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DOI link to article:

https://doi.org/10.1021/acs.est.7b03797

Date deposited:

16/10/2017

Embargo release date:

09 October 2018

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Newcastle University ePrints - eprint.ncl.ac.uk
Antibiotic resistance genes and associated microbial community conditions in aging landfill systems

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ABSTRACT

Landfills receive about 350 million tons of municipal solid wastes (MSWs) per year globally, including antibiotics and other co-selecting agents that impact antimicrobial resistance (AMR). However, little is known about AMR in landfills, especially as a function of landfill ages. Here we quantified antibiotics, heavy metals, and AMR genes (ARGs) in refuse and leachates from landfills of different age (< 3, 10, and > 20 years). Antibiotics levels were consistently lower in refuse and leachates from older landfills, whereas ARG levels in leachates significantly increased with landfill age (One-way ANOVA, F = 10.8, P < 0.01). Heavy metals whose contents increased as landfills age (One-way ANOVA, F = 12.3, P < 0.01) were significantly correlated with elevated levels of ARGs (Mantel test, R = 0.66, P < 0.01) in leachates, which implies greater AMR exposure risks around older landfills. To further explain ARGs distributional mechanisms with age, microbial communities, mobile genetic elements (MGEs) and environmental factors were contrasted between refuse and leachate samples. Microbial communities in the refuse were closely correlated with ARG contents (Procrustes test; M² = 0.37, R = 0.86, P < 0.001), whereas ARG in leachates were more associated with MGEs.
INTRODUCTION

Landfills reportedly receive the largest portion (~350 million tons per year) of municipal solid wastes (MSWs) around the world, which spans countries at all levels of economic development. This estimate may actually be low, given that the USA and China annually sent 262 and 180 million tons of MSWs to landfills (2010-2013), respectively. Even though landfill use has declined in developed countries (e.g. USA) since the 1990s, MSWs landfiling amount is increasing in developing countries, with China predicted to be the largest MSWs generator by 2030. This trend is particularly concerning because contaminants in ill-managed MSWs landfills could be released to neighboring environments in the long-term. Further, inappropriate disposal of antibiotics, detergents and heavy metals have made landfills into harbors and potential sources of antimicrobial resistance (AMR), particularly AMR genes (ARGs).

In general terms, overuse of antibiotics and inadequate residuals management have transformed what was a “health panacea” into a global problem due to increased levels of acquired AMR. The death toll in Europe alone due to AMR bacteria is ~25,000 per year. In United States, ~2,000,000 illnesses are annually caused by AMR. Many AMR studies have been performed on wastewater systems and suggest waste-associated antibiotics and ARGs pose an exposure risk to humans, but very little attention has been paid to the solid waste streams, which may be an important,
but less apparent, gateway for the spread of AMR. Recent work has pointed out that around 2 million people live within a 10 km radius of the 50 largest active dumpsites (unregulated landfills). These are primarily located in SE Asia, Africa, the Subcontinent, and Latin America,\textsuperscript{19} which are all regions where antibiotic use is inefficiently regulated.\textsuperscript{11} In combination, this means densely populated emerging and developing countries are most vulnerable to AMR risks.

Landfilling is a decades long process,\textsuperscript{20, 21} during which the complex and dynamic ecosystems inside the landfill are subject to fluctuations in pH, oxygen level, temperature, carbon and nutrients. As such, the varying habitats may impact microbial AMR development. Previous work has confirmed the release of ARGs, mobile genetic element (MGE)\textsuperscript{22}, AMR bacteria\textsuperscript{10} and antibiotics\textsuperscript{23, 24} from MSW landfills. However, no holistic assessment, including ARGs and MGEs abundance and diversity, has been performed on the age of landfill systems that contrasts physiochemical conditions and microbial communities. Here we quantified antibiotics, heavy metal levels, microbial community conditions, twelve ARG subtypes, and six MGEs in refuse and leachates from landfill sites with three different ages (i.e. <3, 10, and >20 years). The goal was to (i) characterize how the abundance and distribution of ARGs in landfills change over space and time and (ii) to explain how the dynamics of microbial conditions affect the ARGs variation.
MATERIALS AND METHODS

Description of Landfills and Sampling. Sampling was performed at Laogang (LG) landfill in Shanghai, China. This landfill is located in an eastern semi-rural area of Shanghai and receives about 12,000 t/d of MSWs from the city. At LG, on-site landfill operations are separated into five distinct Phases according to operational age. Phases I to III were operated in the 1980s and 1990s, and are currently being reclaimed. Phase IV was operated between 2000 to 2010 and nearly closed, whereas Phase V commenced in 2010 and currently receives > 80% of MSWs at the LG landfill. In this study, landfill refuse (coring to a depth of 1.5 m) and leachate (parallel reservoirs) samples were collected from zones including < 3 yr (Phase V), 10-yr (Phase IV) and > 20 yr zones (Phase I). All cores and leachates were immediately placed in sealed cool boxes after sampling.

Pretreatment of Samples. Sampled leachates (50 mL) were centrifuged at 10000 x g for 8 min. The resulting centrates were passed through sterile 0.45 μm PES membrane filters (MembraneSolutions Allpure/045, Shanghai) to remove residual particles and bacteria. The membrane filters and centrifuge pellets were combined for each sample and frozen at -35°C for subsequent molecular assays. Refuse cores were sub-sampled and frozen at -35°C upon return to the lab. To reduce potential bias caused by heterogeneous composition of MSWs in subsequent analysis, refuse samples were lyophilized (typically ~20 g aliquots) and then sieved through a 40-mesh screen.
Four classes of antibiotics were quantified (Table 1), including tetracyclines (TCs); sulfonamides (SAs); macrolides (MLSs); and beta-lactams (β-lactams). Quantification methods for antibiotics in refuse and landfill leachate samples were optimized according to previous studies using internal standards methods by UPLC-MS/MS.\textsuperscript{12,25} The details of preconditioning, extraction, elution, quality control and ranges of detected antibiotic concentrations are provided in the Supporting Information (SI-1). Metals assessed include Al, As, Fe, Ni, Cr, Cu, Mn, Pb, Zn, Cd and Co, and were quantified using ICP-OES (Agilent 720ES, Agilent Technologies). The detected values (Table S2) and pretreatment methods are described in Supporting Information (SI-1).

Considering that composition of MSWs in Shanghai may have fluctuated over the last 20 years, this study used nitrogen, phosphorus and carbon contents to reflect the changes in MSWs composition during the landfilling process. Specifically, dissolved oxygen (DO) and pH were measured in situ using hand-held field probes (HANNA, Italy). The pH value of pretreated refuse samples were measured in the lab by Soilstik (Spectrum, USA). Ammonia nitrogen (NH\textsubscript{3}-N), nitrate (NO\textsubscript{3}– N), nitrite (NO\textsubscript{2}– N), total nitrogen (TN), total phosphorus (TP), and chemical organic carbon (COD for leachates only) were measured using Smartchem 200 (Alliance, France). Total organic carbon (TOC for refuse only) was measured using Vario TOC Select (Elementar Germany). The quantification methods are described in Supporting Information.
**DNA extraction and quality control.** PowerSoil DNA Isolation Kit (MOBIO, USA) was used for DNA extraction according to the manufacturer’s protocol. The yield and quality of the DNA extractions were verified by spectrophotometry (Merinton 4000, Beijing, China). The obtained DNA extracts with the OD$_{260/280}$ value between 1.8 and 2.0 were used. The qualified DNA extracts were stored at -20 °C for further analysis.

**Real-Time quantitative polymerase chain reaction (qPCR).** To reduce the related interferences to amplification reactions, all DNA samples were diluted by 20%, to concentrations between 5 and 10 ng/µL. The targeted genes (http://ardb.ccbcb.umd.edu/browsegene.shtml) included widespread class A ($\text{bla}_{\text{CTX-M-15}}, \text{bla}_{\text{TEM}}$; the $\text{bla}_{\text{CTX-M-15}}$ is denoted as $\text{bla}_{\text{CTX-M}}$ in the following sections) and D β-lactamase genes ($\text{bla}_{\text{NDM-1}}$ and $\text{bla}_{\text{OXA-4}}$; denoted as $\text{bla}_{\text{NDM}}$ and $\text{bla}_{\text{OXA}}$ respectively in the following sections) resistant to third generation β-lactams ($\text{bla}_{\text{R}}$). Other widespread ARGs include aadA1 and strB, which are resistant to Aminoglycosides ($\text{AgyR}$); $\text{tetM}$ and $\text{tetQ}$, which are resistant to TCs ($\text{tetR}$); $\text{sul1}$ and $\text{sul2}$, which are resistant to SAs ($\text{sulR}$); $\text{ermB}$ and $\text{mefA}$, which are resistant to MLs ($\text{MLsR}$) and multidrug-resistant subtype, $\text{mexF}$. Along with these ARGs, six MGEs markers ($\text{in1}, \text{int2}, \text{IS26}, \text{ISCR}, \text{traA}$ and $\text{trbC}$) were also quantified. The quantification of ARGs was performed on a BioRad CFX96 Touch system (BioRad, USA) in triplicate. All reactions were run simultaneously with seven times serially diluted standards of known quantities ($\times 10^2$ to $\times 10^8$). Quality control, qPCR
protocols, reaction systems and information on primers (Table S3) are detailed in Supporting Information (SI-3).

**Illumina MiSeq sequencing of 16S rRNA gene amplicons.** To assess the diversity and relative abundances of bacteria in all samples, the V1 – V3 region of the bacterial 16S rRNA gene was barcoded, amplified and sequenced on Illumina Miseq platform (PersonalBio, Shanghai). Subsequent sequence data were analyzed using QIIME Pipeline Version 1.7. The data were first filtered to remove reads with sequence lengths less than 150 bp and-or with more than two ambiguous nucleotides (the quality resulting in average nucleotides > Q25). The quality-filtered sequences were then trimmed using USEARCHRDP (http://www.drive5.com/usearch/) for the chimera check. The resulting high quality sequences (400 – 450 bp) were clustered into operational taxonomic units (OTUs) at a 97% identity threshold. Taxonomy was assigned using the Ribosomal Database Project classifier (http://rdp.cme.msu.edu/).

The original Illumina sequencing data are available at Sequence Read Archive (NCBI-PRJNA352879) by the accession No. SRP093020.

**Data Analysis.** The SPSS 22.0 Statistics (IBM Corp, USA) was used to conduct Spearman correlation and other variance analysis, where the significance was always defined by 95% confidence intervals (P < 0.05). Principal component analysis (PCA), redundancy analysis (RDA) and other clustering correlation analyses were performed in R3.2.5. Details of statistical methods are provided in Supporting Information.
RESULTS

Distribution of Target Antibiotics in Landfill Refuse and Leachates. Antibiotics from four different classes were quantified in landfill refuse and leachates with detected levels ranging from 5 to 250 µg/kg in refuse samples and 10 to 1000 ng/L in leachates. Wide variations were noted among samples (Table 1), which reflects innate variability in landfill and leachate contents over space and time. To contrast antibiotic levels between landfills of different ages, mass concentrations of each class were summed to permit statistical comparisons among classes.

The overall trend was lower antibiotic levels in more aged landfill samples. Total antibiotics masses were 314.0 ± 211.1, 292.1±139.2 and 138.1 ± 117.4 µg/kg in < 3yr, 10yr and > 20yr refuse respectively (Kruskal-Wallis (KW), $\chi^2 = 25.5$, P < 0.01), and 1480.0 ± 180.0, 857.3±223.8 and 922.5 ± 179.6 ng/L in < 3yr, 10yr and > 20yr leachates, respectively (KW test, $\chi^2 = 10.2$, P < 0.01). Specifically, mean β-glactam (AMO as the major component) levels ranged from 38.9 ± 10.6 µg/kg (refuse) and 158.6 ± 41.7 ng/L (leachates) in the < 3yr landfill samples to below detection (< 0.1 ng/L; Table 1) in the > 20yr landfill samples. For other antibiotics, TC levels were significantly higher in < 3yr versus > 20yr refuse (69.5 ± 17.2 vs. 12.7 ± 7.9 µg/kg; KW test, $\chi^2 = 11.6$, P < 0.01) and leachates (141 ± 8.9 vs. 10.6 ± 2.2 ng/L; KW test, $\chi^2$
Among them, OTC was the dominant antibiotic with concentrations of 20 µg/kg and 110 ng/L in refuse and leachates, respectively (Table 1). Similarly, MLs were significantly higher in the < 3yr (182 ± 210 µg/kg) versus > 20yr refuse samples (89.1 ± 155 µg/kg; Wilcoxon test, P < 0.05) and they were primarily represented by ETM-H2O. SDZ and SMX were two major antibiotics, contributing mainly to the significant decrease of SAs in leachates (321 ± 67.6 versus 73.8 ± 53.2 ng/L; Wilcoxon test, P < 0.01), and increase in refuse samples (i.e., 22.5 ± 3.7 to 32.8 ± 12.4 µg/kg, Paired t-test, P < 0.05).

**Metals and Physiochemical Properties of Landfill Leachates and Refuse.** Huge variations of target metals and physical properties, which indicate the composition of MSWs (e.g. degradable organics: wood, food residuals; nodegradable organics and inorganics: rubber, plastics, heavy metal, household use coal ashes, etc.) were observed in sampled refuse and leachates. All metals analyzed, except for Co, were detected in all samples (Table S2) with levels ranging from 5 to 500 mg/kg in refuse and 0.001 to 50 mg/L in leachate samples. Overall, metal levels were higher in older landfill samples, and the difference between each ages is statistically significant (One-way ANOVA, F = 12.3, P < 0.01). The most common metals were Zn and Fe, which accounted for ~ 80% of the total metal mass pools.

All refuse and leachate samples had a pH between 6.2 and 6.9, except the > 20yr leachates, which had a mean pH of 8.2 and 7.8 in leachates and refuse, respectively.
(Table 2). This important physiochemical parameter shows samples with landfill age > 20yr were in the “alkaline fermentation” phase, which is also an indicator of a landfill being stable or aged.\textsuperscript{21, 32} In terms of carbon (Table 2), TOC contents in refuse experienced a fourfold reduction from < 3yr (97.5±5.4 g/kg) to > 20yr (One-way ANOVA, F = 164.9, P < 0.01). Likewise, the COD levels in leachates samples significantly decreased from 20000 mg/L to 1000 mg/L (One-way ANOVA, F = 66.9, P < 0.01). Secondary nutrient levels (Table 2), including TN, TP and NH\textsubscript{3}-N, were significantly lower in the > 20yr versus < 3yr landfill samples (KW test, P < 0.05), whereas both the NH\textsubscript{3}-N/TN (KW test, $\chi^2 = 10.4$, P < 0.05) and TN/COD ratios (One-way ANOVA, F = 104.2, P < 0.01) increased with age, which is indicative of aging landfills.\textsuperscript{21}

**Target genes abundances in landfill refuse and leachates.** Distinct clusters of target ARGs and MGEs were visualized as a function of landfill sample age and source (Figure 1). Figure S1 shows that more unique gene clusters are apparent in the landfill refuse samples as compared to the leachate samples, suggesting less homogenization at the source. Figure 1(a) shows that the average contents of all targeted ARGs generally ranged from 6.0 – 6.5 log\textsubscript{10}(copies/ngDNA) in refuse, with the concentrations in the >20 yr samples being the lowest (One-way ANOVA, F = 10.8, P < 0.01). Although target genes’ total abundance of each resistance group fluctuate by ~ 2 orders of magnitude across refuse ages, their subtypes including sul\textsubscript{I},
ermB, aadA1 and bla$_{CTX-M}$ were constantly dominant (Figure 1(a)). Among all target MGEs, intI was the most abundant with a concentration (6.4±0.2 log$_{10}$(copies/ngDNA)) that was consistently 2-3 orders of magnitudes higher than intI2 (3.8±0.3 log$_{10}$(copies/ngDNA)) in refuse. There was no significant difference in total MGE detected across sample ages (KW test, P = 0.06). In contrast, Figure 1(b) shows that ARG levels increased significantly from < 3yr to > 20yr in the leachate samples (One-way ANOVA, F = 76.5, P < 0.01). Among these ARGs, sulI (5.6 ± 0.9 log$_{10}$(copies/ngDNA)), aadA1 (5.5 ± 0.8 log$_{10}$(copies/ngDNA)) and bla$_{CTX-M}$ (4.1 ± 0.7 (log$_{10}$(copies/ngDNA)) were the dominant subtypes. Also, target MGEs were significantly enriched in leachates sampled from older landfills (One-way ANOVA, F = 116.3, P < 0.01), with intI and IS26 (6.5 - 7.5 log$_{10}$(copies/ngDNA)) as major components.

Changes of Bacterial Community During Landfilling Process. The compositions of bacterial communities displayed distinct variations across samples of different landfill ages (Adonis, F = 5.6, P < 0.01). Overall, the predominant bacterial phyla were Proteobacteria, Firmicutes, Chloroflexi, Actinobacteria, Bacteroidetes and Acidobacteria in refuse and leachate samples (Figure S2). Proteobacteria were the largest group, which decreased from ~ 30% (< 3yr) to < 20% (> 20yr) in refuse, but increased by 40% in leachates over this same time period (KW test, $\chi^2 = 6.2$, P < 0.05). The fluctuations in the phylum of Proteobacteria primarily resulted from the
variations of $\alpha$-Proteobacteria and $\gamma$-Proteobacteria (Figure S3). Chloroflexi, the second most abundant phylum in refuse (23.7±7.1%), increased by 5% due to an increase in Anarolineae, which was the most abundant class (~20%) in > 20yr samples. With the progression of landfilling, Actinobacteria also exhibited a stepwise increase to about 15% in aged samples (One-way ANOVA, $P < 0.01$, Figure S2), most of which were contributed by the class of Actinobacteria (Figure S3). Conversely, Firmicutes, primarily represented by the class of Clostridia, decreased by 3% (KW test, $P = 0.24$) and 20% (KW test, $\chi^2 = 19.3$, $P < 0.01$) in refuse and leachates, respectively. Bacteroidetes were dominant in leachate (26.9±18.6%), but experienced a 40% decline from < 3 yr to > 20 yr samples (KW test, $\chi^2 = 8.4$, $P = 0.01$), where its major component at class taxon rank is Bacteroidia.

**DISCUSSION**

In the emerging world, > 65% of MSWs are not systematically collected and then are disposed of at uncontrolled dumpsites, where tens of millions tons of MSWs are estimated to affect the 64 million people’s health. Notably, the rate of antibiotics consumption in these developing countries has been increasing during last decade. It is reasonable to assume that the inefficient MSWs managements and increasing consumptions of antibiotics contribute to the antibiotic residuals entering local wastes discharge and disposal systems. As a result, compositions of antibiotics detected in MSW landfills presumably mirror the domestic antibiotics usage patterns. For
example, household/nonprescription use of β-lactams is a recent practice\textsuperscript{35, 36}, while quite limited decades ago (> 20 yr samples); and in our study, AMO and CFX were exclusively detected in < 3 yr samples (Table 1). Although the effects of these detected β-lactams on the parallel AMR development have not been proved in landfill samples, this might hint that AMR in landfills could possibly evolve along with domestic antibiotic use, and thereby result in a non-static AMR release pattern. Therefore, it is critical for future AMR management to know how ARGs’ contents and their disseminations are associated with aging landfill conditions, which we report for the first time.

**Importance of Antibiotics and Metals to Prevalence of ARGs in Landfills.** As shown in Figure 1(b), aging landfills released increasingly more ARGs, which is in agreement with previous landfill studies.\textsuperscript{9, 22, 24} However, this phenomenon is counterintuitive since the paralleling antibiotics’ levels presumably representing selective pressures are declining (Table 1). Compared to the minimum inhibitory concentrations (MICs) that were detected from anthropogenic wastes impacted environments,\textsuperscript{28, 37} most of target antibiotics’ concentrations in our landfill samples (Table 1) were 2 to 3 orders of magnitude lower. For example, the reported MICs of TC, SMX, AMO and ETM are 30, 64, 18 and 64 mg/L, respectively\textsuperscript{28, 38}, while these target antibiotics were all < 1 mg/L in leachates. This implies that the occurrences of ARGs are probably not directly related to antibiotics disposed in landfills.
Relationships between targeted genes and antibiotics were further delineated by RDA. Figure 2(a) shows that ARGs were poorly correlated with antibiotics in refuse (Mantel test, $P = 0.74$) and their abundances appeared to be negatively affected by the detected antibiotics (Table S4). Although antibiotics-ARGs relationship became more closely associated in leachates (Mantel test, $R = 0.25$, $P = 0.01$), clustered data indicate most significant correlations were negative (Table S5). In contrast, Figure 2(b) shows that metals were more closely correlated with target ARG groups across all samples (RDA, $P < 0.01$, permutations = 999), especially in leachates (Mantel test, $R = 0.66$, $P < 0.01$). In refuse samples, only Ni was significantly correlated with $blaR$ (Spearman, $R = 0.51$, $P < 0.05$; Table S6); and in terms of ARG subtypes, only $mefA$ and $bla_{OXA}$ displayed positive correlations with more than two metals (Spearman, $P < 0.05$). Notably, except for Ni and Pb, all detected metals exhibited significant correlations with more than three ARGs subtypes in landfill leachates (Spearman, $P < 0.05$, Table S7). In previous studies, heavy metals were also found to be the most important factor affecting the pool of ARGs,$^{39,40}$ for imposing longer pressures for resistance selection than other agents in environment,$^{41}$ and the metal resistance are proved having the intimate genetic linkages with AMR.$^{42}$

**Effects of Bacterial Community, MGEs and Environmental Factors on ARGs Distribution.** Previous studies suggest that ARGs in environmental samples are partially structured by the microbial community, although MGEs, nutrients and other
factors may also be determinants.\textsuperscript{10,30} To address key factors here, ARGs - microbial taxa – MGEs linkages were explored using network analysis among samples.\textsuperscript{43} Most ARGs including \textit{blaR (bla\textsubscript{OXA})}, \textit{MLsR (ermB)} and \textit{AgyR (strB)} and \textit{mexF} in refuse samples exhibited high connectivity with the sequenced bacterial community than with MGEs (Figure 3(a)). This suggests that variations of target ARGs in sampled refuse could be more related to the bacterial compositions. The potential refuse bacteria associated with ARGs included Actinobacteria and Firmicutes with \textit{mexF} and \textit{ermB} (Spearman, R\textsuperscript{2} > 0.80, P < 0.05); Synergistetes with \textit{bla\textsubscript{OXA}}, \textit{bla\textsubscript{TEM}}, \textit{strB}, \textit{ermB}; and Thermotogea with \textit{bla\textsubscript{OXA}} and \textit{strB} (Spearman, R\textsuperscript{2} > 0.85, P < 0.05).\textsuperscript{29} For the dominant bacteria like Proteobacteria and Bacteriodetes (Figure S2), Figure 3(a) shows that they had no apparent correlations with any ARG subtypes.

Interestingly, Figure 3(b) shows that, as leachates were discharged from inside landfills, they inherited most exiting ARGs-microbial taxa linkages from refuse, and more connections were constructed indeed. For example, the dominant Proteobacteria and Bacteriodetes established (positive) linkages with ARGs like \textit{ermB}, \textit{mefA}, \textit{mexF} and \textit{tetM} (Spearman, R\textsuperscript{2} > 0.65, P < 0.05). This is presumably because those leachates samples were characterized with higher abundance of \textit{\gamma-Proteobacteria} and Bacteroidia (Figure S3), which often host suites for ARGs conferring multi-resistance.\textsuperscript{45,46} Moreover, MGEs became more closely associated with ARGs in leachates (Figure 3(b)). Among these visualized linkages, the dominant MGEs like
intI1 and IS26 were significantly correlated with all target ARGs. In fact, intI1 (intI2) and traA (marking integrons and plasmids, respectively) linked most ARGs to dominant bacteria (e.g. Proteobacteria and Chloroflexi), which implies that the MGEs may play an important role for the dissemination of ARGs in leachates. Figure 3(b) also shows that MGEs nodes possessing higher ARGs-connectivity degrees, compared to refuse samples, tended to be more inter-correlated. For example, the dominant integron marker gene intI1 (Figure 1) was not only significantly linked to the major transposon markers (insert sequences) IS26, (Spearman, R² = 0.81, P < 0.05), but also was statistically associated with plasmid markers (traA and trbC) in positive ways (Spearman, R² > 0.75, P < 0.05). The increasing MGEs inner-connectivity in leachates (vs. refuse) reflects more complete genetic combinations among them, providing prerequisites for an efficient HGT of ARGs. Linear regression of total ARGs abundance with MGEs generally represents to what extent ARGs were propagated via HGT approach. In the case of landfills, target ARG levels in sampled leachates fluctuated more linearly (vs. refuse) with increasing MGEs contents from < 3yr to > 20yr (Spearman, R = 0.79, P < 0.01). This significant regression provides statistical evidence of leachates ARGs being characterized with high HGT potentials. Further, regarding the distributional structures of ARGs, detected ARG profiles in landfill refuse were significantly correlated to bacterial
compositions (annotated OTUs counts) based on Bray–Curtis distance (Mantel test, $P < 0.01$), and their statistical fitness was validated by Procrustes test ($M^2 = 0.37$, $R = 0.86$, $P < 0.01$, permutations = 999, Figure 4(a)). Similar associations were also observed in solid wastes treatment systems like composting and land application, where microbial communities have been reported as key factors structuring target ARGs or the local resistome.$^{29, 30, 51}$ The variation partitioning analysis (VPA) specifies that microbial community (bacterial composition) defined 29.7% of the variations of target ARGs in refuse (Figure 4(b)), while the effects of ARGs selecting-pressure agents were negligible ($< 1\%$ of explained variations). On the other hand, Figure 4(a) shows that the relationship between ARGs and 16S (rRNA-OTUs) in leachates also passed the goodness-of-fit test (Procrustes test, $M^2 = 0.17$, $R = 0.44$, $P = 0.02$, permutations = 999), but with lower fitness (vs. refuse). Interestingly, following VPA shows that, variations of ARG contents in leachate samples were majorly explained (34.6%) by MGEs (Figure 4(c)), whereas environmental factors, ARGs selecting-pressure agents and microbial community each constituted only $\sim 5\%$ of the total variance.

Implications. This study shows that AMR exists and is readily released from landfills (in leachates). However, the drivers and patterns of release differ as a function of landfill ages. Specifically, younger landfills may release higher levels of antibiotics than older landfills, presumably due to degradation or changes in usage patterns.
However, the fate and release of AMR are not as simple. The contents of selected ARGs are the consequences of evolving microbial events and increasing heavy metal mobilization during landfilling process. Our study confirms that bacterial community composition majorly shapes ARGs profiles in refuse (regardless of landfill age), whereas the HGT conducted by MGEs and presences of heavy metals is more important to ARGs in leachates.

Further, this work hints MSW landfills may pose a much greater AMR risk than is believed, especially within the global context that landfills with “old landfill” traits (e.g. dumpsites),\textsuperscript{52} featuring the possible release of untreated leachates, are still very common in the emerging world.\textsuperscript{19} Prospective worldwide studies, which utilize metagenomics to detect the resistome, explore co-occurrence of ARGs and HGT related genetic elements and identify ARGs-carrying bacteria, are encouraged to better the understanding of ARGs dissemination in landfill systems and neighboring environments.

**ASSOCIATED CONTENT**

The Supporting Information (SI) is comprised of four sections: SI-1) antibiotics and metal measurement; SI-2) physicochemical composition of landfill samples; SI-3) molecular tests information and SI-4) statistics. This material is available free of charge via the Internet at http://pubs.acs.org.
ACKNOWLEDGMENTS

This work is supported by the Natural Science Foundation of China (21577038, 31370510), East China Normal University Outstanding Doctoral Dissertation Cultivation Plan of Action (PY2015034), and Shanghai Tongji Gao-Tingyao Environmental Science & Technology Development Foundation (STGEF2017).

Authors thank editors and reviewers for their constructive suggestions and comments on manuscript revisions. Dong Wu thanks Mgr. H. Huang (LG Wastes Disposal Co. LTD), Ms. Y. Cao, Dr. H.C. Zhao (East China Normal University) and Dr. M.C. McLaughlin (Colorado State University, USA) for their supports in sampling, TOC tests, molecular tests and manuscript language revision, respectively. Dong Wu would specifically like to thank his new-married wife, Ting Wang, for her ongoing and unwavering love.
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**LEGEND OF TABLES**

Table 1. Concentration of antibiotics in landfill refuse and leachates as a function of landfill age

Table 2. Physiochemical properties of sampled landfill leachates and refuse

**LEGEND OF FIGURES**

Figure 1. ARG profiles (log10 transformed values) in refuse (a) and leachates (b) within landfills as a function of age. Samples are grouped into three ages, including: < 3yr, 10 yr and > 20yr. The clusters were calculated based on the on standardized Euclidean clustering – distance.

Figure 2. Redundancy analysis (RDA) identified the correlation between sum of each group of antibiotics (a), metals (b) and sum of each group of ARGs in samples collected from landfill refuse (circle) and leachates (square). The details of correlation coefficient were provided in support information (SI-4). *sulR, AygR, MLsR, tetR* and *blaR* represent ARGs link to SAs, aminoglycosides, MLs, TCs and β- Lactams respectively; MDR is *mexF*.

Figure 3 The network analysis revealing the correlations between ARG and MGEs subtypes in refuse (a) and leachates (b). The circle and triangle nodes indicate target ARGs and MGEs respectively. The 16S sequenced bacteria (at phylum level) were denoted as square nodes. All data were scaled (mean = 0) prior to analysis. Each
connection represents a strong (Spearman; coefficient $R^2 > 0.65$) and significant ($P < 0.05$) correlation. The node size and edge width were weighted according to their degrees of connectivity and coefficients respectively. Grey-solid and green-dash lines depict positive and negative relationships respectively.

**Figure 4** Associations between microbial community conditions and target ARGs in landfill samples. (a) Procrustes analysis of significant correlation between target ARGs contents and bacterial composition (16S rRNA gene OTUs data) on the basis of Bray–Curtis dissimilarity metrics. The $M^2$ and $R$ represent sum of squares and correlation coefficient respectively. Variation partitioning analysis (VPA) differentiating effects of bacterial community, environmental factors, ARGs selecting pressure agents and mobile genetic elements (MGEs) on the target ARGs alteration in landfill (b) refuse and (c) leachates. The explanatory factor with values less than 0.01 (explained < 1% of total ARGs variations) was removed from VPA results, such as “Selecting Pressure” in refuse.
Table 1 Concentration of antibiotics in landfill refuse and leachates as a function of landfill age

<table>
<thead>
<tr>
<th>Samples¹</th>
<th>Tetracyclines (TCs)</th>
<th>Sulfonamides (SAs)</th>
<th>Macrolides (MLs)</th>
<th>β- Lactams</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OTC</td>
<td>TC</td>
<td>DXC</td>
<td>SMZ</td>
</tr>
<tr>
<td>Refuse (µg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 3 yr</td>
<td>20.6 (13.8)²</td>
<td>4.7 (0.8)</td>
<td>13.1 (7.8)</td>
<td>8.7 (5.0)</td>
</tr>
<tr>
<td>(n = 12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 yr</td>
<td>47.9 (54.1)</td>
<td>6.7 (6.0)</td>
<td>31.4 (23.5)</td>
<td>9.2 (10.1)</td>
</tr>
<tr>
<td>(n= 12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;20 yr</td>
<td>6.6 (4.7)</td>
<td>4.2 (5.4)</td>
<td>2.8 (1.4)</td>
<td>5.6 (1.4)</td>
</tr>
<tr>
<td>(n = 18)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>19.4 (16.7)</td>
<td>5.0 (4.6)</td>
<td>15.1 (30.4)</td>
<td>7.2 (6.9)</td>
</tr>
</tbody>
</table>

| Leachate (soluble form; ng/L) |          |                   |                  |            |            |            |            |           |           |            |            |            |
|                              | Oxytetracycline [OTC], tetracycline [TC], deoxytetracycline [DXC]; sulfamethazine [SMZ], sulfadiazine [SDZ], sulfamethoxazole [SMX] and trimethoprim [TMP]; roxithromycin [RTM] and Erythromycin-H₂O [ETM-H₂O]; cephalaxin [CFX] and amoxicillin [AMO].² Standard deviation in parentheses; ³ NA = Not available; ND = Not detected |
| < 3 yr                       | 1070 (314) | 90.4 (60.8)       | 472 (379)       | 25.4       | 450        | 222        | 182 (247)  | 215       | 1620 (1700)| 28.2 (44.6) | 151 (147)  |
| (n = 12)                     |                     |                    |                  |            |            |            |            |           |           |            |             |
| 10 yr                        | 56.7 (13.6)       | 35.9 (11.2)       | 60.8 (14.3)     | 38.1       | 152        | 16.1       | 23.9 (52.3) | 20.3      | 630 (828)  | ND (11.3)  |            |
| (n= 12)                      |                     |                    |                  |            |            |            |            |           |           |            |             |
| >20 yr                       | ND (115)          | 2.9 (1.2)         | 22.6 (113.9)    | 35.6       | 84         | 8.5        | 19.8 (34.2) | 36.6      | 804 (187)  | ND (NA)    |            |
| (n = 12)                     |                     |                    |                  |            |            |            |            |           |           |            |             |
| Mean (SD)                    | 115 (289)         | 19.8 (12.8)       | 71.3 (114.6)    | 91.4       | 147        | 42.8       | 29.4 (98.6) | 39.4      | 887 (503)  | 28.2 (44.6) | 72.2 (124) |

¹ Oszytetracycline [OTC], tetracycline [TC], deoxytetracycline [DXC]; sulfamethazine [SMZ], sulfadiazine [SDZ], sulfamethoxazole [SMX] and trimethoprim [TMP]; roxithromycin [RTM] and Erythromycin-H₂O [ETM-H₂O]; cephalaxin [CFX] and amoxicillin [AMO].² Standard deviation in parentheses; ³ NA = Not available; ND = Not detected
Table 2 Physiochemical properties of sampled landfill leachates and refuse as a function of landfill age

<table>
<thead>
<tr>
<th>Samples</th>
<th>Basic Properties</th>
<th>Nutrients (µg/kg-dry refuse)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Conductivity (EC µs/cm)</td>
</tr>
<tr>
<td>Refuse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 3 yr</td>
<td>6.1 (0.5)</td>
<td>126.4 (27.8)</td>
</tr>
<tr>
<td>(n = 12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 yr</td>
<td>7.2 (0.1)</td>
<td>291 (38.9)</td>
</tr>
<tr>
<td>(n = 12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 20 yr</td>
<td>7.8 (0.1)</td>
<td>2.8 (1.4)</td>
</tr>
<tr>
<td>(n = 18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)³</td>
<td>6.8 (0.1)</td>
<td>178.2 (61.7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Leachates</th>
<th>DO</th>
<th>pH</th>
<th>Conductivity (EC µs/cm)</th>
<th>NH₃-N (mg/L)</th>
<th>NO₂⁻/NO₃⁻ N (mg/L)</th>
<th>TN (mg/L)</th>
<th>TP (mg/L)</th>
<th>COD (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 3 yr</td>
<td>NA</td>
<td>6.2 (0.9)</td>
<td>1470 (78.6)</td>
<td>1370 (91.4)</td>
<td>23.1 (13.0)</td>
<td>3620 (545)</td>
<td>135 (5.3)</td>
<td>22200 (500)</td>
</tr>
<tr>
<td>(n = 12)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>10 yr</td>
<td>0.03 (0.01)</td>
<td>6.5 (0.3)</td>
<td>425 (49.8)</td>
<td>114 (178)</td>
<td>19.6 (7.2)</td>
<td>2920 (113)</td>
<td>50.3 (21.0)</td>
<td>11500 (231)</td>
</tr>
<tr>
<td>(n = 12)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 20 yr</td>
<td>0.5 (0.1)</td>
<td>8.2 (0.3)</td>
<td>109 (4.4)</td>
<td>616 (41.8)</td>
<td>34.5 (26.8)</td>
<td>933 (52.8)</td>
<td>4.5 (1.9)</td>
<td>984 (67.0)</td>
</tr>
<tr>
<td>(n = 12)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>0.28 (0.27)</td>
<td>6.9 (0.7)</td>
<td>546 (428)</td>
<td>1510 (970)</td>
<td>27.7 (20.9)</td>
<td>2970 (533)</td>
<td>29.4 (25.5)</td>
<td>7420 (9760)</td>
</tr>
</tbody>
</table>

All data shown are average or mean; data in the parentheses are standard deviation (SD)
Figure 1 ARG profiles (log10 transformed values) in refuse (a) and leachates (b) within landfills as a function of age. Samples are grouped into three ages, including: < 3yr, 10 yr and > 20yr. The clusters were calculated based on the standardized Euclidean clustering – distance.
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*All materials used in the Table of Content were originally from authors.