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Article

Ionic Liquid Enriches the Antibiotic Resistome, Especially Efflux Pump Genes, Before Significantly Affecting Microbial Community Structure

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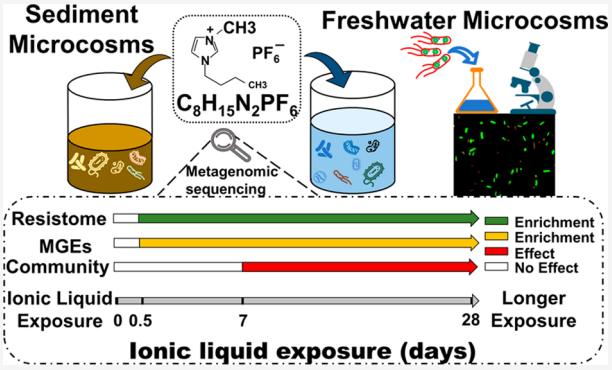
ABSTRACT: An expanding list of chemicals may permeabilize bacterial cells and facilitate horizontal gene transfer (HGT), which enhances propagation of antibiotic resistance genes (ARGs) in the environment. Previous studies showed that 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIm][PF6]), an ionic liquid, can facilitate HGT of some ARGs among bacteria. However, the dynamic response of a wider range of ARGs and associated mobile genetic elements (MGEs) in different environments is unknown. Here, we used metagenomic tools to study shifts of the resistome and microbiome in both sediments and freshwater microcosms exposed to [BMIm][PF6]. Exposure for 16 h to 0.1 or 1.0 g/L significantly enriched more than 207 ARG subtypes primarily encoding efflux pumps in freshwater microcosms as well as cultivable antibiotic-resistant bacteria. This resistome enrichment was attributed to HGT facilitated by MGEs (428 plasmids, 61 integron-integrase genes, and 45 gene cassettes were enriched) as well as to HGT-related functional genes. Interestingly, resistome enrichment occurred fast (within 16 h) after [BMIm][PF6] exposure, before any significant changes in bacterial community structure. Similar ARG enrichment occurred in sediment microcosms exposed to [BMIm][PF6] for 28 d, and this longer exposure affected the microbial community structure (e.g., *Proteobacteria* abundance increased significantly). Overall, this study suggests that [BMIm][PF6] releases could rapidly enrich the antibiotic resistome in receiving environments by increasing HGT and fortuitously selecting for efflux pump genes, thus contributing to ARG propagation.



INTRODUCTION

Antibiotic resistance propagation is a growing concern due to its projected negative impact on global health and the economy.[1] According to a 2017 report from the U.S. Centers for Disease Control and Prevention, antibiotic resistant bacteria (ARB) cause more than 2 million illness and about 23000 deaths each year in U.S. alone.[2] The dissemination of ARB and antibiotic resistance genes (ARGs) worldwide is associated with misuse or overuse of antibiotics in human medicine and animal production.[3,4] The acquisition of antibiotic resistance genes (ARGs) by bacteria is probably caused by three principal mechanisms: genetic mutation, recombination, and horizontal gene transfer (HGT).[5−8] In addition to clinical settings, ARGs are also found in various environments such as soil,[9−11] wastewater,[12−14] and surface water,[15−17] and direct transfer of ARGs from the environments to human pathogens has been reported.[18,19] Thus, a thorough understanding of ARG dissemination in either clinical, gastrointestinal, agricultural, or natural environments is critical for effectively managing and mitigating this grand challenge.

The list of anthropogenic chemicals released to the environment continues to increase, and some xenobiotics may enhance ARG propagation by inducing microbial population shifts and fortuitously selecting for resistant populations. For example, enrichment of the antibiotic resistome was reported after drinking water chlorination.[20] Prior studies also found that some chemicals facilitate HGT and ARG propagation among different bacterial species. Nanoparticles (e.g., nano-Al_2O_3 and nano-ZnO),[21−23] antimicrobial agents (e.g., triclosan),[24] and antiepileptic drugs (e.g., carbamazepine)[25] were reported to enhance the conjugative transfer of multiresistance genes via increasing reactive oxygen species, the SOS response, cell membrane permeability, and pilus generation. Nevertheless, the dynamic reaction of complex microbial communities to xenobiotics and

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the associated response of the antibiotic resistome and MGEs are not fully understood.

Ionic liquids (ILs), a class of safe and environmentally friendly organic solvents, have been widely used in industrial reactions due to its chemical and thermal stability.[26,27] Several studies have addressed the toxicity and biodegradability of ILs,[28] but their influence on antibiotic resistance propagation is far less understood. In our previous studies,[29,30] ILs promoted integrons- or conjugative plasmids-mediated ARG propagation by increasing the cell membrane permeability and expression level of pilin, which are vital for HGT by conjugation. Nonetheless, the environmental resistome response to IL exposure was not characterized. The rapid development of high-throughput DNA sequencing techniques represents a great opportunity to discern the influence of environmental pollutants on the environmental resistome, bacterial microbiome, and associated functional genes.

In this study, freshwater and sediment microcosms were spiked with 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIm][PF6]) at 0.1 or 1.0 g/L, and these microcosms were incubated in 2-L flasks at room temperature. Using metagenomics sequencing, antibiotic resistomes, bacterial communities, and MGEs were quantified relative to [BMIm]-[PF6]-free controls. The culturable bacteria resistant to tetracycline, ampicillin, ciprofloxacin, erythromycin, and streptomycin were also isolated before and after [BMIm][PF6] exposure. Conjugative transfer of antibiotic multiresistance RK2 plasmid between *Escherichia coli* and natural water microbiota, and quantitative polymerase chain reaction (qPCR) measurement of an important MGE (Class 1 integron) were combined to confirm the impact of [BMIm]-[PF6] on HGT. Overall, this unprecedented study informs on the potential impacts of [BMIm][PF6] releases on the antibiotic resistome and bacterial communities in freshwater and sediment environments.

MATERIALS AND METHODS

Setup of Freshwater and Sediment Microcosms. Fresh water, as well as the sediment, were collected from Water Park in Tianjin, China (39°05′N, 117°09′E) on September 20, 2013. The collected water samples were allowed to stand for 3 h at 4 °C in a refrigerator to remove suspended particles, and the supernatant was collected for the subsequent experiment. The detailed water quality parameters are given in Table S1 of the Supporting Information. Three groups of freshwater microcosms including two separate treatments spiked with [BMIm][PF6] (purity >99%) at the final concentrations of 0.1 and 1.0 g/L,[29,30] and one [BMIm][PF6]-free control, were prepared in triplicate in 1.5 L of water in 2-L flasks. Given that "typical" [BMIm][PF6] concentrations in impacted environments are still unknown, the [BMIm][PF6] concentrations selected were based on prior IL toxicity studies, and similar or even lower concentrations were used.[31−33] After a 16 h exposure, water samples (1.0 L) collected from each microcosm were separately passed through 0.22 μm filters to obtain bacterial biomass. All filters were stored at −20 °C for further analyses.

To assess the effect of [BMIm][PF6] on plasmid conjugative transfer, *E. coli* K12::Td-Tomato carrying broad-host range plasmid RK2::EGFP[34] was used as a donor (Table S2). The chromosome and plasmid of this strain are labeled differentially with red fluorescence protein gene Td-Tomato and green fluorescence protein gene EGFP, respectively. This facilitates tracking and quantifying conjugative transfer events in the microbial community. Specifically, since the donor strain harbors a transferable GFP-labeled plasmid, the transconjugants (N_T) emit single green fluorescence after acquiring the plasmid, which is different from the donor strains (N_D) that emit both green and red fluorescence. Therefore, transconjugants and donor bacteria can be differentiated and automatically counted by ImageJ (1.52a). The transfer frequency (f) was then calculated using the formula $f = N_T$ (the number of transconjugants in LSCM)/N_D(the number of donors in LSCM).[35]

The donor cells were inoculated into LB media with 50 μg/mL apramycin. After overnight incubation, bacterial cultures were centrifuged and washed with sterile PBS for three times to remove residual antibiotics. Finally, bacterial pellets were resuspended in 10 mL of sterile PBS and the suspensions were immediately mixed with 90 mL of collected fresh water spiked with [BMIm][PF6] to reach final concentrations of 0.1 or 1.0 g/L in triplicate 250 mL beakers. The final concentration of the *E. coli* donor was 10^7 CFU/mL, which is typical bacterial density in natural river water.[15,36] The obtained freshwater microcosms were vortexed briefly and cultivated at 30 °C for 16 h under light condition. After cultivation, a laser scanning confocal microscope (LSCM) (Leica TCS SP8) was used to confirm the HGT and calculate the conjugation transfer frequency in the freshwater microcosms.

Similar to the setup of freshwater microcosm, sediment microcosms (500.0 g) were also conducted at [BMIm][PF6] concentrations of 0, 0.08, 0.8 g/kg, which corresponds to the amended IL mass in water microcosm. Each treatment set of three replicates and all sediment microcosms were incubated in a stationary manner in the dark at 30 °C up to 28 d without shaking. Periodic sediment samples (2.0 g) of each microcosm were taken at 0, 0.5, 2, 4, 7, 14, and 28 d and stored in −20 °C for further analyses.

Antibiotic Susceptibility Assay. The number of total culturable bacteria as well as bacteria resistant to ampicillin (100 mg/L), tetracycline (10 mg/L), streptomycin (30 mg/L), erythromycin (50 mg/L), or ciprofloxacin (10 mg/L) were quantified in both freshwater and sediment samples. These antibiotic concentrations were selected based on previous studies that similarly screened for the presence of ARB in environmental samples.[36,37] Approximately 100 mL water samples were filtered through 0.22 μm filters, and the filters were immersed in 10 mL of ultrapure water and sonicated for 15 min to detach the bacterial cells.[38] The collected microbial cells were diluted in phosphate-buffered saline (PBS) to make serial 10-fold dilutions. Subsequently, 100 μL of dilute solutions were spread on LB agar plates in the absence or presence of the antibiotic described above.[39] CFU (colony-forming units) were counted after a 3 d incubation at 30 °C.

DNA Extraction. DNA was extracted from collected filters and sediment samples using the E.N.Z.A. Water DNA kit (Omega Biotek, CA) and the QIAamp DNeasy PowerSoil Kit (QIAamp, German) according to the instructions of the manufacturer, respectively. Microspectrophotometry (NanoDrop Technologies, DE) and gel electrophoresis (Bio-Rad, CA) were used to measure DNA concentration, purity, and integrity.

Metagenomic Sequencing and Data Analysis. About 5 μg of freshwater DNA was sent to the Beijing Genomics Institute for sequencing using the Illumina Hiseq 2000 platform with the sequencing strategy of index PE101 +

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8+101 cycle (paired-end sequencing, 101-bp reads and 8-bp index sequence). Insert size of the library was about 170 bp. The sequencing data were first trimmed by removing reads with bases of quality score lower than 30 or containing more than three ambiguous bases. The raw data consisted of 76.1 million reads for the 3 samples. The raw sequences were first quality-filtered to remove adapters and low-quality sequences using Trimmomatic version 0.36,[40] resulting in 75.1 million clean reads with average 6.2 Gb clean reads for each sample. The clean reads were subjected to align against 16S Greengene nonredundant 85 OTUs database and a widely accepted antibiotic resistance proteins database Structured Antibiotic Resistance Genes (SARGs v2.0) in ARG-OAPs using BLASTX with *E*-value $<10^{-7}$, sequence identity >80%, and alignment length >25 amino acids.[41] SARG contains 4049 manually curated ARG reference sequences comprising 24 ARGs types and 1209 ARG subtypes in a hierarchical structure.[42] "ARG subtypes" represent individual ARG in one "ARG type", for example, "tetracycline resistance genes" an "ARG type", whereas "*tet*A " is a tetracycline resistance subtypes.[43] The abundance of each ARG was calculated using the method by Li et al.[44]

$$\text{Abundance} = \sum_{1}^{n} \frac{N_{ARG-likesequence} \times L_{reads}/L_{ARGreferencesequence}}{N_{16Ssequence} \times L_{reads}/L_{16Ssequence}} \quad (1)$$

in which $N_{ARG\text{-}like\ sequence}$ is the number of the ARG-like sequence annotated as one specific ARG reference sequence; $L_{ARG\ reference\ sequence}$ is the sequence length of the corresponding specific ARG reference sequence; $N_{16S\ sequence}$ is identified from the metagenomic data; $L_{16S\ sequence}$ is the average length of the 16S sequence in the Greengenes database; n is the number of the mapped ARG reference sequence belonging to the ARG subtype; L_{reads} is the sequence length of the Illumina reads (100 nt) that was used in the present study.

The clean reads were queried against plasmid sequences in the Reference Sequences in NCBI and integrons from the Integral database.[45] A read was identified as integron or gene cassettes if the BLAST hit (E-value cutoff of 10^{-5}) had a sequence identity above 90% over a length of at least 50 bp.[46] The threshold of identified plasmids was determined as the BLAST hits (E-value cutoff of 10^{-5}) with a nucleotide sequence identity above 95% over a length of at least 90 bp.[46] Finally, to explore the bacterial composition profile, the pair-end reads were merged to tags of 150−190 bp with FLASH,[47] then compared with the SILVA SSU database, and assigned to taxonomy with MEGAN (version 5.7).

For the functional assignment, the clean reads were assembled using IDBA_UD with default k-mer.[48] Assembled contigs longer than 500 bp were used for downstream analysis. In total, 36697, 42583, and 41266 contigs were obtained for the control, IL 0.1 g/L, and IL 1.0 g/L treatments, respectively. ORFs within contigs were predicted using MetaGeneMark (Georgia Institute of Technology, Atlanta, Georgia).[49] Then, the nucleotide sequences of the ORFs were compared to the nonredundant (nr) database (NCBI). The results were then assigned to the SEED database using MEGAN (version 5.7).[50] The SEED database is the subsystems annotated across genomes and are based on biochemical pathways, fragments of pathways, and clusters of genes that function together or any group of genes considered to be related.

To compare functional annotations in metagenomic data sets of IL-exposed treatments versus IL-free controls, we randomly sampled with replacement one million reads from the comparison data set to generate a distribution for each data set, and the number of annotations from 10000 trials was counted. Subsequently, the Z-score was calculated to determine whether changes in the number functional genes due to IL exposure were significant, as follows[51]

$$Z = \left| \frac{(x - \mu)}{\delta} \right| \quad (2)$$

where x is the raw number of functional annotations in treatment A, μ is the mean number of functional annotations in distribution for treatment B, and δ is the standard deviation of the distribution of treatment B. Using this approach, the differences between IL-free control and IL-exposed treatments were assessed for statistical significance. When comparing the IL-free control to the IL-exposed treatment, the mean and standard deviations of IL-exposed treatment were used. Similarly, when comparing IL-exposed treatment to IL-free control, the mean and standard deviations of IL-free control were used. Therefore, two Z-scores and two standard deviations were used, and the minimum Z-score was conservatively selected for statistical inference. Since random resampling with replacement would be normally distributed, differences were considered as significant if the minimum Z score was larger than 1.65 ($p < 0.05$).

High-Throughput Sequencing of 16S rRNA Gene and Real-Time PCR. To quantify the abundance of bacteria and ARGs in the sediment, DNAs were sent for high-throughput sequencing of 16S rRNA gene and real-time PCR. The V4 region was amplified using the universal primers 515F and 806R. Library preparation and 16S rRNA sequencing on an Illumina MiSeq instrument were performed by Beijing Genomics Institute (Wuhan, China). The amplified products were purified and recovered using 1.0% agarose gel electrophoresis. The raw tags were obtained by merged the paired-end reads using FLASH (v1.2.7). The raw tags were then filtered and clustered. The merged tags were compared to the primers, while the FASTX-Toolkit was used to discard the tags with more than six mismatches. Tags with an average quality score <20 in a 50 bp sliding window were truncated using Trimmomatic and tags shorter than 350 bp were removed. We identified possible chimeras by employing UCHIME, a tool included in Mothur. The denoised sequences were clustered using USEARCH (version 10.0) and tags with similarity ≥97% were regarded as an OTU. Taxonomy was assigned to all OTUs by searching against the Silva databases using the uclust within QIIME. Principal coordinate analysis (PCoA) was performed to visualize the β diversity among samples based on the weighted unifrac distance.[52]

qPCR amplifications were performed using a Bio-Rad iQ5 instrument (Bio-Rad, Hercules, CA). Calibration standard curves for positive controls were generated as described previously.[15,53] Negative controls contained all the components of the PCR mixture without the DNA template. The details of qPCR assays, absolute abundance of ARGs in sediment (Table S3), plasmid copy number detection, and primers used in the plasmid copy number detection (Table S4) are described in the Supporting Information (SI).

Statistical Analysis. Downstream statistical analysis was performed using MEGAN, Microsoft Excel, STAMP,[54] and R

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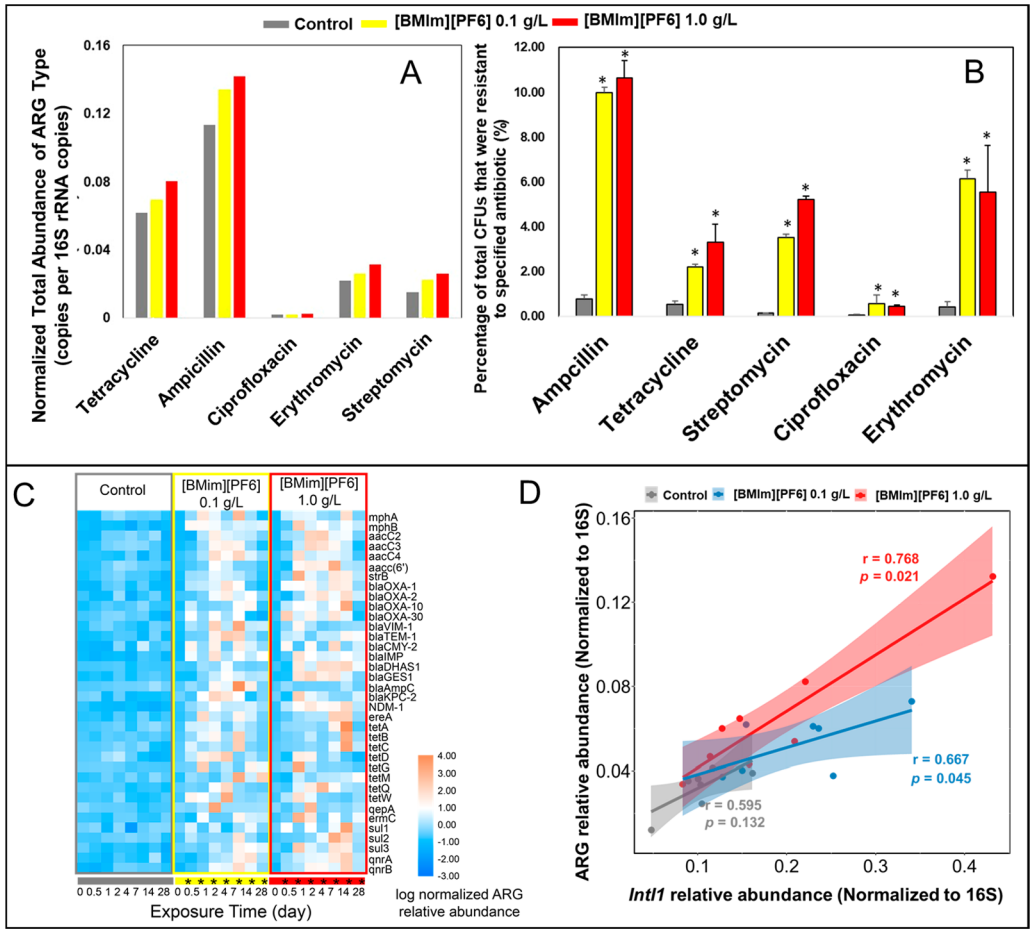

Figure 1. Enrichment of ARGs and relative abundance of antibiotic resistant bacteria in freshwater (parts A and B) or sediment ([arts C and D) microcosm exposed to [BMIm][PF6] at 0.1 g/L or 1.0 g/L. The total abundance of ARG type (A) categorized by different antibiotics, and by the percentage of total CFUs that were resistant to antibiotics (Tetracycline, Ampicillin, Ciprofloxacin, Erythromycin or Streptomycin) after exposure to [BMIm][PF6] for 16 h in freshwater microcosms (B). Each value is shown as mean ± SD of three biological replicates. Significant differences between groups exposed to ionic liquid versus unexposed controls were determined with the Student's *t* test. (*, $p < 0.05$) (C) The dynamics of ARG relative abundance (normalized to 16S rRNA) shown as heatmap. Significant differences between groups exposed to ionic liquid (0.1 g/L, yellow, 1.0 g/L, red) versus corresponding unexposed controls (gray) following different exposure duration (0.5, 1, 2, 4, 7, 14, 28 days) were determined with nonparametric Kruskal–Wallis tests. Asterisks (*) indicate significant differences ($p < 0.05$) of ARG relative abundance between ionic liquid exposed groups and the unexposed controls. (D) Positive correlation between Class 1 integron and total ARG relative abundance in sediment microcosms exposed to [BMIm][PF6] for 28 d. The plot includes all data collected at different sampling times (0, 0.5, 1, 2, 4, 7, 14, and 28 days). Smoothing curves based on linear model separately for each control or treatment group are shown in gray with 95% confidence intervals. Spearman's r and *P* values are depicted.

package ggplot2.[55] Fisher's exact test was applied to analyze changes in microbial community composition.[56] Variation partitioning analysis (VPA) was conducted to determine the contributions of MGEs versus bacterial community structure (i.e., population shifts) to the variations of ARGs.[57,58] Student's *t* test and nonparametric Kruskal–Wallis tests in R package vegan or Fisher's exact test in STAMP software was used to discern significant differences between sample data at the 95% confidence level.

Nucleotide Sequence Accession Numbers. All the nucleotide sequence data used in the present study have been deposited in the NCBI SRA database under the project ID PRJNA552296.

RESULTS AND DISCUSSION

Exposure to [BMIm][PF6] Rapidly Enriched Antibiotic Resistomes in Both Freshwater and Sediment Microcosms. [BMIm][PF6]-amended freshwater and sediment microcosms were set up to discern the effect of [BMIm][PF6] on antibiotic resistomes and bacterial communities in different natural environments. In freshwater microcosms, 285 ARG subtypes were detected and divided into categories according to antibiotics types; the most enriched five ARG categories are shown in Figure 1A. The relative abundance of most ARGs was enriched following [BMIm][PF6] exposure for 16 h, with the exception of ciprofloxacin resistance genes (Figure 1A). Enrichment as a result of [BMIm][PF6] exposure was concentration dependent. For example, the Z-scores of ampicillin resistance genes were 2.34 and 3.15 after 16 h exposure to [BMIm][PF6] concentration of 0.1 and 1.0 g/L, respectively, suggesting significant enrichment of ampicillin resistance genes.

Using cultivation methods, tetracycline-, ampicillin-, ciprofloxacin-, erythromycin-, and streptomycin-resistant bacteria were also isolated. The tested [BMIm][PF6] concentrations did not significantly alter the number of culturable bacteria in

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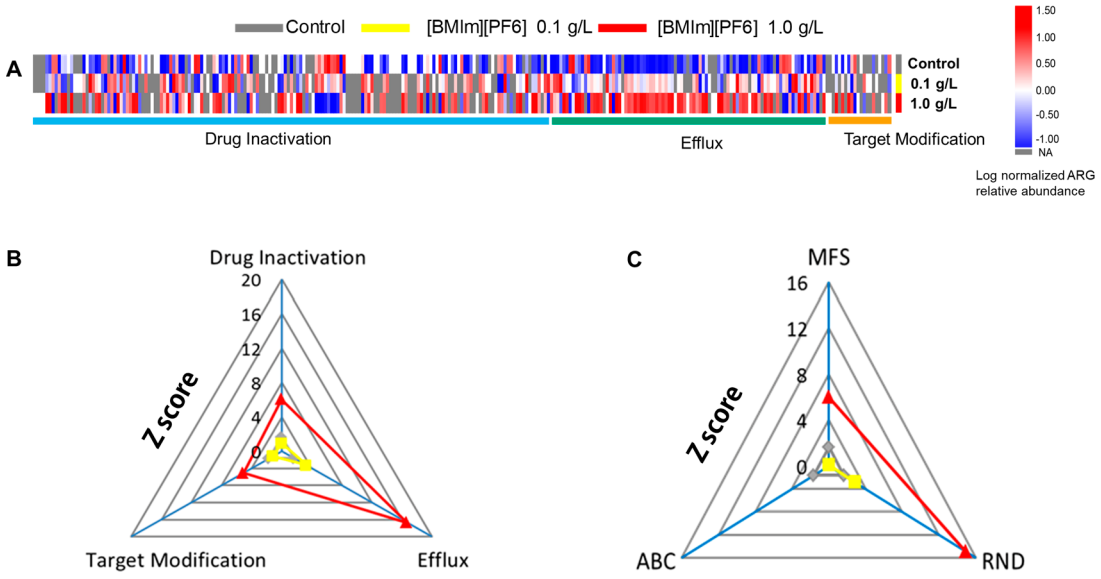

Figure 2. Enriched ARGs in freshwater microcosms exposed to [BMIm][PF6] 0.1 g/L (yellow) or 1.0 g/L (red) for 16 h. (A) Enrichment of ARG relative abundance as categorized by resistance mechanisms. The scale bar depicts ARG relative abundance (log normalized by 16s rRNA abundance); (parts B and C) Z-score are shown for sequencing reads annotated as ARGs in freshwater microcosm from 0.1 g/L exposure and 1.0 g/L exposure. The enrichment of ARGs categorized by resistance mechanisms (B) and subtypes of efflux pump genes (C). Abbreviations: ABC, ATP-binding cassette; RND, Resistance-nodulation-cell division; MFS, Major facilitator superfamily.

freshwater (Figure 1B). Specifically, the total culturable bacteria concentration in control ([BMIm][PF6]-free) freshwater microcosms was $(5.5 \pm 2.2) \times 10^6$ CFU/mL, which was not significantly different from that in the [BMIm][PF6]-exposed treatments ($(6.1 \pm 1.9) \times 10^6$ and $(6.7 \pm 1.6) \times 10^6$ CFU/mL for 0.1 and 1.0 g/L [BMIm][PF6], respectively). However, the relative abundance of culturable bacteria that became resistant to the tested antibiotics (as well as their total CFU concentrations) increased significantly after [BMIm][PF6] exposure (Figure 1B and Figure S1). Among the antibiotics considered, enrichment of tetracycline-resistant bacteria was most pronounced, increasing by 5.5-fold and 10.0-fold after 16-h exposure to 0.1 and 1.0 g/L [BMIm][PF6], respectively ($p < 0.05$, *t*-test).

Significant ARG enrichment was also observed in sediment microcosms after [BMIm][PF6] exposure for different durations (nonparametric Kruskal–Wallis tests, $p < 0.05$) (Figure 1C). Twelve-hour exposure to [BMIm][PF6] at 0.1 g/L increased ARG abundance by 1.12 to 4.34-fold, while exposure to 1.0 g/L resulted in increases of 1.35 to 3.05-fold. Except for *tet*A, *bla*AmpC, *bla*OXA-30, *sul*1, *mph*A, and *aac*C6, enrichment of 30 ARGs persisted for up to 28 d, when 1.25 to 4.25-fold and 1.33 to 4.74-fold increases were observed at that time in treatments with 0.1 and 1.0 g/L [BMIm][PF6], respectively (Figure S2). This implies that short-term exposure to [BMIm][PF6] causes long-lasting enrichment of most of ARGs in sediments. Consistently, a positive correlation was observed between ARGs and Class 1 integron concentrations in the treatment group (Figure 1D, Spearman correlation $p < 0.05$) but not in the control ($p > 0.05$). Although correlation does not prove causation, this finding suggests that Class 1 integron (an important MGE) was enriched in sediments after [BMIm][PF6] exposure.

Efflux Pump ARGs Were Preferentially Enriched Following [BMIm][PF6] Exposure. Metagenomic analysis was used to further parse the antibiotic resistome response to [BMIm][PF6] in freshwater microcosms. A total of 156 ARGs were significantly enriched following 16-h exposure to [BMIm][PF6], which was indicated by a Z-score of 3.5 for 0.1 and 18.3 for 1.0 g/L of [BMIm][PF6]. The detected 285 ARG subtypes were categorized according to their resistant mechanisms (i.e., efflux pumps, drug inactivation, and target modification/protection).[59] ARGs conferring these resistance mechanisms were significantly enriched after 1.0 g/L [BMIm][PF6] treatment ($Z > 1.65$, Figure 2A,B). The most significant enrichment was found for ARGs that encode efflux pumps (Z-score of 3.3 and 16.7 for [BMIm][PF6] concentration at 0.1 and 1.0 g/L, respectively (Figure 2B)). This might reflect fortuitous selection of this category of ARGs since enrichment of efflux pumps that extrude toxicants (and possibly ILs[60]) from the cell is a common defense of bacteria against toxic compounds.[61,62]

It is well established that multidrug-resistance efflux pumps can confer bacterial resistance to both clinically used antibiotics and chemicals produced by the host, including bile, hormones, and host defense molecules.[63] Bacterial drug efflux systems have been categorized into five classes; i.e., the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family.[63] The detected efflux pump ARGs in this study primarily belong to MFS and RND family (Figure 2C), and [BMIm][PF6] exposure most significantly enriched the RND efflux system (Z-score was 2.8 and 14.9 for 0.1 and 1.0 g/L [BMIm][PF6], respectively). As one representative efflux in RND family, the three-component efflux pump *AcrAB-TolC* has a very broad range of antibiotic substrates including tetracycline, macrolide, aminoglycoside, rifamycin, and carbapenem, and it plays a vital role in bacterial antibiotic resistance.[61] We also observed high abundance and significantly enriched *AcrAB-TolC* in [BMIm][PF6]-spiked treatments (Z-score = 6.6 for 1.0 g/L [BMIm][PF6]). A similar response was observed for another IL of

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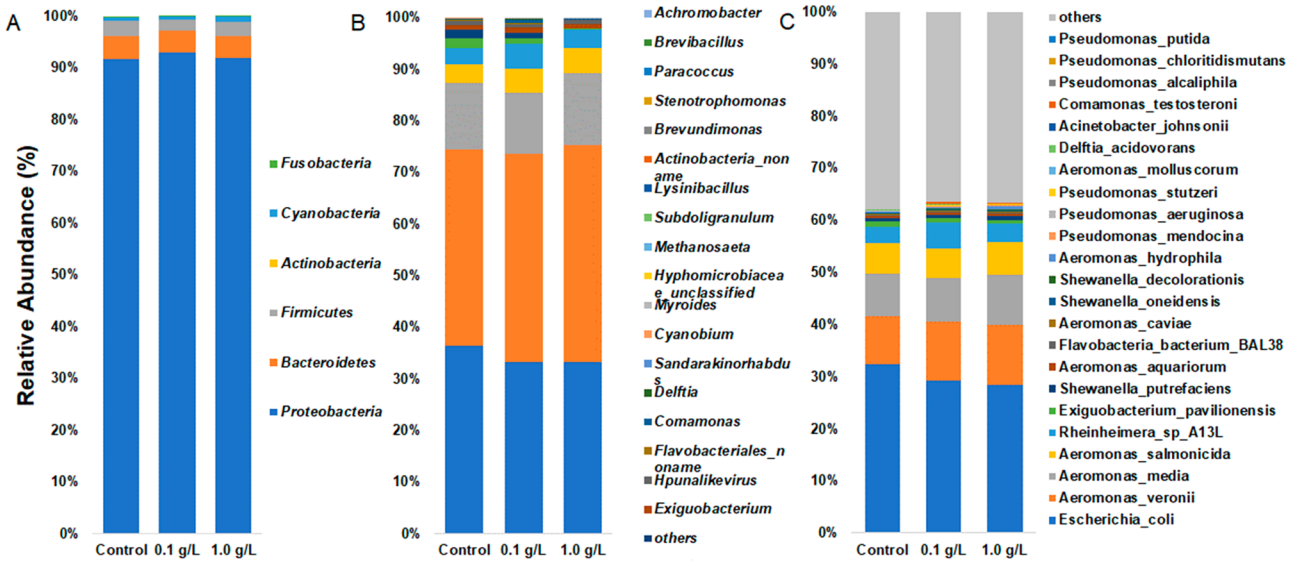

Figure 3. Insignificant effect on bacterial communities in freshwater microcosm at phylum (A), genus (B), and (C) species levels following short-term (16 h) exposure to [BMIm][PF6]. Fisher's exact test, $p > 0.05$.

$[C_2mim]Cl$, which simulated *AcrAB-TolC* efflux pump expression in *Enterobacter lignolyticus*.[60]

Overall, [BMIm][PF6] rapidly (16 h) enriched ARGs abundance, especially efflux pump genes. Efflux pumps of the RND family, whose enrichment was most pronounced, are known to be mainly expressed by Gram-negative bacteria.[64] This suggests that [BMIm][PF6] might fortuitously select antibiotic resistant Gram-negative bacteria.

Enrichment of Mobile Genetic Elements Rather than Growth of Specific Bacteria Contributed to the Rapid Propagation of ARGs. To explore the potential mechanism for the observed [BMIm][PF6]-induced ARG enhancement, we examined shifts in bacterial populations and MGEs in the freshwater microcosms for 16 h. Using metagenomics sequencing, we found that the dominant bacterial phyla were *Proteobacteria* (91.1−91.8%) regardless of the absence or presence of [BMIm][PF6] (Figure 3A). Predominant genera were *Escherichia*, *Aeromonas*, and *Pseudomonas* (Figure 3B). Compared with the control, relative abundances of *Proteobacteria* and *Firmicutes* were slightly higher in the 0.1 g/L [BMIm][PF6]-exposed group, while the abundance of *Bacteroidetes* decreased in [BMIm][PF6]-exposed groups. These effects were slightly higher for the 1.0 g/L [BMIm][PF6] treatment. However, all changes in microbial community after a 16 h exposure at both phylum and genus levels were not statistically significant (Fisher's exact test, $p > 0.05$). Metaphlan2 (which relies on unique clade-specific marker genes to obtain the species-level bacterial community) was also used to parse the bacterial community at the species level. Similar to the phylum- and genus-level analyses, there was no significant difference observed at the species level (Fisher's exact test, $p > 0.05$) between the [BMIm][PF6]-exposed group and control (Figure 3C). Although many studies have shown that the chemical pollution-induced microbial community shifts could contribute to ARG dissemination in water systems,[20,65] our results indicate that short-term exposure of [BMIm][PF6] at concentrations of 0.1 and 1.0 g/L would not significantly change bacterial community structure (Figure 3). Therefore, the observed enrichment of ARGs cannot be attributed to phylogenetic shifts.

In contrast, the abundance of MGEs, especially plasmids, was highly enriched after treatment in freshwater microcosms (Figure 4A). The mean plasmid relative abundance in the

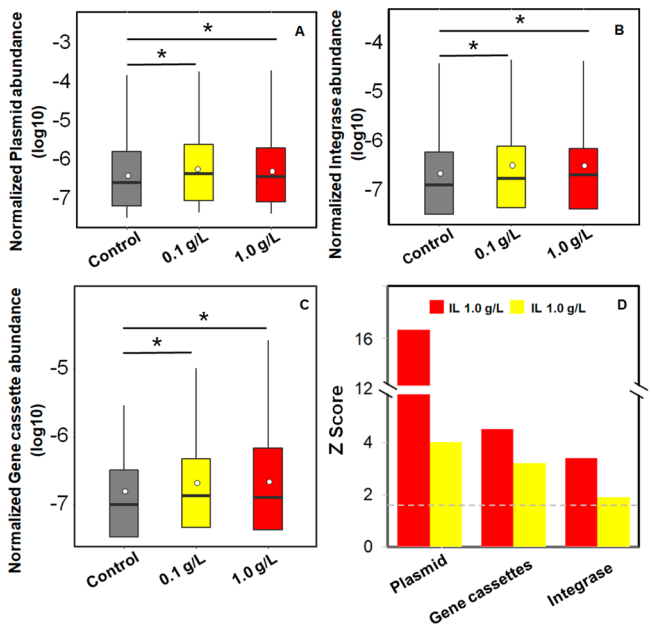

Figure 4. Enrichment of MGEs (A) Plasmid, (B) Integrase gene, and (C) Gene cassette after exposure to [BMIm][PF6] (0.1 g/L or 1.0 g/L). The colors indicate different groups (gray, control group; yellow, 0.1 g/L [BMIm][PF6], red, 1.0 g/L [BMIm][PF6]). Significant differences were determined with the Z-score. (*, Z-score > 1.65). In boxplots, the lower hinge represents 25% quantile, upper hinge 75% quantile, and center line the median. And the white dot represents the mean. (D) Z scores are shown for enrichment of MGEs (Plasmid, Gene cassettes, and Integrase) from 1.0 g/L [BMIm][PF6]-treated (red) and 0.1 g/L [BMIm][PF6]-treated (yellow) freshwater microcosms in comparison with the (untreated) control group. The dashed line shows Z-score = 1.65, and Z scores >1.65 are considered significantly enriched.

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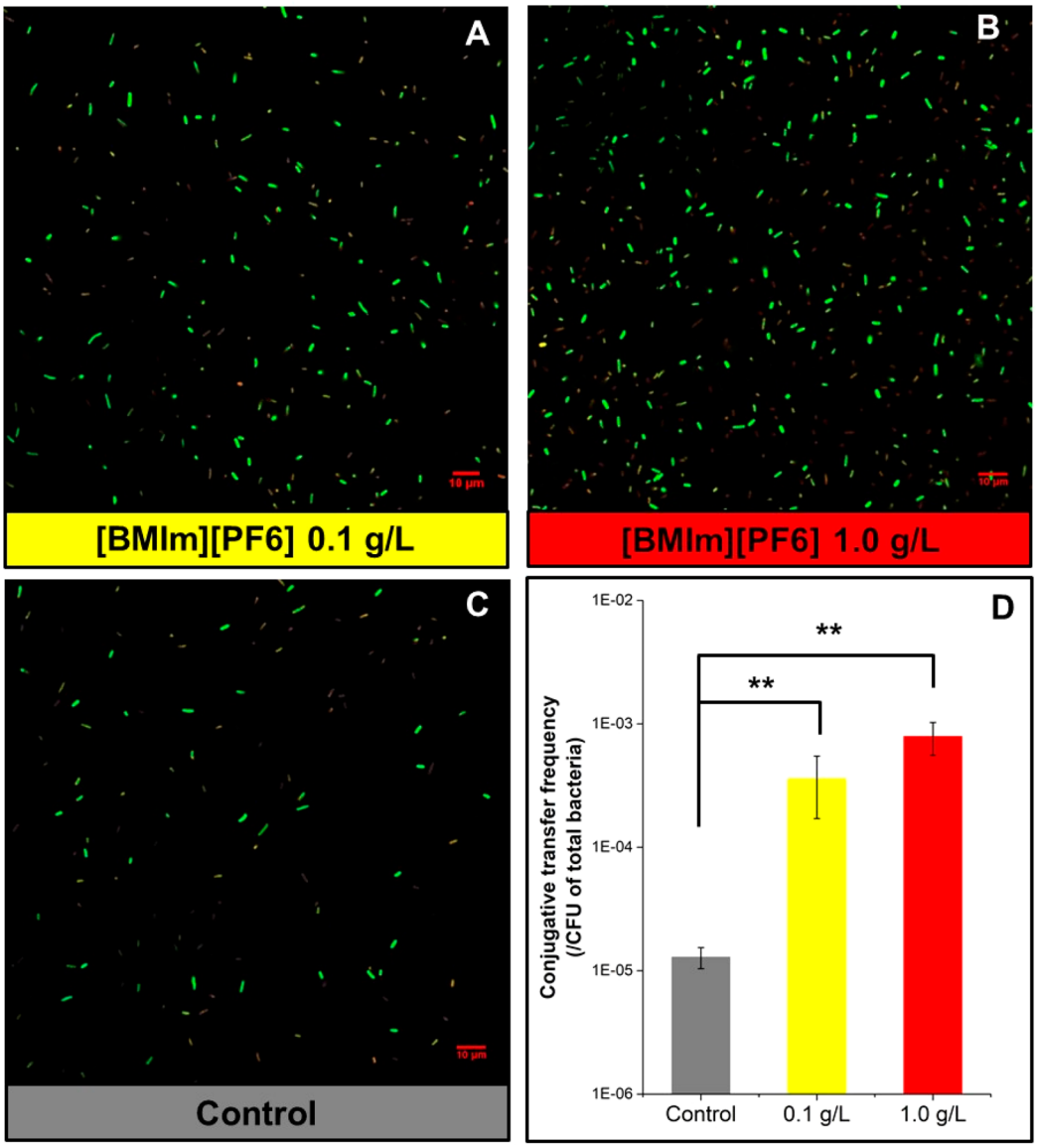

Figure 5. Confocal fluorescent images showed enrichment of RK2 plasmid in different treatment group (A) [BMIm][PF6] 0.1 g/L exposed group, (B) [BMIm][PF6] 1.0 g/L exposed group, and (C) Control group. The green one showed the transconjugants. (D) The conjugative transfer frequency of RK2 plasmid based on the result of confocal fluorescence images, calculated by transconjugants to total bacteria of the freshwater microcosm. Significant differences between ionic liquids with the control were tested with Student's *t* test. (**, $P < 0.01$).

[BMIm][PF6]-exposed treatments was 1.44×10^5 copies/16S for 0.1 g/L and 1.97×10^5 copies/16S for 1.0 g/L, which reflects significant enrichment (Z-score = 4.0 for 0.1 g/L and Z = 16.8 for 1.0 g/L [BMIm][PF6]) compared to the [BMIm][PF6]-free control (1.07×10^5 copies/16S) in freshwater microcosms (Figure 4D). In the alignments between the clean reads in the present research and plasmids in the Genbank Reference Sequences, up to 428 different plasmids were enriched, among which 66% (283 plasmids) were associated with resistance to antibiotics and/or heavy metals, involving 37 antibiotics and 8 heavy metals. This highly enriched plasmid abundance after exposure to [BMIm][PF6] represents a greater opportunity for ARGs transmission. In addition to plasmids, 61 integron-integrase genes were also enriched in the [BMIm][PF6]-exposed microcosms. Relative to the control group, exposure to [BMIm][PF6] led to significant enrichment of integron-integrase gene (Figure 4B, Z-score = 1.9 for 0.1 g/L and Z = 3.4 for 1.0 g/L [BMIm][PF6], Figure 4D). Gene cassettes within integrons that often contain various multidrug resistance ARGs[66] were also significantly enriched in the [BMIm][PF6]-exposed groups (Figure 4C,D, Z-score = 3.2 for 0.1 g/L and Z = 4.5 for 1.0 g/L [BMIm][PF6], Figure 3D).

Variation partitioning analysis (VPA) was also conducted to discern the contributions of MGEs versus bacterial community structure to ARGs variance. VPA infers that MGE enrichment after [BMIm][PF6] exposure contributed to 86.9% of the resistome variation, which is much higher than the 0.5% attributed to bacterial population shifts (Figure S3). Thus, MGE enrichment rather than growth of specific bacteria contributed significantly to the rapid propagation of ARGs after [BMIm][PF6] exposure. Class 1 integrons are known to trigger the transfer and/or incorporation of various ARGs and are particularly suitable for the dissemination of antibiotic resistance.[67,68] A significantly positive relationship between class 1 integron and ARG abundance was also observed in

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sediment microcosms, suggesting consistent response of bacterial communities in both water and sediment to IL exposure. The underlying mechanisms of enhanced HGT following exposure to some pollutants is often attributed to intracellular SOS response and cell membrane permeabilization.[21−25] Previous studies showed that [BMIm][PF6] significantly increases cell membrane permeability, contributing to ARG dissemination mediated by conjugative plasmid transfer. Apparently, the SOS response may be activated after ionic liquid exposure, thus facilitating HGT via mobile genetic elements and contributing to dissemination of ARGs.

To track the potential effect on plasmid-mediated HGT in freshwater microcosms exposed to [BMIm][PF6], *E. coli* K12::Td-Tomato cells carrying broad-host range plasmid RK2::EGFP were used as donors. The RK2 plasmid used in the present study is a representative conjugative plasmid of the Inc.P-1 family, which was detected in bacteria from diverse water environments (including sewage water,[69] freshwater,[70] and river water[71]). This plasmid is tagged with EGFP, so single green fluorescence is expressed in transconjugants. Confocal fluorescence imaging showed an increase in abundance of transconjugants by exposure to [BMIm][PF6] (both 0.1 and 1.0 g/L) compared to the control group (Figure 5A,C), verifying that conjugative plasmid transfer was facilitated by [BMIm][PF6]. Compared to the control group, the calculated conjugation frequency in 1.0 g/L treatments was 60.3-fold higher ($p < 0.05$, Student's t test, Figure 5D). These results demonstrate that [BMIm][PF6] can facilitate plasmid-mediated HGT, which corroborates the observed MGE enrichment induced by [BMIm][PF6] exposure. To exclude the increase in plasmid copy numbers due to a stress response, *E. coli DH5α* carrying a conjugative plasmid (RP4) and *E. coli DH5α* carrying a nonconjugative plasmid (pBR322) were selected to compare changes in plasmid copy number after exposure to [BMIm][PF6]. The results showed that the plasmid copy number was not significantly changed after a 16 h exposure to [BMIm][PF6] (Table S5). This excludes the increase in plasmid copy numbers after a [BMIm][PF6] exposure.

Note that several enriched genera in the freshwater microcosms such as the *Aeromonas* spp. and the *Pseudomonas* spp. are indigenous inhabitants of natural waters[72] with some species acting as opportunistic pathogens. For example, *Aeromonas hydrophila* and *Aeromonas veronii* are associated with septicemia and gastroenteritis,[73] and *Pseudomonas aeruginosa* can cause nosocomial infections.[73] Thus, MGEs enrichment by [BMIm][PF6] exposure increases the risk of human pathogens acquiring antibiotic resistance. Taking integrons as an example, the high capacity for acquiring and integrating xenogeneic elements into the gene cassettes makes them as efficient platforms for ARG transfer.[74] As such, the Class 1 integron was recently proposed as a proxy for indicating anthropogenic pollution, and higher Class 1 integron abundance could indicate a greater frequency of ARG dissemination.[68]

Longer Exposure to [BMIm][PF6] Reshaped the Sediments Bacterial Community. No significant bacterial community shift was observed in sediments exposed to [BMIm][PF6] for 12 h (weighted Unifrac Distance, Figures S4 and S5, Fisher's exact test, $p > 0.05$). However, longer exposure time reshaped the bacterial community in a time-dependent manner (Figure S5). For the 1.0 g/L treatment, 12 bacterial phyla significantly changed their abundance with a 7 d exposure (Figure S4, Fisher's exact test, $p < 0.05$), and these changes were more pronounced at 28 d (Figure S4, Fisher's exact test, $p < 0.05$). Specifically, the abundance of *Firmicutes* decreased by 21.4% (relative to unexposed control at same time) after a 7 d exposure and by 41.3% after a 28 d exposure, while *Proteobacteria* abundance increased by 15.4% (7 d) and 20.4% (28 d). In contrast, MGEs showed an early response to [BMIm][PF6] exposure (rapidly becoming enriched within 12 h) and their abundance stabilized until the end of the experiment. Overall, these observations corroborate that MGE enrichment, which is known to accelerate bacterial adaptation to environmental stresses and expand metabolic niches via the sharing of genes,[75] is an important determinant in reshaping the microbial community metagenome.[76] MGEs in both freshwater and sediment showed a rapid response to [BMIm][PF6] (enriched within 12 h), whereas bacterial phylogeny was unchanged during that time. A bacterial population shift was observed in the sediment after a 7 d exposure, suggesting that HGT mediated by MGEs has the potential to reshape the bacterial community. The resistome enrichment occurred fast (within 16 h) after [BMIm][PF6] exposure, before any significant changes in bacterial community structure. This highlights the potential for the rapid and fortuitous long-lasting enrichment of antibiotic resistomes in environments impacted by releases of the IL [BMIm][PF6].

Environmental Implications. This comprehensive metagenomics study shows that freshwater resistomes can be enriched by exposure to 0.1 and 1.0 g/L [BMIm][PF6] for as little as 16 h, which was mainly due to HGT rather than changes in bacterial community composition. Similarly, short-term (12 h) exposure to [BMIm][PF6] enriched ARGs and HGT in sediment microcosm without significantly affecting bacterial community structure. However, the bacterial community in the sediments eventually changed after exposure for 7 d, and some potential opportunistic pathogens became more abundant.

The potential for rapid and fortuitous long-lasting enrichment of antibiotic resistomes in environments impacted by releases of the IL [BMIm][PF6] deserves attention, both from the perspectives of mitigating proliferation of antibiotic resistance as well as risk assessment of the life cycle of ILs. This study underscores the need for the judicious use of [BMIm][PF6] and the mitigation of its incidental or accidental releases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b04116.

Additional methodological details and results about the influence of [BMIm][PF6] exposure on plasmid replication, quantification of the target genes, and enrichment of functional genes confirming HGT (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) O'Neill, J. *Review on antimicrobial resistance: tackling a crisis for the health and wealth of nations*. 2014. HM Government: London,**2016**.

(2) Control, C. f. D.; *Prevention, Antibiotic use in the United States, 2017: progress and opportunities*. US Department of Health and Human Services, Atlanta 2017.

(3) Xiong, W.; Wang, Y.; Sun, Y.; Ma, L.; Zeng, Q.; Jiang, X.; Li, A.; Zeng, Z.; Zhang, T. Antibiotic-mediated changes in the fecal microbiome of broiler chickens define the incidence of antibiotic resistance genes. *Microbiome* **2018**, *6* (1), 34.

(4) Toh, S. M.; Xiong, L.; Arias, C. A.; Villegas, M. V.; Lolans, K.; Quinn, J.; Mankin, A. S. Acquisition of a natural resistance gene renders a clinical strain of methicillin-resistant Staphylococcus aureus resistant to the synthetic antibiotic linezolid. *Mol. Microbiol.* **2007**, *64* (6), 1506−14.

(5) Allen, H. K.; Donato, J.; Wang, H. H.; Cloud-Hansen, K. A.; Davies, J.; Handelsman, J. Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.* **2010**, *8* (4), 251−259.

(6) Martínez, J. L. Antibiotics and antibiotic resistance genes in natural environments. *Science* **2008**, *321* (5887), 365−367.

(7) Blair, J. M.; Webber, M. A.; Baylay, A. J.; Ogbolu, D. O.; Piddock, L. J. Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* **2015**, *13* (1), 42.

(8) Berendonk, T. U.; Manaia, C. M.; Merlin, C.; Fatta-Kassinos, D.; Cytryn, E.; Walsh, F.; Burgmann, H.; Sørum, H.; Norstrom, M.; Pons, M.-N.; Kreuzinger, N.; Huovinen, P.; Stefani, S.; Schwartz, T.; Kisand, V.; Baquero, F.; Martinez, J. L. Tackling antibiotic resistance: the environmental framework. *Nat. Rev. Microbiol.* **2015**, *13* (5), 310−317.

(9) Smith, D. L.; Harris, A. D.; Johnson, J. A.; Silbergeld, E. K.; Morris, J. G. Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99* (9), 6434−6439.

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

(11) Wu, N.; Qiao, M.; Zhang, B.; Cheng, W.-D.; Zhu, Y.-G. Abundance and diversity of tetracycline resistance genes in soils adjacent to representative swine feedlots in China. *Environ. Sci. Technol.* **2010**, *44* (18), 6933−6939.

(12) Zhang, X.-X.; Zhang, T. Occurrence, abundance, and diversity of tetracycline resistance genes in 15 sewage treatment plants across China and other global locations. *Environ. Sci. Technol.* **2011**, *45* (7), 2598−2604.

(13) Calero-Caceres, W.; Melgarejo, A.; Colomer-Lluch, M.; Stoll, C.; Lucena, F.; Jofre, J.; Muniesa, M. Sludge as a potential important source of antibiotic resistance genes in both the bacterial and bacteriophage fractions. *Environ. Sci. Technol.* **2014**, *48* (13), 7602−7611.

(14) 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.

(15) Luo, Y.; Mao, D.; Rysz, M.; Zhou, Q.; Zhang, H.; Xu, L.; Alvarez, J. J. P. Trends in antibiotic resistance genes occurrence in the Haihe River, China. *Environ. Sci. Technol.* **2010**, *44* (19), 7220−7225.

(16) Pruden, A.; Pei, R. T.; Storteboom, H.; Carlson, K. H. Antibiotic resistance genes as emerging contaminants: Studies in northern Colorado. *Environ. Sci. Technol.* **2006**, *40* (23), 7445−7450.

(17) Su, H.-C.; Ying, G.-G.; Tao, R.; Zhang, R.-Q.; Zhao, J.-L.; Liu, Y.-S. Class 1 and 2 integrons, sul resistance genes and antibiotic resistance in Escherichia coli isolated from Dongjiang River, South China. *Environ. Pollut.* **2012**, *169*, 42−49.

(18) Poirel, L.; Rodriguez-Martinez, J. M.; Mammeri, H.; Liard, A.; Nordmann, P. Origin of plasmid-mediated quinolone resistance determinant QnrA. *Antimicrob. Agents Chemother.* **2005**, *49* (8), 3523−5.

(19) Poirel, L.; Kämpfer, P.; Nordmann, P. Chromosome-encoded Ambler class A beta-lactamase of Kluyvera georgiana, a probable progenitor of a subgroup of CTX-M extended-spectrum beta-lactamases. *Antimicrob. Agents Chemother.* **2002**, *46* (12), 4038−40.

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

(21) Qiu, Z.; Yu, Y.; Chen, Z.; Jin, M.; Yang, D.; Zhao, Z.; Wang, J.; Shen, Z.; Wang, X.; Qian, D.; Huang, A.; Zhang, B.; Li, J. W. Nanoalumina promotes the horizontal transfer of multiresistance

genes mediated by plasmids across genera. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (13), 4944−9.
(22) Zhang, S.; Wang, Y.; Song, H.; Lu, J.; Yuan, Z.; Guo, J. Copper nanoparticles and copper ions promote horizontal transfer of plasmid-mediated multi-antibiotic resistance genes across bacterial genera. *Environ. Int.* **2019**, *129*, 478−487.
(23) Wang, X.; Yang, F.; Zhao, J.; Xu, Y.; Mao, D.; Zhu, X.; Luo, Y.; Alvarez, P. J. J. Bacterial exposure to ZnO nanoparticles facilitates horizontal transfer of antibiotic resistance genes. *NanoImpact* **2018**, *10*, 61−67.
(24) Lu, J.; Wang, Y.; Li, J.; Mao, L.; Nguyen, S. H.; Duarte, T.; Coin, L.; Bond, P.; Yuan, Z.; Guo, J. Triclosan at environmentally relevant concentrations promotes horizontal transfer of multidrug resistance genes within and across bacterial genera. *Environ. Int.* **2018**, *121*, 1217−1226.
(25) Wang, Y.; Lu, J.; Mao, L.; Li, J.; Yuan, Z.; Bond, P. L.; Guo, J. Antiepileptic drug carbamazepine promotes horizontal transfer of plasmid-borne multi-antibiotic resistance genes within and across bacterial genera. *ISME J.* **2019**, *13* (2), 509−522.
(26) Earle, M. J.; Seddon, K. R. Ionic liquids. Green solvents for the future. *Pure Appl. Chem.* **2000**, *72* (7), 1391−1398.
(27) Rogers, R. D.; Seddon, K. R. Ionic liquids–solvents of the future? *Science* **2003**, *302* (5646), 792−793.
(28) Pham, T. P. T.; Cho, C.-W.; Yun, Y.-S. Environmental fate and toxicity of ionic liquids: a review. *Water Res.* **2010**, *44* (2), 352−372.
(29) Wang, Q.; Mao, D.; Luo, Y. Ionic Liquid Facilitates the Conjugative Transfer of Antibiotic Resistance Genes Mediated by Plasmid RP4. *Environ. Sci. Technol.* **2015**, *49* (14), 8731−8740.
(30) Luo, Y.; Wang, Q.; Lu, Q.; Mu, Q.; Mao, D. An Ionic Liquid Facilitates the Proliferation of Antibiotic Resistance Genes Mediated by Class I Integrons. *Environ. Sci. Technol. Lett.* **2014**, *1* (5), 266−270.
(31) Docherty, K. M.; Kulpa, J. C. F. Toxicity and antimicrobial activity of imidazolium and pyridinium ionic liquids. *Green Chem.* **2005**, *7* (4), 185−189.
(32) Bittermann, K.; Goss, K.-U. Assessing the toxicity of ionic liquids − Application of the critical membrane concentration approach. *Chemosphere* **2017**, *183*, 410−418.
(33) Yu, J.; Zhang, S.; Dai, Y.; Lu, X.; Lei, Q.; Fang, W. Antimicrobial activity and cytotoxicity of piperazinium- and guanidinium-based ionic liquids. *J. Hazard. Mater.* **2016**, *307*, 73−81.
(34) Fu, J.; Yang, D.; Jin, M.; Liu, W.; Zhao, X.; Li, C.; Zhao, T.; Wang, J.; Gao, Z.; Shen, Z.; Qiu, Z.; Li, J.-W. Aquatic animals promote antibiotic resistance gene dissemination in water via conjugation: Role of different regions within the zebra fish intestinal tract, and impact on fish intestinal microbiota. *Mol. Ecol.* **2017**, *26* (19), 5318−5333.
(35) Nancharaiah, Y. V.; Wattiau, P.; Wuertz, S.; Bathe, S.; Mohan, S. V.; Wilderer, P. A.; Hausner, M. Dual labeling of Pseudomonas putida with fluorescent proteins for in situ monitoring of conjugal transfer of the TOL plasmid. *Appl. Environ. Microbiol.* **2003**, *69* (8), 4846−4852.
(36) Yang, F.; Mao, D.; Zhou, H.; Wang, X.; Luo, Y. Propagation of New Delhi metallo-β-lactamase genes (bla NDM-1) from a wastewater treatment plant to its receiving river. *Environ. Sci. Technol. Lett.* **2016**, 3 (4), 138−143.
(37) Mao, D.; Yu, S.; Rysz, M.; Luo, Y.; Yang, F.; Li, F.; Hou, J.; Mu, Q.; Alvarez, P. J. J. Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. *Water Res.* **2015**, *85*, 458−466.
(38) Chao, Y.; Ma, L.; Yang, Y.; Ju, F.; Zhang, X.-X.; Wu, W.-M.; Zhang, T. Metagenomic analysis reveals significant changes of microbial compositions and protective functions during drinking water treatment. *Sci. Rep.* **2013**, *3*, 3550 DOI: 10.1038/srep03550.
(39) Gao, P.; Mao, D.; Luo, Y.; Wang, L.; Xu, B.; Xu, L. Occurrence of sulfonamide and tetracycline-resistant bacteria and resistance genes in aquaculture environment. *Water Res.* **2012**, *46* (7), 2355−2364.
(40) Bolger, A. M.; Lohse, M.; Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **2014**, *30* (15), 2114−2120.
(41) Yin, X.; Jiang, X.-T.; Chai, B.; Li, L.; Yang, Y.; Cole, J. R.; Tiedje, J. M.; Zhang, T. ARGs-OAP v2.0 with an expanded SARG database and Hidden Markov Models for enhancement characterization and quantification of antibiotic resistance genes in environmental metagenomes. *Bioinformatics* **2018**, *34* (13), 2263−2270.
(42) Yin, X.; Jiang, X. T.; Chai, B.; Li, L.; Yang, Y.; Cole, J. R.; Tiedje, J. M.; Zhang, T. ARGs-OAP v2.0 with an expanded SARG database and Hidden Markov Models for enhancement characterization and quantification of antibiotic resistance genes in environmental metagenomes. *Bioinformatics* **2018**, *34* (13), 2263−2270.
(43) Yang, Y.; Jiang, X.; Chai, B.; Ma, L.; Li, B.; Zhang, A.; Cole, J. R.; Tiedje, J. M.; Zhang, T. ARGs-OAP: online analysis pipeline for antibiotic resistance genes detection from metagenomic data using an integrated structured ARG-database. *Bioinformatics* **2016**, *32* (15), 2346−2351.
(44) Li, B.; Yang, Y.; Ma, L.; Ju, F.; Guo, F.; Tiedje, J. M.; Zhang, T. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. *ISME J.* **2015**, *9* (11), 2490−2502.
(45) Moura, A.; Soares, M.; Pereira, C.; Leitão, N.; Henriques, I.; Correia, A. INTEGRALL: a database and search engine for integrons, integrases and gene cassettes. *Bioinformatics* **2009**, *25* (8), 1096−1098.
(46) Kristiansson, E.; Fick, J.; Janzon, A.; Grabic, R.; Rutgersson, C.; Weijdegård, B.; Söderström, H.; Larsson, D. J. Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. *PLoS One* **2011**, *6* (2), e17038.
(47) Magoc, T.; Salzberg, S. L. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **2011**, *27* (21), 2957−2963.
(48) Peng, Y.; Leung, H. C.; Yiu, S.-M.; Chin, F. Y. IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics* **2012**, *28* (11), 1420−1428.
(49) Zhu, W.; Lomsadze, A.; Borodovsky, M. Ab initio gene identification in metagenomic sequences. *Nucleic Acids Res.* **2010**, *38* (12), e132−e132.
(50) Huson, D. H.; Auch, A. F.; Qi, J.; Schuster, S. C. MEGAN analysis of metagenomic data. *Genome Res.* **2007**, *17* (3), 377−386.
(51) Modi, S. R.; Lee, H. H.; Spina, C. S.; Collins, J. J. Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. *Nature* **2013**, *499* (7457), 219.
(52) Zhou, H.; Wang, X.; Li, Z.; Kuang, Y.; Mao, D.; Luo, Y. Occurrence and Distribution of Urban Dust-Associated Bacterial Antibiotic Resistance in Northern China. *Environ. Sci. Technol. Lett.* **2018**, *5* (2), 50−55.
(53) Luo, Y.; Yang, F.; Mathieu, J.; Mao, D.; Wang, Q.; Alvarez, P. J. J. Proliferation of Multidrug-Resistant New Delhi Metallo-β-lactamase Genes in Municipal Wastewater Treatment Plants in Northern China. *Environ. Sci. Technol. Lett.* **2014**, *1* (1), 26−30.
(54) Parks, D. H.; Tyson, G. W.; Hugenholtz, P.; Beiko, R. G. STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics* **2014**, *30* (21), 3123−3124.
(55) Dixon, P. VEGAN, a package of R functions for community ecology. *J. Veg Sci.* **2003**, *14* (6), 927−930.
(56) Jiang, X.; Li, X.; Yang, L.; Liu, C.; Wang, Q.; Chi, W.; Zhu, H. How Microbes Shape Their Communities? A Microbial Community Model Based on Functional Genes. *Genomics, Proteomics Bioinf.* **2019**, *17* (1), 91−105.
(57) Jia, S.; Shi, P.; Hu, Q.; Li, B.; Zhang, T.; Zhang, X. X. Bacterial Community Shift Drives Antibiotic Resistance Promotion during Drinking Water Chlorination. *Environ. Sci. Technol.* **2015**, *49* (20), 12271−9.
(58) Zhu, B.; Chen, Q.; Chen, S.; Zhu, Y.-G. Does organically produced lettuce harbor higher abundance of antibiotic resistance genes than conventionally produced? *Environ. Int.* **2017**, *98*, 152−159.
(59) Levy, S. B.; Marshall, B. Antibacterial resistance worldwide: causes, challenges and responses. *Nat. Med.* **2004**, *10*, S122−S129.
(60) Khudyakov, J. I.; D'haeseleer, P.; Borglin, S. E.; DeAngelis, K. M.; Woo, H.; Lindquist, E. A.; Hazen, T. C.; Simmons, B. A.; Thelen,

https://dx.doi.org/10.1021/acs.est.9b04116
Environ. Sci. Technol. 2020, 54, 4305−4315

M. P. Global transcriptome response to ionic liquid by a tropical rain forest soil bacterium, Enterobacter lignolyticus. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (32), E2173−E2182.
(61) Poole, K. Efflux-mediated antimicrobial resistance. *J. Antimicrob. Chemother.* **2005**, *56* (1), 20−51.
(62) Li, X.-Z.; Plésiat, P.; Nikaido, H. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clin. Microbiol. Rev.* **2015**, *28* (2), 337−418.
(63) Piddock, L. J. V. Multidrug-resistance efflux pumps ? not just for resistance. *Nat. Rev. Microbiol.* **2006**, *4* (8), 629−636.
(64) Piddock, L. J. V. Multidrug-resistance efflux pumps ? not just for resistance. *Nat. Rev. Microbiol.* **2006**, *4*, 629.
(65) Wang, J.; Wang, J.; Zhao, Z.; Chen, J.; Lu, H.; Liu, G.; Zhou, J.; Guan, X. PAHs accelerate the propagation of antibiotic resistance genes in coastal water microbial community. *Environ. Pollut.* **2017**, *231*, 1145−1152.
(66) Ma, L.; Li, A.-D.; Yin, X.-L.; Zhang, T. The prevalence of integrons as the carrier of antibiotic resistance genes in natural and man-made environments. *Environ. Sci. Technol.* **2017**, *51* (10), 5721−5728.
(67) Gillings, M. R. Integrons: past, present, and future. *Microbiol. Mol. Biol. Rev.* **2014**, *78* (2), 257−277.
(68) Gillings, M. R.; Gaze, W. H.; Pruden, A.; Smalla, K.; Tiedje, J. M.; Zhu, Y.-G. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME J.* **2015**, *9* (6), 1269−1279.
(69) Bahl, M. I.; Burmølle, M.; Meisner, A.; Hansen, L. H.; Sørensen, S. J. All IncP-1 plasmid subgroups, including the novel ε subgroup, are prevalent in the influent of a Danish wastewater treatment plant. *Plasmid* **2009**, *62* (2), 134−139.
(70) Haines, A. S.; Akhtar, P.; Stephens, E. R.; Jones, K.; Thomas, C. M.; Perkins, C. D.; Williams, J. R.; Day, M. J.; Fry, J. C. Plasmids from freshwater environments capable of IncQ retrotransfer are diverse and include pQKH54, a new IncP-1 subgroup archetype. *Microbiology* **2006**, *152* (9), 2689−2701.
(71) De la Cruz Barrón, M.; Merlin, C.; Guilloteau, H.; Montargès-Pelletier, E.; Bellanger, X. Suspended Materials in River Waters Differentially Enrich Class 1 Integron- and IncP-1 Plasmid-Carrying Bacteria in Sediments. *Front. Microbiol.* **2018**, *9* (1443), 1443 DOI: 10.3389/fmicb.2018.01443.
(72) Altwegg, M.; Geiss, H. K.; Freij, B. J. Aeromonas as a human pathogen. *CRC Critical Reviews in Microbiology* **1989**, *16* (4), 253−286.
(73) Merino, S.; Rubires, X.; Knøchel, S.; Tomás, J. M. Emerging pathogens: Aeromonas spp. *Int. J. Food Microbiol.* **1995**, *28* (2), 157−168.
(74) Stalder, T.; Barraud, O.; Casellas, M.; Dagot, C.; Ploy, M.-C. Integron involvement in environmental spread of antibiotic resistance. *Front. Microbiol.* **2012**, *3*, 119.
(75) Hall, J. P. J.; Wood, A. J.; Harrison, E.; Brockhurst, M. A. Source−sink plasmid transfer dynamics maintain gene mobility in soil bacterial communities. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113* (29), 8260−8265.
(76) Smets, B. F.; Barkay, T. Horizontal gene transfer: perspectives at a crossroads of scientific disciplines. *Nat. Rev. Microbiol.* **2005**, *3*, 675.

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