Metagenomic Profiles of Antibiotic Resistance Genes (ARGs) between Human Impacted Estuary and Deep Ocean Sediments

ARTICLE *in* ENVIRONMENTAL SCIENCE & TECHNOLOGY · OCTOBER 2013

Impact Factor: 5.33 · DOI: 10.1021/es403818e · Source: PubMed

6 AUTHORS, INCLUDING:

BaoWei Chen
The Hong Kong Polytechnic University
20 PUBLICATIONS **224** CITATIONS
SEE PROFILE

Ying Yang
The University of Hong Kong
10 PUBLICATIONS **122** CITATIONS
SEE PROFILE

Tong Zhang
The University of Hong Kong
167 PUBLICATIONS **5,007** CITATIONS
SEE PROFILE

Xiang-Dong Li
The Hong Kong Polytechnic University
175 PUBLICATIONS **10,062** CITATIONS
SEE PROFILE

Environmental Science & Technology

Article

pubs.acs.org/est

Metagenomic Profiles of Antibiotic Resistance Genes (ARGs) between Human Impacted Estuary and Deep Ocean Sediments

Baowei Chen,[†] Ying Yang,[‡] Ximei Liang,[§] Ke Yu,[‡] Tong Zhang,[‡,*] and Xiangdong Li[†,*]

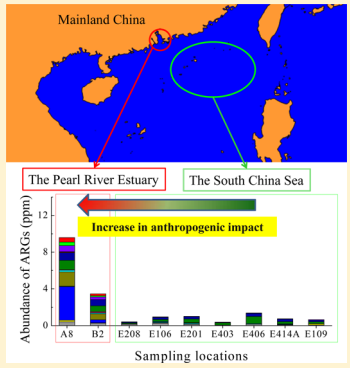
[†]Department of Civil and Environmental Engineering, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong

[‡]Environmental Biotechnology Laboratory, The University of Hong Kong, Hong Kong

[§]South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China

Supporting Information

ABSTRACT: Knowledge of the origins and dissemination of antibiotic resistance genes (ARGs) is essential for understanding modern resistomes in the environment. The mechanisms of the dissemination of ARGs can be revealed through comparative studies on the metagenomic profiling of ARGs between relatively pristine and human-impacted environments. The deep ocean bed of the South China Sea (SCS) is considered to be largely devoid of anthropogenic impacts, while the Pearl River Estuary (PRE) in south China has been highly impacted by intensive human activities. Commonly used antibiotics (sulfamethazine, norfloxacin, ofloxacin, tetracycline, and erythromycin) have been detected through chemical analysis in the PRE sediments, but not in the SCS sediments. In the relatively pristine SCS sediments, the most prevalent and abundant ARGs are those related to resistance to macrolides and polypeptides, with efflux pumps as the predominant mechanism. In the contaminated PRE sediments, the typical ARG profiles suggest a prevailing resistance to antibiotics commonly used in human health and animal farming (including sulfonamides, fluoroquinolones, and aminoglycosides), and higher diversity in both genotype and resistance mechanism than those in the SCS. In particular, antibiotic inactivation significantly contributed to the resistance to aminoglycosides, β-lactams, and macrolides observed in the PRE sediments. There was a significant correlation in the levels of abundance of ARGs and those of mobile genetic elements (including integrons and plasmids), which serve as carriers in the dissemination of ARGs in the aquatic environment. The metagenomic results from the current study support the view that ARGs naturally originate in pristine environments, while human activities accelerate the dissemination of ARGs so that microbes would be able to tolerate selective environmental stress in response to anthropogenic impacts.



INTRODUCTION

The discovery of antibiotics commenced a new era of innovative drugs for human and animal health as well as agriculture. However, a serious setback has occurred in that resistance to antibiotics is now prevalent in modern environmental and human commensal microbes.[1−4] This raises an important question of whether antibiotic resistance is only a modern phenomenon caused by the widespread use of antibiotics by humans or whether it is an ancient phenomenon.

Studies have demonstrated the existence of antibiotic resistance in natural environments. Microbes can produce antibiotics that kill or inhibit neighboring microbes to preserve resources. As a dynamic competitive mode between microorganisms, antibiotics and antibiotic synthetic pathways have evolved over millions of years.[5] Interestingly, bacteria in soils also exhibit the capacity to subsist on antibiotics as their sole source of carbon.[6] Antibiotic producers or utilizers generally carry ARGs for self-protection from antibiotics.[7−9] Previous studies have shown that ARGs are diverse and abundant in 30 000-year-old Beringian permafrost sediments.[10] It has also been reported that ARGs have indeed been found in the microbes that inhabit natural environments where there has been no anthropogenic impact, such as isolated caves,[11] deep oceans,[12] and the deep terrestrial subsurface.[13] Therefore, such evidence supports the concept that antibiotic resistance is a natural and ancient phenomenon.

However, the high levels and prevalence of antibiotic resistance found to date are also a modern phenomenon that is related to human activity. Collections of microbes that predate the antibiotic era are highly susceptible to antibiotics, and mobile genetic elements (MGEs) are largely devoid of resistance genes.[14,15] In a remote environment with limited human occupation and presumably a low level of exposure to antibiotics, antibiotic resistance was found absent in the microflora that had been isolated from terrestrial animals.[16] The presence of antibiotics has been demonstrated as an important form of selective stress in driving the evolution, proliferation, and spread of ARGs, and can significantly contribute to the elevated levels of antibiotic resistance in the modern environment.[17] These studies strongly support the hypothesis that the high level of antibiotic resistance in human-

Received: August 28, 2013
Revised: October 14, 2013
Accepted: October 14, 2013

   dx.doi.org/10.1021/es403818e | Environ. Sci. Technol. XXXX, XXX, XXX−XXX

influenced environments is a modern phenomenon directly linked to the widespread use of antibiotics in the last half century.

A declining gradient in the determinants of antibiotic resistance from human-impacted areas to relatively pristine environments has been observed in previous studies.[18−20] Nevertheless, a key issue is how to find a contemporary environment that is completely unexposed to anthropogenic antibiotics and ARGs. Such a survey of ARGs in a pristine environment would improve our understanding of the origins of antibiotic resistance in the environment. The South China Sea (SCS) sediments at a depth several thousands of meters may be considered to be devoid of human impacts, where environmental bacteria have never been exposed to modern antibiotics and ARGs. Although the importance of aquatic environments in the dissemination of antibiotic resistance has been established, comprehensive information on the occurrence and prevalence of antibiotic resistance in the deep sea environment is limited.[20,21] Much-needed information about the deep ocean and comparisons with heavily impacted coastal zones could be very helpful in elucidating the provenance of antibiotic resistance in modern resistomes.

The objectives of the present study are to uncover broader profiles of ARGs in the sediments collected from the highly human-impacted Pearl River Estuary (PRE) and the relatively pristine deep SCS using a metagenomic approach, and to understand the emergence of antibiotic resistance in the less-human-impacted environment as well as the role of antibiotic use in accelerating the dissemination of ARGs in the human-impacted aquatic environments.

MATERIALS AND METHODS

Site Selection and Sampling. Sediment samples were collected from the PRE in June 2011 and from the SCS during a cruise of the northern South China Sea (NSCS) in August 2011. Figure 1 shows the sampling sites in the PRE and SCS.

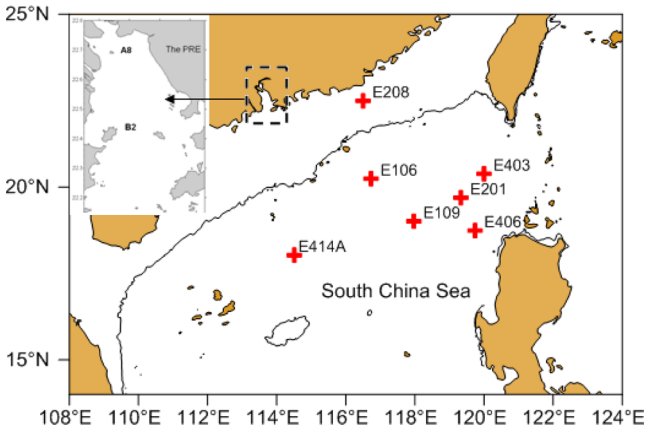


Figure 1. Map showing the sampling sites in the PRE and SCS.

Two sediment samples were collected from PRE that had been heavily impacted by rapid urbanization and industrialization that had taken place nearby. Site A8 was located at the river mouth of the Shiziyang Channel, and site B2 was in the middle of the PRE. The depth of the water at these two sites was lower than 7 m. There were seven sampling locations in the SCS, with varying distances to the Chinese mainland and different water depths. The information on the sampling sites is presented in Table S1, Supporting Information (SI). One sample (E208) was collected at an offshore area close to the coast of Guangdong Province (water depth of 35 m), and another sample (E106) was collected at a location between the offshore area and the continental shelf (water depth of 717 m). Five sediment samples were collected in the deep SCS, at an average depth of more than 3000 m. Sediment samples were taken using a grab sampler, and stored in polyethylene plastic bags. Following their collection, all sediment samples were immediately kept in a refrigerator at 4−6 °C until they reached the laboratory and were subjected to DNA extraction and chemical analysis for antibiotics.

Chemicals and Materials. Antibiotic standards were purchased from Sigma-Aldrich (St. Louis, MO, U.S.), including sulfadiazine (SDZ), sulfamethazine (SMZ), sulfamethoxazole (SMX), norfloxacin (NOR), ofloxacin (OFL), enrofloxacin (ENR), tetracycline (TC), erythromycin (ETM), and roxithromycin (RTM). $^{13}C_3$-caffeine was used as the surrogate standard and purchased from Cambridge Isotope Laboratories (1 mg/mL in methanol, U.S.). Methanol (MeOH) and acetonitrile (ACN) were obtained from Merck (Darmstadt, Germany). Milli-Q water was prepared using a Milli-Q water purification system (Millipore, U.S.).

Stock solutions of individual antibiotics (100 mg/L) were prepared in methanol. Stock solutions of fluoroquinolones were prepared in methanol containing 0.5% NaOH (1 M), and erythromycin-H_2O (ETM-H_2O) was prepared by acidification according to the reported procedure.[22] All stock solutions were stored in the dark at −20 °C. Working solutions were freshly prepared daily for analysis.

Chemical Analysis of Antibiotics. Details of the pretreatment and the methods of analyzing the antibiotics in the sediments have been described in our previous publications.[20,23] In brief, antibiotics were extracted from sediments using tandem solid-phase extraction (SPE) on SAX (6 mL, 200 mg, CNW, Germany) and HLB cartridges (6 mL, 500 mg, Waters, U.K.). The final extract was analyzed using an Agilent HP1100 liquid chromatography (Agilent, Palo Alto, CA, U.S.) coupled with Applied Biosystems API 4000 tandem mass spectrometry. The mass spectrometer was equipped with an electrospray ionization source and operated in the positive mode. In the present study, nine common antibiotics, including norfloxacin (NOR), enrofloxacin (ENR), ofloxacin (OFL), sulfadiazine (SDZ), sulfadimidine (SMZ), sulfamethoxazole (SMX), erythromycin (ETM), roxithromycin (RTM), and tetracycline (TC), were categorized into four groups (fluoroquinolones, sulfonamides, macrolides, and tetracyclines). The chromatographic separation of antibiotics was achieved using an Agilent ZORBAX C18 column (2.1 × 150 mm, 5 μm particle size). The limits of quantification (LOQ) of the antibiotics are presented in Table S2 of the SI.

DNA Preparation and High-throughput Sequencing. All of the DNA in the sediment samples was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) according to the manufacturer's protocol. Due to the low level of DNA in marine sediments, the process of preparing the DNA was performed multiple times, and combined to eliminate heterogeneity in sediment samples and to avoid potential bias during the DNA extraction process. The purity and yield of the DNA were determined using a Thermo Scientific NanoDrop 1000 Spectrophotometer. About 6 μg of DNA from each sample were submitted to the Beijing Genomics Institute (BGI) (Shenzhen, China). Sequence libraries of ~180 bp DNA fragments were prepared and then sequenced using Illumina

dx.doi.org/10.1021/es403818e | Environ. Sci. Technol. XXXX, XXX, XXX−XXX

HiSeq 2000. Twelve DNA samples were laden on one lane, and the total data output per lane is more than 30 Gb.

Bioinformatic Analysis. The raw reads from each of the samples (100 bp in length) were trimmed to remove low-quality reads that contained ambiguous nucleotides or had a quality value lower than 20. The local BLASTX programs were employed to align trimmed clean reads of each data set against an antibiotic resistance genes database (ARDB).[24] A read was annotated as an ARG-like sequence if the best BLAST hit (blastx) had a sequence identity of higher than 90% and an alignment length of at least 25 amino acids (aa).[25]

Our data were also aligned against the databases of integrons and plasmids to characterize these two kinds of MGEs in the PRE and SCS sediments. A database of integrons was developed on the basis of the nucleotide sequences for all integrases available in the INTEGRALL database (1447 integrase genes and 8053 gene cassettes).[26] In addition, a plasmid database was built using the plasmid sequences available in the NCBI RefSeq database (2465 sequences).[25] A read was annotated as an integron sequence if the best BLAST hit (blastn) had a nucleotide sequence identity higher than 90% over an alignment length of at least 50 bp.[27] Plasmid sequences were determined for the alignments with a nucleotide sequence identity above 95% over a length of at least 90 bp.

RESULTS AND DISCUSSION

Antibiotic Concentrations in Sediments. The concentrations of nine common antibiotics in the PRE and SCS sediments were measured using LC-MS-MS. The results are summarized in Table S2 of the SI. All of the investigated antibiotics were undetectable in the SCS sediments. Common antibiotics, including sulfamethazine, norfloxacin, ofloxacin, tetracycline, and erythromycin, were detected in the PRE sediments, possibly due to their wide use in the surrounding areas of the PRE.[20,23] The total concentrations of antibiotics were obtained by summing the concentrations of all measured antibiotics. The total concentrations of antibiotics at sites A8 and B2 in the PRE were 7.41 and 5.89 ng/g, respectively. Significant differences in antibiotic concentration between the PRE and the deep SCS reflects the degree to which those two areas were impacted by human activities related to antibiotic use. Extensive use of antibiotics results in the elevated levels of antibiotics in the PRE, while the deep SCS is obviously much less-impacted.

Total Abundance of ARGs. In order to avoid the bias caused by different sequencing depths among the samples, the total hit reads of the ARGs in the sediment samples were normalized to the size of the sequencing data. The total levels of abundance of ARG-like reads in the samples are shown in Figure 2. Triplicate (A8) and duplicate (E208 and E201) analyses demonstrated good repeatability in the metagenomic profiling of ARGs in the sediments, with a relative standard deviation (RSD) lower than 10%. The total abundance of ARG-like reads in sediments substantially decreased from the mouth of the Pearl River (A8) to the middle of the PRE (B2), and on to the SCS, which was in good accordance with antibiotic concentrations. The total abundance of ARG-like reads in the sediments of the Pearl River mouth was at least 7 times higher than those in the SCS sediments. Within the SCS area, no distinct spatial pattern could be discerned in the total abundance of ARGs in sediments, such as a significant relationship with the depth of the water or with the distance to the continent. With one exception, the total abundance of

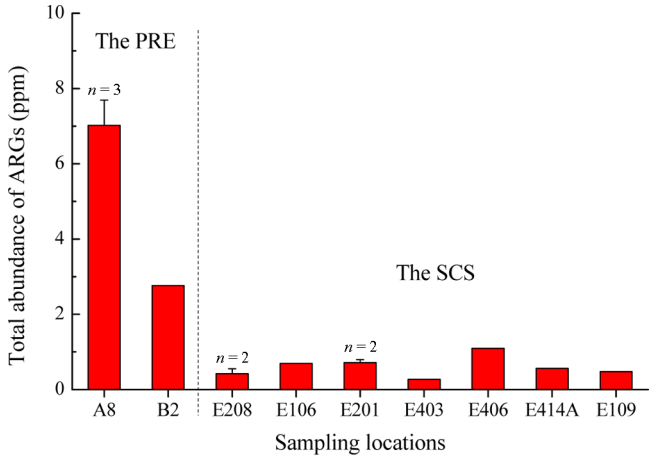


Figure 2. Total abundance of ARGs in the PRE and SCS sediments. The total hit read of ARGs in the sediments was normalized to the size of the sequencing data. "ppm" means one ARG-like read in one million metagenomic sequencing reads. "*n*" indicates sample number recorded in high-throughput sequencing analysis. Error bar represents one standard deviation. Sampling sites are generally shown in the order of increasing water depth.

ARG-like reads at E406 site was higher than those at other sites in the SCS possibly due to the proximity of this site to the Philippines.

Diversity and Abundance of the Resistance Type, Subtype, and Resistance Mechanism of ARGs. The ARGs in the PRE and SCS sediments were categorized according to their resistance types, viz., the types of antibiotics to which they are related (Figure 3A, B and Table S3). ARGs encoding resistances to 10 groups of antibiotics were found in both the PRE and SCS sediments, including aminoglycosides, β-lactams, chloramphenicols, fluoroquinolones, fosfomycins, fosmidomycins, macrolides, polypeptides, sulfonamides, and tetracyclines. Some ARGs were associated with the resistance to multiple antibiotics. Nevertheless, the abundance of each type of ARG in sediments was much lower in the SCS than that in the PRE. Figure 3B shows that genes that encode resistance to macrolides and polypeptides were the most abundant in the SCS sediments. In comparison, three most abundant ARGs in the PRE sediments were related to commonly used antibiotics including sulfonamides, fluoroquinolones, and aminoglycosides. For instance, the abundance of resistance genes to sulfonamides, fluoroquinolones, and aminoglycosides at the A8 site (the PR) were 3.66, 1.54, and 1.54 ppm, respectively. In comparison, the abundance of resistance genes to polypeptides (1.02) and macrolides (0.83 ppm) was lower.

A summary of the resistance type, subtype, and sequence diversity of ARGs in the PRE and SCS sediments is given in Figure 3C. Subtype represents the genotype of resistance genes according to the description in the ARDB database, and sequence diversity represents the number of reference sequences from the ARDB database in each subtype identified in our data sets. In general, the amounts of subtype and the sequence diversity of the ARGs in the PRE sediments were substantially higher than those in the SCS sediments. In the areas that were highly impacted by human activities (e.g., site A8 in the PRE), the resistance to a single type of antibiotic was probably encoded by different ARGs, and the subtypes of ARGs also exhibited a high degree of diversity in their gene sequences. By contrast, ARGs with less diversity of genotype and gene

dx.doi.org/10.1021/es403818e | Environ. Sci. Technol. XXXX, XXX, XXX−XXX

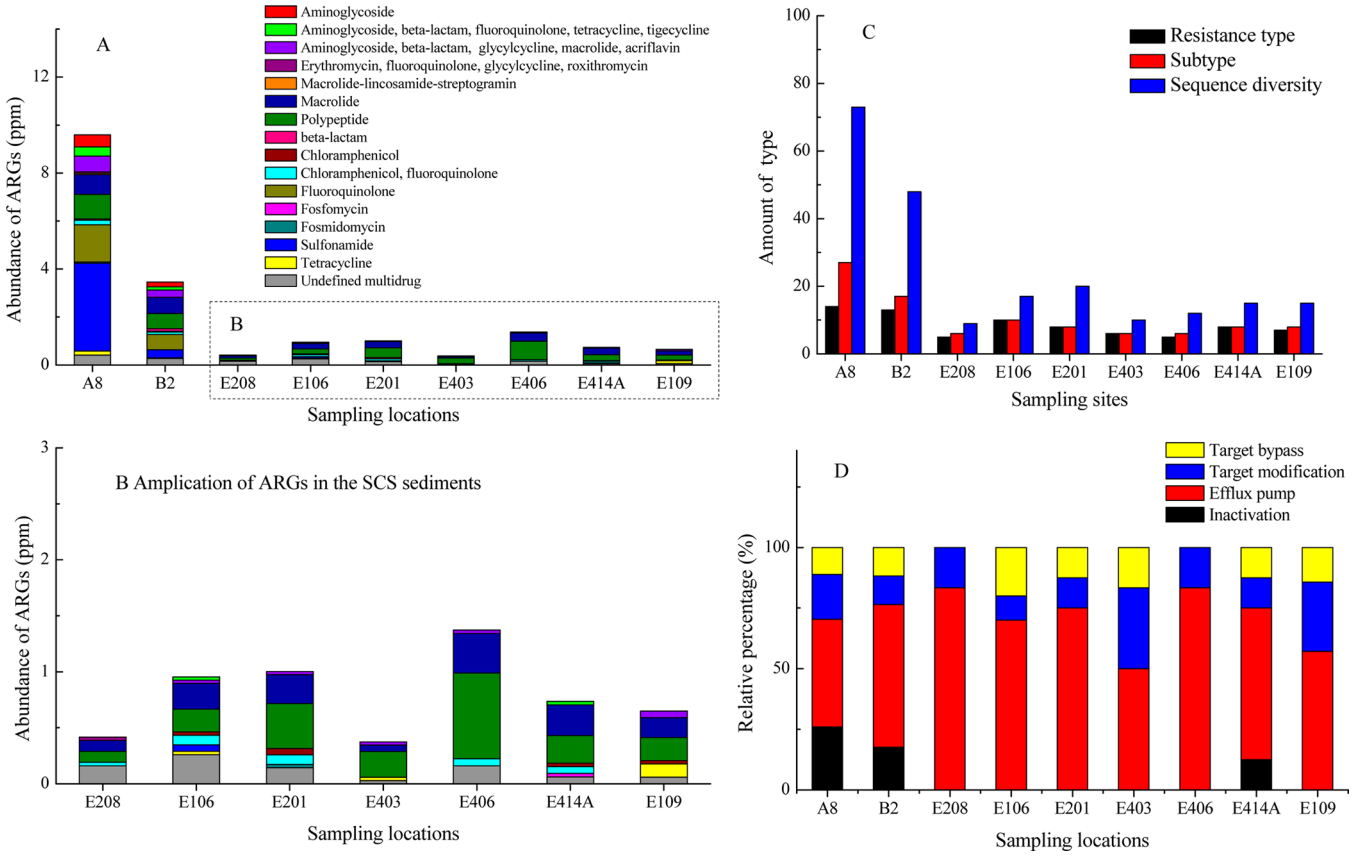


Figure 3. Diversity and abundance of ARGs in the PRE and SCS sediments. First, ARGs are categorized according to the type of antibiotics to which they are related (A). (B) shows an amplification of ARGs in the SCS sediments. Subtype represents the genotype of the resistance genes according to the description in the ARDB database. Sequence diversity represents the number of reference sequences from the ARDB database in each subtype identified in our data sets. A summary of the resistance type, subtype, and sequence diversity of ARGS in the PRE and SCS sediments is given in (C). The resistance genes were also classified according to their general mechanisms of resistance, including the inactivation of antibiotics, efflux pumps, target modification, and bypass (D).

sequences were identified in the SCS sediments. Figure 3D shows the relative percentages of resistance mechanisms in the PRE and SCS sediments. Efflux pumps were the predominant resistance mechanism in all of the PRE and SCS sediments. Distinct from the SCS sediments, a significant fraction of ARGs in the PRE sediments acts by the mechanism of antibiotic inactivation, which is mainly associated with resistance to common anthropogenic antibiotics, such as aminoglycosides, β-lactams, and macrolides.

Genotype Distribution of Macrolide and Polypeptide Resistance Genes. Since ARGs related to macrolides and polypeptides are the most widely distributed among all ARGs in the SCS sediments, a further comparison was made on the abundance and diversity of subtypes of macrolide and polypeptide resistance genes between the PRE and SCS sediments (Figure 4A). Regarding macrolide resistance genes, the predominant genotype in the SCS sediments was *mac*B that encodes a macrolide-specific efflux transporter. The *acr*B gene associated with multidrug resistance efflux pump was detected in most of the SCS sediments, but it was much less abundant than *mac*B. In the PRE sediments, *mac*B and *acr*B were also the major genotypes, but the relative percentage of *acr*B increased significantly. Moreover, the diversity of resistance genes to macrolides was highest at the mouth of the Pearl River (site A8), and subtypes of macrolide resistance genes involved with other mechanisms of resistance, i.e., inactivation (*ere*A) and rRNA methylation (*erm*X), were also found. Figure 4B shows that the total abundance of resistance genes to polypeptides was generally higher in the PRE sediments than in the SCS. Only two genotypes (*bac*A and *arn*A) were found, which are responsible for the resistance to bacitracin and polymyxin, respectively. The *arn*A gene was detectable in all sediments, but there is no clear relationship between *arn*A abundance and anthropogenic impacts. In contrast, the *bac*A gene was significantly more abundant in the PRE sediments than in the SCS sediments, suggesting that the abundance of *bac*A is potentially related to the impact of human activities.

Correlations between ARGs and MGEs. Both the total abundance and diversity of integrons and plasmids in the PRE sediments far exceeded those in the SCS sediments (Table S4 of the SI). For instance, the total abundance of integrons at site A8 in the PRE was generally five times greater than those in the SCS with the exception of E406, and two times for plasmids (Table S4 of the SI). Figure 5 shows the relationship between ARGs and MGEs in the PRE and SCS sediments. The abundance of ARGs significantly correlated with the abundance of the two MGEs (e.g., integrons and plasmids) on the level of $p < 0.01$ in the PRE and SCS sediments, and a relationship of significance was also observed for the diversity of ARGs and MGEs in sediments ($p < 0.01$). These results strongly suggest that MGEs play an important role in the dissemination of ARGs in the aquatic environment.

Discussion. A large variety of antibiotics have been widely used to prevent and treat bacterial infectious diseases.[28,29]

dx.doi.org/10.1021/es403818e | *Environ. Sci. Technol.* XXXX, XXX, XXX−XXX

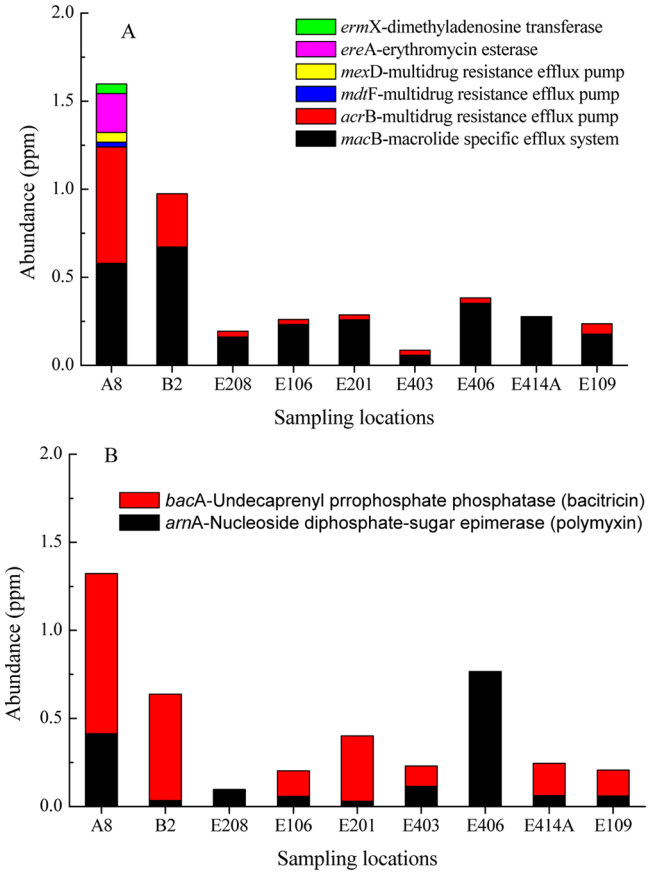


Figure 4. Diversity and abundance of subtypes of resistant genes to marcolides (A) and polypeptides (B) in the PRE and SCS sediments.

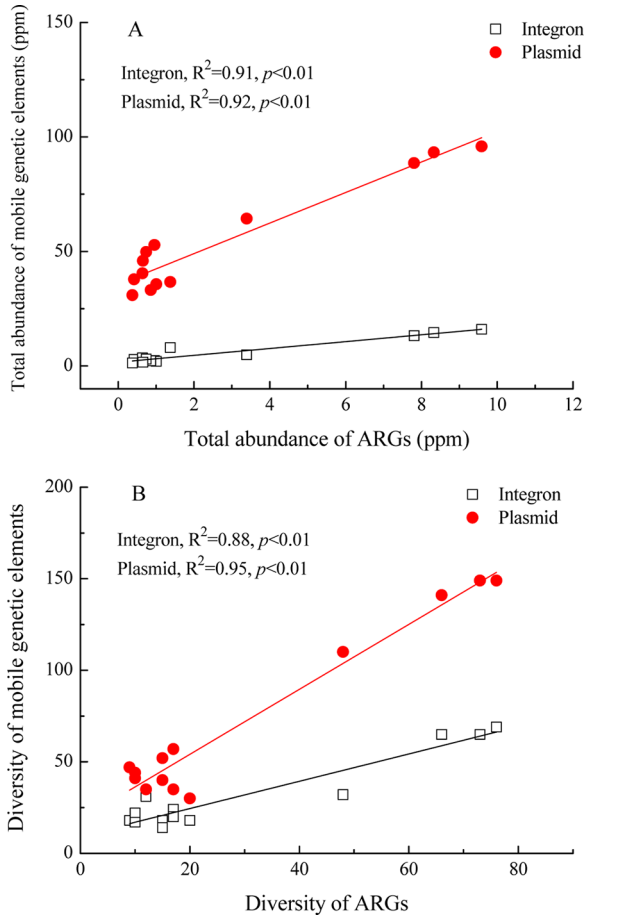


Figure 5. Correlations of abundance (A) and diversity (B) between ARGs and mobile genetic elements in the PRE and SCS sediments.

Consequently, it is very difficult to find a pristine modern environment that has never been exposed to anthropogenic antibiotics or resistance genes evolving in response to the use of anthropogenic antibiotics. The occurrence of ARGs in environmental niches completely devoid of anthropogenic impacts (e.g., isolated caves,[11] the terrestrial subsurface,[13,30] and permafrost sediments[10]) supports the view that antibiotic resistance is naturally originated. The deep sea sediment is also considered to be a locale isolated from anthropogenic impacts, which is supported by the absence of commonly used antibiotics in the SCS sediments. The existence of ARGs in deep sea sediments supports to a growing body of evidence that naturally originated antibiotic resistance is widespread in pristine natural environments.

The presence of antibiotic resistance in deep sea bacteria has been demonstrated in a few studies.[12,31,32] However, previous studies only focused on a single type of ARG using culture-dependent methods or PCR-based approaches. Although these studies are helpful in understanding the antibiotic resistance of cultivable bacteria or targeted genes, or the linkages between them, cultivable bacteria represent less than 1% of the microorganism population in natural environments and PCR approach only targets limited well-studied ARGs.[33] There is a lack of knowledge about the comprehensive profile of antibiotic resistance in the deep sea environment, which can now be achieved using high-throughput sequencing and metagenomic analysis.[19,27] In spite of their low levels of abundance and occurrence, ARGs that are present in deep sea sediments are resistant to major antibiotics in the human arsenal against bacterial infection. The genes encoding resistance to macrolides and polypeptides were the most prevalent and abundant in the SCS sediments.

Macrolides and polypeptides interfere with the essential biological processes that make it possible for bacteria to live. A macrolide antibiotic can interfere with bacterial protein biosynthesis by binding reversibly to the P site on the subunit 50S of the bacterial ribosome.[34] In the SCS sediments, the resistance to macrolides is mainly achieved by a macrolide-specific efflux transporter on the cell membrane encoded by the *mac*B gene,[35,36] which has been found in various potential pathogens, such as *Enterobacter aerogenes*,[37] *Escherichia coli*,[38] *Klebsiella pneumonia*,[39] and *Salmonella typhimurium*.[40] Polypeptide antibiotics can inhibit cell wall and peptidoglycan synthesis (bacitracin),[41] and destabilize the outer membrane (polymyxin).[42,43] In the SCS sediments, the *bac*A gene product can bypass the inhibition of the dephosphorylation of the isoprenyl pyrophosphate caused by bacitracin,[44] and the *arn*A gene confers resistance to polymyxin by a modified arabinose.[45,46]

There are four different mechanisms of antibiotic resistance, including efflux pumps, target modification, target bypass, and the inactivation of antibiotics.[21] The major mechanism of resistance in the SCS sediments is efflux pumps capable of reducing intracellular concentrations of antibiotics. The mechanism of efflux pumps is also important for microbes to fight against heavy metals or other toxins/environmental stresses (e.g., high salinity).[47−52] Given the limited nutrients in extreme environments, possessing the efflux pump would be one of the most efficient ways of fighting against various

dx.doi.org/10.1021/es403818e | Environ. Sci. Technol. XXXX, XXX, XXX−XXX

environmental stresses in terms of both maintenance cost and functional versatility.[8,53]

Comparative studies of scenarios in which the anthropogenic impacts vary in extent have shed light on the role of antibiotic use in the dissemination and proliferation of ARGs in various environments.[54−57] The levels of ARGs in the modern environment exhibit good correlation with human impacts,[20,58] especially to such significant sources of pollution as wastewater treatment plants,[59] animal husbandry,[60] and aquaculture.[61] In turn, the molecular signature of ARGs serves as a suitable marker to indicate the degree of anthropogenic impacts.[20,54] Metagenomic profiling of ARGs is a good approach to characterize the unique molecular trait of antibiotic resistance in different scenarios since it can provide broader profiles of ARGs. In the present study, one feature of the profiles of ARGs in the PRE sediments which is thoroughly distinct from those in the SCS sediments was the high abundance of ARGs related to commonly used antibiotics (e.g., sulfonamides, fluoroquinolones, and aminoglycosides). Our results suggest anthropogenic antibiotics might promote the acquisition and retention of resistance genes in indigenous microbes in highly human-impacted environments, as well as in exogenous microbes from typical sources of pollution.[62] It is very difficult to differentiate the contributions of the above potential causes for increased ARG levels in the human-impacted environments. Antibiotic concentrations are generally low in the environments; even near sources of pollution. Such subinhibitory levels of antibiotics could not be expected to exert a significant stress for selecting ARB and ARGs in the ambient environments. Alternatively, it is considered that the release of bacteria from the human and/or farmed animal flora is the predominant reason for the wide dissemination of ARGs in the human-impacted environments.[20,54] ARGs can also be horizontally transferred between microbes through MGEs, which act as carriers.[63,64] In our study, we observed significant correlations of both abundance and diversity between ARGs and MGEs. More abundant and diverse MGEs were identified in the PRE, which would greatly facilitate the transfer of ARGs among different microorganisms in various niches, such as environmental mediums and animal intestines. As a result, microbes can collectively tolerate the harsh selective pressure that they had never faced before the era of antibiotics.[65]

It is highly possible that microbes develop new resistance genes, mechanisms, or gene mutations to deal with the higher selective pressure exerted by various contaminants in human-impacted environments.[66−68] In the present study, it was shown that the resistance genotype and mechanisms were more diverse in the polluted PRE sediments than in the relatively pristine SCS sediments. For instance, more diverse resistance mechanisms and genotypes of ARGs conferring resistance to macrolides were identified in sediment at the mouth of the Pearl River (site A8) in comparison to a single resistance mechanism in the SCS sediments, probably due to high selective stress or to a significant release of ARGs from major sources of pollution in this region. ARGs are also recombined in a gene cassette that can simultaneously confer the resistance to different groups of antibiotics.[69,70] The evolution, horizontal transfer, and recombination of ARGs are essentially driven by the highly complex kinds of environmental stress that microbes need to face if they are to stand a chance of surviving in such highly human-impacted environments.

In summary, various ARGs were found in the deep ocean sediments of the SCS thoroughly isolated from anthropogenic impacts, which mainly encoded resistance to macrolides and polypeptides with efflux pumps as the predominant mechanism. The data suggest that ARGs are originated naturally in pristine environments. In comparison, the ARG genotypes and resistance mechanisms were much more abundant and diverse in the anthropogenic-influenced PRE sediments than in the SCS sediments. The resistance profiles in the PRE sediments were characterized by resistance to commonly used antibiotics, such as sulfonamides, fluoroquinolones, and aminoglycosides. Anthropogenic impacts, especially the wide use of anthropogenic antibiotics, are an important determinant in the dissemination of ARGs in human-impacted aquatic environments.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The sampling sites, the concentrations of antibiotics, the metagenomic profiles of ARGs, and the abundance and diversity of MGEs in sediments. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +852-28578551 (T.Z.); +852-27666041 (X.D.L.). Fax: +852-25595337 (T.Z.); +852-23346389 (X.D.L.). E-mail: zhangt@hku.hk (T.Z.); cexdli@polyu.edu.hk (X.D.L.).

Author Contributions

B.W.C. and Y.Y. contributed equally to this work. X.D.L., B.W.C., and T.Z. designed the study; B.W.C., X.M.L., and K.Y. collected the samples in the field; B.W.C. and X.M.L. measured antibiotic concentrations; B.W.C., Y.Y., and K.Y. analyzed the samples and data; B.W.C. and X.D.L. wrote the paper; all of the authors contributed to revising the manuscript and approved the final version.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported by the Area of Excellence (AoE) project under Grant No. AoE/P-04/2004 from the University Grants Council of Hong Kong, and the CAS/SAFEA International Partnership Program for Creative Research Teams (No. KZCX2-YW-T001). We thank Xiaoping Huang, Zhen Shi, and Feng Ye from the South China Sea Institute of Oceanology, the Chinese Academy of Sciences, for their assistance in the field sampling program.

■ REFERENCES

(1) Sommer, M. O.; Dantas, G.; Church, G. M. Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* **2009**, *325* (5944), 1128−31.

(2) Livermore, D. M. Has the era of untreatable infections arrived? *J. Antimicrob. Chemoth.* **2009**, *64* (4), 29−36.

(3) D'Costa, V. M.; McGrann, K. M.; Hughes, D. W.; Wright, G. D. Sampling the antibiotic resistome. *Science* **2006**, *311* (5759), 374−377.

(4) Forsberg, K. J.; Reyes, A.; Bin, W.; Selleck, E. M.; Sommer, M. O. A.; Dantas, G. The shared antibiotic resistome of soil bacteria and human pathogens. *Science* **2012**, *337* (6098), 1107−1111.

(5) Baltz, R. H. Renaissance in antibacterial discovery from actinomycetes. *Curr. Opin. Pharmacol.* **2008**, *8* (5), 557−563.

(6) Dantas, G.; Sommer, M. O. A.; Oluwasegun, R. D.; Church, G. M. Bacteria subsisting on antibiotics. *Science* **2008**, *320* (5872), 100−103.

(7) Cordero, O. X.; Wildschutte, H.; Kirkup, B.; Proehl, S.; Ngo, L.; Hussain, F.; Le Roux, F.; Mincer, T.; Polz, M. F. Ecological populations of bacteria act as socially cohesive units of antibiotic production and resistance. *Science* **2012**, *337* (6099), 1228−31.
(8) Wright, G. D. The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat. Rev. Microbiol.* **2007**, *5* (3), 175−186.
(9) Wright, G. D. The antibiotic resistome. *Expert. Opin. Drug. Dis.* **2010**, *5* (8), 779−788.
(10) D'Costa, V. M.; King, C. E.; Kalan, L.; Morar, M.; Sung, W. W. L.; Schwarz, C.; Froese, D.; Zazula, G.; Calmels, F.; Debruyne, R.; Golding, G. B.; Poinar, H. N.; Wright, G. D. Antibiotic resistance is ancient. *Nature* **2011**, *477* (7365), 457−461.
(11) Bhullar, K.; Waglechner, N.; Pawlowski, A.; Koteva, K.; Banks, E. D.; Johnston, M. D.; Barton, H. A.; Wright, G. D. Antibiotic resistance is prevalent in an isolated cave microbiome. *PLoS One* **2012**, *7* (4), e34953.
(12) Toth, M.; Smith, C.; Frase, H.; Mobashery, S.; Vakulenko, S. An antibiotic-resistance enzyme from a deep-sea bacterium. *J. Am. Chem. Soc.* **2010**, *132* (2), 816−823.
(13) Brown, M. G.; Balkwill, D. L. Antibiotic resistance in bacteria isolated from the deep terrestrial subsurface. *Microbial. Ecol.* **2009**, *57* (3), 484−493.
(14) Hughes, V. M.; Datta, N. Conjugative plasmids in bacteria of the pre-antibiotic era. *Nature* **1983**, *302* (5910), 725−726.
(15) Davis, C. E.; Anandan, J. Evolution of R factor . A study of a preantibiotic community in Borneo. *New. Engl. J. Med.* **1970**, *282* (3), 117−122.
(16) Thaller, M. C.; Migliore, L.; Marquez, C.; Tapia, W.; Cedeno, V.; Rossolini, G. M.; Gentile, G. Tracking acquired antibiotic resistance in commensal bacteria of Galápagos Land Iguanas: No man, no resistance. *PLoS One* **2010**, *5* (2), e8989.
(17) Knapp, C. W.; Dolfing, J.; Ehlert, P. A. I.; Graham, D. W. Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environ. Sci. Technol.* **2010**, *44* (2), 580−587.
(18) Durso, L. M.; Miller, D. N.; Wienhold, B. J. Distribution and quantification of antibiotic resistant genes and bacteria across agricultural and non-agricultural metagenomes. *PLoS One* **2012**, *7* (11), e48325.
(19) Port, J. A.; Wallace, J. C.; Griffith, W. C.; Faustman, E. M. Metagenomic profiling of microbial composition and antibiotic resistance determinants in Puget Sound. *PLoS One* **2012**, *7* (10), e48000.
(20) Chen, B. W.; Liang, X. M.; Huang, X. P.; Zhang, T.; Li, X. D. Differentiating anthropogenic impacts on ARGs in the Pearl River Estuary by using suitable gene indicators. *Water Res.* **2013**, *47* (8), 2811−2820.
(21) Zhang, X. X.; Zhang, T.; Fang, H. H. Antibiotic resistance genes in water environment. *Appl. Microbiol. Biotechnol.* **2009**, *82* (3), 397−414.
(22) Xu, W. H.; Zhang, G.; Zou, S. C.; Li, X. D.; Liu, Y. C. Determination of selected antibiotics in the Victoria Harbour and the Pearl River, South China using high-performance liquid chromatography-electrospray ionization tandem mass spectrometry. *Environ. Pollut.* **2007**, *145* (3), 672−679.
(23) Liang, X. M.; Chen, B. W.; Nie, X. P.; Shi, Z.; Huang, X. P.; Li, X. D. The distribution and partitioning of common antibiotics in water and sediment of the Pearl River Estuary, South China. *Chemosphere* **2013**, *92*, 1410−1416.
(24) Liu, B.; Pop, M. ARDB-antibiotic resistance genes database. *Nucleic Acids Res.* **2009**, *37*, D443−D447.
(25) Yang, Y.; Li, B.; Ju, F.; Zhang, T. Exploring variation of antibiotic resistance genes in activated sludge over a four-year period through a metagenomic approach. *Environ. Sci. Technol.* **2013**, *47* (18), 10197−10205.
(26) Moura, A.; Soares, M.; Pereira, C.; Leitao, N.; Henriques, I.; Correia, A. INTEGRALL: A database and search engine for integrons, integrases and gene cassettes. *Bioinformatics* **2009**, *25* (8), 1096−1098.
(27) Zhang, T.; Zhang, X. X.; Ye, L. Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge. *PLoS One* **2011**, *6* (10), e26041.
(28) Landers, T. F.; Cohen, B.; Wittum, T. E.; Larson, E. L. A review of antibiotic use in food animals: Perspective, policy, and potential. *Public Health Rep.* **2012**, *127* (1), 4−22.
(29) Zinner, S. H. Antibiotic use: Present and future. *New Microbiol.* **2007**, *30* (3), 321−325.
(30) Fredrickson, J. K.; Hicks, R. J.; Li, S. W.; Brockman, F. J. Plasmid incidence in bacteria from deep subsurface sediments. *Appl. Environ. Microb.* **1988**, *54* (12), 2916−2923.
(31) Kurusu, Y.; Yoshimura, S.; Tanaka, M.; Nakamura, T.; Maruyama, A.; Higashihara, T. Genetic transformation system for a psychrotrophic deep-sea bacterium: Isolation and characterization of a psychrotrophic plasmid. *Mar. Biotechnol.* **2001**, *3* (2), 96−99.
(32) Hughes, C. C.; Kauffman, C. A.; Jensen, P. R.; Fenical, W. Structures, reactivities, and antibiotic properties of the marinopyrroles A−F. *J. Org. Chem.* **2010**, *75* (10), 3240−3250.
(33) Amann, R. I.; Ludwig, W.; Schleifer, K. H. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **1995**, *59* (1), 143−69.
(34) Tenson, T.; Lovmar, M.; Ehrenberg, M. The mechanism of action of macrolides, lincosamides and streptogramin B reveals the nascent peptide exit path in the ribosome. *J. Mol. Biol.* **2003**, *330* (5), 1005−1014.
(35) Kobayashi, N.; Nishino, K.; Yamaguchi, A. novel macrolide-specific ABC-type efflux transporter in *Escherichia coli*. *J. Bacteriol.* **2001**, *183* (19), 5639−44.
(36) Modali, S. D.; Zgurskaya, H. I. The periplasmic membrane proximal domain of MacA acts as a switch in stimulation of ATP hydrolysis by MacB transporter. *Mol. Microbiol.* **2011**, *81* (4), 937−51.
(37) Shin, S. H.; Kim, S.; Kim, J. Y.; Lee, S.; Um, Y.; Oh, M. K.; Kim, Y. R.; Lee, J.; Yang, K. S. Complete genome sequence of *Enterobacter aerogenes* KCTC 2190. *J. Bacteriol.* **2012**, *194* (9), 2373−2374.
(38) Husain, F.; Nikaido, H. Substrate path in the AcrB multidrug efflux pump of *Escherichia coli*. *Mol. Microbiol.* **2010**, *78* (2), 320−330.
(39) Padilla, E.; Llobet, E.; Domenech-Sanchez, A.; Martinez-Martinez, L.; Bengoechea, J. A.; Alberti, S. *Klebsiella pneumoniae* AcrAB efflux pump contributes to antimicrobial resistance and virulence. *Antimicrob. Agents Chemother.* **2010**, *54* (1), 177−183.
(40) Lacroix, F. J. C.; Cloeckaert, A.; Grepinet, O.; Pinault, C.; Popoff, M. Y.; Waxin, H.; Pardon, P. Salmonella typhimurium acrB-like gene: Identification and role in resistance to biliary salts and detergents and in murine infection. *Fems. Microbiol. Lett.* **1996**, *135* (2−3), 161−167.
(41) Stone, K. J.; Stroming, J. Mechanism of action of bacitracin—complexation with metal ion and C55-isoprenyl pyrophosphate. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68* (12), 3223−3227.
(42) Hsuchen, C. C.; Feingold, D. S. Mechanism of polymyxin-B-action and selectivity toward biologic membranes. *Biochemistry* **1973**, *12* (11), 2105−2111.
(43) Schindler, P. R.; Teuber, M. Action of polymyxin B on bacterial membranes: morphological changes in the cytoplasm and in the outer membrane of *Salmonella typhimurium* and *Escherichia coli* B. *Antimicrob. Agents Ch.* **1975**, *8* (1), 95−104.
(44) El Ghachi, M.; Bouhss, A.; Blanot, D.; Mengin-Lecreulx, D. The bacA gene of *Escherichia coli* encodes an undecaprenyl pyrophosphate phosphatase activity. *J. Biol. Chem.* **2004**, *279* (29), 30106−13.
(45) Gatzeva-Topalova, P. Z.; May, A. P.; Sousa, M. C. Crystal structure of *Escherichia coli* ArnA (PmrI) decarboxylase domain. A key enzyme for lipid A modification with 4-amino-4-deoxy-L-arabinose and polymyxin resistance. *Biochemistry* **2004**, *43* (42), 13370−9.
(46) Gatzeva-Topalova, P. Z.; May, A. P.; Sousa, M. C. Structure and mechanism of ArnA: conformational change implies ordered dehydrogenase mechanism in key enzyme for polymyxin resistance. *Structure* **2005**, *13* (6), 929−42.
(47) Silver, S. Bacterial resistances to toxic metal ions - A review. *Gene* **1996**, *179* (1), 9−19.

(48) Rosen, B. P. Bacterial resistance to heavy metals and metalloids. *J. Biol. Inorg. Chem.* **1996**, *1* (4), 273−277.
(49) Koronakis, V.; Eswaran, J.; Hughes, C. Structure and function of tolC: The bacterial exit duct for proteins and drugs. *Annu. Rev. Biochem.* **2004**, *73*, 467−489.
(50) Taylor, D. L.; Bina, X. W. R.; Bina, J. E. *Vibrio cholerae vexH* encodes a multiple drug efflux pump that contributes to the production of cholera toxin and the toxin co-regulated pilus. *PLoS One* **2012**, *7* (5), e38208.
(51) Ryan, B. M.; Dougherty, T. J.; Beaulieu, D.; Chuang, J.; Dougherty, B. A.; Barrett, J. F. Efflux in bacteria: What do we really know about it? *Exp. Opin. Invest. Drugs* **2001**, *10* (8), 1409−1422.
(52) Zhou, A. F.; Baidoo, E.; He, Z. L.; Mukhopadhyay, A.; Baumohl, J. K.; Benke, P.; Joachimiak, M. P.; Xie, M.; Song, R.; Arkin, A. P.; Hazen, T. C.; Keasling, J. D.; Wall, J. D.; Stahl, D. A.; Zhou, J. Z. Characterization of NaCl tolerance in *Desulfovibrio vulgaris* Hildenborough through experimental evolution. *ISME J.* **2013**, *7* (9), 1790−1802.
(53) Fredrickson, J. K.; Garland, T. R.; Hicks, R. J.; Thomas, J. M.; Li, S. W.; Mcfadden, K. M. Lithotrophic and heterotrophic bacteria in deep subsurface sediments and their relation to sediment properties. *Geomicrobiol. J.* **1989**, *7* (1−2), 53−66.
(54) Storteboom, H.; Arabi, M.; Davis, J. G.; Crimi, B.; Pruden, A. Tracking antibiotic resistance genes in the South Platte River Basin using molecular signatures of urban, agricultural, and pristine sources. *Environ. Sci. Technol.* **2010**, *44* (19), 7397−7404.
(55) Graham, D. W.; Olivares-Rieumont, S.; Knapp, C. W.; Lima, L.; Werner, D.; Bowen, E. Antibiotic resistance gene abundances associated with waste discharges to the Almendares River near Havana, Cuba. *Environ. Sci. Technol.* **2011**, *45* (2), 418−424.
(56) Song, J. S.; Jeon, J. H.; Lee, J. H.; Jeong, S. H.; Jeong, B. C.; Kim, S. J.; Lee, J. H.; Lee, S. H. Molecular characterization of TEM-type beta-lactamases identified in cold-seep sediments of Edison seamount (south of Lihir Island, Papua New Guinea). *J. Microbiol.* **2005**, *43* (2), 172−178.
(57) Hall, B. G.; Barlow, M. Evolution of the serine beta-lactamases: Past, present and future. *Drug Resist. Update* **2004**, *7* (2), 111−123.
(58) Pruden, A.; Arabi, M.; Storteboom, H. N. Correlation between upstream human activities and riverine antibiotic resistance genes. *Environ. Sci. Technol.* **2012**, *46* (21), 11541−11549.
(59) Negreanu, Y.; Pasternak, Z.; Jurkevitch, E.; Cytryn, E. Impact of treated wastewater irrigation on antibiotic resistance in agricultural soils. *Environ. Sci. Technol.* **2012**, *46* (9), 4800−4808.
(60) Zhu, Y. G.; Johnson, T. A.; Su, J. Q.; Qiao, M.; Guo, G. X.; Stedtfeld, R. D.; Hashsham, S. A.; Tiedje, J. M. Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110* (9), 3435−3440.
(61) Shah, S. Q. A.; Colquhoun, D. J.; Nikuli, H. L.; Sorum, H. Prevalence of antibiotic resistance genes in the bacterial flora of integrated fish farming environments of Pakistan and Tanzania. *Environ. Sci. Technol.* **2012**, *46* (16), 8672−8679.
(62) Baquero, F.; Negri, M. C.; Morosini, M. I.; Blazquez, J. Antibiotic-selective environments. *Clin. Infect. Dis.* **1998**, *27* (Suppl 1), S5−11.
(63) Normark, B. H.; Normark, S. Evolution and spread of antibiotic resistance. *J. Intern. Med.* **2002**, *252* (2), 91−106.
(64) Maiden, M. C. J. Horizontal genetic exchange, evolution, and spread of antibiotic resistance in bacteria. *Clin. Infect. Dis.* **1998**, *27*, S12−S20.
(65) Mazel, D.; Davies, J. Antibiotic resistance in microbes. *Cell. Mol. Life Sci.* **1999**, *56* (9−10), 742−754.
(66) Pu, X. Y.; Zhang, Q. J.; Pan, J. C.; Shen, Z. Q.; Zhang, W. Spontaneous mutation frequency and molecular mechanisms of *Shigella flexneri* fluoroquinolone resistance under antibiotic selective stress. *World J. Microb. Biot.* **2013**, *29* (2), 365−371.
(67) Woodford, N.; Ellington, M. J. The emergence of antibiotic resistance by mutation. *Clin. Microbiol. Infec.* **2007**, *13* (1), 5−18.
(68) Martinez, J. L.; Baquero, F. Mutation frequencies and antibiotic resistance. *Antimicrob. Agents Ch.* **2000**, *44* (7), 1771−1777.
(69) Doublet, B.; Weill, F. X.; Fabre, L.; Chaslus-Dancla, E.; Cloeckaert, A. Variant Salmonella genomic island 1 antibiotic resistance gene cluster containing a novel 3′-*N*-aminoglycoside acetyltransferase gene cassette, aac(3)-id, in Salmonella enterica serovar Newport. *Antimicrob. Agents Ch.* **2004**, *48* (10), 3806−3812.
(70) Venturini, C.; Beatson, S. A.; Djordjevic, S. P.; Walker, M. J. Multiple antibiotic resistance gene recruitment onto the enterohemorrhagic *Escherichia coli* virulence plasmid. *Faseb. J.* **2010**, *24* (4), 1160−1166.