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Exploration of the antibiotic resistome in a wastewater treatment plant by a nine-year longitudinal metagenomic study

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ABSTRACT

The spread of antibiotic resistance genes (ARGs) is a growing global problem. Activated sludge (AS) in wastewater treatment plants (WWTPs) has been proposed as a hotspot for ARGs. However, few studies have been conducted to uncover the temporal dynamics of the resistome of AS in WWTPs by long-term longitudinal sampling. In this study, we quantified ARGs and identified their host microbiome in a Hong Kong WWTP in 97 monthly AS samples spanning 9 years. Throughout this analysis, we demonstrated that both the abundance and structures of the resistome changed significantly every two to three years, implying that there was a successive selection of resistomes in the AS system over the study period. The detection of genes of antibiotic-resistant pathogens that are emerging major threats to public health in the AS samples, including *mcr*, CRE (carbapenem-resistant *Enterobacteriaceae*) and MRSA (methicillin-resistant *Staphylococcus aureus*)-related genes, highlight the role of WWTPs as reservoirs of ARGs. In addition, the core resistome (abundant and persistent genes) in AS were found to overlap with those in other ecosystems such as urban sewage, livestock feces, and fishpond sediments, revealing the broad dissemination of ARGs in WWTPs and other environments. Annual variation of resistomes were explained via structural equation modeling (SEM), which deciphered the structural linkages of determining factors such as the operational parameters, microbial community composition and horizontal gene transfer (HGT). Specifically, potentially relevant antibiotic resistance bacteria (ARBs) were explored and discussed based on assembly-based analyses and network correlations. Moreover, consistent with the clear relationship between resistomes and mobile genetic elements (MGEs), it was found that there was a relatively high potential for gene exchange in AS in comparison with soil genomes, which could be explained by the engineering features of WWTPs. Based on these findings, longitudinal monitoring of WWTPs is warranted for risk assessment to reveal emerging ARGs, resistome evolution, correlations with ARBs, and the potential for spread in downstream environments and concomitant exposure risks for humans.

1. Introduction

The spread of antibiotic resistance genes (ARGs) has become one of the biggest health concerns in the 21st century. According to a review commissioned by the UK government in 2016, the annual total of worldwide deaths caused by resistance infections is ≥700,000 (O'Neill, 2016). ARGs are more than a clinical problem, and they have been detected in many different environments, such as drinking water (Ma et al., 2019), lakes (Czekalski et al., 2014, Di Cesare et al., 2015), rivers (Liu et al., 2018a), soil (Chen et al., 2016), wastewater treatment plants (WWTPs) (Yang et al., 2013), and plastics (Yang et al., 2019). In December 2017, the United Nations Environment Programme (UNEP) in its Frontier Report listed antimicrobial resistance (AMR) as the first out of six emerging issues of environmental concern (UNEP, 2017). WWTPs have been proposed to be hotspots for ARGs because bacteria/genes and antibiotic residues piped from various sources were collected in wastewater (Karkman et al., 2017). Moreover, the high biomass concentration and diverse bacterial communities (Ju and Zhang, 2015) of

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activated sludge (AS) systems in WWTPs could supply ideal conditions for the horizontal gene transfer (HGT) of ARGs. Our previous study showed that overall ARG pollution in AS is more serious than that in rivers and soils and is of the same order of magnitude as that in human feces (Li et al., 2015b). The persistence and mobility of ARGs exacerbates the risks these genes pose to human health in the post-antibiotic era (Lamba and Ahammad, 2017; Liu et al., 2018b; Lorenzo et al., 2018).

Several studies in the past decade have characterized ARGs and their relevant dissemination potential in AS samples. A plethora of spatiotemporal AS samples are needed to delineate the association of bacterial hosts and the resistome. Several studies were conducted using geographically distributed sample analysis (Hendriksen et al., 2019; Ju et al., 2018); however, few studies have been based on time-series datasets. Lamba and Ahammad (2017) applied qPCR to study four specific genes encoding resistance against extended-spectrum beta lactam- and carbapenem-resistant bacteria over two seasons. An et al. (2018) and Jiao et al. (2018) profiled dozens of ARGs in different seasons by high-throughput qPCR. However, improved studies are needed, as described below.

First, previous studies were only conducted on samples collected in very short periods at low sampling frequencies, which may not accurately reveal the dynamic changes in the resistome over a long period. Second, traditional PCR-based methods were limited by the availability of primers and by amplification bias, which was especially limiting given that a comprehensive overview is needed on the diversity and phylogenetic distribution of ARGs and ARG-carrying bacteria (Li et al., 2015b). In this study, we conducted a successive nine-year analysis of the same sampling site in an aeration tank to derive a high-resolution temporal profile of AS resistomes. In addition, a high-throughput sequencing (HTS)-based method was utilized to afford an unbiased and broad-spectrum survey of the ARG pollution, as it enabled quantification, characterization, and parallel comparison of a wide range of ARG types (Boolchandani et al., 2019). Metagenomic assembly was also used to determine the in-depth correlation of the resistome microbial community and the occurrence of gene exchange within the complex communities in AS (Li et al., 2015b; Schmieder and Edwards, 2011; Yang et al., 2013).

The high-throughput sequencing technology and customized analysis pipeline of this study enabled the application of both read-based and assembly-based approaches to search the whole resistome with an integrated database. This revealed the comprehensive dynamics of ARGs over an unbroken nine-year period, identified the bacterial hosts of the resistome in the AS of a local municipal WWTP, and located the core resistome in AS samples, highlighting the role of WWTPs as reservoirs for ARG proliferation and dissemination. Mobile genetic elements (MGEs) were also scrutinized in genomic contexts to probe the potential for the spread of ARGs. Taken together, the findings of this study provide new insights into gene types or ARG-carrying species that could threaten human health.

2. Materials and methods

2.1. Sampling and DNA extraction

The sampling site was Shatin wastewater treatment plant, which is the largest secondary WWTP in Hong Kong. This WWTP uses an anoxic/oxic (A/O) process with a treatment capacity of 216,000 m^3 day^{-1} from a population of 630,000. AS samples were collected monthly from the aeration tank from June 2007 to December 2015 (Table S1). Samples were mixed with absolute ethanol at a volume ratio of 1:1 for biomass fixation, then stored in a refrigerator at −20 °C. A 1-mL subsample of each sample was centrifuged to obtain a pellet of ~200 mg, which was subject to DNA extraction with a FastDNA™ Spin Kit for Soil (MP Biomedicals).

2.2. Metagenomic sequencing

The construction and sequencing of shotgun libraries were conducted on all DNA samples on an Illumina HiSeq4000 platform by the Beijing Genomics Institute (BGI). A collection of 97 datasets with a range of 3.5 to 7.7 Gbp was obtained and the total sequence length was 539.9 Gbp, which was the largest temporal sequence dataset of AS samples reported thus far. All data were uploaded onto the NCBI database and are labeled with Bioproject accession number PRJNA432264. The data has been published before in our recent paper themed on virome in WWTPs (Wang et al., 2018), the theme of which is totally different from this study.

2.3. Quantification of ARGs, bacteria and MGEs

Quality control of reads was conducted by fastp (v0.12.1) with default filtering parameters, and then filtered reads were used as inputs to the ARG-analysis pipeline ARGs-OAP v2.0, which integrated detection of ARGs using the reference database SARG v2.0. Quantification of ARGs as a unit of ARG copies per cell was calculated by normalizing ARG abundance to the cell number, which was derived from essential single-copy marker gene-estimation (Chen et al., 2018; Yin et al., 2018). The quantification in this unit reflected the concentration levels of the resistome in the microbial community more directly than the previously used unit, copy number of ARGs per copy number of 16S rRNA, which was obtained by examining the 16S copy number in different bacteria (Yang et al., 2016). For example, an abundance at 0.2 copy of ARGs per cell means that one resistance gene occurred in every five bacterial cells on average. However, for parallel comparison with other studies, the abundance information of ARGs was also determined in other units (ppm; copy number of ARGs per copy number of 16S) (see Supplementary Files).

For taxonomic classification, Kraken2 (v2.0.7), together with a customized complete genome *k*-mer database, was applied to clean reads (Wood and Salzberg, 2014). The classification results were further passed through Bracken 2.0 for relative abundance estimation of the taxa in each sample (Lu et al., 2017). To quantify MGEs in the samples, ARGs-OAP v2.0 was used, but the reference database was replaced by a recently published MGEs database (Parnanen et al., 2018). The unit in the quantification of MGEs was copy number of MGEs per cell.

2.4. Ordination and correlation analysis

Principal Coordinates Analysis (PCoA) was conducted on all samples with ARG abundance in subtype level using Bray-Curtis distance. Redundancy analysis (RDA) was conducted on the abundance of ARG types and all environmental parameters (such as temperature, salinity, pH, dissolved oxygen (DO), hydraulic retention time (HRT), mean cell-residence time (MCRT), mixed-liquor suspended solids (MLSS), bleach, and concentrations of N and P). Before RDA, Hellinger transformation was conducted on the ARG abundance and variance inflation was conducted on environmental parameters to remove redundancy. RDA was validated by assessing the significance of the constraints using a permutation test. Variation Partition Analysis (VPA) was used for interpretation of ARG types from bacterial communities at the class level and by MGE abundance (Jia et al., 2015). Bacterial abundance and MGE abundance were Hellinger transformed. R packages vegan (Oksanen et al., 2019), BiodiversityR (Kindt, 2016), and plotly (Sievert, 2019) were used to conduct the above analysis. Correlations of the resistome and bacterial composition were investigated using Procrustes analysis (9999 permutations) to correlate the ARG-subtype abundance profile with the relative abundance of species in each sample, supported by tool QIIME (Forsberg et al., 2014). A structural equation model (SEM) was constructed to evaluate the linkages between MCRT, bacterial abundance, MGE abundance, and ARG abundance using R package lavaan (Rosseel et al., 2019). The input SEM was a matrix of

the pairwise correlations between variables from a Mantel test conducted using the vegan package (Oksanen et al., 2019) in R. MCRT was an independent variable, while the bacterial and MGE abundances were two intermediate variables, and the ARG abundance was a response variable. A maximum likelihood estimation method was used to estimate model parameters. The best fit model was examined by the following fit statistics: chi square ($\chi^2 > 0.05$), goodness of fit (GFI > 0.95), normed fit index (NFI > 0.95), and root-mean-square error of approximation (RMSEA < 0.008) (Hu et al., 2016; Wang et al., 2019).

2.5. Detection of co-localizations of ARGs and MGEs on bacterial assembly

Clean shotgun sequencing reads were *de novo* assembled for each sample on CLC Genomic Workbench (v12.0, QIAGEN Bioinformatics) with default parameters. Kraken2 was applied on ARG-carrying contigs for taxonomic annotation (Wood and Salzberg, 2014). Pathogenic hosts of ARGs were identified by reference to a customized pathogen list (Li et al., 2015a; Table S2). The open reading frames (ORFs) on the assembled contigs were predicted using Prodigal (v2.6.3) (Hyatt et al., 2010). Afterwards, predicted ORFs were searched for ARGs using BLAST against the SARG v2.0 database (*e*-value: 1e−5, identity: 0.7, query length ratio: 0.8). Integrases were identified by BLAST against a reference database in the Integron Visualization and Identification Pipeline (I-VIP) (*e*-value: 1e−5, identity: 0.8, length ratio: 0.5) (Zhang et al., 2018b). Additionally, other MGEs were identified by keyword search (including integron, transposase, transposon, conjugative, conjugal, recombinase, recombination, mobilization and plasmid) after alignment against the NBCI-NR database (Forsberg et al., 2012).

2.6. Network analysis

The enrichment of ARGs in taxonomy was evaluated on ARG-carrying contigs by Fisher's exact test and *P* values were adjusted by using Benjamini-Hochberg methods to control the false discovery rate (Benjamini and Hochberg, 1995). Only significantly correlated pairs of taxonomy-ARGs ($P < 0.05$) were extracted for demonstration on networks using Cytoscape (v3.7.1) (Shannon et al., 2003).

2.7. Assessment of HGT-potential in AS

The incidence of ARGs encountering MGEs were compared between bacterial contigs in this study with three categories of complete genomes, i.e., human pathogens, non-pathogenic AS, and non-pathogenic soil. The calculation method was based on reported methods (Forsberg et al., 2014; Li et al., 2017). A list of complete pathogen and soil genomes listed on the NCBI was obtained from a recent paper (Forsberg et al., 2014), and a list of AS genomes was obtained from the IMG database (September 2019), with "Activated Sludge" in the "Ecosystem Type" field and "Sludge, Wastewater" in the "Habitat" field (Table S3). ARGs and MGEs were identified with the same method used for identifying assembled contigs. Then, the number of MGEs within the assigned distance (200–100,000 bp) from each ARG were counted, and the average count for each genome group was denoted the incidence of its encountering MGEs. Fisher's exact test was then used to evaluate whether the pairwise difference between genome groups is significant in the potential increase in HGT along the determined distances.

3. Results

3.1. The long-term occurrence and diversity of ARGs in AS

The extent of resistome richness was shown by the identification of 19 ARG types (based on the antibiotic the genes conferred resistance to) and 384 ARG subtypes (genotypes). The total count of ARG subtypes varied from 92 to 157 (Fig. 1a) across all sampling dates. The total abundance of ARGs also differed in samples, ranging from 0.144 to 0.358 copy of ARGs per cell. A generally decreasing trend was observed, with the average abundance of ARGs per cell being 0.283 copy in 2007 to 0.191 copy in 2015, which was consistent with the trend observed in our previous study (Yang et al., 2013).

PCoA analysis afforded yearly clustering data, showing the resistome composition of four consecutive time ranges were distinctly from each other, including Cluster 1 (2007–2009), Cluster 2 (2010.1–2011.5), Cluster 3 (2011.6–2014.9), and Cluster 4 (2014.10–2015.12) (Fig. 1b). However, no clear seasonal clustering was observed (Fig. S3).

3.2. The long-term dynamics profiles of ARGs related to widely used antibiotics types

As shown in Fig. 1, Figs. S4 and S5, significant differences in the abundance of most ARG types existed between 2009 (the end-year of cluster 1) and 2010 (the start of cluster 2), such as in genes for resistance to aminoglycosides, bacitracin, beta-lactams, chloramphenicol, tetracycline, and MLS (Mann-Whitney test, $P < 0.05$, Table S4). The abundance of bacitracin-resistance, which contributed 15% to the total number of ARGs, significantly increased in cluster 2; however, it returned in cluster 3 & 4 to the same level as in cluster 1, thus demonstrating a relatively stable trend over the sampling time range.

A continuous decrease in the abundance of aminoglycoside, chloramphenicol, and tetracycline resistance genes was observed from 2009 to 2011, with these three ARG types decreasing by 72%, 85%, and 77%, respectively. In contrast, the abundance of beta-lactam resistance genes fluctuated over time, increasing significantly in 2010 (the start of cluster 2), decreasing significantly in 2012 (the start of cluster 3), and then increasing again in 2014 (cluster 3), with an overall increase of 43%. More interestingly, the abundance of MLS and quinolone resistance genes exhibited seasonal variation. The abundance of MLS resistance genes reached a maximum in September–November in each year and dropped to a minimum abundance in January in each year, while the maximum abundance of quinolone resistance genes occurred in February or March in each of four years.

3.3. The persistent and abundant resistome identification

The 384 ARG subtypes detected in the AS samples were partitioned according to the frequency of their occurrence (Fig. 2). The category of greatest concern for human health was the persistent resistome, defined here as those ARG subtypes that occurred in over 95% of samples. This category comprised 41 subtypes of 13 different ARG types (listed in Table S5). Moreover, the persistent resistome tended to be more abundant than the less frequently occurring ARGs, as illustrated in Fig. 2d. Specifically, a small portion of the persistent resistome accounted for an average of 79% of the overall abundance of ARGs, and this proportion changed little over time (Fig. S6). This was consistent with a previous report that the core resistome over a two-year period accounted for an average of 70% of the whole sample ARG abundance (Munck et al., 2015). These highly persistent and abundant ARG subtypes were defined as the core resistome. The dynamics of each of these 41 core subtypes are shown in Figs. S7 and S8.

3.4. Detection of particular ARGs of emerging concern

A set of ARGs of particular concern were also detected, which included very recently discovered ARGs (the *sul4* gene (Razavi et al., 2017) and the *mcr*-1 gene and its variants (Feßler et al., 2018; Liu et al., 2016; Tijet et al., 2017)) and genes of other antibiotic-resistant pathogens that are emerging major threats to public health (MRSA-related genes such as *mecA, qacA, qacB, norA* (Garcia-Alvarez et al., 2011; Hryniewicz, 1999; Noguchi et al., 2005; Fischbach and Walsh, 2009) and carbapenem-resistant *Enterobacteriaceae* [CRE]-related genes such as KPC, NDM, OXA-48 and its variants, IMI-1, SME-1, IMP-1, VIM-1,

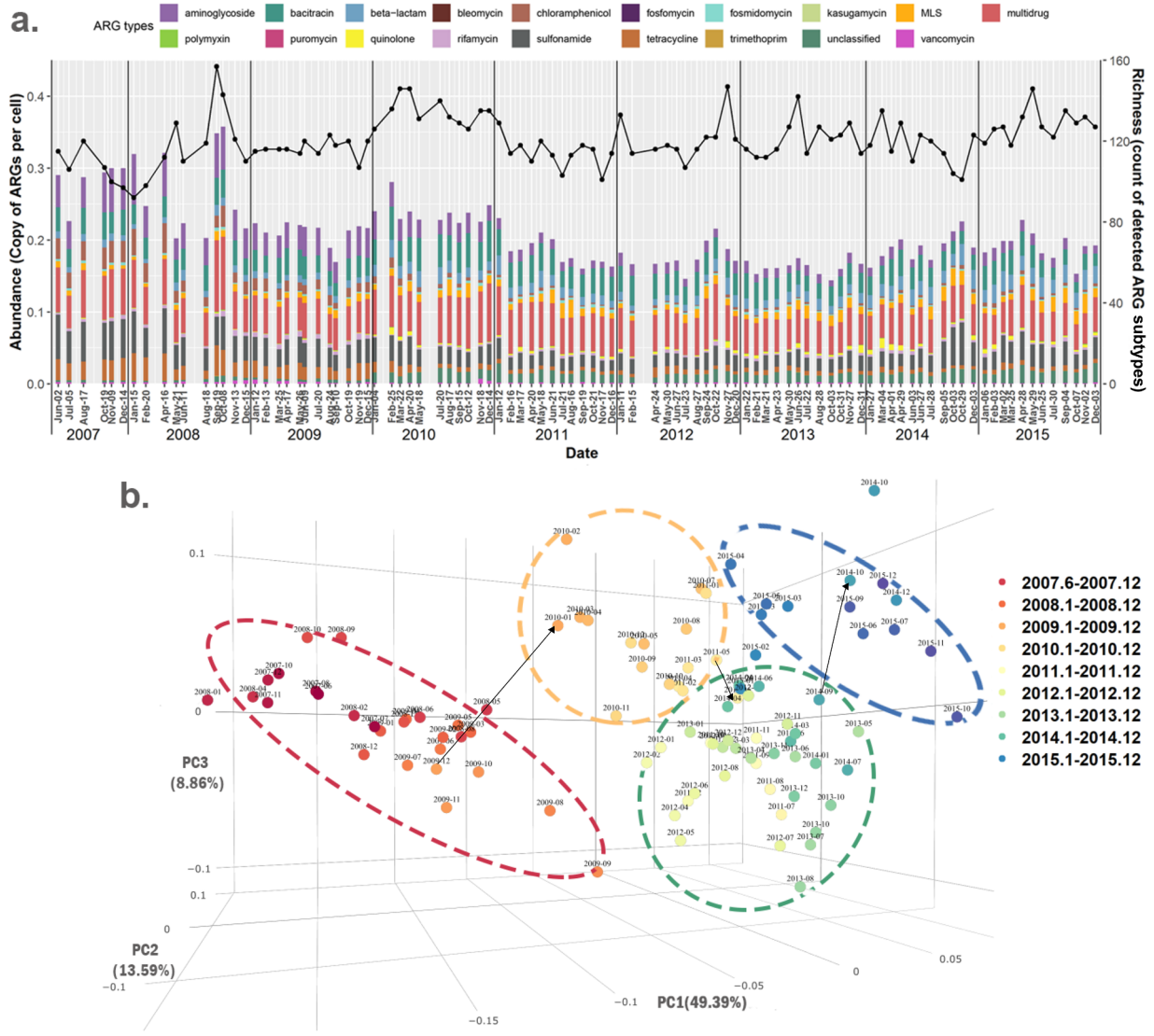

Fig. 1. The profile of monthly variations of ARG abundance in AS samples. a. Occurrence: abundance of ARGs, in copy of ARGs per cell (bar chart) and count of detected ARGs subtypes (line chart) per month. b. Structure: PCoA clustering of all samples based on the Bray-Curtis distance. MLS = Macrolide-Lincosamide-Steptogramin.

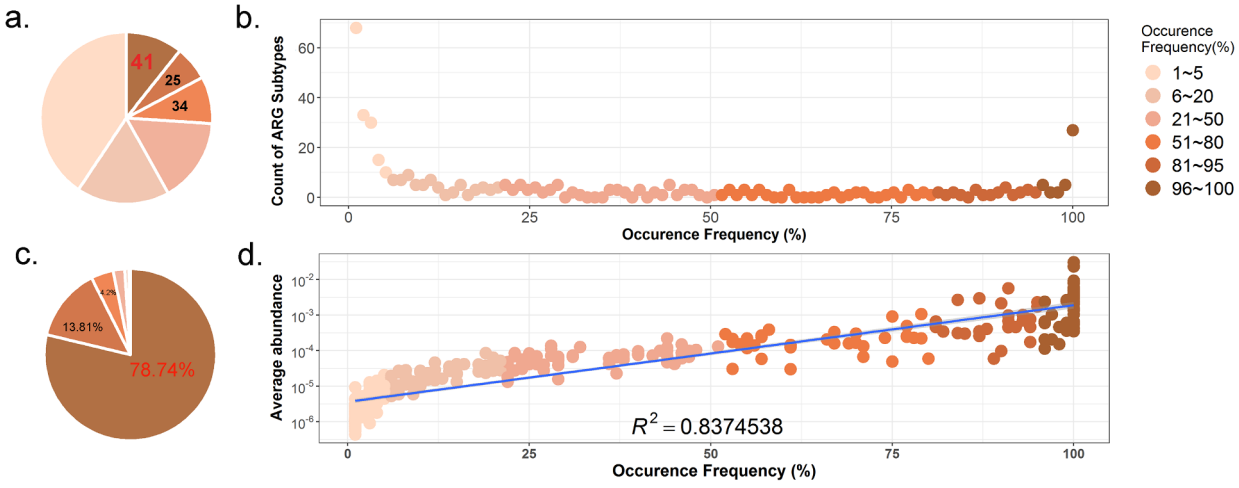

Fig. 2. Characterization of the resistome persistence over the nine-year study period. Pie charts summarize the percentages of six occurrence-frequency groups in terms of their total (a) count and (c) abundance. Each dot in (b) and (d) represents one detected ARG subtype in AS, with its intensity of color being proportional to its occurrence frequency. Occurrence frequency was defined as the number of occurrences divided by the total sample number (97). The R^2 value is 0.84 for the linear regression of (d).

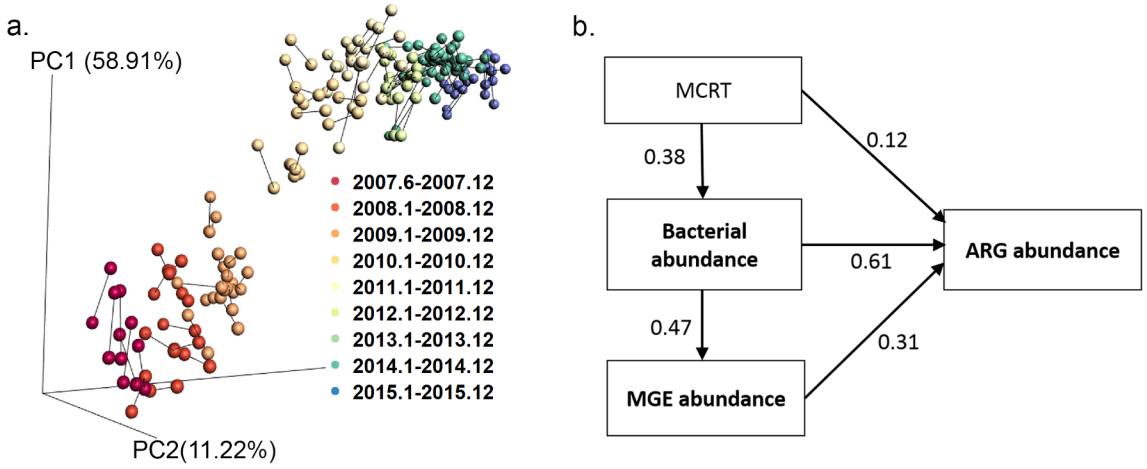

Fig. 3. The relationships between determining factors and the resistome in AS. a. Procrustes analysis of ARG subtypes with bacterial species (N = 97, $P < 0.001$, $M^2 = 0.194$, based on 9999 permutations). b. Structural equation models showing the connections between operational parameters (MCRT), bacterial abundance (class), MGEs and the ARG abundance (type) (N = 97, $\chi^2 = 0.499$). All shown arrows are significant ($P < 0.05$) and represent positive effects, and the numbers along the arrows represent their standardized regression weights.

IND-1, ccrA, GOB-1, and FEZ-1 (Bush and Jacoby, 2010; CDC, 2016; Evans and Amyes, 2014; Logan, 2012)).

3.5. The relationships between contributing factors to the resistome

The relationship between the environmental parameters and the resistome were analyzed by RDA. The results showed that MCRT significantly affected ARG composition (Fig. S11a, P < 0.05). In addition, Procrustes analysis revealed a significant correlation between the bacterial taxonomy and the resistome (Fig. 3a, Figs. S9 and S10, $P < 0.0001$, $M^2 = 0.194$ based on 9999 permutations), indicating that bacterial taxonomy had an effect on the resistome in AS. Furthermore, variation partitioning analysis (VPA) indicated that the bacterial community shift and MGEs contributed 34.9% and 2.2% to the resistome variation, respectively, while the combined effects of these factors had the greatest effect (39.8%) on shaping the resistome in AS (Fig. S11b).

To construct a model of the structural relationships between contributing factors and the resistome, structural equation modeling (SEM) was conducted. This showed that these factors had both direct and indirect effects on the abundance of ARGs (Fig. 3b, $\chi^2 = 0.499$, $p < 0.05$).

3.6. The correlation of the bacterial community with the resistome

To further explore the specific relationships between bacterial hosts and the ARG subtypes, the assembled contigs of the individual samples were subjected to ARG annotation and host identification. More than 20 Gb assembled contigs were obtained, and the N50 length was 1550 ± 431 bp. Within these, 1870 ARG-carrying contigs were identified, 174 of which contained more than one ARG. After host identification using *k*-mer-based methodology, the taxa of 85% and 57% of contigs carrying ARGs were predicted at their specific phyla and species levels, respectively.

Fisher's exact test was used to screen for host-resistome pairs with significant correlations, as demonstrated in Fig. 4a (phyla or classes of *Proteobacteria* – ARG types) and Fig. 4b (family/genus/species – ARG subtypes). In general, no ARG types were conserved in any specific phylum; the γ-, β- and α-*Proteobacteria*, which were most dominant in terms of relative abundance in the AS, hosted a wide range of ARGs. However, significance levels were different for some bacteria hosting specific types of ARGs. For example, aminoglycoside resistance genes were mostly detected in γ-*Proteobacteria*, while sulfonamide resistance genes correlated mostly with α-*Proteobacteria*. In addition, seven aminoglycoside genotypes were detected (Fig. 4b), and three genes (*aadA, aph(6)-I* and *aph(3′)-I*) that produce streptomycin adenylyltransferase or aminoglycoside phosphotransferase were found in the pathogens *Escherichia coli* and *Pseudomonas putida*. Four sulfonamide resistance genotypes (*sul1, sul2, sul3,* and *sul4*) were detected in different taxa, among which *sul1* was carried by both pathogenic species in the γ-*Proteobacteria* class (*Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) and the nonpathogenic species in an α-*Proteobacteria* class (*Martelella* sp. AD-3, *Zymomonas mobilis*, and *Novosphingobium resinovarum*).

3.7. The HGT potential in AS

Of the 1104 ARG-carrying contigs whose taxa were predicted into 23 species, 9 species (166 contigs) simultaneously harbored ARGs and MGEs and thus had a high potential for HGT (Fig. S12). To reduce the bias from random occurrence, only prevalent cases of co-occurrence of ARGs and MGEs were further examined (Fig. S12), where prevalence was arbitrarily defined as ARG-carrying species that were detected more than eight times. There was a wide host-range for contigs carrying both ARGs and MGEs, especially for the classes of *Proteobacteria*. ARG-carrying contigs belonging to *Actinobacteria* and *Deinococcus-Thermus* carried no MGEs, while those detected in γ-*Proteobacteria* had a high percentage of co-localization between ARGs and MGEs. There were four species where > 75% of ARG-carrying contigs also contained MGEs: *Pseudomonas putida*, *Yangia* sp. CCB-MM3, *Geobacter lovleyi*, and *Slinimonas* sp. N102, the first of which was a potential pathogen.

To further resolve the co-occurrence of ARGs and MGEs, the incidence of ARGs encountering HGT signatures was calculated. As expected, there was an increasing incidence of MGE encounters as the distance from an ARG increased (Fig. 5). It is interesting to note that there was a significantly higher HGT potential in AS than in soil along the tested distances over 1.2 kbp from ARGs ($P < 0.05$, Fisher's exact test). However, AS displayed a slower increase in HGT potential than pathogens at all distances over 0.8 kbp ($P < 0.05$, Fisher's exact test).

4. Discussion

A comparison of AS with other ecotypes listed by Li et al. (2015b) showed that the average abundance of ARGs in AS (0.2 copy of ARGs per cell; the units used in the following data are the same) was far below that in fecal samples (5.94) and untreated sewage (1.93, which was considered to represent a large and mostly healthy population),

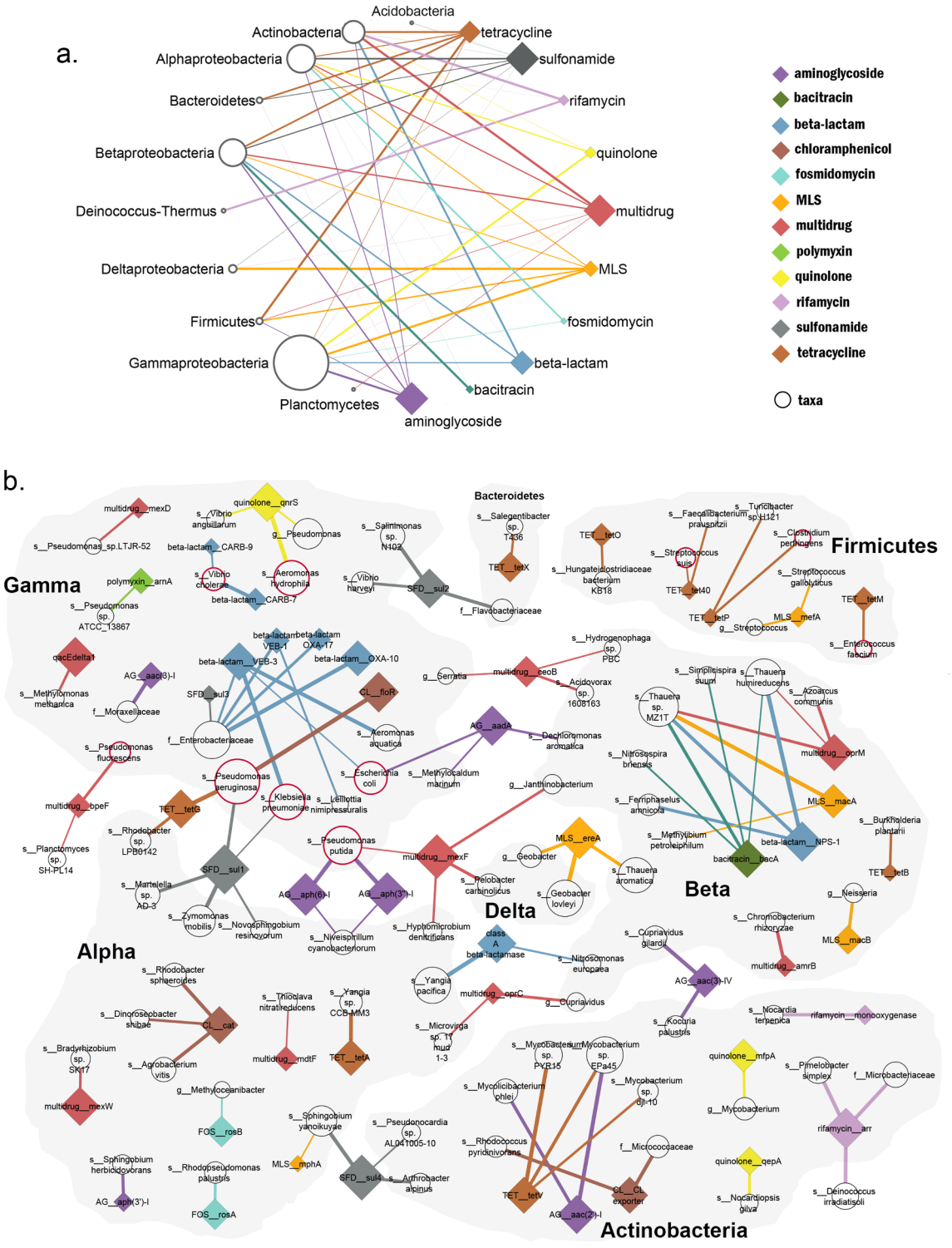

Fig. 4. The predicted antibiotic-resistant bacteria classified according to different taxonomy levels: a. phyla (or class for *Proteobacteria*) and b. species/genus/family level (diversity). The node size of networks indicates relative abundance, and the node shape indicates specific ARGs (diamond) or taxa (circle). The width of edge on networks represents the level of significance of the correlation (Fisher's exact test). All shown correlations are significant ($P < 0.05$). Pathogens are highlighted as red circles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

while comparable to environmental systems such as WWTPs effluent (0.44), anaerobic digested sludge (ADS) (0.43), drinking water (0.35), river water (0.20), and sediment (0.24) (Fig. S13) (Hendriksen et al., 2019; Li et al., 2015b). This indicated that the pollution levels of ARGs in AS were similar to those in other natural samples.

The data suggest that the core resistome should be examined for persistent and abundant ARG subtypes, as this would aid characterization of resistant profiles in AS in terms of variation over time, especially when compared with other ecotypes. The core resistome comprised 41 genotypes resistant to most widely used antibiotics (Table S5) and, intriguingly, extensively overlapped with core ARGs in other ecotype systems mentioned above (Figs. S13 and S14). Almost all of these ARGs were abundant in both human and livestock fecal samples (Figs. S13 and S14), while genes conferring resistance to

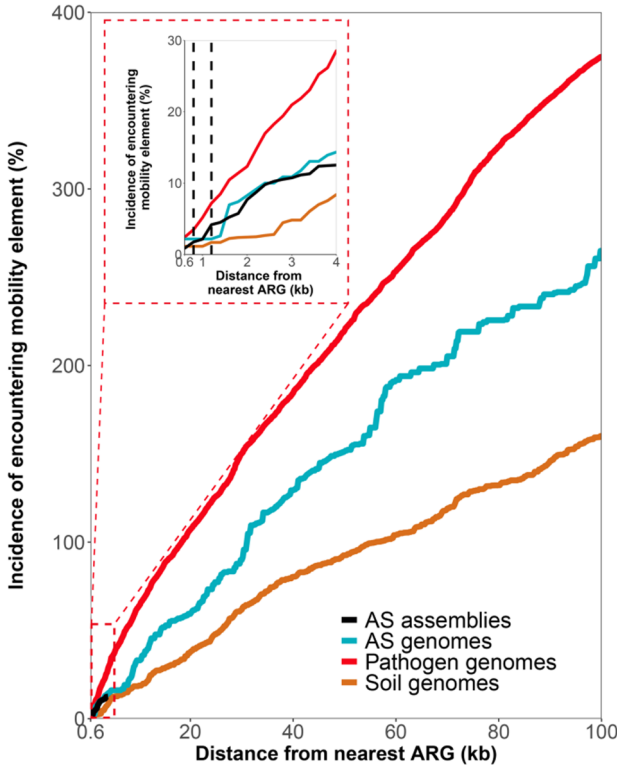

Fig. 5. The potential of ARGs to encounter MGEs in this study and in publicly available complete genomes. AS showed a significantly increased HGT potential than soil at all distances over 1.2 kbp (right dashed-line in inset, $P < 0.05$) but a slower increased HGT potential than pathogens at all distances over 0.8 kbp (left dashed-line, $P < 0.05$).

bacitracin, MLS, multidrug, and vancomycin have been widely detected in natural environments including fishpond sediment and river and drinking water (Figs. S13 and S14). This indicates that AS may disseminate ARGs throughout a wide range of environments, underscoring the profound health threats of AS being a reservoir of resistomes (Gouliouris et al., 2019).

A longitudinal variation in the quantity and diversity of the resistome was observed over the study period. The ARG abundance range was 0.144–0.358 copy of ARGs per cell. A Mann-Whitney test revealed that the overall abundance of ARGs changed significantly every two to three years ($P < 0.01$, Table S3). Similarly, ordination analysis of resistome composition (ARGs subtypes) by PCoA showed that the composition of the resistome changed every two to three years. This annual change in both the abundance and the structure revealed the longitudinal evolution of the resistome in the AS samples, highlighting the need for more comprehensive temporal monitoring studies to definitively explore fluctuations in the makeup and function of the AS resistome.

Seasonal clustering in the overall structure of the resistome types was not observed (Fig. S3), which might have been due to the small variations of temperatures in Hong Kong over different seasons. Nevertheless, the abundance of two ARG types (MLS and quinolone) showed that some clear seasonal dynamic regularity existed. The maximum abundance of MLS was in September–November in each year and the lowest abundance was in January. Furthermore, the most dominant MLS resistance gene, *ereA*, exhibited similar variation trends (Fig. S8). The quinolone resistance genes *qnrS* reached a maximum in February or March in four of the study years.

However, seasonal resistome variations were not always confirmed in AS in previous studies. Jiao *et al.* reported no seasonal variation of ARGs in a high-throughput qPCR analysis of excess sludge from a city with an annual temperature range of 10–35 °C (Jiao et al., 2018). Lamba and Ahammad (2017) found higher abundances of ARB and ARGs in winter than in summer in AS in New Delhi. An et al. (2018) reported a significant difference between winter and summer levels of ARGs in AS in their study. However, these previous studies involved sampling within only one year or only in several paired months across multiple years, which meant that conclusions were based on data biased by limited sampling time points and narrow time-spans. However, we minimized bias by conducting our study with a high-resolution sampling over a very long period (nine years), and our assembly-based network analysis enabled exploration of the ARBs relevant to the seasonally varying levels of *ereA* and *qurS* genes (Fig. 4b). Thus, the most important *ereA*-carrying bacteria in AS was found to be *Geobacter*, whose abundance variation showed similar seasonal trends (Fig. S15), while the fluctuation in abundance of *qnrS* was attributed largely to the variation in the abundance of *Vibrio anguillarum* (Fig. S16).

The examination of ARGs that constitute emerging concerns to human health assisted in risk evaluations of AS. For example, the *sul4* gene was first discovered in 2017 in integron amplifications of mobile gene cassettes from Indian river sediment and was soon found to have spread across Asia and Europe (Razavi et al., 2017). This study has now confirmed the long-term occurrence of *sul4* in sewage sludge in Hong Kong, with an increasing abundance of *sul4* after 2012 (Fig. S8, Mann-Whitney test, $P < 0.05$). Examples of the diverse *sul4*-carrying species were α-*Proteobacteria* (*Sphingobium yanoikuyae*) and *Actinobacteria* (*Pseudonocardia* sp. AL041005-10 and *Arthrobacter alpinus*), as illustrated in the network analysis in Fig. 4b, which further highlighted the mobility of *sul4* across phylogeny. MRSA-related genes were rarely detected after 2009 (Fig. S18), in line with a worldwide clinical survey that the prevalence of MRSA declined after 2008 (Diekema et al., 2019). Other emerging ARGs (*mcr-1* and CRE related genes) that were only recently detected in clinical isolates were also found in AS from the Shatin WWTP, demonstrating that genetic exchange had possibly occurred between clinical and WWTPs microbiota, thus contributing to the increased levels of health-threatening bacteria.

As determined by VPA and SEM analysis, a bacterial community is the most influential factor shaping AS resistomes and can do so in both direct and indirect ways. Although the resistomes in AS were generally shared with other ecotypes, the hosts for ARG-carrying contigs differentiated AS from other environments. To be specific, in comparing AS with wastewater samples, 50% of tetracycline resistance genes were located in *Clostridium* genus in *Firmicutes* phyla in wastewater (Jia et al., 2017). However, in AS, *Firmicutes* harbored the most diverse tetracycline resistance genes from different species not limited to the genus *Clostridium*, while the most common tetracycline resistance gene was detected to be *tetG* in *Pseudomonas aeruginosa* species in γ-*Proteobacteria*. This was consistent with the reported association of *tetG* with *Pseudomonas* from stress incubation experiments (Li et al., 2013). Bacitracin resistance genes were reported to be widely detected in *Proteobacteria* based on an intensive search of the whole genome database using the ARGs-OSP platform (Zhang et al., 2018a). However, analysis of the AS samples in this study showed that *β-Proteobacteria* were almost the only class that was predicted to be the host of bacitracin resistance genes. Further analysis showed it was the gene *bacA* (Fig. 4b), one of genotype of bacitracin resistance genes, correlated with *β-Proteobacteria* such as species *Thauera* sp. MZ1T, *Nitrosospira briensis*, *Simplicispira suum*, and *Thauera humireducens*. All of the phenomena discussed above indicated that the wide hosting tendencies of the resistome could probably be further diversified by the characteristically dense and rich bacterial community of AS (Figs. S9 and S10).

To validate the host of ARG-carrying contigs predicted using network analysis, we used the NCBI whole genome database as a reference. Some pairs of the resistome taxonomy were confirmed by genetic screening of genomes or isolates recovered from aquatic environments or clinics, such as *Vibrio cholerae* with CARB-7 and CARB-9 (beta-lactam) (Melano et al., 2002; Petroni et al., 2004), *Pseudomonas aeruginosa* with *tetG*, *floR*, and *sul1* (Coyne et al., 2010), and *Pseudomonas*

putida with *mexF* (Nelson et al., 2002). Nevertheless, other occurrences of bacterial species carrying specific ARGs have not been detailed in published genomes or isolates. For example, *sul3*-carrying contigs were merely predicted at the family level (*Enterobacteriaceae*) due to the unavailability of *k*-mers of *sul3*-carrying genomes in current genome databases. It was recently reported that *sul3* was abundant in total assemblage but not in colony-forming bacteria (Suzuki et al., 2019). These findings imply that further complementary work on currently uncultured bacteria or undrafted genomes is required to determine other possibly complex ARG-host correlations present in AS.

Genetic exchange across phylogenies is another factor that shapes the AS resistome, although in this study, the resistome characteristics were more likely the result of combined effects also resulting from interactions with the bacterial community (Fig. 3b, Fig. S11). To determine the overall potential for HGT in AS, the incidence of ARGs encountering MGEs in AS was compared with that in pathogenic and non-pathogenic soil. The pathogens investigated were those that are detrimental to human health and that reflect the range of microbes in clinical settings. In addition, ARGs are known to spread widely between such pathogens via HGT (Forsberg et al., 2014). A lower potential for HGT in AS was expected as a result of the lower level of exposure to antibiotics in AS than in clinical settings (Fig. 5). Interestingly, however, a higher HGT potential was found in AS than in soil. Both of these media contain a diverse range of bacteria (George et al., 2019) and a huge diversity of ARGs (Forsberg et al., 2014). However, the function of AS as a concentrated treatment point of sewage from cities means that it contains a highly dense microbial community (2–10 g/L), has a shorter generation time for bacteria (15–30 days), has a large volume flux (216,000 m^3 day^{-1}), and enables bacterial contact within flocs (Ju and Zhang, 2015). These features were hypothesized to be perfect environments for HGT in AS comparing to the relative still conditions within soil.

Aside from the microbial community composition and potential HGT in the ARG-carrying bacteria, there are other factors that shaped the variation of resistomes, such as the environmental parameters of WWTPs. Specifically, this study found that MCRT positively correlated with ARG abundance (Fig. 3b), for reasons still obscure that longer MCRT might select resistant bacteria (Neyestani, 2016). Besides, bleaching was applied in the aeriation tank at the end of 2009 with the purpose of removing detrimental microbes to maintain treatment efficiency. This led to sharp changes of bacterial composition (Figs. S9 & S10), and furtherly contributed to the distinct dissimilarity on ARGs' structures between cluster 1 and cluster 2 in Fig. 1b.

In addition, although the selection pressure of antibiotic residues has also been proposed to shape the AS resistome, results in the recent literature have been contradictory. Bengtsson-Palme et al. (2016) inferred from their data that there was no direct selection for ARGs in response to any antibiotics in WWTPs, while Gao et al. (2012) observed a significant correlation between certain resistance genes and antibiotic types (e.g., *sul1* correlated with abundance of sulfonamide but *tetO/tetW* did not correlate with tetracycline). Novo et al. (2013) concluded that tetracycline residues were significantly responsible for higher total resistance, but not for tetracycline resistance specifically. These results may be due to the relatively low abundance of antibiotics in wastewater (normally in the level of ng/L), which is below the minimum selective concentration (Greenfield et al., 2018; Li et al., 2009) and which is explained by a recently proposed theory that there is reduced selection pressure for antibiotic resistance in complex communities than within single species in vitro (Klümper et al., 2019). However, this study did not involve collection of antibiotic concentration data, and thus, no similar conclusions can be drawn; further dose-response experiments are needed to examine these possibilities.

Longitudinal sampling of the same site in WWTPs can be considered as a general strategy to obtain deeper sequencing of an identical sample. All shotgun-sequencing reads from 97 sampling time points, totaling 539.9 Gbp, were taken as a whole sample for rarefaction curve analysis (Fig. 6). For the first time, this clearly demonstrated that sequencing depth would strongly bias quantification of ARGs. The results showed that the proper sequencing depth for ARG quantification analysis by shotgun whole-genomic sequencing of AS samples is 60 Gbp; at depths < 60 Gbp, both the count of detected ARGs and the total abundance of the resistome kept increasing, while at depths > 60 Gbp, only rare ARGs were retrieved. Thus, it can be seen that the sequencing depths in previous studies, which ranged from 1 to 11 Gbp (Bengtsson-Palme et al., 2016; Guo et al., 2017; Ju et al., 2018; Ng et al., 2019), were insufficient to determine the range of ARGs in AS, and may have underestimated or not detected ARGs in AS from less abundant bacterial groups such as *Enterobacteriaceae* (Narciso-da-Rocha et al., 2018).

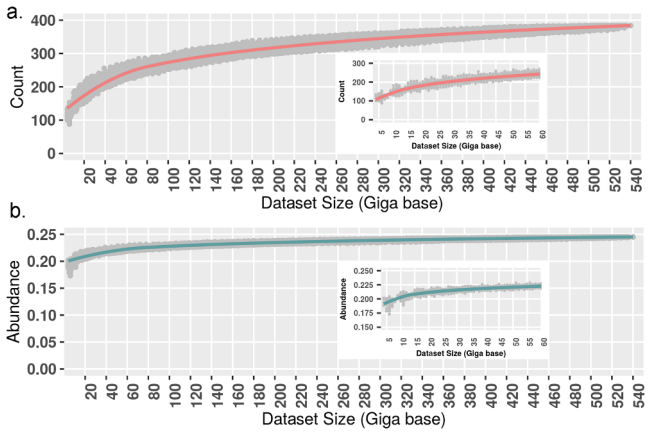

Fig. 6. The special rarefaction curve formed by taking 539.9 Gbp as a whole. The gray shadow represents 300 rounds of Monte Carlo simulations, and the smoothed curve was created by the local regression function in Rstudio. The inset delineates the count or total abundance of detected ARGs at a sequencing depth < 60 Gbp. The unit of ARG abundance is copies of ARGs per cell.

5. Conclusions

In this study, profiling of ARGs in AS samples from a local WWTP was conducted in high resolution (monthly) and over a long term (over nine years) using high-throughput sequencing technology with powerful analytical data-mining methods. Overall, the whole dataset of shotgun metagenomic reads was 539.9 Gbp. This WWTP harbored 120 ± 12 ARG genotypes with a total abundance of 0.207 ± 0.0443 copy of ARGs per cell. Notably, the intensive longitudinal survey revealed significant changes every two to three years in both the abundance and composition of ARGs, which further revealed the evolution of the resistome in the AS samples.

Moreover, the core resistome comprised dominant and stable ARGs that persisted throughout the entire sampling process and were ascribable to 41 genotypes resistant to most widely used antibiotics. These ARGs have also been found to be broadly disseminated in other ecotype systems such as human feces, livestock feces, and natural environments. In addition, the prevalence of newly emerging ARGs such as *sul4*, *mcr*, and CRE genes was observed over the nine-year study period. These are human health-threatening ARGs of particular concern, highlighting the importance of WWTPs as reservoirs of ARGs and the need for further studies. A further correlation analysis enabled the elaboration of the resistome characteristics in terms of its operational determinants, bacterial phylogeny, and HGTs based on a large assembly reservoir (over 20 Gb assembled contigs) from AS samples with dense and diverse microbes.

In summary, the strategies used and results found in this study have provided a dynamic perspective of ARGs and their host bacteria from multiple biological viewpoints, underscoring the health-threatening challenges arising from chronic resistome evolution and highlighting the analytical power of longitudinal monitoring of the resistome of AS.

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 material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105270.

References

An, X.L., Su, J.Q., Li, B., Ouyang, W.Y., Zhao, Y., Chen, Q.L., Cui, L., Chen, H., Gillings, M.R., Zhang, T., Zhu, Y.G., 2018. Tracking antibiotic resistome during wastewater treatment using high throughput quantitative PCR. Environ. Int. 117, 146–153.

Bengtsson-Palme, J., Hammaren, R., Pal, C., Ostman, M., Bjorlenius, B., Flach, C.F., Fick, J., Kristiansson, E., Tysklind, M., Larsson, D.G.J., 2016. Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. Sci. Total Environ. 572, 697–712.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. Roy. Stat. Soc.: Ser. B (Methodol.) 57 (1), 289–300.

Boolchandani, M., D'Souza, A.W., Dantas, G., 2019. Sequencing-based methods and resources to study antimicrobial resistance. Nat. Rev. Genet. 20, 356–370.

Bush, K., Jacoby, G.A., 2010. Updated functional classification of beta-lactamases. Antimicrob. Agents Chemother. 54 (3), 969–976.

CDC, 2016. Carbapenem-resistant Enterobacteriaceae in Healthcare Settings. Available access: https://www.cdc.gov/hai/organisms/cre/index.html.

Chen, Q., An, X., Li, H., Su, J., Ma, Y., Zhu, Y.G., 2016. Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil. Environ. Int. 92–93, 1–10.

Chen, S., Zhou, Y., Chen, Y., Gu, J., 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics (Oxford, England) 34 (17), i884–i890.

Coyne, S., Courvalin, P., Galimand, M., 2010. Acquisition of multidrug resistance transposon Tn6061 and IS6100-mediated large chromosomal inversions in Pseudomonas aeruginosa clinical isolates. Microbiology 156 (5), 1448–1458.

Czekalski, N., Díez, E.G., Bürgmann, H., 2014. Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. ISME J. 8 (7), 1381.

Diekema, D.J., Hsueh, P.R., Mendes, R.E., Pfaller, M.A., Rolston, K.V., Sader, H.S., Jones, R.N., 2019. The microbiology of bloodstream infection: 20-year trends from the SENTRY antimicrobial surveillance program. Antimicrob. Age. Chemother. 63 (7), e00355–19.

Di Cesare, A., Eckert, E.M., Teruggi, A., Fontaneto, D., Bertoni, R., Callieri, C., Corno, G., 2015. Constitutive presence of antibiotic resistance genes within the bacterial community of a large subalpine lake. Mol. Ecol. 24 (15), 3888–3900.

Evans, B.A., Amyes, S.G., 2014. OXA beta-lactamases. Clin. Microbiol. Rev. 27 (2), 241–263.

Feßler, A.T., Lübke-Becker, A., Feudi, C., Michael, G.B., Eichhorn, I., Schwarz, S., Shen, J., Wang, Y., Kaspar, H., 2018. Identification of novel variants of the colistin resistance gene mcr-3 in Aeromonas spp. from the national resistance monitoring programme GERM-Vet and from diagnostic submissions. J. Antimicrob. Chemother. 73 (5), 1217–1221.

Fischbach, M.A., Walsh, C.T., 2009. Antibiotics for emerging pathogens. Science 325 (5944), 1089.

Forsberg, K.J., Patel, S., Gibson, M.K., Lauber, C.L., Knight, R., Fierer, N., Dantas, G., 2014. Bacterial phylogeny structures soil resistomes across habitats. Nature 509 (7502), 612–616.

Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O.A., Dantas, G., 2012. The shared antibiotic resistome of soil bacteria and human pathogens. Science 337 (6098), 1107.

Gao, P., Munir, M., Xagoraraki, I., 2012. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. Sci. Total Environ. 421, 173–183.

Garcia-Alvarez, L., Holden, M.T.G., Lindsay, H., Webb, C.R., Brown, D.F.J., Curran, M.D., Walpole, E., Brooks, K., Pickard, D.J., Teale, C., Parkhill, J., Bentley, S.D., Edwards, G.F., Girvan, E.K., Kearns, A.M., Pichon, B., Hill, R.L.R., Larsen, A.R., Skov, R.L., Peacock, S.J., Maskell, D.J., Holmes, M.A., 2011. Meticillin-resistant Staphylococcus aureus with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study. Lancet Infect. Dis. 11 (8), 595–603.

George, P.B.L., Lallias, D., Creer, S., Seaton, F.M., Kenny, J.G., Eccles, R.M., Griffiths, R.I., Lebron, I., Emmett, B.A., Robinson, D.A., Jones, D.L., 2019. Divergent national-scale trends of microbial and animal biodiversity revealed across diverse temperate soil ecosystems. Nat. Commun. 10 (1), 1107.

Gouliouris, T., Raven, K.E., Moradigaravand, D., Ludden, C., Coll, F., Blane, B., Naydenova, P., Horner, C., Brown, N.M., Corander, J., Limmathurotsakul, D., Parkhill, J., Peacock, S.J., 2019. Detection of vancomycin-resistant Enterococcus faecium hospital-adapted lineages in municipal wastewater treatment plants indicates widespread distribution and release into the environment. Genome Res. 29 (4), 626–634.

Greenfield, B.K., Shaked, S., Marrs, C.F., Nelson, P., Raxter, I., Xi, C., McKone, T.E., Jolliet, O., 2018. Modeling the emergence of antibiotic resistance in the environment: an analytical solution for the minimum selection concentration. Antimicrob. Age. Chemother. 62 (3), e01686–17.

Guo, J., Li, J., Chen, H., Bond, P.L., Yuan, Z., 2017. Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic resistance genes and mobile genetic elements. Water Res. 123, 468–478.

Hendriksen, R.S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., Roder, T., Nieuwenhuijse, D., Pedersen, S.K., Kjeldgaard, J., Kaas, R.S., Clausen, P., Vogt, J.K., Leekitcharoenphon, P., van de Schans, M.G.M., Zuidema, T., de Roda Husman, A.M., Rasmussen, S., Petersen, B., Global Sewage Surveillance project, C., Amid, C., Cochrane, G., Sicheritz-Ponten, T., Schmitt, H., Alvarez, J.R.M., Aidara-Kane, A., Pamp, S.J., Lund, O., Hald, T., Woolhouse, M., Koopmans, M.P., Vigre, H., Petersen, T.N., Aarestrup, F.M., 2019. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. Nat. Commun. 10 (1), 1124.

Hryniewicz, W., 1999. Epidemiology of MRSA. Infection 27 (2), S13.

Hu, H.W., Wang, J.T., Li, J., Li, J.J., Ma, Y.B., Chen, D., He, J.Z., 2016. Field-based evidence for copper contamination induced changes of antibiotic resistance in agricultural soils. Environ. Microbiol. 18 (11), 3896–3909.

Hyatt, D., Chen, G.-L., Locascio, P.F., Land, M.L., Larimer, F.W., Hauser, L.J., 2010. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinf. 11, 119.

Jia, S.Y., Shi, P., Hu, Q., Li, B., Zhang, T., Zhang, X.X., 2015. Bacterial community shift drives antibiotic resistance promotion during drinking water chlorination. Environ. Sci. Technol. 49 (20), 12271–12279.

Jia, S., Zhang, X.X., Miao, Y., Zhao, Y., Ye, L., Li, B., Zhang, T., 2017. Fate of antibiotic resistance genes and their associations with bacterial community in livestock breeding wastewater and its receiving river water. Water Res. 124, 259–268.

Jiao, Y.N., Zhou, Z.C., Chen, T., Wei, Y.Y., Zheng, J., Gao, R.X., Chen, H., 2018. Biomarkers of antibiotic resistance genes during seasonal changes in wastewater treatment systems. Environ. Pollut. 234, 79–87.

Ju, F., Beck, K., Yin, X., Maccagnan, A., McArdell, C.S., Singer, H.P., Johnson, D.R., Zhang, T., Burgmann, H., 2018. Wastewater treatment plant resistomes are shaped by bacterial composition, genetic exchange, and upregulated expression in the effluent microbiomes. ISME J. 13, 346–360.

Ju, F., Zhang, T., 2015. Bacterial assembly and temporal dynamics in activated sludge of a full-scale municipal wastewater treatment plant. ISME J. 9 (3), 683–695.

Karkman, A., Do, T.T., Walsh, F., Virta, M.P.J., 2017. Antibiotic-Resistance Genes in Waste Water. Trends Microbiol. 26 (3), 220–228.

Kindt, R., 2016. BiodiversityR: Package for Community Ecology and Suitability Analysis version 2.7-2.

Klümper, U., Recker, M., Zhang, L., Yin, X., Zhang, T., Buckling, A., Gaze, W., 2019. Selection for antibiotic resistance is reduced when embedded in a natural microbial community. bioRxiv 529651.

Lamba, M., Ahammad, S.Z., 2017. Sewage treatment effluents in Delhi: a key contributor of beta-lactam resistant bacteria and genes to the environment. Chemosphere 188, 249–256.

Li, B., Zhang, T., Xu, Z., Fang, H.H.P., 2009. Rapid analysis of 21 antibiotics of multiple classes in municipal wastewater using ultra performance liquid chromatography-tandem mass spectrometry. Anal. Chim. Acta 645 (1–2), 64–72.

Li, B., Ju, F., Cai, L., Zhang, T., 2015a. Profile and fate of bacterial pathogens in sewage treatment plants revealed by high-throughput metagenomic approach. Environ. Sci. Technol. 49 (17), 10492–10502.

Li, B., Yang, Y., Ma, L., Ju, F., Guo, F., Tiedje, J.M., Zhang, T., 2015b. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. ISME J. 9 (11), 2490–2502.

Li, B., Zhang, X.X., Guo, F., Wu, W.M., Zhang, T., 2013. Characterization of tetracycline resistant bacterial community in saline activated sludge using batch stress incubation with high-throughput sequencing analysis. Water Res. 47 (13), 4207–4216.

Li, L.-G., Xia, Y., Zhang, T., 2017. Co-occurrence of antibiotic and metal resistance genes revealed in complete genome collection. ISME J. 11, 651.

Liu, T., Zhang, A.N., Wang, J., Liu, S., Jiang, X., Dang, C., Ma, T., Liu, S., Chen, Q., Xie, S., Zhang, T., Ni, J., 2018a. Integrated biogeography of planktonic and sedimentary bacterial communities in the Yangtze River. Microbiome 6 (1), 16.

Liu, X., Lu, S., Guo, W., Xi, B., Wang, W., 2018b. Antibiotics in the aquatic environments: a review of lakes, China. Sci. Total Environ. 627, 1195–1208.

Liu, Y.-Y., Wang, Y., Walsh, T.R., Yi, L.-X., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B., Huang, X., Yu, L.-F., Gu, D., Ren, H., Chen, X., Lv, L., He, D., Zhou, H., Liang, Z.,

Liu, J.-H., Shen, J., 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect. Dis. 16 (2), 161–168.
Logan, L.K., 2012. Carbapenem-resistant enterobacteriaceae: an emerging problem in children. Clin. Infect. Dis. 55 (6), 852–859.
Lorenzo, P., Adriana, A., Jessica, S., Caries, B., Marinella, F., Marta, L., Luis, B.J., Pierre, S., 2018. Antibiotic resistance in urban and hospital wastewaters and their impact on a receiving freshwater ecosystem. Chemosphere 206, 70–82.
Lu, J., Breitwieser, F.P., Thielen, P., Salzberg, S.L., 2017. Bracken: estimating species abundance in metagenomics data. PeerJ Comput. Sci. 3, e104.
Ma, L., Li, B., Zhang, T., 2019. New insights into antibiotic resistome in drinking water and management perspectives: a metagenomic based study of small-sized microbes. Water Res. 152, 191–201.
Melano, R., Petroni, A., Garutti, A., Saka, H.A., Mange, L., Pasterán, F., Rapoport, M., Rossi, A., Galas, M., 2002. New carbenicillin-hydrolyzing beta-lactamase (CARB-7) from Vibrio cholerae non-O1, non-O139 strains encoded by the VCR region of the V. cholerae genome. Antimicrob. Age. Chemother. 46 (7), 2162–2168.
Munck, C., Albertsen, M., Telke, A., Ellabaan, M., Nielsen, P.H., Sommer, M.O., 2015. Limited dissemination of the wastewater treatment plant core resistome. Nat. Commun. 6, 8452.
Narciso-da-Rocha, C., Rocha, J., Vaz-Moreira, I., Lira, F., Tamames, J., Henriques, I., Martinez, J.L., Manaia, C.M., 2018. Bacterial lineages putatively associated with the dissemination of antibiotic resistance genes in a full-scale urban wastewater treatment plant. Environ. Int. 118, 179–188.
Nelson, K.E., Weinel, C., Paulsen, I.T., Dodson, R.J., Hilbert, H., Martins dos Santos, V.A., Fouts, D.E., Gill, S.R., Pop, M., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R.T., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Chris Lee, P., Holtzapple, E., Scanlan, D., Tran, K., Moazzez, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D., Moestl, D., Wedler, H., Lauber, J., Stjepandic, D., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J.A., Timmis, K.N., Dusterhoft, A., Tummler, B., Fraser, C.M., 2002. Complete genome sequence and comparative analysis of the metabolically versatile Pseudomonas putida KT2440. Environ. Microbiol. 4 (12), 799–808.
Neyestani, M., 2016. Occurrence and proliferation of antibiotics and antibiotic resistance in wastewater treatment plants.
Ng, C., Tan, B., Jiang, X.T., Gu, X., Chen, H., Schmitz, B.W., Haller, L., Charles, F.R., Zhang, T., Gin, K., 2019. Metagenomic and resistome analysis of a full-scale municipal wastewater treatment plant in singapore containing membrane bioreactors. Front. Microbiol. 10, 172.
Noguchi, N., Suwa, J., Narui, K., Sasatsu, M., Ito, T., Hiramatsu, K., Song, J.-H., 2005. Susceptibilities to antiseptic agents and distribution of antiseptic-resistance genes qacA/B and smr of methicillin-resistant Staphylococcus aureus isolated in Asia during 1998 and 1999. J. Med. Microbiol. 54 (6), 557–565.
Novo, A., Andre, S., Viana, P., Nunes, O.C., Manaia, C.M., 2013. Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. Water Res. 47 (5), 1875–1887.
Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2019. vegan: Community Ecology Package version 2.5-6.
O'Neill, J., 2016. The Review on Antimicrobial Resistance. Available access: https://amr-review.org/.
Parnanen, K., Karkman, A., Hultman, J., Lyra, C., Bengtsson-Palme, J., Larsson, D.G.J., Rautava, S., Isolauri, E., Salminen, S., Kumar, H., Satokari, R., Virta, M., 2018. Maternal gut and breast milk microbiota affect infant gut antibiotic resistome and mobile genetic elements. Nat. Commun. 9 (1), 3891.
Petroni, A., Melano, R.G., Saka, H.A., Garutti, A., Mange, L., Pasterán, F., Rapoport, M., Miranda, M., Faccone, D., Rossi, A., Hoffman, P.S., Galas, M.F., 2004. CARB-9, a Carbenicillinase encoded in the VCR Region of *Vibrio cholerae* Non-O1, Non-O139 Belongs to a family of cassette-encoded β-lactamases. Antimicrob. Age. Chemother. 48 (10), 4042.
Razavi, M., Marathe, N.P., Gillings, M.R., Flach, C.-F., Kristiansson, E., Joakim Larsson, D.G., 2017. Discovery of the fourth mobile sulfonamide resistance gene. Microbiome 5 (1), 160.
Rosseel, Y., Oberski, D., Byrnes, J., Vanbrabant, L., Savalei, V., Merkle, E., Hallquist, M., 2019. lavaan: Latent Variable Analysis version 0.6-5.
Schmieder, R., Edwards, R., 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27 (6), 863–864.
Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13 (11), 2498–2504.
Sievert, C., 2019. plotly. for R.
Suzuki, S., Nakanishi, S., Tamminen, M., Yokokawa, T., Sato-Takabe, Y., Ohta, K., Chou, H.Y., Muziasari, W.I., Virta, M., 2019. Occurrence of sul and tet(M) genes in bacterial community in Japanese marine aquaculture environment throughout the year: profile comparison with Taiwanese and Finnish aquaculture waters. Sci. Total Environ. 669, 649–656.
Tijet, N., Faccone, D., Rapoport, M., Seah, C., Pasterán, F., Ceriana, P., Albornoz, E., Corso, A., Petroni, A., Melano, R.G., 2017. Molecular characteristics of mcr-1-carrying plasmids and new mcr-1 variant recovered from polyclonal clinical Escherichia coli from Argentina and Canada. PLoS ONE 12 (7), e0180347.
UNEP, 2017. Frontiers 2017: Emerging Issues of Environmental Concern. Available access: https://www.unenvironment.org/resources/frontiers-2017-emerging-issues-environmental-concern.
Wang, Y., Cadotte, M.W., Chen, Y., Fraser, L.H., Zhang, Y., Huang, F., Luo, S., Shi, N., Loreau, M., 2019. Global evidence of positive biodiversity effects on spatial ecosystem stability in natural grasslands. Nat. Commun. 10 (1), 3207.
Wang, Y., Jiang, X., Liu, L., Li, B., Zhang, T., 2018. High-resolution temporal and spatial patterns of virome in wastewater treatment systems. Environ. Sci. Technol. 52 (18), 10337–10346.
Wood, D.E., Salzberg, S.L., 2014. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol. 15 (3), R46.
Yang, Y., Jiang, X.T., Chai, B.L., Ma, L.P., Li, B., Zhang, A.N., Cole, J.R., Tiedje, J.M., Zhang, T., 2016. ARGs-OAP: online analysis pipeline for antibiotic resistance genes detection from metagenomic data using an integrated structured ARG-database. Bioinformatics 32 (15), 2346–2351.
Yang, Y., Li, B., Ju, F., Zhang, T., 2013. Exploring variation of antibiotic resistance genes in activated sludge over a four-year period through a metagenomic approach. Environ. Sci. Technol. 47 (18), 10197–10205.
Yang, Y., Liu, G., Song, W., Ye, C., Lin, H., Li, Z., Liu, W., 2019. Plastics in the marine environment are reservoirs for antibiotic and metal resistance genes. Environ. Int. 123, 79–86.
Yin, X., Jiang, X.T., Chai, B., Li, L., Yang, Y., Cole, J.R., Tiedje, J.M., Zhang, T., 2018. ARGs-OAP v2.0 with an expanded SARG database and hidden markov models for enhancement characterization and quantification of antibiotic resistance genes in environmental metagenomes. Bioinformatics 34 (13), 2263–2270.
Zhang, A.N., Hou, C.-J., Li, L.-G., Zhang, T., 2018a. ARGs-OSP: online searching platform for antibiotic resistance genes distribution in metagenomic database and bacterial whole genome database. bioRxiv 337675.
Zhang, A.N., Li, L.G., Ma, L., Gillings, M.R., Tiedje, J.M., Zhang, T., 2018b. Conserved phylogenetic distribution and limited antibiotic resistance of class 1 integrons revealed by assessing the bacterial genome and plasmid collection. Microbiome 6 (1), 130.