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Nanopore sequencing analysis of integron gene cassettes in sewages and soils

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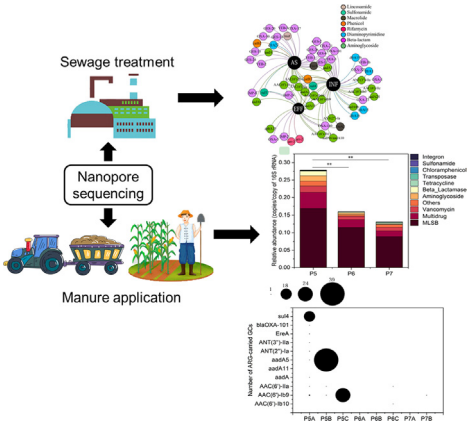
HIGHLIGHTS

- Single manure application exerted a long-term effect on the enrichment of resistance cassettes.
- Antibiotics and heavy metals showed no impact on the resistome profile in soils.
- Sewage treatment could efficiently remove integrase genes and resistance cassettes.
- Gene cassettes mainly carried genes coding resistance to aminoglycoside, and beta-lactams.

GRAPHICAL ABSTRACT



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ABSTRACT

Integrons are genetic elements that can facilitate rapid spread of antibiotic resistance by insertion and removal of genes. However, knowledge about the diversity and distribution of gene cassettes embedded in class 1 integron is still limited. In this study, we sequenced integron gene cassettes using nanopore sequencing and quantified antibiotic resistance genes (ARGs) and integrase genes in the manured soils and sewages of a bioreactor. The results showed that class 1 integron integrase genes were the most abundant in soils and sewages compared with class 2 and class 3 integrase genes. Long-term manure application exacerbated the enrichment of total ARGs, integrase genes and antibiotic resistance-associated gene cassettes, while antibiotics and heavy metals showed no impact on the overall resistome profile. Sewage treatment could efficiently remove the absolute abundance of integrase genes (~3 orders of magnitude, copies/L) and antibiotic resistance gene cassettes. The resistance gene cassettes mainly carried the ARGs conferring resistance to aminoglycoside and beta-lactams in soils and sewages, some of which were persistent during the sewage treatment. This study underlined that soil and sewage were potential reservoirs for integron-mediated ARGs transfer, indicating that anthropogenic activity played a vital role in the prevalence and diversity of resistance gene cassettes in integrons.

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1. Introduction

Infections caused by antibiotic resistance bacteria are a growing public health concern worldwide, challenging the efficacy of antibiotic treatment. Integrons are genetic elements that facilitate the horizontal gene transfer (including antibiotic resistance transfer), allowing bacteria to capture and express diverse exogenous genes (Nemergut et al., 2008; Gillings, 2018). Studies have revealed that integrons could be detected in various anthropogenic environments, e.g. wastewater treatment plants (Gatica et al., 2016; An et al., 2018a; Marathe et al., 2019), soils (Ghaly et al., 2019), biofilms (Gillings et al., 2009), hospital sewage (Gillings et al., 2009), and freshwater (Amos et al., 2018; Dias et al., 2021). These integrons are located on chromosome or plasmid, and they, especially class 1 integrons, enable the exchange of antibiotic resistance determinants between pathogens, exacerbating the global propagation of antibiotic resistance genes (ARGs) (Stalder et al., 2012). Therefore, understanding the dynamic dissemination of ARGs associated with integrons is crucial for the mitigation and control of antibiotic resistance (Gillings et al., 2009).

Integrons contain three key components: an integron-integrase gene (*intI*) and its promoter for transcription (P_{intI}), an *attI* integration site, and a constitutive promoter (Pc) for integrating gene cassettes (GCs) at the *attI* site (Mazel, 2006; Partridge, 2011; Gillings, 2014). Gene cassette is another component of integrons, and typical GCs couple an open reading frame (ORF) with an *attC* recombination site. Generally, the integrons located on plasmids and transposons are considered as "mobile integrons", which often carry antibiotic resistance-associated gene cassettes in pathogenic bacteria (Stalder et al., 2012; Cury et al., 2016; Buongermino et al., 2020). These so-called mobile integrons use the *IntI* integrase-mediated site-specific recombination mechanism to insert/excise GCs, shuffling the GC arrays and thus fueling the recombination of ARG-carried GCs. Based on the amino acid sequences of IntI integrases, mobile integrons can be classified into five classes, namely class 1–5 integron (integrase genes defined as *intI1-intI5*), in which class 1 integron is the most prevalent in the environmental niches (Stalder et al., 2012). Owing to its wide distribution, linkage with the genes conferring resistance to antibiotics, disinfectants and heavy metals, and rapid change in the abundance, *intI1* has been used as a good proxy for anthropogenic pollution (Gillings et al., 2015). With the development of sequencing techniques, gene cassettes in class 1 integrons have been widely described to involve in the resistance to the majority of antibiotic families, including aminoglycosides, trimethoprim, beta-lactams, fosfomycin, chloramphenicol, rifampicin and quinolones. Previous studies have showed that soil and sewage were the typical environments for the reservoirs of antibiotic resistance with the strong human driven disturbances (Forsberg et al., 2012; Su et al., 2017). Anthropogenic activities (e.g., sewage discharge, manure application, heavy metal pollution) were closely associated with ARG (Su et al., 2017; Yang et al., 2021) dissemination in soils and sewages (Stalder et al., 2012; Chen et al., 2016; Hu et al., 2016; Hu et al., 2017; An et al., 2018b). Moreover, integron-intergrase genes were commonly detected in these environments with the high prevalence (Jechalke et al., 2014; Gillings et al., 2015). Therefore, soil and sewage possibly represent the vast and diverse pools of antibiotic resistance gene cassettes in integrons. However, the abundance and diversity of the class 1 integrons and their associated antibiotic resistance gene cassettes in soil and sewage is still elusive.

Sequencing-based detection of integron and gene cassette array has largely depended on Sanger sequencing of clone libraries (An et al., 2018a; Gatica et al., 2016) and high-throughput shotgun sequencing (Ma et al., 2017; Ghaly et al., 2019). However, low-throughput property (Sanger sequencing) or limited assembly efficiencies (second-generation sequencing) has limited the uncovering of the comprehensive profiles of class 1 integrons and their carried antibiotic resistance (Yang et al., 2021). Third-generation sequencing technologies, including Oxford Nanopore sequencing and Pacific Biosciences (PacBio) generate long reads that can span GC array sequences and allow to explore the linkage the ARGs and their flanking regions, overcoming these above mentioned limitations (Che et al., 2019). Compared with the PacBio sequencing, nanopore sequencing may enable the rapid data generation with a lower cost and provide a time-saving and portable framework. Previous studies have successfully recovered sequences of ARGs and mobile genetic elements (MGEs, e.g. plasmids, transposons and integrons) from the Nanopore sequencing of plasmidome and metagenome (Helm et al., 2017; Che et al., 2019; Kirstahler et al., 2021), and demonstrated the profound merits of such technology in expanding our current knowledge of the ARGs dissemination mediated by integrons.

Hence, this study applied nanopore sequencing for the variable regions for class 1 integrons to explore the contents of gene cassette pool from sewage and manure-amended soil, characterizing the gene cassettes associated with antibiotic resistance and understanding the impact of anthropogenic activities on the integron-mediated ARG dissemination. Additionally, qPCR assay was applied to investigate the variation in the abundance of integrase genes, and high-throughput quantitative PCR (HT-qPCR) was used to evaluate the number, abundance and diversity of ARGs and identified the potential drivers shaping the resistome in soils.

2. Materials and methods

2.1. Sample collection and DNA extraction

Paddy soil was collected from a field experiment in the experiment station of Zhejiang Academy of Agricultural Sciences (30° 49′N 120° 42′E, Jiaxing, China), and soil samples (0–20 cm) were collected in November 2017. Pig manure and chemical fertilizer have been applied to soils since 2007, aiming to explore the effects of long-term manure application on microbiome and resistome in paddy soils. Three treatments were set, including the soils with manure (P5), chemical fertilizer (P6), and control (P7). Each treatment has three plots except the control treatment (two plots). For each plot, one composite soil was collected, which was made by physically mixing five soil cores taken within a plot area (2.5 m × 2.5 m) into one homogenous sample. The collected samples were transported to the laboratory on ice within 24 h and stored at −20 °C before DNA extraction.

One hundred liters of raw sewage samples were collected from a municipal wastewater treatment (Xiamen, China) in 2018. Triplicate samples of each influent, sludge (anoxic, aerobic and activated sludge) and effluent were collected from a membrane bioreactor (MBR) running for 7 day. Bacterial assemblages were collected by filtering 200 mL of influents and 600 mL of effluents through 0.22-μm cellulose nitrate filters (Milipore, USA) and by centrifuging 2 mL of sludge at 10000 ×*g* and 4 °C for 20 min. The filters and the sludge pellets were kept at −20 °C until DNA extraction.

DNA extraction was performed by using FastDNA SPIN Kits for soil (MP Biomedicals, USA) according to the manufacturer's instructions. DNA concentration was measured using Qubit 4 Flurometer with a Quant-iT™ PicoGreen dsDNA Assay Kit (ThermoFishier, USA).

2.2. Determination of heavy metals and antibiotics in soils

The contents of heavy metals, including chromium (Cr), manganese (Mn), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd) and lead (Pb) were determined using inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo iCAP 6000, UK). Briefly, the soil samples were dried at 105 °C and homogenized by sieving through a 0.15-mm mesh. Approximately 1 g of soils was acid-digested with an HCl-HNO_3 mixture, and all determinations were performed in triplicate.

The antibiotic residues, including ten sulfonamides, five fluoroquinolones, three macrolides, three chloramphenicols, six non-steroidal anti-inflammatory drugs and one beta-lactam were extracted using the mixture of acetonitrile: $Mg(NO_3)^{2-}$ $NH_3{\cdot}H_2O$ (v/v, 3: 1) basing on a solid-phase extraction method (Table 1) (Huang et al., 2013). The extraction was analyzed using an LC-MS/MS (Agilent 7000D GC/TQ, USA).

Table 1
Concentrations of heavy metals and antibiotics in soils. ND, not detected; a and b represent the significant difference by One-way ANOVA analysis.

Heavy metals & antibiotics	mg/kg	P5	P6	P7
Heavy metals	Cr	36.09 ± 21.83	62.00 ± 1.01	60.54 ± 1.09
	Mn	518.12 ± 0.23	517.93 ± 0.13	517.77 ± 0.03
	Ni	30.47 ± 2.03	30.33 ± 1.47	29.97 ± 0.04
	Cu	56.29 ± 34.63	29.96 ± 11.96	22.07 ± 2.01
	Zn	159.58 ± 76.43	84.44 ± 27.11	69.80 ± 0.24
	As	5.37 ± 7.25	15.19 ± 1.99	12.54 ± 1.39
	Cd	0.25 ± 0.02[a]	0.15 ± 0.03[b]	0.17 ± 0.05[b]
	Pb	14.43 ± 15.39	28.36 ± 0.57	27.96 ± 1.53
Sulfonamides	Sulfamethoxazole	0.04 ± 0.07	ND	ND
	Sulfamethazine	0.504 ± 0.18[a]	ND[b]	ND[b]
	Sulfamonomethoxine	0.18 ± 0.168	ND	ND
	Sulfadiazine	ND	ND	ND
	Sulfamerazine	ND	ND	ND
	Sulfaquinoxaline	ND	ND	ND
	Sulfadimethoxine	ND	ND	ND
	Sulfameter	ND	ND	ND
	Sulfaclozine	ND	ND	ND
	Sulfathiazole	ND	ND	ND
	Trimethoprim	ND	ND	ND
Fluoroquinolones	Norfloxacin	0.32 ± 0.558	ND	0.26 ± 0.37
	Ciprofloxacin	1.07 ± 0.59[a]	0.11 ± 0.11[b]	0.12 ± 0.17[b]
	Enrofloxacin	9.85 ± 7.71	0.02 ± 0.04	ND
	Difloxacin	0.07 ± 0.06	0.06 ± 0.06	0.03 ± 0.01
	Ofloxacin	ND	ND	ND
Macrolides	Erythromycin-H_2O	ND	ND	ND
	Roxithromycin	0.337 ± 0.57	ND	ND
	Tylosin	ND	ND	ND
Chloramphenicols	Chloramphenicol	0.72 ± 0.35	0.47 ± 0.41	1.4 ± 1.98
	Thiamphenicol	ND	ND	ND
	Florfenicol	ND	ND	ND
Beta-lactam	Penzylpenicillin	1.34 ± 1.35	0.44 ± 0.77	ND

2.3. qPCR analysis for integrase genes

Class 1, class 2 and class 3 integron-integrase genes (*intI1*, *intI2*, and *intI3*) and 16S rRNA gene were quantified as previously reported (An et al., 2018a). Briefly, qPCR assays were conducted in triplicate by using SYBR® Premix ExTaq™ II (Takara, Japan) on a Roche 480 (Roche Inc., USA). Negative control was included in each run. The primers and amplification reaction conditions were used according to the previously reported (Stubner, 2002; Barraud et al., 2013). Standard curve for each gene was constructed using the 10-fold serial dilutions of the plasmid carrying the corresponding gene target. The Ct values were used to obtain the absolute abundance (copies/L) of all target genes based on standard curve with R^2 values more than 0.99. Quantifications for integrase genes were normalized (relative abundance, copies/copy of 16S rRNA gene) by dividing the absolute abundance of each gene by the absolute quantification of 16S rRNA gene.

2.4. High-throughput qPCR (HT-qPCR) assays for ARGs in paddy soils

ARGs quantification for paddy soils was performed using a WaferGen Smart-Chip Real-Time PCR system (WaferGen Inc. USA), as described previously (Wang et al., 2014). Briefly, 5184 100-nL reactions were dispensed into the SmartChip using a SmartChip Multisample Nanodispenser, and three technical replicates were included for each sample-primer set combination. Sterilized water was used as a negative control. Amplification conditions for HT-qPCR and data analysis were performed according to the description by Wang et al. (2014). A threshold cycle of 31 was set as the detection limit, and genes detected in all tree technical replicates were considered true positives. Calculations for ΔC_t and relative abundance were conducted as previously reported (Zhu et al., 2013).

2.5. MinION sequencing for class 1 integron gene cassettes

The gene cassette arrays of class 1 integron were amplified using the reported primers MRG284 and MRG285, which target the variables between *intI1* and immediately after the final *attC* site in the array (Gillings et al., 2009). The resulting amplicons were purified using a universal DNA purification kit (Tiangen, China) and a KAPA Pure Beads (Roche, Switzerland). The concentration of the purified PCR products was evaluated by a QubitTM dsDNA Assay Kit (Invitrogen, USA).

1D ligation library approach from Oxford Nanopore Technology (ONT) was applied for library preparation. One ug purified amplicons for each sample were used without fragmentation. End repair and dA-tailing were performed using NEBNext FFPE DNA Repair Mix (New England BioLabs, 6630) and NEBNext End repair/dA-tailing Module (New England BioLabs, 7546) reagents in accordance with nanopore's protocol. Native barcodes and adapters were ligated for each sample using NEBNext Quick Ligation Module (New England BioLabs, 6056) and native barcoding genomic DNA kit (SQK-LSK-109, Oxford Nanopore Technologies). Flowcell priming and library loading were conducted according to the kit's protocol (SQK-LSK-109, Oxford Nanopore Technologies). Constructed library sequencing was run using a MinION sequencer from ONT equipped with a flowcell of FLO-MIN106 R9.4.1.

Basecalling of nanopore reads were performed in MinKNOW with the high accuracy model (Q-score cut-off >7). Barcoding/demultiplexing was performed according to Guppy's protocol (v3.2.4), and adapters on the ends of reads were trimmed off and reads with internal adapters were discarded using porechop (version 0.2.3) with the default parameters. The potential ARGs were annotated by BLASTN 2.9.0+ against CARD database (e-value = 1e−10; identity ≥ 90%; coverage ≥ 90%) (https://card.mcmaster.ca/?q=CARD/ontology/35506). Integron_Finder (v1.5) was applied to detect *attC* sites with the use of cmsearch (v1.1.4), hmmsearch (v3.1b1) and infernal (v1.5.1) profiles against an attC database with the "–local_max" option (https://integronfinder.readthedocs.io/en/latest/user_guide/introduction.html) (Cury et al., 2016). The putative gene cassettes and gene cassette arrays were interrogated by searching against INTEGRALL database (http://integrall.bio.ua.pt/) using a BLAST algorithm.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) and significance testing were performed using SPSS v26.0 (IBM, USA), and nonparametric Kruskal-

Wallis tests were applied to assess any statistically significant ($P < 0.05$) differences when data were from a suspected non-normal population. PCA analysis based on Bray-Curtis dissimilarity and Adonis test were performed in R 3.2.3 (R Foundation for Statistical Computing: Vienna, Austria, 2014) with vegan 2.0–10. Network was visualized using Gephi 0.9.1 software.

3. Results

3.1. Heavy metals, antibiotics and ARGs in soils

No distinct variation in the concentrations of heavy metals was observed among the manure-applied (P5), chemical fertilizer-applied (P6) and control (P7) soils except Cd concentration. Higher Cd concentrations were detected in the manure-amended soils than the no-manure applied treatments (Table 1). Ten antibiotics were detected in all samples, including three sulfonamides (sulfamethoxazole, sulfamethazine and sulfamonomethoxine), four fluoroquinolones (norfloxacin, ciprofloxacin, enrofloxacin and difloxacin), chloramphenicol, roxithromycin and penzylpenicillin. Manure-applied soils were found to have higher concentrations of sulfamethazine and ciprofloxacin compared with inorganic fertilizer-applied and control treatments.

A total of 138 ARGs and 5 MGEs were detected from all soils with the relative abundance ranging from 0.04 to 0.60 copies/copy of 16S rRNA. The detected ARG number (Kruskal-Wallis test, $P < 0.05$) and abundance (one-way ANOVA, $P > 0.05$) were increased with the application of manure (Fig. 1a and b). ARGs conferring resistance to macrolide-lincosamide-streptogramin B, multidrug and vancomycin were the most dominant in soils. PCA analysis showed that no distinct cluster of resistome was observed with the treatments (Fig. 1c), and Adonis test indicated no significant correlation between environmental factors (heavy metals or antibiotics) and ARG distribution.

3.2. Abundance of integrase-genes

The *intI1*, *intI2* and *intI3* were detected in all analyzed sewage or soil samples, and *intI1* and *intI2* were more prevalent than *intI3* (Fig. 2). The absolute abundances of *intI1* (one-way ANOVA, $P < 0.05$) and *intI2* (one-way ANOVA, $P < 0.05$) were significantly increased with the manure application in soils, while the *intI3* abundance in the manure-applied soils was decreased when comparing with the no-manure applied soils (one-way ANOVA, $P < 0.05$) (Fig. 2a and b). For the sewage samples, the sewage treatment reduced 94.1%–99.9% of the absolute abundance of integrase-genes (~3 orders of magnitude, copies/L) (Kruskal-Wallis test, $P < 0.05$), whereas a considerable amount of integrase-genes were detected in the effluents (Fig. 2c and d). Approximately 99.3% of bacterial abundance (16S rRNA gene, copies/L) was also removed through the sewage treatment process (Kruskal-Wallis test, $P < 0.05$), corresponding to ~2 orders of magnitude in its concentrations.

The relative abundances of *intI1* (one-way ANOVA, $P < 0.05$) and *intI2* (one-way ANOVA, $P < 0.05$) were also significantly increased in the manure-applied soils, ranging from 2.0×10^{-5} to 0.2 copies/copy of 16S rRNA. The relative abundance of *intI3* was decreased with the addition of manure into soils (one-way ANOVA, $P < 0.05$), with the range from 8.3×10^{-9} to 6.1×10^{-7} copies/copy of 16S rRNA (Fig. 2b). The relative abundances of integrase-genes were distinctly decreased across the sewage treatment (~1 orders of magnitude, copies/copy of 16S rRNA) (Kruskal-Wallis test, $P < 0.05$), ranging from 2.8×10^{-6} to 0.2 copies/copy of 16S rRNA (Fig. 2d).

3.3. Diversity of class 1 integron gene cassettes and gene cassette arrays

Nanopore sequencing generated a total of 82,795 reads from the sewage and soil samples, and read length N_{50} was 1628 bp (Table S1). After quality trimming and sequence demultiplexing, median read number for each sample was 2437, ranging from 321 to 27,821. A high proportion of reads (32.1%) were unable to be demultiplexed into specific samples, for example, no reads could be classified into the sample ADS2 according to the barcode sequences (Table S2).

Since ARGs are of major concern in class 1 integrons, only ARGs embedded in the variable regions were annotated, and the arrangement of ARG cassettes was characterized. Approximately 2.2%–13.7% of trimmed and demultiplexed reads carried ≥1 resistance gene cassette in sewages, and sewage treatment decreased the percentage of antibiotic resistance-associated sequences with the highest proportion (9.6% of total reads) of ARG-carried sequences in the influents (Table S3). A total of 58 unique ARG cassettes were found in sewages, in which 38 unique resistance cassettes were detected in influents, and 23 unique cassettes were found in effluents (Fig. 3b). Ten ARGs-associated gene cassettes were observed to persist during the sewage treatment in the bioreactor. These functionally annotated ARGs were mostly affiliated with the resistance to aminoglycoside, beta-lactam, diaminopyrimidine, lincosamide, macrolide, phenicol, rifamycin, and sulfonamide, of which aminoglycoside and beta-lactam resistance genes were the predominant in sewages, taking accounting for 81.2%–96.2% of total ARGs (Fig. 3a). Influents harbored a high proportion of diaminopyrimidine resistance genes (8.3%), and the genes encoding rifamycin (6.7%) and sulfonamide (6.5%) resistance were dominant in effluents.

For soil samples, the ARG cassettes-carried sequences took a small proportion of all trimmed and demultiplexed sequences, ranging from 0% to 1.0%. The manure-applied soils were observed to harbor a higher percentage of ARG cassette-associated sequences (0.6%) compared with the control soils (0.03%) and inorganic fertilizer applied soils (0.01%). Eleven unique ARGs-related cassettes were detected in the soils, of which ten unique resistance cassettes were found in manured soils and three unique cassettes were observed in soils without manure (Fig. 3c and Table S4). These annotated ARG cassettes were related to the resistance to aminoglycoside, macrolide, beta-lactam and sulfonamide, and sulfonamide and aminoglycoside resistance cassettes were more prevalent than macrolide and beta-lactam resistance-associated cassettes (Fig. 3b).

A majority of ARG cassettes were present in sequences in the form of single resistance cassette instead of resistance gene cassette array, and most of them were not matched with the INTEGRALL database. For

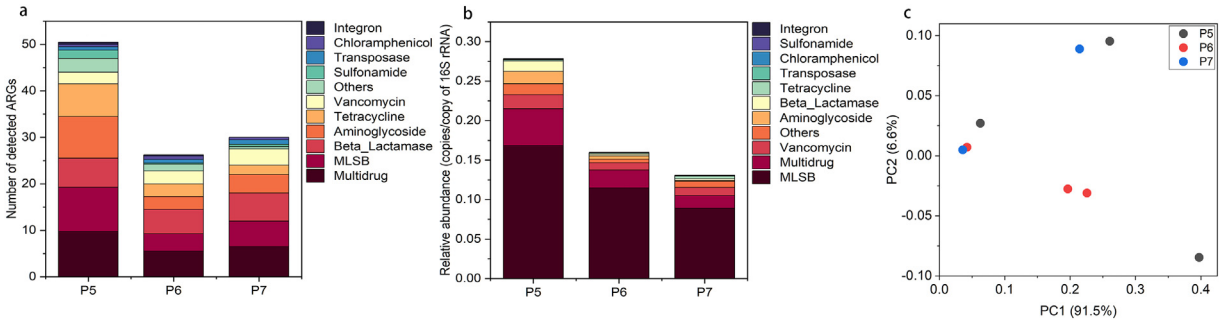

Fig. 1. Distribution of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in manure-applied (P5), chemical fertilizer-applied (P6) and control (P7) soils. (a) the detected number of ARGs in soils; (b) relative abundance (copies/copy of 16S rRNA) of ARGs in soils; (c) PCA analysis showed the distribution of ARGs in soils.

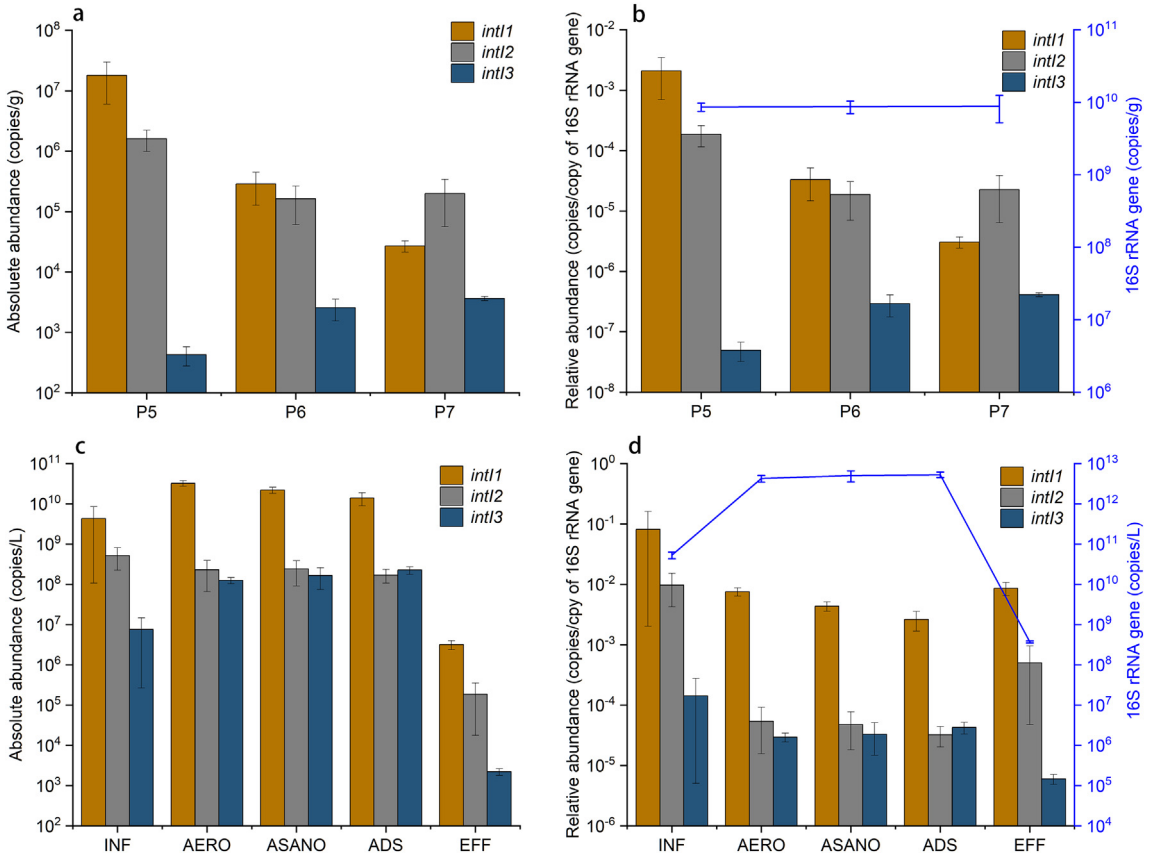

Fig. 2. Abundance of integrase genes in soils and sewages. (a) and (b) absolute abundance (copies/g) and relative abundance (copies/copy of 16s rRNA gene) of integrase genes in soils; (c) and (d) absolute abundance (copies/L) and relative abundance (copies/copy of 16s rRNA gene) of integrase genes in sewages.

example, single resistance gene cassettes of *aadA8b* (a hybrid cassette), *catB2* (a plasmid-encoded variant of the *cat* gene) and *Sul4* (a new sulfonamide resistance gene) were not detected in the INTEGRALL database. Additionally, forty unique resistance gene cassette arrays were identified in sewages and soils, of which 37 unique GC arrays carried two resistance cassettes, three unique arrays contained three GCs, and no array carried >3 gene cassettes (Table 2). ARGs conferring resistance to aminoglycoside were frequently found in the resistance GC arrays and mostly were the first GC in the arrays (60% of 65 gene arrays). The array |AAC(6′)-Ib9|AAC(6′)-Ib9| was most frequently detected in sewage samples. The arrangement of most ARG cassette arrays was not observed in the INTEGRALL database, e.g., |AAC(6′)-Ib9|AAC(6′)-Ib9|aadA11| and |sul4|catB3|AAC(6′)-Ib9|.

4. Discussion

Integrons are bacterial genetic elements involving in the environmental dissemination of antibiotic resistance, enabling to acquire, exchange and express ARGs embedded within gene cassettes. In this study, we focused on the contents of gene cassettes in the sewage and soils and the impact of human activities (e.g. sewage treatment and manure application) on the antibiotic resistance carried by class 1 integrons. HT-qPCR analysis for soil resistome suggested that long-term manure amendment significantly facilitated the enrichment of ARGs, indicating that manure application exacerbated the augment and persistence of ARGs in the agricultural soils. These data highlight that prudent manure application is essential for alleviating the spread of ARGs when providing available nutrients from manure for crop growth in the soils (Heuer et al., 2011; Chen et al., 2016). Laboratory experiments have demonstrated that the accompanying antibiotics and heavy metals provided a selective pressure on the occurrence of ARGs in the manure amended soils, increasing the accumulation of resistance genes and resistant bacteria (Chen et al., 2016; Ruuskanen et al., 2016; Liu et al., 2021). Although the concentrations of heavy metals and antibiotics were increased in the manured soils, these factors showed no association with the distribution profile of ARGs in this study, which could be attributed to the low bioavailability of antibiotic and heavy metal residues owing to their strong adsorption to particular matters in soils (Song et al., 2017; Rutgersson et al., 2020). Other antibiotic resistance determinants, e.g. mobile genetic elements (integrons, plasmids and transposons) and microbial community, possibly facilitate spread and acquisition of diverse ARGs and influence the resistome profile in soils (Domingues et al., 2012; Partridge et al., 2018).

Class 1 integron-integrase gene (*intI1*) was more prevalent than *intI2* and *intI3* in either soil or sewage. The possible explanation was that *intI1* was more active than *intI2* and *intI3* and thus considered as a proxy for anthropogenic pollution. Previous studies showed that integrons could come with bacteria from manure into soils and colonized the soils (Heuer et al., 2011). Our study found that the abundance of *intI1* and *intI2* significantly increased in the manured soils. This indicated that manure application possibly facilitated the colonization and proliferation of *intI1*/*intI2*-carrying bacteria in the overall bacterial community, since the total bacterial abundance showed no variation in soils. The *intI3* abundance was observed to decrease with the manure amendment. However, no possible explanation could be deduced to elucidate the low prevalence of *intI3* in the manure-applied soils owing to the limited studies on class 3 integrons. For sewage samples, the treatment process could effectively remove the class 1, 2 and 3 integrons with ~3 orders of magnitude, which was consistent with our previous study of municipal wastewater treatment plants (An et al., 2018a). The distinct reduction of bacterial biomass largely contributed to the variations in the integron abundance in effluent. However, massive

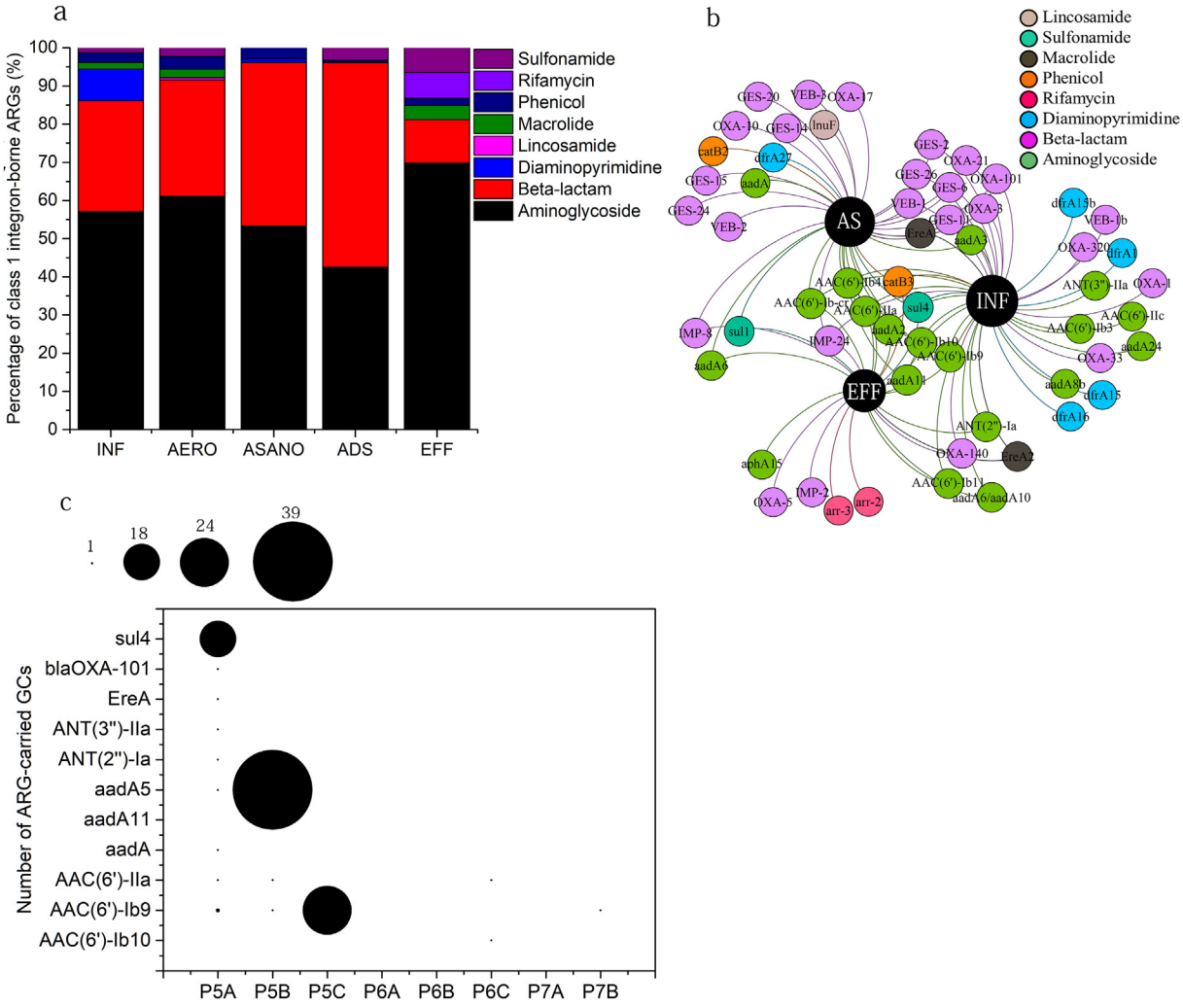

Fig. 3. Distribution of ARGs embedded in gene cassettes in the sewage and paddy soils environment. (a) Proportion of different ARGs in gene cassettes during the sewage treatment. (b) Diversity and persistence of antibiotic resistance gene cassettes across the sewage treatment. One ARG was considered to be present in one treatment process when the gene was detected in any one of parallel samples. The circle nodes stand for different ARGs carried by gene cassettes. INF: influent; EFF: effluent; AS: sludge. (c) Detection number and distribution of ARGs embedded in gene cassettes. The size of circle represents the detected number of antibiotic resistance-associated gene cassettes.

integrons were persisted in the effluent, representing potential risks of ARGs-carried integrons spreading into downstream environments (Ma et al., 2017).

Integrons are considered to be the vital vectors of ARGs, whereas it is changeling to monitor the ARGs-associated gene cassettes in complex environmental compartments owing to limitations of Sanger Sequencing or Next Generation Sequencing (Gatica et al., 2016). In this study, we applied Nanopore sequencing for long DNA or RNA fragments to uncover the contents of gene cassettes in class 1 integrons. A large diversity of resistance gene cassettes was observed in sewages. Antibiotic resistance cassettes-associated sequences took account for a higher proportion in sewage (2.3%–13.7%) than in soils (0%–1.0%), which was consistent with previous observations in the wastewater treatment plants (Gatica et al., 2016; An et al., 2018a) and soils (An et al., 2018b). These data further indicated that sewage was the important reservoir for the gene cassettes encoding antibiotic resistance. The aminoglycoside and beta-lactam resistance-associated gene cassettes were the most prevalent in the sewages, which were also frequently detected in other environments, e.g., effluents in sewage treatment plants (Gatica et al., 2016; An et al., 2018a; Yang et al., 2021), polluted marine sediments (Elsaied et al., 2010), and soils (Holmes et al., 2003). Comparison of GCs in the influent and effluent revealed a decreased shift in the prevalence of ARGs-associated gene cassettes, indicating that sewage treatment removed these ARGs-gene cassettes. However, a portion (6.2%) of resistance gene cassettes-carried sequences was persistent in effluents. Although previous studies suggested that the low survival rate of bacteria harboring effluent-derived resistance gene cassettes possible caused the limited dissemination of integron-harboring mobile genetic elements to soil microbiome (Gatica et al., 2016; An et al., 2018a), there still existed a potential risk of the spread of ARG-harboring cassettes from effluent to the receiving soil or aquatic environments.

Manure is a vast ARG reservoir, and recent studies have highlighted that manure amendment practice increased the mobile genetic resistance elements pool (Binh et al., 2008; Heuer et al., 2011; Chen et al., 2016). In this study, the proportion of ARG cassettes-barboring sequences and the detected number of antibiotic resistance-associated GCs were increased in the amended soils. These data were consistent with previous studies, indicating that the long-term organic fertilizer application increased the diversity of ARGs in the gene cassettes and might drive the shuffling of antibiotic resistance gene cassettes (Binh et al., 2009; An et al., 2018b). This also further underlined the effect of anthropogenic activities on the dissemination of antibiotic resistance in soils. Therefore, direct manure application should be prudently considered owing to the potential spread of ARGs mediated by integrons from manure to soils. Unlike sewage samples, aminoglycoside

Table 2
Antibiotic resistance-carried gene cassette arrays in class 1 integrons detected in soils and sewages based on Nanopore sequencing.

Synonym of GC array in INTEGRALL	GC array	Sample	Detected number
|AacA4|AacA4|	|AAC(6′)-Ib10|AAC(6′)-Ib10|	EFF1	1
|AacA4|aadA11|	|AAC(6′)-Ib10|aadA11|[a]	AERO1	1
|AacA4|aadB|	|AAC(6′)-Ib10|ANT(2″)-Ia|	P6C; EFF2	5
|AacA4|blaIMP-2|	|AAC(6′)-Ib10|blaIMP-2|[a]	EFF1; EFF2	2
|AacA4|blaIMP-24|	|AAC(6′)-Ib10|blaIMP-24|[a]	AERO1	1
|AacA4|AacA4|	|AAC(6′)-Ib4|AAC(6′)-Ib4|	AERO4	1
|AacA4|AacA4|	|AAC(6′)-Ib4|AAC(6′)-Ib9|	AERO1	1
|AacA4|AacA4|	|AAC(6′)-Ib9|AAC(6′)-Ib4|	ADS1	1
|AacA4|AacA4|	|AAC(6′)-Ib9|AAC(6′)-Ib9|	INF2; AERO1; AERO2; ADS1; ASA1	10
|AacA4|AacA4|aadA11|	|AAC(6′)-Ib9|AAC(6′)-Ib9|aadA11|[a]	EFF3	1
|AacA4|aadA11|	|AAC(6′)-Ib9|aadA11|[a]	EFF1; EFF3	2
|AacA4|aphA15|	|AAC(6′)-Ib9|aphA15|	EFF3	1
|AacA4|catB3|	|AAC(6′)-Ib9|catB3|	INF1; ASA3	2
|AacA4|EreA|	|AAC(6′)-Ib9|EreA|	INF1	1
|AacA4|blaIMP-24|	|AAC(6′)-Ib9|blaIMP-24|[a]	EFF3; ADS1; ADS2	6
|AacA4|blaIMP-8|	|AAC(6′)-Ib9|blaIMP-8|	AERO1	1
|AacA4|aadA6|aadA10|	|AAC(6′)-Ib-cr|aadA6|aadA10|[a]	EFF2	1
|AacA4|catB2|EreA|	|AAC(6′)-Iia|catB2|EreA|[a]	AERO3	1
|aadA11|AacA4|	|aadA11|AAC(6′)-Ib9|[a]	INF2	1
|aadA11|sul4|	|aadA11|sul4|[a]	P5B	1
|aadA3|blaOXA-3|	|aadA3|blaOXA-3|[a]	ADS1	1
|aadA6|AacA4|	|aadA6|AAC(6′)-Ib-cr|[a]	EFF2	1
|aadA|sul4|	|aadA|sul4|[a]	ADS1	1
|arr-2|sul1|	|arr-2|sul1|[a]	EFF1	1
|arr-3|sul1|	|arr-3|sul1|[a]	EFF3	1
|catB3|AacA4|	|catB3|AAC(6′)-Ib10|[a]	EFF2	1
|catB3|AacA4|	|catB3|AAC(6′)-Ib-cr|[a]	EFF1	1
|EreA|AacA4|	|EreA|AAC(6′)-Ib3|[a]	INF1	1
|EreA|AacA4|	|EreA|AAC(6′)-Ib9|[a]	P5A	1
|blaIMP-8|AacA4|	|blaIMP-8|AAC(6′)-Ib9|	EFF3; AERO2	2
|blaOXA-21|AacA4|	|blaOXA-21|AAC(6′)-Ib9|[a]	AERO2	1
|blaOXA-21|aadA2|	|blaOXA-21|aadA2|[a]	ASA2	1
|blaOXA-3|AacA4|	|blaOXA-3|AAC(6′)-Iic|[a]	INF1	1
|blaOXA-3|aadA2|	|blaOXA-3|aadA2|[a]	AERO1; ADS1; ASA2	4
|blaOXA-3|aadA3|	|blaOXA-3|aadA3|[a]	AERO1	1
|blaOXA-5|AacA4|	|blaOXA-5|AAC(6′)-Ib-cr|[a]	EFF1	1
|sul4|aadA|	|sul4|aadA|[a]	P5A	1
|sul4|aadA11|	|sul4|aadA11|[a]	AERO3	1
|sul4|AacA4|	|sul4|ANT(3″)-Iia|[a]	P5A	1
|sul4|catB3|AacA4|	|sul4|catB3|AAC(6′)-Ib9|[a]	EFF3	1

[a] Indicated that the resistance gene cassettes were not found in INTEGRALL database.

and sulfonamide resistance gene cassettes were predominant in soils. One possible explanation is that aminoglycosides and sulfonamides are still broadly used in animals for treatment, growth promotion, and prophylactic purposes (Razavi et al., 2017). The antibiotic residues would have the potential of promoting the transfer of ARGs by integrons.

Numerous resistance gene cassettes and gene cassette arrays were not matched against the INTEGRALL database, implying that the pool of ARG cassettes and their arrays remained largely unexplored and recombination of gene cassette stayed active in environments (especially sewages) (Huang et al., 2009; An et al., 2018a; Yang et al., 2021). For example, *aadA8b* is created by a recombination event between *aadA1* and *aadA2* (Gestal et al., 2005), and *Sul4* (the fourth mobile sulfonamide resistance gene) is a recently described novel resistance genes (Razavi et al., 2017; Marathe et al., 2019), which was first described from sewages and soils. In this study, aminoglycoside resistance cassettes were frequently located in class 1 integrons (mostly the closest to *intI1*), and a limited number of resistance gene cassettes (usually 2 or 3 cassettes) were carried by class 1 integrons. The cost of class 1 integrons could explain the frequency, location and number of resistance gene cassettes in class 1 integrons. Previous studies showed that the gene cassette contents and numbers affected the cost of class 1 integron, and the aac(6′)-Ib gene cassette was the costliest (Lacotte et al., 2017).

5. Conclusions

This study provided a profile of the abundance of ARGs and integrase genes, and the contents and diversity of resistance gene cassettes in sewages and soils. Long-term manure application could increase the abundance of the total ARGs and the diversity of ARGs embedded in gene cassettes. This highlighted that the agricultural practice of manure amendment should be prudently considered due to the raising risk of ARGs dissemination before direct applying into soils. Sewage treatment could significantly remove the ARGs abundance and the relative abundance of resistance gene cassettes-carrying sequences, while numerous ARGs and resistance gene cassettes were still persistent in effluents, posing a potential risk of ARGs spreading downstream. Therefore, integron-borne antibiotic resistance genes should be focused when developing the sewage treatment technology. These data would help deeply understand the dissemination of ARGs in environments, and provide the basis for guiding and monitoring efforts for antibiotic resistance in environments.

Data availability

The raw amplicons sequences were submitted to Sequence Read Archive (SRA) with the accession number PRJNA758193.

CRediT authorship contribution statement

Xin-Li An and **Olusegun K. Abass**: Methodology, Investigation, Visualization, Data curation, Writing-Original draft preparation. **Qiang Pu** and **Ting Pan**: Methodology, Formal analysis. **Cai-Xia Zhao** and **Mei-Rong Xu:** Validation, Resources. **Hu Li** and **Hu Liao**: Software. **Jian-Qiang Su** and **Yong-Guan Zhu**: Conceptualization, Supervision, Writing-Reviewing and Editing, Project administration.

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.scitotenv.2021.152766.

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