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**Antibiotic resistance genes and associated microbial
community conditions in aging landfill systems**

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23 ABSTRACT

24 Landfills receive about 350 million tons of municipal solid wastes (MSWs) per year
25 globally, including antibiotics and other co-selecting agents that impact antimicrobial
26 resistance (AMR). However, little is known about AMR in landfills, especially as a
27 function of landfill ages. Here we quantified antibiotics, heavy metals, and AMR
28 genes (ARGs) in refuse and leachates from landfills of different age (< 3, 10, and > 20
29 years). Antibiotics levels were consistently lower in refuse and leachates from older
30 landfills, whereas ARG levels in leachates significantly increased with landfill age
31 (One-way ANOVA, $F = 10.8$, $P < 0.01$). Heavy metals whose contents increased as
32 landfills age (One-way ANOVA, $F = 12.3$, $P < 0.01$) were significantly correlated with
33 elevated levels of ARGs (Mantel test, $R = 0.66$, $P < 0.01$) in leachates, which implies
34 greater AMR exposure risks around older landfills. To further explain ARGs
35 distributional mechanisms with age, microbial communities, mobile genetic elements
36 (MGEs) and environmental factors were contrasted between refuse and leachate
37 samples. Microbial communities in the refuse were closely correlated with ARG
38 contents (Procrustes test; $M^2 = 0.37$, $R = 0.86$, $P < 0.001$), whereas ARG in leachates
39 were more associated with MGEs.

40 INTRODUCTION

41 Landfills reportedly receive the largest portion (~350 million tons per year) of
42 municipal solid wastes (MSWs) around the world, which spans countries at all levels
43 of economic development.¹ This estimate may actually be low, given that the USA
44 and China annually sent 262 and 180 million tons of MSWs to landfills (2010-2013),²
45 ³ respectively. Even though landfill use has declined in developed countries (e.g. USA)
46 since the 1990s,^{1,4} MSWs landfilling amount is increasing in developing countries^{1,3},
47 with China predicted to be the largest MSWs generator by 2030⁵. This trend is
48 particularly concerning because contaminants in ill-managed MSWs landfills could be
49 released to neighboring environments in the long-term.⁶⁻⁸ Further, inappropriate
50 disposal of antibiotics, detergents and heavy metals have made landfills into harbors
51 and potential sources of antimicrobial resistance (AMR), particularly AMR genes
52 (ARGs).^{9,10}

53 In general terms, overuse of antibiotics and inadequate residuals management have
54 transformed what was a “health panacea” into a global problem,^{11,12} due to increased
55 levels of acquired AMR.^{13,14} The death toll in Europe alone due to AMR bacteria is ~
56 25,000 per year.¹⁵ In United States, ~ 2,000,000 illnesses are annually caused by
57 AMR.¹⁶ Many AMR studies have been performed on wastewater systems and suggest
58 waste-associated antibiotics and ARGs pose an exposure risk to humans,^{17,18} but very
59 little attention has been paid to the solid waste streams, which may be an important,

60 but less apparent, gateway for the spread of AMR. Recent work has pointed out that
61 around 2 million people live within a 10 km radius of the 50 largest active dumpsites
62 (unregulated landfills). These are primarily located in SE Asia, Africa, the
63 Subcontinent, and Latin America,¹⁹ which are all regions where antibiotic use is
64 inefficiently regulated.¹¹ In combination, this means densely populated emerging and
65 developing countries are most vulnerable to AMR risks.

66 Landfilling is a decades long process,^{20, 21} during which the complex and dynamic
67 ecosystems inside the landfill are subject to fluctuations in pH, oxygen level,
68 temperature, carbon and nutrients. As such, the varying habitats may impact microbial
69 AMR development. Previous work has confirmed the release of ARGs, mobile
70 genetic element (MGE)²², AMR bacteria¹⁰ and antibiotics^{23, 24} from MSW landfills.
71 However, no holistic assessment, including ARGs and MGEs abundance and diversity,
72 has been performed on the age of landfill systems that contrasts physiochemical
73 conditions and microbial communities. Here we quantified antibiotics, heavy metal
74 levels, microbial community conditions, twelve ARG subtypes, and six MGEs in
75 refuse and leachates from landfill sites with three different ages (i.e. <3, 10, and >20
76 years). The goal was to (i) characterize how the abundance and distribution of ARGs
77 in landfills change over space and time and (ii) to explain how the dynamics of
78 microbial conditions affect the ARGs variation.

79 MATERIALS AND METHODS

80 **Description of Landfills and Sampling.** Sampling was performed at Laogang (LG)
81 landfill in Shanghai, China. This landfill is located in an eastern semi-rural area of
82 Shanghai and receives about 12,000 t/d of MSWs from the city. At LG, on-site landfill
83 operations are separated into five distinct Phases according to operational age. Phases
84 I to III were operated in the 1980s and 1990s, and are currently being reclaimed.
85 Phase IV was operated between 2000 to 2010 and nearly closed, whereas Phase V
86 commenced in 2010 and currently receives > 80% of MSWs at the LG landfill. In this
87 study, landfill refuse (coring to a depth of 1.5 m) and leachate (parallel reservoirs)
88 samples were collected from zones including < 3 yr (Phase V), 10-yr (Phase IV) and >
89 20 yr zones (Phase I). All cores and leachates were immediately placed in sealed cool
90 boxes after sampling.

91 **Pretreatment of Samples.** Sampled leachates (50 mL) were centrifuged at 10000 x g
92 for 8 min. The resulting centrates were passed through sterile 0.45 μm PES membrane
93 filters (MembraneSolutions Allpure/045, Shanghai) to remove residual particles and
94 bacteria. The membrane filters and centrifuge pellets were combined for each sample
95 and frozen at -35°C for subsequent molecular assays. Refuse cores were sub-sampled
96 and frozen at -35°C upon return to the lab. To reduce potential bias caused by
97 heterogeneous composition of MSWs in subsequent analysis, refuse samples were
98 lyophilized (typically ~20 g aliquots) and then sieved through a 40-mesh screen.⁹

99 **Measurement of Antibiotics, Metals and Other Physicochemical Parameters.**

100 Four classes of antibiotics were quantified (**Table 1**), including tetracyclines (TCs);
101 sulfonamides (SAs); macrolides (MLSs); and beta-lactams (β -lactams). Quantification
102 methods for antibiotics in refuse and landfill leachate samples were optimized
103 according to previous studies using internal standards methods by UPLC-MS/MS.^{12, 25}
104 The details of preconditioning, extraction, elution, quality control and ranges of
105 detected antibiotic concentrations are provided in the Supporting Information (SI-1).
106 Metals assessed include Al, As, Fe, Ni, Cr, Cu, Mn, Pb, Zn, Cd and Co, and were
107 quantified using ICP-OES (Agilent 720ES, Agilent Technologies). The detected
108 values (**Table S2**) and pretreatment methods are described in Supporting Information
109 (SI-1).
109 Considering that composition of MSWs in Shanghai may have fluctuated over the last
110 20 years, this study used nitrogen, phosphorus and carbon contents to reflect the
111 changes in MSWs composition during the landfilling process. Specifically, dissolved
112 oxygen (DO) and pH were measured in situ using hand-held field probes (HANNA,
113 Italy). The pH value of pretreated refuse samples were measured in the lab by Soilstik
114 (Spectrum, USA). Ammonia nitrogen ($\text{NH}_3\text{-N}$), nitrate ($\text{NO}_3^- - \text{N}$), nitrite ($\text{NO}_2^- - \text{N}$),
115 total nitrogen (TN), total phosphorus (TP), and chemical organic carbon (COD for
116 leachates only) were measured using Smartchem 200 (Alliance, France). Total organic
117 carbon (TOC for refuse only) was measured using Vario TOC Select (Elementar
118 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.cbcb.umd.edu/browsegene.shtml>) included widespread class A (*bla*_{CTX-M-15}, *bla*_{TEM}; the *bla*_{CTX-M-15} is denoted as *bla*_{CTX-M} in the following sections) and D β-lactamase genes (*bla*_{NDM-1} and *bla*_{OXA-1}; denoted as *bla*_{NDM} and *bla*_{OXA} respectively in the following sections) resistant to third generation β-lactams (*bla*_R).²⁶⁻²⁸ Other widespread ARGs include *aadA1* and *strB*, which are resistant to Aminoglycosides (*AgyR*); *tetM* and *tetQ*, which are resistant to TCs (*tetR*); *sulI* and *sul2*, which are resistant to SAs (*sulR*); *ermB* and *mefA*, which are resistant to MLs (*MLsR*) and multidrug-resistant subtype, *mexF*.²³ Along with these ARGs, six MGEs markers (*int1*, *int2*, *IS26*, *ISCR*, *traA* and *trbC*) were also quantified.²² 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

139 protocols, reaction systems and information on primers (**Table S3**) are detailed in
140 Supporting Information (SI-3).

141 **Illumina MiSeq sequencing of 16S rRNA gene amplicons.** To assess the diversity
142 and relative abundances of bacteria in all samples, the V1 – V3 region of the bacterial
143 16S rRNA gene was barcoded, amplified and sequenced on Illumina Miseq platform
144 (PersonalBio, Shanghai). Subsequent sequence data were analyzed using QIIME
145 Pipeline Version 1.7. The data were first filtered to remove reads with sequence
146 lengths less than 150 bp and-or with more than two ambiguous nucleotides (the
147 quality resulting in average nucleotides > Q25). The quality-filtered sequences were
148 then trimmed using USEARCHRDP (<http://www.drive5.com/usearch/>) for the
149 chimera check. The resulting high quality sequences (400 – 450 bp) were clustered
150 into operational taxonomic units (OTUs) at a 97% identity threshold. Taxonomy was
151 assigned using the Ribosomal Database Project classifier (<http://rdp.cme.msu.edu/>).
152 The original Illumina sequencing data are available at Sequence Read Archive
153 (NCBI-[PRJNA352879](https://www.ncbi.nlm.nih.gov/sra/PRJNA352879)) by the accession No. SRP093020.

154 **Data Analysis.** The SPSS 22.0 Statistics (IBM Corp, USA) was used to conduct
155 Spearman correlation and other variance analysis, where the significance was always
156 defined by 95% confidence intervals ($P < 0.05$). Principal component analysis (PCA),
157 redundancy analysis (RDA) and other clustering correlation analyses were performed
158 in R3.2.5.²⁹⁻³¹ Details of statistical methods are provided in Supporting Information

159 (SI-4).

160 RESULTS

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

168 The overall trend was lower antibiotic levels in more aged landfill samples. Total
169 antibiotics masses were 314.0 ± 211.1 , 292.1 ± 139.2 and 138.1 ± 117.4 $\mu\text{g/kg}$ in < 3yr,
170 10yr and > 20yr refuse respectively (Kruskal-Wallis (KW), $\chi^2 = 25.5$, $P < 0.01$), and
171 1480.0 ± 180.0 , 857.3 ± 223.8 and 922.5 ± 179.6 ng/L in < 3yr, 10yr and > 20yr
172 leachates, respectively (KW test, $\chi^2 = 10.2$, $P < 0.01$). Specifically, mean β -lactam
173 (AMO as the major component) levels ranged from 38.9 ± 10.6 $\mu\text{g/kg}$ (refuse) and
174 158.6 ± 41.7 ng/L (leachates) in the < 3yr landfill samples to below detection (< 0.1
175 ng/L ; **Table 1**) in the > 20yr landfill samples. For other antibiotics, TC levels were
176 significantly higher in < 3yr versus > 20yr refuse (69.5 ± 17.2 vs. 12.7 ± 7.9 $\mu\text{g/kg}$;
177 KW test, $\chi^2 = 11.6$, $P < 0.01$) and leachates (141 ± 8.9 vs. 10.6 ± 2.2 ng/L ; KW test, χ^2

178 = 15.2, $P < 0.01$). Among them, OTC was the dominant antibiotic with concentrations
179 of 20 $\mu\text{g/kg}$ and 110 ng/L in refuse and leachates, respectively (**Table 1**). Similarly,
180 MLs were significantly higher in the $< 3\text{yr}$ ($182 \pm 210 \mu\text{g/kg}$) versus $> 20\text{yr}$ refuse
181 samples ($89.1 \pm 155 \mu\text{g/kg}$; Wilcox test, $P < 0.05$) and they were primarily represented
182 by ETM- H_2O . SDZ and SMX were two major antibiotics, contributing mainly to the
183 significant decrease of SAs in leachates (321 ± 67.6 versus $73.8 \pm 53.2 \text{ ng/L}$; Wilcox
184 test, $P < 0.01$), and increase in refuse samples (i.e., 22.5 ± 3.7 to $32.8 \pm 12.4 \mu\text{g/kg}$,
185 Paired t-test, $P < 0.05$).

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

196 All refuse and leachate samples had a pH between 6.2 and 6.9, except the $> 20\text{yr}$
197 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.^{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 $\text{NH}_3\text{-N}$, were significantly lower in the > 20yr versus < 3yr landfill samples (KW test, $P < 0.05$), whereas both the $\text{NH}_3\text{-N}/\text{TN}$ (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.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 $\log_{10}(\text{copies/ngDNA})$ 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 *sulI*,

218 *ermB*, *aadA1* and *bla*_{CTX-M} were constantly dominant (**Figure 1(a)**). Among all target
219 MGEs, *intl1* was the most abundant with a concentration (6.4 ± 0.2
220 $\log_{10}(\text{copies/ngDNA})$) that was consistently 2-3 orders of magnitudes higher than
221 *intl2* ($3.8 \pm 0.3 \log_{10}(\text{copies/ngDNA})$) in refuse. There was no significant difference in
222 total MGE detected across sample ages (KW test, $P = 0.06$). In contrast, **Figure 1(b)**
223 shows that ARG levels increased significantly from $< 3\text{yr}$ to $> 20\text{yr}$ in the leachate
224 samples (One-way ANOVA, $F = 76.5$, $P < 0.01$). Among these ARGs, *sul1* (5.6 ± 0.9
225 $\log_{10}(\text{copies/ngDNA})$), *aadA1* ($5.5 \pm 0.8 \log_{10}(\text{copies/ngDNA})$) and *bla*_{CTX-M} ($4.1 \pm$
226 $0.7 \log_{10}(\text{copies/ngDNA})$) were the dominant subtypes. Also, target MGEs were
227 significantly enriched in leachates sampled from older landfills (One-way ANOVA, F
228 $= 116.3$, $P < 0.01$), with *intl1* and *IS26* ($6.5 - 7.5 \log_{10}(\text{copies/ngDNA})$) as major
229 components.

230 **Changes of Bacterial Community During Landfilling Process.** The compositions
231 of bacterial communities displayed distinct variations across samples of different
232 landfill ages (Adonis, $F = 5.6$, $P < 0.01$). Overall, the predominant bacterial phyla
233 were Proteobacteria, Firmicutes, Chloroflexi, Actinobacteria, Bacteroidetes and
234 Acidobacteria in refuse and leachate samples (**Figure S2**). Proteobacteria were the
235 largest group, which decreased from $\sim 30\%$ ($< 3\text{yr}$) to $< 20\%$ ($> 20\text{yr}$) in refuse, but
236 increased by 40% in leachates over this same time period (KW test, $\chi^2 = 6.2$, $P < 0.05$).
237 The fluctuations in the phylum of Proteobacteria primarily resulted from the

238 variations of α -Proteobacteria and γ -Proteobacteria (**Figure S3**). Chloroflexi, the
239 second most abundant phylum in refuse ($23.7 \pm 7.1\%$), increased by 5% due to an
240 increase in Anarolineae, which was the most abundant class ($\sim 20\%$) in > 20 yr
241 samples. With the progression of landfilling, Actinobacteria also exhibited a stepwise
242 increase to about 15% in aged samples (One-way ANOVA, $P < 0.01$, **Figure S2**),
243 most of which were contributed by the class of Actinobacteria (**Figure S3**).
244 Conversely, Firmicutes, primarily represented by the class of Clostridia, decreased by
245 3% (KW test, $P = 0.24$) and 20% (KW test, $\chi^2 = 19.3$, $P < 0.01$) in refuse and leachates,
246 respectively. Bacteroidetes were dominant in leachate ($26.9 \pm 18.6\%$), but experienced
247 a 40% decline from < 3 yr to > 20 yr samples (KW test, $\chi^2 = 8.4$, $P = 0.01$), where its
248 major component at class taxon rank is Bacteroidia.

249 **DISCUSSION**

250 In the emerging world, $> 65\%$ of MSWs are not systematically collected and then are
251 disposed of at uncontrolled dumpsites, where tens of millions tons of MSWs are
252 estimated to affect the 64 million people's health¹⁹. Notably, the rate of antibiotics
253 consumption in these developing countries has been increasing during last decade.^{1, 33}
254 It is reasonable to assume that the inefficient MSWs managements and increasing
255 consumptions of antibiotics contribute to the antibiotic residuals entering local wastes
256 discharge and disposal systems.³⁴ As a result, compositions of antibiotics detected in
257 MSW landfills presumably mirror the domestic antibiotics usage patterns. For

example, household/nonprescription use of β -lactams is a recent practice^{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.^{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,^{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^{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

298 factors may also be determinants.^{10, 30} To address key factors here, ARGs - microbial
299 taxa – MGEs linkages were explored using network analysis among samples.⁴³ Most
300 ARGs including *blaR* (*bla*_{OXA}), *MLsR* (*ermB*) and *AgyR* (*strB*) and *mexF* in refuse
301 samples exhibited high connectivity with the sequenced bacterial community than
302 with MGEs (**Figure 3(a)**). This suggests that variations of target ARGs in sampled
303 refuse could be more related to the bacterial compositions. The potential refuse
304 bacteria associated with ARGs included Actinobacteria and Firmicutes with *mexF* and
305 *ermB* (Spearman, $R^2 > 0.80$, $P < 0.05$)⁴⁴; Synergistetes with *bla*_{OXA}, *bla*_{TEM}, *strB*,
306 *ermB*; and Theromotogea with *bla*_{OXA} and *strB* (Spearman, $R^2 > 0.85$, $P < 0.05$).²⁹ For
307 the dominant bacteria like Proteobacteria and Bacteriodetes (**Figure S2**), **Figure 3(a)**
308 shows that they had no apparent correlations with any ARG subtypes.

309 Interestingly, **Figure 3(b)** shows that, as leachates were discharged from inside
310 landfills, they inherited most exiting ARGs-microbial taxa linkages from refuse, and
311 more connections were constructed indeed. For example, the dominant Proteobacteria
312 and Bacteriodetes established (positive) linkages with ARGs like *ermB*, *mefA*, *mexF*
313 and *tetM* (Spearman, $R^2 > 0.65$, $P < 0.05$). This is presumably because those leachates
314 samples were characterized with higher abundance of γ -Proteobacteria and
315 Bacteroidia (**Figure S3**), which often host suites for ARGs conferring
316 multi-resistance.^{45, 46} Moreover, MGEs became more closely associated with ARGs in
317 leachates (**Figure 3(b)**). Among these visualized linkages, the dominant MGEs like

318 *intl1* and *IS26* were significantly correlated with all target ARGs. In fact, *intl1* (*intl2*)
319 and *traA* (marking integrons and plasmids, respectively) linked most ARGs to
320 dominant bacteria (e.g. Proteobacteria and Chloroflexi), which implies that the MGEs
321 may play an important role for the dissemination of ARGs in leachates.^{22, 47} **Figure**
322 **3(b)** also shows that MGEs nodes possessing higher ARGs-connectivity degrees,
323 compared to refuse samples, tended to be more inter-correlated. For example, the
324 dominant integron marker gene *intl1* (**Figure 1**) was not only significantly linked to
325 the major transposon markers (insert sequences) *IS26*, (Spearman, $R^2 = 0.81$, $P <$
326 0.05), but also was statistically associated with plasmid markers (*traA* and *trbC*) in
327 positive ways (Spearman, $R^2 > 0.75$, $P < 0.05$). The increasing MGEs
328 inner-connectivity in leachates (vs. refuse) reflects more complete genetic
329 combinations among them, providing prerequisites for an efficient HGT of ARGs.^{22, 48,}
330 ⁴⁹

331 Linear regression of total ARGs abundance with MGEs generally represents to what
332 extent ARGs were propagated via HGT approach.^{31, 50} In the case of landfills, target
333 ARG levels in sampled leachates fluctuated more linearly (vs. refuse) with increasing
334 MGEs contents from $< 3\text{yr}$ to $> 20\text{yr}$ (Spearman, $R = 0.79$, $P < 0.01$). This significant
335 regression provides statistical evidence of leachates ARGs being characterized with
336 high HGT potentials. Further, regarding the distributional structures of ARGs,
337 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-and 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.

358 However, the fate and release of AMR are not as simple. The contents of selected
359 ARGs are the consequences of evolving microbial events and increasing heavy metal
360 mobilization during landfilling process. Our study confirms that bacterial community
361 composition majorly shapes ARGs profiles in refuse (regardless of landfill age),
362 whereas the HGT conducted by MGEs and presences of heavy metals is more
363 important to ARGs in leachates.

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

372 **ASSOCIATED CONTENT**

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

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550 **LEGEND OF TABLES**

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

553 **Table 2.** Physiochemical properties of sampled landfill leachates and refuse

554 **LEGEND OF FIGURES**

555 **Figure 1.** ARG profiles (log10 transformed values) in refuse (a) and leachates (b)
556 within landfills as a function of age. Samples are grouped into three ages, including: <
557 3yr, 10 yr and > 20yr. The clusters were calculated based on the on standardized
558 Euclidean clustering – distance.

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

565 **Figure 3** The network analysis revealing the correlations between ARG and MGEs
566 subtypes in refuse (a) and leachates (b). The circle and triangle nodes indicate target
567 ARGs and MGEs respectively. The 16S sequenced bacteria (at phylum level) were
568 denoted as square nodes. All data were scaled (mean = 0) prior to analysis. Each

569 connection represents a strong (Spearman; coefficient $R^2 > 0.65$) and significant ($P <$
570 0.05) correlation. The node size and edge width were weighted according to their
571 degrees of connectivity and coefficients respectively. Grey-solid and green-dash lines
572 depict positive and negative relationships respectively.

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

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

Samples ¹	Tetracyclines (TCs)				Sulfonamides (SAs)			Macrolides (MLs)		β- Lactams	
Refuse (µg/kg)	OTC	TC	DXC	SMZ	SDZ	SMX	TMP	RTM	ETM-H ₂ O	CFX	AMO
< 3 yr (n = 12)	20.6 (13.8) ²	4.7 (0.8)	13.1 (7.8)	8.7 (5.0)	46.7 (4.6)	29.9 (15.7)	5.0 (4.6)	14.8 (9.5)	223 (266)	13.2 (3.6)	36.4 (18.9)
10 yr (n= 12)	47.9 (54.1)	6.7 (6.0)	31.4 (23.5)	9.2 (10.1)	23.4 (12.7)	5.2 (3.9)	3.0 (2.4)	7.8 (10.9)	111 (73.6)	NA ³	10.9 (11.3)
>20 yr (n = 18)	6.6 (4.7)	4.2 (5.4)	2.8 (1.4)	5.6 (4.9)	ND	39.8 (6.7)	22.6 (17.9)	ND	49.8 (55.6)	ND	NA
Mean (SD)	19.4 (16.7)	5.0 (4.6)	15.1 (30.4)	7.2 (6.9)	37.4 (33.7)	21.3 (37.2)	11.6 (14.4)	7.5 (9.5)	162 (152)	13.2 (3.6)	15.1 (17.4)
Leachate (soluble form; ng/L)											
< 3 yr (n = 12)	1070 (314)	90.4 (60.8)	472 (379)	25.4 (19.8)	450 (31.5)	222 (247)	182 (168)	215 (168)	1620 (1700)	28.2 (44.6)	151 (147)
10 yr (n = 12)	56.7 (13.6)	35.9 (11.2)	60.8 (14.3)	38.1 (5.5)	152 (85.5)	16.1 (52.3)	23.9 (21.0)	20.3 (4.2)	630 (828)	ND	15 (11.3)
>20 yr (n = 12)	ND	2.9 (1.2)	22.6 (136.9)	35.6 (17.1)	84 (34.2)	8.5 (1.6)	19.8 (5.6)	36.6 (6.7)	804 (187)	ND	NA
Mean (SD)	115 (289)	19.8 (12.8)	71.3 (114.6)	91.4 (121)	147 (107)	42.8 (98.6)	29.4 (49.5)	39.4 (57.3)	887 (503)	28.2 (44.6)	72.2 (124)

584 ¹ Oxytetracycline [OTC], tetracycline [TC], deoxytetracycline [DXC]; sulfamethazine [SMZ], sulfadiazine [SDZ], sulfamethoxazole [SMX] and trimethoprim [TMP] ; roxithromycin [RTM]
585 and Erythromycin-H₂O [ETM-H₂O]; cephalexin [CFX] and amoxicillin [AMO].² Standard deviation in parentheses; ³ NA = Not available; ND = Not detected

586 **Table 2 Physiochemical properties of sampled landfill leachates and refuse as a function of landfill age**

Samples	Basic Properties			Nutrients (µg/kg-dry refuse)				
Refuse		pH	Conductivity (EC µs/cm)	NH ₃ -N (µg/kg)	NO ₂ ⁻ /NO ₃ ⁻ -N (µg/kg)	TN (mg/kg)	TP (mg/kg)	TOC (g/kg)
< 3 yr (n = 12)		6.1 (0.5)	126.4 (27.8)	103 (50.3)	7.0 (6.3)	7.1 (1.1)	11.3 (4.6)	97.5 (5.4)
10 yr (n= 12)		7.2 (0.1)	291 (38.9)	354 (92.1)	0.87 (0.5)	8.6 (2.4)	9.9 (0.4)	57.1 (6.3)
> 20 yr (n = 18)		7.8 (0.1)	2.8 (1.4)	102 (43.8)	3.5 (1.2)	4.2 (1.3)	5.4 (2.9)	28.9 (10.0)
Mean (SD) ^a		6.8 (0.1)	178.2 (61.7)	163 (113)	3.2 (2.9)	6.3 (2.4)	7.8 (3.2)	55.5 (28.1)
Leachates	DO	pH	Conductivity (EC µs/cm)	NH ₃ -N (mg/L)	NO ₂ ⁻ /NO ₃ ⁻ -N (mg/L)	TN (mg/L)	TP (mg/L)	COD (mg/L)
< 3 yr (n = 12)	NA	6.2 (0.9)	1470 (78.6)	1370 (91.4)	23.1 (13.0)	3620 (545)	135 (5.3)	22200 (500)
10 yr (n = 12)	0.03 (0.01)	6.5 (0.3)	425 (49.8)	114 (178)	19.6 (7.2)	2920 (113)	50.3 (21.0)	11500 (231)
> 20 yr (n = 12)	0.5 (0.1)	8.2 (0.3)	109 (4.4)	616 (41.8)	34.5 (26.8)	933 (52.8)	4.5 (1.9)	984 (67.0)
Mean (SD)	0.28 (0.27)	6.9 (0.7)	546 (428)	1510 (970)	27.7 (20.9)	2970 (533)	29.4 (25.5)	7420 (9760)

587 All data shown are average or mean; data in the parentheses are standard deviation (SD)

588

589

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

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592

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

Figure 3 The network analysis revealing the correlations between ARG and MGE 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.

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

618 **TABLE OF CONTENT (TOC)**

620 *All materials used in the Table of Content were originally from authors.

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