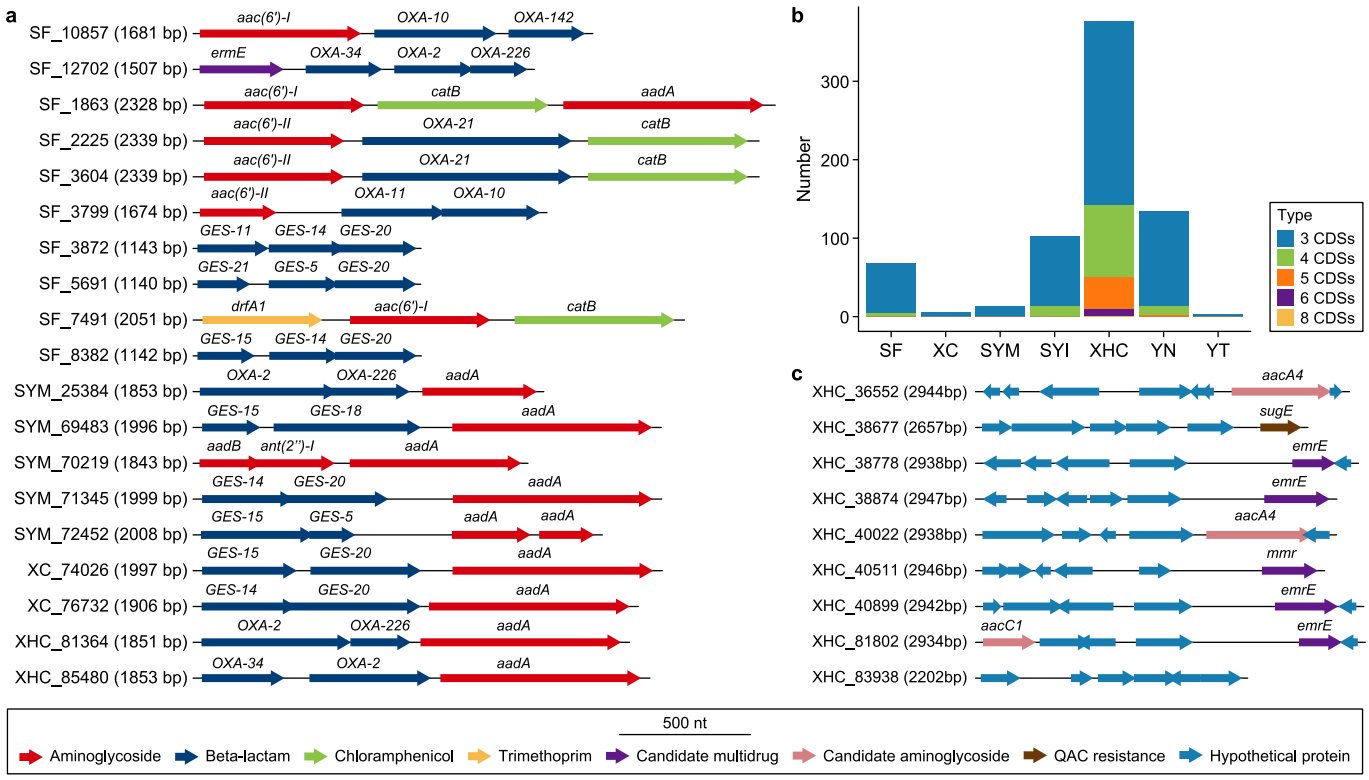

Fig. 6. Pairwise co-occurrence of genes on the class 1 integron-associated gene cassette arrays. **a**, Co-occurrence of different antibiotic resistance gene (ARG) subtypes (inter-subtype) on the multi-resistant (≥3 ARGs) arrays. **b**, The number of arrays carrying above three other genes (non-ARG CDSs). **c**, Representative gene arrangement with six and eight other genes (non-ARG CDSs). CDSs: coding sequences. QAC: quaternary ammonium compound.

multidrug resistances. In summary, this study provides valuable insights into the study of environmental integron-associated ARGs, and an effective approach for future environmental integron research.

4. Conclusion

This study investigated the compositional characteristics of ARGs and gene cassette arrays associated with class 1 integrons in municipal and industrial WWTPs, utilizing a proposed strategy that combines nested-like HT-qPCR and PacBio sequencing. Industrial WWTPs exhibited a higher abundance of class 1 integrons; however, the relative abundance of ARGs on these integrons were lower in industrial WWTPs than in municipal ones. Aminoglycoside was the predominant ARG type on class 1 integrons in both municipal and industrial WWTPs. The relatively lower abundance of aminoglycoside ARGs was a key factor contributing to the diminished abundance of ARGs on class 1 integrons in industrial WWTPs compared to municipal ones. Additionally, the proportion of ARGs in the detected CDSs and the diversity of ARG array arrangements were relatively lower in industrial WWTPs than in municipal WWTPs. Conversely, the abundance of non-ARG CDS and non-ARG CDS gene cassette arrays was higher in industrial WWTPs. The class 1 integrons in industrial WWTPs appear to integrate a greater diversity of exogenous genes beyond ARGs in response to more complex water quality conditions and diverse selective pressures. This study highlights the distinct characteristics of class 1 integrons between industrial and municipal WWTPs and provides an effective strategy for investigating environmental integrons.

CRediT authorship contribution statement

Yan Zhang: Writing - Original Draft, Methodology, Funding Acquisition, Data Curation, Conceptualization. **Zhiguo Su:** Writing - Original Draft, Methodology, Investigation, Formal Analysis. **Xuyang Qiu:** Investigation, Formal Analysis, Data Curation. **He Liu:** Supervision, Project Administration, Writing - Review & Editing. **Donghui Wen:** Project Administration, Funding Acquisition, Writing - Review & Editing. **Lyujun Chen:** Supervision.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ese.2025.100586.

References

[1] N.Q. Balaban, S. Helaine, K. Lewis, M. Ackermann, B. Aldridge, D.I. Andersson, M.P. Brynildsen, D. Bumann, A. Camilli, J.J. Collins, C. Dehio, S. Fortune, J.-M. Ghigo, W.-D. Hardt, A. Harms, M. Heinemann, D.T. Hung, U. Jenal, B. R. Levin, J. Michiels, G. Storz, M.-W. Tan, T. Tenson, L. Van Melderen, A. Zinkernagel, Definitions and guidelines for research on antibiotic persistence, Nat. Rev. Microbiol. 17 (2019) 441–448, https://doi.org/10.1038/s41579-019-0196-3.

[2] S. Kim, T.D. Lieberman, R. Kishony, Alternating antibiotic treatments constrain evolutionary paths to multidrug resistance, P. Natl. Acad. Sci. USA 111 (2014) 14494–14499, https://doi.org/10.1073/pnas.1409800111.

[3] F. Ju, K. Beck, X.L. Yin, A. Maccagnan, C.S. McArdell, H.P. Singer, D.R. Johnson, T. Zhang, H. Burgmann, Wastewater treatment plant resistomes are shaped by bacterial composition, genetic exchange, and upregulated expression in the effluent microbiomes, ISME J. 13 (2019) 346–360, https://doi.org/10.1038/s41396-018-0277-8.

[4] M.R. Gillings, Integrons: Past, Present, and Future, Microbiol. Mol. Biol. Rev. 78 (2014) 257–277, https://doi.org/10.1128/mmbr.00056-13.

[5] Y.-G. Zhu, M. Gillings, P. Simonet, D. Stekel, S. Banwart, J. Penuelas, Microbial mass movements, Sci. Technol. Humanit. 357 (2017) 1099–1100, https://doi.org/10.1126/science.aao3007.

[6] D.Q. Chen, Y.T. Jiang, D.H. Feng, S.X. Wen, D.H. Su, L. Yang, Integron mediated bacterial resistance and virulence on clinical pathogens, Microb. Pathog. 114 (2018) 453–457, https://doi.org/10.1016/j.micpath.2017.12.029.

[7] W.L. Zheng, J.Q. Huyan, Z. Tian, Y. Zhang, X.H. Wen, Clinical class 1 integron-integrase gene - a promising indicator to monitor the abundance and elimination of antibiotic resistance genes in an urban wastewater treatment plant, Environ. Int. 135 (2020) 105372, https://doi.org/10.1016/j.envint.2019.105372.

[8] M.R. Gillings, W.H. Gaze, A. Pruden, K. Smalla, J.M. Tiedje, Y.-G. Zhu, Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution, ISME J. 9 (2015) 1269–1279, https://doi.org/10.1038/ismej.2014.226.

[9] N.A. Sabri, S. van Holst, H. Schmitt, B.M. van der Zaan, H.W. Gerritsen, H.H. M. Rijnaarts, A.A.M. Langenhoff, Fate of antibiotics and antibiotic resistance genes during conventional and additional treatment technologies in wastewater treatment plants, Sci. Total Environ. 741 (2020) 140199, https://doi.org/10.1016/j.scitotenv.2020.140199.

[10] S.J. Sun, Q. Wang, N. Wang, S.J. Yang, H. Qi, High-risk antibiotics positively correlated with antibiotic resistance genes in five typical urban wastewater, J. Environ. Manag. 342 (2023) 118296, https://doi.org/10.1016/j.jenvman.2023.118296.

[11] X.-L. An, Q.-L. Chen, D. Zhu, Y.-G. Zhu, M.R. Gillings, J.-Q. Su, Impact of wastewater treatment on the prevalence of integrons and the genetic diversity of integron gene cassettes, Appl. Environ. Microbiol. 84 (2018) e02766, https://doi.org/10.1128/aem.02766-17.

[12] J. Gatica, V. Tripathi, S. Green, C.M. Manaia, T. Berendonk, D. Cacace, C. Merlin, N. Kreuzinger, T. Schwartz, D. Fatta-Kassinos, L. Rizzo, C.U. Schwermer, H. Garelick, E. Jurkevitch, E. Cytryn, High throughput analysis of integron gene cassettes in wastewater environments, Environ. Sci. Technol. 50 (2016) 11825–11836, https://doi.org/10.1021/acs.est.6b03188.

[13] L. Ma, A.-D. Li, X.-L. Yin, T. Zhang, The prevalence of integrons as the carrier of antibiotic resistance genes in natural and man-made environments, Environ. Sci. Technol. 51 (2017) 5721–5728, https://doi.org/10.1021/acs.est.6b05887.

[14] J. Bengtsson-Palme, M. Milakovic, H. Svecová, M. Ganjto, V. Jonsson, R. Grabic, N. Udikovic-Kolic, Industrial wastewater treatment plant enriches antibiotic resistance genes and alters the structure of microbial communities, Water Res. 162 (2019) 437–445, https://doi.org/10.1016/j.watres.2019.06.073.

[15] H.J. Ding, M. Qiao, J.Y. Zhong, Y.G. Zhu, C.J. Guo, Q.Q. Zhang, P. Yang, L. Han, W. H. Zhang, Y.X. Wu, J.T. Liu, L.T. Zhang, J.H. Sun, Characterization of antibiotic resistance genes and bacterial community in selected municipal and industrial sewage treatment plants beside Poyang Lake, Water Res. 174 (2020) 115603, https://doi.org/10.1016/j.watres.2020.115603.

[16] X.Y. Fan, J.F. Gao, K.L. Pan, D.C. Li, H.H. Dai, X. Li, Functional genera, potential pathogens and predicted antibiotic resistance genes in 16 full-scale wastewater treatment plants treating different types of wastewater, Bioresour. Technol. 268 (2018) 97–106, https://doi.org/10.1016/j.biortech.2018.07.118.

[17] J.Q. Huyan, Z. Tian, Y. Zhang, H. Zhang, Y.H. Shi, M.R. Gillings, M. Yang, Dynamics of class 1 integrons in aerobic biofilm reactors spiked with antibiotics, Environ. Int. 140 (2020) 105816, https://doi.org/10.1016/j.envint.2020.105816.

[18] Y. Zhang, Y.A. Su, X.Y. Qiu, Q.Y. Mao, H.B. Liu, H. Liu, D.H. Wen, Z.G. Su, Temperature affects variations of class 1 integron during sludge anaerobic digestion, Bioresour. Technol. 364 (2022) 128005, https://doi.org/10.1016/j.biortech.2022.128005.

[19] C. Levesque, L. Piche, C. Larose, P.H. Roy, PCR mapping of integrons reveals several novel combinations of resistance genes, Antimicrob. Agents Chemother. 39 (1995) 185–191, https://doi.org/10.1128/aac.39.1.185.

[20] T. Stalder, O. Barraud, T. Jove, M. Casellas, M. Gaschet, C. Dagot, M.-C. Ploy, Quantitative and qualitative impact of hospital effluent on dissemination of the integron pool, ISME J. 8 (2014) 768–777, https://doi.org/10.1038/ismej.2013.189.

[21] A.T. Adesoji, A.A. Ogunjobi, I.O. Olatoye, Molecular characterization of selected multidrug resistant *Pseudomonas* from water distribution systems in southwestern Nigeria, Ann. Clin. Microbiol. Antimicrob. 14 (2015) 39, https://doi.org/10.1186/s12941-015-0102-4.

[22] A.N. Zhang, J.M. Gaston, C.Z.L. Dai, S.J. Zhao, M. Poyet, M. Groussin, X.L. Yin, L. G. Li, M.C.M. van Loosdrecht, E. Topp, M.R. Gillings, W.P. Hanage, J.M. Tiedje, K. Moniz, E.J. Alm, T. Zhang, An omics-based framework for assessing the health risk of antimicrobial resistance genes, Nat. Commun. 12 (2021) 4765, https://doi.org/10.1038/s41467-021-25096-3.

[23] A. Fiorentino, A. Di Cesare, E.M. Eckert, L. Rizzo, D. Fontaneto, Y. Yang, G. Corno, Impact of industrial wastewater on the dynamics of antibiotic resistance genes in a full-scale urban wastewater treatment plant, Sci. Total Environ. 646 (2019) 1204–1210, https://doi.org/10.1016/j.scitotenv.2018.07.370.

[24] S.R. Partridge, G. Tsafnat, E. Coiera, J.R. Iredell, Gene cassettes and cassette arrays in mobile resistance integrons, FEMS Microbiol. Rev. 33 (2009) 757–784, https://doi.org/10.1111/j.1574-6976.2009.00175.x.

[25] N. Makowska, R. Koczura, J. Mokracka, Class 1 integrase, sulfonamide and tetracycline resistance genes in wastewater treatment plant and surface water, Chemosphere 144 (2016) 1665–1673, https://doi.org/10.1016/j.chemosphere.2015.10.044.

[26] C. Narciso-da-Rocha, A.R. Varela, T. Schwartz, O.C. Nunes, C.M. Manaia, bla_{TEM} and *vanA* as indicator genes of antibiotic resistance contamination in a hospital-urban wastewater treatment plant system, J. Glob. Antimicrob. Resist. 2 (2014) 309–315, https://doi.org/10.1016/j.jgar.2014.10.001.

[27] Z.G. Su, A.L. Li, J.Y. Chen, B. Huang, Q.L. Mu, L.J. Chen, D.H. Wen, Wastewater discharge drives ARGs spread in the coastal area: a case study in Hangzhou Bay, China, Mar. Pollut. Bull. 151 (2020) 110856, https://doi.org/10.1016/j.marpolbul.2019.110856.

[28] P.A. White, C.J. McIver, W.D. Rawlinson, Integrons and gene cassettes in the *Enterobacteriaceae*, Antimicrob. Agents Chemother. 45 (2001) 2658–2661, https://doi.org/10.1128/aac.45.9.2658-2661.2001.

[29] L. Vinué, Y. Sáenz, S. Somalo, E. Escudero, M.A. Moreno, F. Ruiz-Larrea, C. Torres, Prevalence and diversity of integrons and associated resistance genes in faecal *Escherichia coli* isolates of healthy humans in Spain, J. Antimicrob. Chemother. 62 (2008) 934–937, https://doi.org/10.1093/jac/dkn331.

[30] H.Y. Kang, Y.S. Jeong, J.Y. Oh, S.H. Tae, C.H. Choi, D.C. Moon, W.K. Lee, Y.C. Lee, S.Y. Seol, D.T. Cho, J.C. Lee, Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from humans and animals in Korea, J. Antimicrob. Chemother. 55 (2005) 639–644, https://doi.org/10.1093/jac/dki076.

[31] K.J. Forsberg, A. Reyes, W. Bin, E.M. Selleck, M.O.A. Sommer, G. Dantas, The shared antibiotic resistome of soil bacteria and human pathogens, Sci. Technol. Humanit. 337 (2012) 1107–1111, https://doi.org/10.1126/science.1220761.

[32] G.C.A. Amos, S. Ploumakis, L.H. Zhang, P.M. Hawkey, W.H. Gaze, E.M. H. Wellington, The widespread dissemination of integrons throughout bacterial communities in a riverine system, ISME J. 12 (2018) 681–691, https://doi.org/10.1038/s41396-017-0030-8.