Intracellular and Extracellular Antibiotic Resistance Genes in Airborne $PM_{2.5}$ for Respiratory Exposure in Urban Areas

Tangtian He, Ling Jin, Jiawen Xie, Siyao Yue, Pingqing Fu, Xiangdong Li*

ABSTRACT: The current research paradigm for the environmental dissemination of antibiotic resistance genes (ARGs) focuses on those harbored within bacteria (*i.e.*, the intracellular fraction). Understanding both intracellular and extracellular ARGs has particular implications for the flux and fate of ARGs airborne via fine particulate matter ($PM_{2.5}$) from the ambient atmosphere to the human airway. In this study, we developed an operationally defined protocol for urban $PM_{2.5}$ to quantify the abundance of ARGs occurring in free and phage-associated DNA, in addition to their counterparts commonly analyzed within bacterial cells. The results demonstrated that the extracellular fraction comprises a significant proportion of total ARGs in $PM_{2.5}$, with subtype-specific dominance in either free or phage-associated DNA. A comparison between temperate and subtropical cities revealed geographical disparities of $PM_{2.5}$-associated intracellular and extracellular ARGs due to the influences of regional meteorological factors and oxidative gases. Considering the potential for horizontal gene transfers and the efficiency of respiratory deposition, the extracellular fraction could represent >60% of the modeled inhalational intake of most of the analyzed ARGs in some cities. The present study highlights the importance of the dynamics of ARGs in airborne $PM_{2.5}$ and their health implications across climate zones and pollution

gradients.

INTRODUCTION

Antimicrobial resistance (AMR) poses a huge challenge to the health of populations around the world. It is estimated that if not effectively decelerated, the continued rise of drug-resistant infections will claim 10 million lives a year and cost a cumulative 100 trillion USD in economic output by 2050.[1] The environment alone has been recognized as the single largest reservoir of antibiotic resistance genes (ARGs), the genetic entities that confer AMR.[2-7] There have been a multitude of studies on the environmental dissemination of ARGs in both natural environments[2,3] and engineered ecosystems,[4,5] with implications for their consequent contribution to human exposure.[6,7] Airborne bacteria, which are an integral part of airborne fine particulate matter ($PM_{2.5}$), are an important pathway for the dissemination of ARGs.[8] $PM_{2.5}$ can penetrate deep into the alveolar region of human lungs; therefore, its presence has strong implications for human exposure to environmentally disseminated ARGs.

The current DNA extraction protocol for studying ARGs in environmental matrices, including $PM_{2.5}$, often only involves collecting intracellular (bacteria-associated) ARGs on 0.2-μm filters, while leaving out extracellular fractions (free and phage-associated ARGs). Neglect of the extracellular fractions may result in the overlooking of horizontal gene transfer (HGT) processes other than that of conjugation via cell-to-cell contact, such as transformation via the direct uptake of extracellular free DNA and

transduction via bacteriophage.[9,10] Recent reports on the prevalence of free and phage-associated ARGs in various matrices of surface environments hint at the significance of these fractions, with potential for transformation and transduction in the environmental transmission of AMR.[11,12] The occurrence and implications of extracellular ARGs in airborne $PM_{2.5}$ have yet to be explored.

Airborne bacterial communities are influenced by a range of meteorological factors and gaseous and particulate constituents.[13,14] It is reasonable to consider that ARG fractions in airborne $PM_{2.5}$ are governed by atmospheric conditions. Oxidative gases (*i.e.*, O_3, NO_x) are able to degrade proteins/polypeptides to form amino acids,[15-17] and induce bacterial lysis,[18,19] leading to the release of free DNA to the extracellular environment. Free ARGs may thus be influenced by oxidative gases and ultraviolet radiation, which can act in a way similar to disinfection techniques.[20,21] Phage-associated ARGs are generated in metabolically active hosts during a viral infection, and assemble to transfer ARGs among hosts.[22] Relative humidity (RH) is an important factor in modulating the survival/infectivity of viruses,[23,24] which may have implications for phage-associated ARGs. However, the mechanisms for the "partitioning" of three ARG fractions in a natural or engineered environment would differ from one another, especially for the more complex atmospheric dynamics and prevailing meteorology in the air. The effects of atmospheric influencing factors on intracellular and extracellular fractions of ARGs in airborne $PM_{2.5}$ warrant investigations using geographically comparative sites with contrasting atmospheric conditions.

In this study, we first developed a protocol to extract bacteria-associated, free, and phage-associated DNA in an operationally defined manner for airborne $PM_{2.5}$ samples. Focusing on the temperate inland city of Beijing in northern China and the subtropical coastal cities of Hangzhou in eastern China and Hong Kong in southern China, we then investigated the seasonal evolution and geographical differentiation of the intracellular and extracellular profiles of selected ARGs in $PM_{2.5}$ in relation to meteorological parameters and PM and gas components. We further estimated the city-specific daily inhalational intake of $PM_{2.5}$-associated intracellular and extracellular ARGs, taking into consideration their HGT potential and respiratory deposition efficiency. Overall, our aim was to gain an enhanced understanding of the fate and health implications of total ARGs in airborne $PM_{2.5}$ in relation to large urban populations.

MATERIALS AND METHODS

$PM_{2.5}$ Sampling

$PM_{2.5}$ samples were collected at an urban site in Beijing, Hangzhou, and Hong Kong (**Table S1 of SI**) with high-volume (1000 L min^{-1}) samplers (TH-1000C II, Wuhan Tianhong Instruments Co., Ltd.), using quartz microfiber filters (QMA, 203 mm × 254 mm, Whatman 1851-65; prebaked at 400 °C for 4 h). Samples accumulated on a 24 h basis (from 8:00 a.m. to 8:00 a.m.) were collected from March 2016 to May 2017 in Beijing, and from December 2016 to November 2017 in Hangzhou and Hong Kong, respectively, and were stored at -80 °C before the extraction of DNA. Our control

experiments verified that the freezing and subsequent thawing of the samples did not contribute significantly to lysis of the cells and the release of free DNA (Figure S1 of SI). A field blank filter was placed in an air sampler accessory box before, during, and at the end of the sampling campaign. Data on meteorological parameters and trace gas concentrations were accessed from the closest weather station (Table S2).

DNA Extraction

For Beijing and Hangzhou, where sample amounts were limited, a quarter of each individual quartz filter was cut and four individual subsamples within the same month were pooled for DNA extraction and subsequent analysis. For Hong Kong, each full-size filter sample was used, with a total of six individual samples in each season. In the pretreatment process, the samples were sonicated with sterilized 1× phosphate-buffered saline (PBS).[6,25] The particulates suspended in a PBS buffer were then filtered through a 0.2 μm PES membrane disc filter (47 mm, Pall). The disc filters were then used to extract bacteria-associated DNA, while the filtrates were collected to extract free and phage-associated DNA. Our control experiments verified that the sonication of the samples did not contribute significantly to lysis of the cells and the release of free DNA (Figure S1).

Bacteria-associated DNA were extracted from the disc filters using methods that have been described in previous studies.[6,25] The procedures for the extraction of extracellular DNA and quality control were developed based on previous studies[11,26-30] with some

111 modifications (Figure 1). Please see the detailed methods in Section 1 of SI.

112

113 **Real-time Quantitative Polymerase Chain Reaction**

114 Several selected ARGs (*bla*$_{TEM-1}$, *erm*B, *lnu*A, *qnr*S, *sul*1, *tet*A, *tet*M, and *tet*W) and
115 MGEs (*int*I1, *tnp*A-02, and *tnp*A-04), as well as the 16S rRNA gene, were quantified
116 on a StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA). The 16S
117 rRNA gene is an indicator of total bacterial abundance. The selected ARGs are
118 associated with six major groups of antibiotics commonly used in clinical and
119 agricultural settings. Details of the primer sets, thermocycling protocols, standard
120 constructions, quality-control procedures, and MIQE information[31] are provided in the
121 Supporting Information (Section S2 and Table S4) and Appendix I.

122

123 **Statistical Analysis**

124 A correlation analysis between intracellular/extracellular genes and environmental
125 parameters (meteorological factors and trace gaseous pollutants) was performed using
126 SPSS (IBM, USA). A variation partitioning analysis was performed using RStudio
127 (Package "vegan").

128

129 # RESULTS AND DISCUSSION

130 **Extracellular Fractions as Significant Contributions of Total ARGs in Ambient**
131 **$PM_{2.5}$** The quantitation of the absolute abundance of targeted ARGs revealed gene-
132 specific dominance in intracellular and extracellular DNA (Figures 2a and S2; Tables

S5-S7). For example, *sul*1 was dominant in intracellular fractions, accounting for >60% of the total number of copies in three cities. *qnr*S and *erm*B occurred more frequently in the extracellular free and phage-associated DNA fractions, respectively, in Beijing and Hong Kong. With this fraction taken into account, the total abundance of *qnr*S and *erm*B was generally consistent across seasons. Neglecting the extracellular fractions would result in an underestimation of the atmospheric occurrence of ARGs and an overestimation of their seasonal variability, thus leading to a biased assessment of inhalation exposure. The prevalence of extracellular ARGs in the breathing zone of the ambient atmosphere may also have implications for the multi-pathway HGT of ARGs in interaction with bacterial communities in human respiratory tract upon inhalation.

Compared to surface environmental matrices,[11,31-36] ambient $PM_{2.5}$ is where extracellular ARGs can be particularly enriched with high relative abundance (Figure S3). By contrast, the relative abundance of intracellular ARGs is generally consistent from surface environments to airborne $PM_{2.5}$. The comparison suggests that secondary processes play a role in the generation of extracellular ARGs in the atmosphere, which cannot be explained merely by the preservation of primary emission sources in surface environments. Evidence of secondary processes in the atmosphere to degrade proteins/polypeptides and form amino acids has been found in many previous studies,[15-17] suggesting that atmospheric conditions may favor the lysis of bacteria[18,19] and hence the generation of extracellular ARGs. Interestingly, while the absolute abundance of intracellular 16S rRNA genes is generally higher than that of its extracellular

155 counterpart by up to four orders of magnitude (Figure S4), the absolute abundance of
156 intracellular and extracellular ARGs typically differ only by less than two orders of
157 magnitude (Figure S5). This contrast may be attributable to the relative distribution of
158 ARGs in plasmids and bacterial genomes. Previous studies have suggested that
159 chromosomal DNA (*e.g.*, the 16S rRNA gene), degrades more rapidly than the plasmid
160 DNA that harbors the ARGs.[32,36] Hence, the persistence of free ARGs located on
161 plasmids in $PM_{2.5}$ is expected, and can be confirmed by controlled environmental
162 chamber studies. While normalization of the abundance of ARGs to that of the 16S
163 rRNA gene is a common practice, we acknowledge its limitation as different bacteria
164 species contain varying copies. A monocopy candidate marker (*e.g.*, *rpo*B)[37] needs to
165 be validated to this purpose.
166
167 **Geographical Disparities in the Intracellular-Extracellular Partitioning of the 16S**
168 **rRNA Gene and ARGs**
169 The total abundance of 16S rRNA genes varied across seasons in Beijing and Hong
170 Kong, in contrast to its seasonal consistency in Hangzhou (Figure 2a). In Beijing, there
171 was a wintertime decline in the abundance of 16S rRNA genes by two to three orders
172 of magnitude as compared to other seasons. In Hong Kong, a drop in the abundance of
173 16S rRNA genes by up to one order of magnitude occurred in warm seasons (spring
174 and summer) as compared to cooler season (winter). These trends were also reflected
175 in a drastic change in the structure of the bacterial community in wintertime in Beijing,
176 a gradual shift from spring to winter in Hong Kong, and seasonal consistency in

177 Hangzhou (Figure S6). While there was a lower total abundance of 16S rRNA genes in
178 Hong Kong than in other two cities by two orders of magnitude, a much higher free
179 fraction was observed in Hong Kong, especially in spring and summer when it
180 accounted for approximately 50% of the total abundance of 16S rRNA genes. In Beijing
181 and Hangzhou, the free fraction of the 16S rRNA gene was generally negligible (<5%
182 across seasons). Such a contrast highlights differences in the life and death of bacteria
183 in the atmosphere across geographic locations.
184
185 Clear differences were observed among the three cities in the distribution of ARGs in
186 intracellular and extracellular fractions. In Hangzhou, most of the studied ARGs were
187 bacteria-associated, with a minor contribution from extracellular fractions (<10% in
188 general for free ARGs and negligible for phage-associated ARGs). There was seasonal
189 consistency in the absolute abundance of the studied ARGs, which again agreed with
190 the seasonally consistent structure of the bacterial community (Figure S6). In contrast,
191 extracellular fractions occurred frequently in Beijing and Hong Kong. Free fractions
192 dominated extracellular ARGs in Beijing and both free and phage-associated fractions
193 contributed significantly in Hong Kong. On average, the percentage of free *qnr*S in
194 Beijing and free $bla_{\text{TEM-1}}$ in Hong Kong (over 40% on average across seasons in both
195 cities) was much higher than that in Hangzhou (<10% across seasons). Of all analyzed
196 ARGs, *erm*B in winter in Beijing and *qnr*S and *tet*W in summer in Hong Kong occurred
197 with the highest abundance in the phage-associated fraction, which was consistent with
198 the results in previous studies on feces and sludge,[26,29] highlighting the role of

bacteriophages in the airborne dissemination of ARGs.

A variation partitioning analysis indicated differential influences from meteorological factors and oxidative gases on the city-specific profiles of intracellular and extracellular 16S rRNA genes and ARGs (Figure 2b and Tables S8-S14). The two categories of environmental factors explained >60% of the total variance in the intracellular-extracellular partitioning of the 16S rRNA gene and ARGs in the three studied cities. Meteorological factors exhibited stronger independent influences than oxidative gaseous components. While the fraction explained solely by meteorological factors increased from northern to southern cities (Beijing: 25.5%; Hangzhou: 42.2%; Hong Kong: 45.3%), the interactive effects of meteorological factors and oxidative gaseous components decreased (Beijing: 51.4%; Hangzhou: 34.7%; Hong Kong: 13.3%).

In Beijing, temperature, RH, and O_3 were shown to be predominant factors in association with intracellular-extracellular ARG ratios (Tables S12-S14). The strong interactive effects of these factors may reflect the interdependence between the prevailing meteorology and photochemical pollution, which may jointly drive the seasonal succession of bacteria-associated, free, and phage-associated ARGs. In Hangzhou, wind speed and rainfall in interaction with O_3 were found to be associated with the dominance of bacteria-associated 16S rRNA genes and ARGs (Table S12). Hong Kong had a lower level of O_3 and higher ranges of meteorological parameters (*e.g.*, temperature, UV radiation, RH, and wind speed) compared to other two cities. As

such, the set of meteorological factors may dictate the intracellular-extracellular partitioning of the 16S rRNA gene and ARGs. The above statistical modeling results hint at the differential influences of meteorological factors and oxidative gases on city-specific dynamics of bacteria and ARGs in airborne $PM_{2.5}$. Large-scale field studies in combination with controlled laboratory experiments are needed to decipher the geographically dependent interactive effects of prevailing meteorology and oxidative gases on the dynamics of airborne intracellular and extracellular ARGs.

Modeled Inhalational Intake of Transferable ARG Fractions

The statistical correlation between the total free or phage-associated ARGs and the total MGEs, *int*I1, *tnp*A-02, and *tnp*A-04, was generally maintained at a 1:1 ratio, which is similar to the ratio for bacteria-associated ARGs in this study (Figure S7) and other studies.[6,7,38] Previous studies demonstrated that MGEs, *e.g., int*I1, are involved in HGT by carrying ARGs for mobilization under environmental stressors.[30,39,40] The co-existence of ARGs and MGEs in free and phage-associated fractions suggests that free and phage-associated MGEs can be involved in transformation and transduction processes,[30,39] respectively, and further supports the view that free and phage-associated ARGs are available sources for airborne bacterial HGT.

A synthesis of the data in the literature on conjugation, transformation, and transduction frequencies in aquatic and terrestrial environments (Figure S8) revealed comparability among the three HGT processes. Therefore, ARGs in free and phage-associated

243 fractions in the atmosphere may have a similar possibility of being acquired by bacterial
244 communities in the human airway upon inhalation. A recent study showed that bacteria
245 positively uptakes free DNA using pilus, showing that transformation is of great
246 importance for the environmental HGT of ARGs.[41] Another study also indicated that
247 the administration of antibiotics increased the potential mobility of ARGs in the gut of
248 the *Piaractus mesopotamicus*, a freshwater fish species, while phage played an
249 important role during the process.[42] Recent studies also uncovered gene transfer agents
250 (GTAs) for high-frequency transduction in various environments.[43-45]
251
252 We predicted the daily intake of ARGs and the lung deposition of each ARG fraction
253 (Section S3). The daily intake of free and phage-associated ARG and MGE fractions in
254 Beijing, Hangzhou, and Hong Kong was estimated to account for up to 90%, 50%, or
255 90% of the total inhalational ARG loadings (Figures S9 and S10), respectively, which
256 is comparable to or more dominant than that of the bacteria-associated fraction. The
257 total ARGs and MGEs of bacteria-associated fractions are anticipated to be dominant
258 in lung extrathoracic regions in the three cities, but in bronchial and alveolar regions,
259 the free and phage-associated fractions are also anticipated to be responsible for up to
260 70% of the total deposition due to the high deposition efficiency of the size of the
261 particulates (Figures 3 and S10). The model results also indicate that variations in gene-
262 specific lung deposition can be anticipated. For instance, free and phage-associated
263 $bla_{\text{TEM-1}}$, *erm*B, and *tet*W are predicted to be dominant sources for the bronchial and
264 alveolar regions (Figure S9). Therefore, the modeling results suggest that extracellular

ARGs may be an integral part of the total ARGs associated with airborne $PM_{2.5}$ for spatiotemporal dissemination, atmospheric HGT, and human lung exposure. By comparing a number of exposure pathways for the intake of intracellular ARGs (*e.g.*, drinking water and food ingestion) in China and the U.S.A, our previous study[7] identified the region-specific relative importance of inhalation in China as well as country-specific exposure scenarios. The inhalation of ambient $PM_{2.5}$-associated ARGs means a unique pathway for the human intake of ARGs when compared to other exposure pathways (*e.g.*, ingestion through food/water). When data on extracellular ARGs in media of direct relevance to human exposure (*e.g.*, drinking water and food) become available, such comparisons can be made to establish the full picture of human exposure to exogenous ARGs, including both intracellular and extracellular fractions.

The size of free and phage-associated ARGs can be at the quasi-ultrafine particle $PM_{0.1}$ level, which is much smaller than that of bacteria-associated ARGs. The prevalence of these extracellular ARGs may provide additional sources of ARGs in airborne $PM_{2.5}$ to human pulmonary bacterial communities for HGT. Future studies are warranted to examine the genetic exchange/HGT of ARGs in airborne $PM_{2.5}$ upon inhalation, for example by using *in vitro* simulated lung fluid or *in vivo* animal experiments.

Linking the intracellular-extracellular partitioning of ARGs to prevailing meteorology and air pollution puts the environmental dissemination of antibiotic resistance in the context of evolving global climate and pollution scenarios. Geographically comparative

studies coupled with controlled laboratory experiments are needed to decipher the mechanisms by which atmospheric processes can modify the profiles of ARGs in airborne $PM_{2.5}$ in the bacteria-virus-free DNA triad. Addressing this issue would be a significant step toward a more comprehensive understanding of geographically driven human health impacts via inhalable airborne microbiomes.

ASSOCIATED CONTENT

The Supporting Information is available free of charge at XXX.

- Additional information on the sampling sites; data on meteorological parameters and criteria air pollutant concentrations; QA/QC for DNA extraction; qPCR specifics; data on gene abundance; correlation analysis; and algorithms for estimating the daily inhalational intake and respiratory deposition of ARGs (PDF).
- Details requested in the MIQE guidelines for qPCR data reporting (XLSX)

AUTHOR INFORMATION

Corresponding Author

Xiangdong Li - *Department of Civil and Environmental Engineering, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong; The Hong Kong Polytechnic University Shenzhen Research Institute, Shenzhen 518057, China*; http://orcid.org/0000-0002-4044-2888; Email: cexdli@polyu.edu.hk

Authors

Tangtian He - *Department of Civil and Environmental Engineering, The Hong*

Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong; The Hong Kong Polytechnic University Shenzhen Research Institute, Shenzhen 518057, China; http://orcid.org/0000-0002-7422-4950

Ling Jin - *Department of Civil and Environmental Engineering, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong; The Hong Kong Polytechnic University Shenzhen Research Institute, Shenzhen 518057, China*; http://orcid.org/0000-0003-1267-7396

Jiawen Xie - *Department of Civil and Environmental Engineering, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong; The Hong Kong Polytechnic University Shenzhen Research Institute, Shenzhen, China*; http://orcid.org/0000-0001-6461-4464

Siyao Yue - *State Key Laboratory of Atmospheric Boundary Layer Physics and Atmospheric Chemistry, Institute of Atmospheric Physics, Chinese Academy of Sciences, Beijing 100029, China*; https://orcid.org/0000-0003-1320-9279

Pingqing Fu - *Institute of Surface-Earth System Science, Tianjin University, Tianjin 300072, China*; http://orcid.org/0000-0001-6249-2280

Author Contributions

T.T.H. and L.J. contributed equally to this work. T.T.H., L.J. and X.D. L. designed the research. J.W.X., T.T.H., S.Y.Y. and P.Q.F. conducted the field work. T.T.H., J.W.X. and J.L. performed the laboratory experiments and model work. T.T.H., L.J., J.W.X., and X.D. L. wrote the paper. All authors have given approval to the final version of the manuscript.

Notes

The authors declare that they have no competing financial interests.

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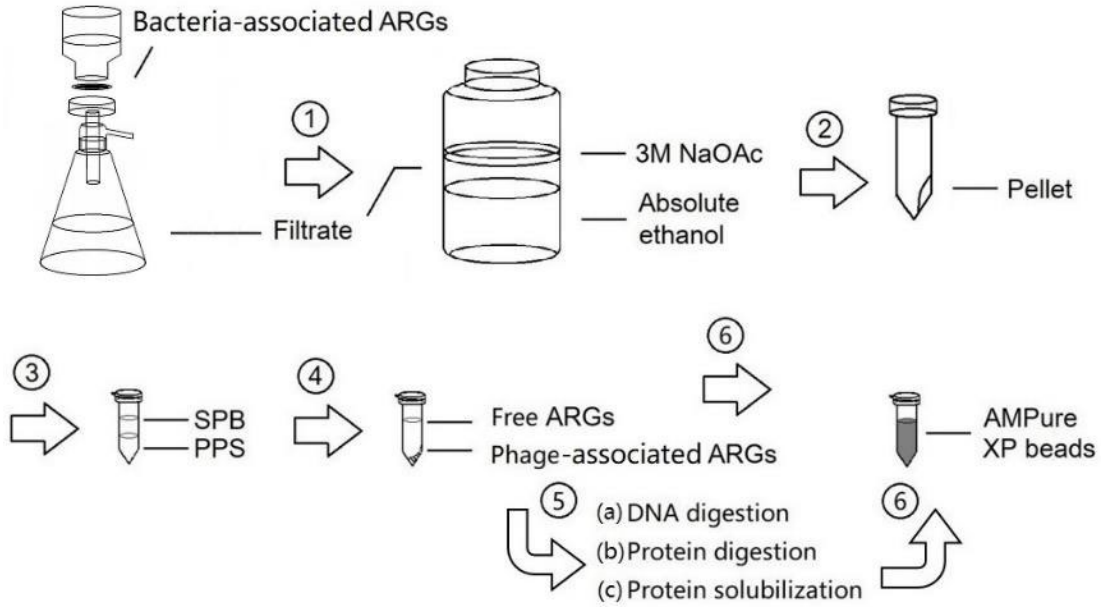

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468 **Figure 1.** Schematic of the method for extracting intracellular and extracellular DNA
469 (free and phage-associated DNA). 1) filtration through a 0.2 μm PES membrane filter;
470 2) precipitation and centrifugation; 3) dissolution of pellets in a Sodium Phosphate
471 Buffer (SPB) and Protein Precipitation Solution (PPS); 4) centrifugation to collect the
472 supernatant (containing free DNA) and pellets (containing virion particles); 5)
473 separation of free and phage-associated DNA; and (6) purification of free and phage-
474 associated DNA.

Figure 2. (A) Comparison of the seasonal abundance of 16S rRNA genes and three representative ARGs (*sul*1: bacterial-associated fraction dominant; *qnr*S and *erm*B: free and phage-associated fraction dominant) and the percentage contribution of intracellular (bacteria-associated) and extracellular (free and phage-associated) fractions to each ARG in $PM_{2.5}$ in Beijing, Hangzhou, and Hong Kong (from top to bottom). The error bars represent the standard deviation in the monthly samples within a season. The data on the other analyzed ARGs can be found in Figure S2. (B) Variation partitioning showing the associations (%) of meteorological factors (temperature, UV index, relative humidity, wind speed, and rainfall) [a], trace gases (NO_2 and O_3) [b] and joint effects [c] with the partitioning of the 16S rRNA gene and ARGs between intracellular and extracellular fractions in Beijing, Hangzhou, and Hong Kong. Detailed results on the correlation between each parameter (meteorological factors or trace gases) and the percentage contribution of 16S rRNA in bacteria-associated or free fractions and ARGs in bacteria-associated, free, or phage-associated fractions can be found in Tables S8-S14 of SI.

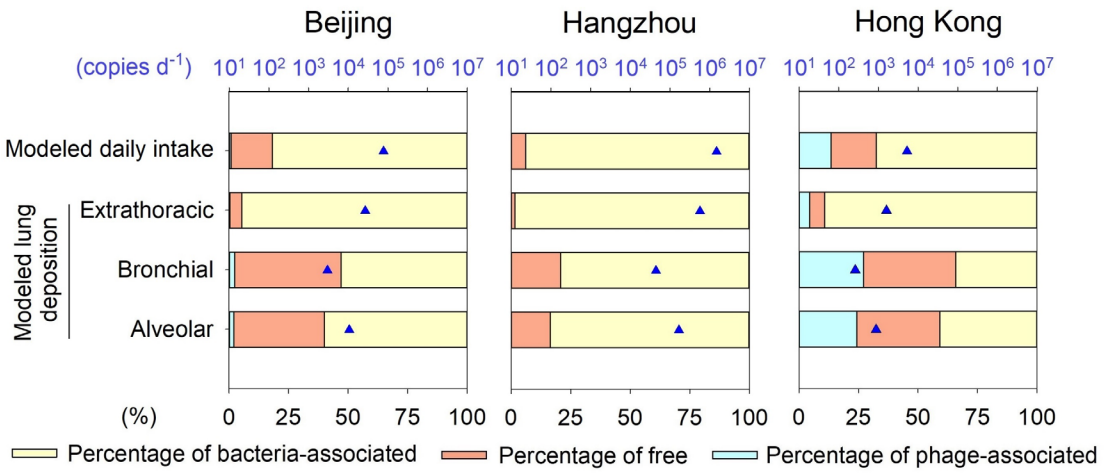

Figure 3. Dots represent the modeled daily intake and lung deposition (*i.e.*, extrathoracic, bronchial, and alveolar regions) of ARGs from bacteria-associated, free, and phage-associated fractions, and bars represent the percentage of each fraction in Beijing, Hangzhou, and Hong Kong.

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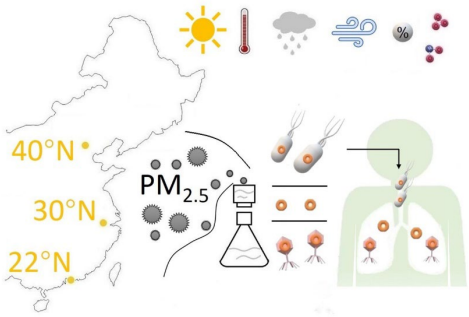

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