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Long-Term Exposure to Benzalkonium Chloride Disinfectants Results in Change of Microbial Community Structure and Increased Antimicrobial Resistance

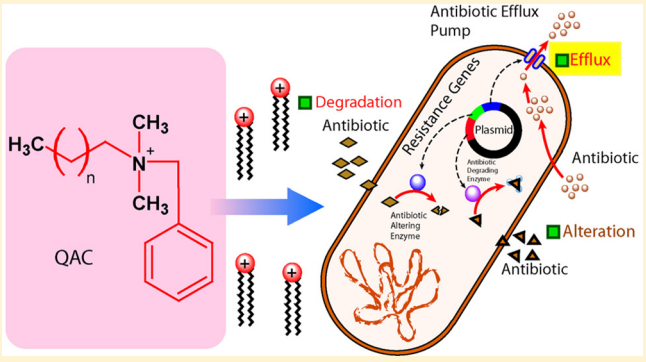
Madan Tandukar,[†] Seungdae Oh,[†] Ulas Tezel,[†,§] Konstantinos T. Konstantinidis,[†,‡] and Spyros G. Pavlostathis*[,†]

[†]School of Civil and Environmental Engineering and [‡]School of Biology, Georgia Institute of Technology, Atlanta Georgia 30332-0512, United States

[§]The Institute of Environmental Sciences, Bogazici University, Bebek, Istanbul, 34342, Turkey

Supporting Information

ABSTRACT: The effect of benzalkonium chlorides (BACs), a widely used class of quaternary ammonium disinfectants, on microbial community structure and antimicrobial resistance was investigated using three aerobic microbial communities: BACs-unexposed (DP, fed a mixture of dextrin/peptone), BACs-exposed (DPB, fed a mixture of dextrin/peptone and BACs), and BACs-enriched (B, fed only BACs). Long-term exposure to BACs reduced community diversity and resulted in the enrichment of BAC-resistant species, predominantly *Pseudomonas* species. Exposure of the two microbial communities to BACs significantly decreased their susceptibility to BACs as well as three clinically relevant antibiotics (penicillin G, tetracycline, ciprofloxacin). Increased resistance to BACs and penicillin G of the two BACs-exposed communities is predominantly attributed to degradation or transformation of these compounds, whereas resistance to tetracycline and ciprofloxacin is largely due to the activity of efflux pumps. Quantification of several key multidrug resistance genes showed a much higher number of copies of these genes in the DPB and B microbial communities compared to the DP community. Collectively, our findings indicate that exposure of a microbial community to BACs results in increased antibiotic resistance, which has important implications for both human and environmental health.



INTRODUCTION

Bacterial resistance to antimicrobials has become a global problem with significant consequences for both human and environmental health.[1] Excessive use of antimicrobial compounds leads to the development and persistence of antibiotic resistance in environmental and clinical settings. Although several published surveys have reported that exposure to disinfectants and antiseptics resulted in increased tolerance to these antimicrobials without evidence of increased antibiotic resistance,[2,3] a substantial body of work suggests otherwise.[4,5] Quaternary ammonium compounds (QACs) are cationic surfactants extensively used in various domestic, industrial, and medical applications, primarily as disinfectants. QACs are predominant micropollutants found in both engineered and natural systems.[6−8] The bacteriocidal modes of action of QACs are still largely unclear, but are thought to include mechanisms such as the destruction of the lipid bilayer in bacterial cell membrane, dissipation of proton motive force, and interference with the activities of membrane-bound enzymes.[9] QACs have recently been implicated in the proliferation (coselection) of antibiotic resistance,[10−12] which is recognized as a growing and pressing problem for human and environmental health. Exposure of microbes to QACs potentially makes them resistant to these compounds as well as a wide range of clinically important antibiotics by virtue of acquisition and proliferation of antibiotic resistance genes, which are associated with a number of antibiotic resistant mechanisms, including multidrug efflux pumps.[4,5,10−12] Multidrug efflux pumps are not specific to substrates and thus are largely responsible for cross- and coresistance. Quantification of key genetic elements responsible for antibiotic resistance, along with physiological evidence, provides additional proof of the involvement of specific resistance mechanisms in affected bacterial communities.

Most research on the emergence, development, and transfer of antibiotic resistance has been conducted with bacteria isolates.[4,5] However, from an environmental perspective, it is important to assess the effect of antimicrobial selective pressure on the community structure in addition to the development

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and dissemination of antibiotic resistance in complex microbial communities. Understanding the interactions and roles of different microbial species in a community under the environmental stress brought about by antimicrobial compounds such as QACs is vital. The objective of the work presented here was to systematically assess the effect of benzalkonium chlorides (BACs), a widely used class of QACs, on the structure of three aerobic microbial communities as well as their antimicrobial resistance.

MATERIALS AND METHODS

Microbial Community Development. Three aerobic microbial communities were developed and maintained in the laboratory for over 4 years with different carbon and energy sources and/or a BAC mixture: (a) dextrin/peptone 50:50 (w/w) mixture (DP); (b) dextrin/peptone plus BAC mixture (DPB); and (c) BAC mixture only (B). The DP microbial community was developed from an inoculum of a contaminated sediment sample collected at the Bayou d'Inde, a tributary of the Calcasieu River, near Lake Charles, LA. After one year of maintaining the DP community, the DPB community was developed with inoculum from the DP community, which in turn was subsequently used as inoculum to develop the B community. The B community was enriched and has been maintained for over 4 years with the BAC mixture as the sole carbon/energy source, supplemented with NH_4NO_3 as the nitrogen source. The BAC mixture consisted of 60:40 (w/w) dodecyl benzyl dimethyl ammonium chloride (C_{12}BDMA-Cl; $C_{21}H_{38}NCl$) and tetradecyl benzyl dimethyl ammonium chloride (C_{14}BDMA-Cl; $C_{23}H_{42}NCl$) purchased from Sigma Aldrich (St. Louis, MO). The three communities were maintained at room temperature (22−24 °C) in aerated, fed-batch reactors with a residence time of 14 days. One-fourth of the culture volume was wasted and replenished twice a week with autoclaved mineral medium along with dextrin/peptone (DP and DPB communities) and/or BAC mixture (DPB and B communities). The medium contained the following (in g/L): K_2HPO_4, 0.6; KH_2PO_4, 0.34; $CaCl_2{\cdot}2H_2O$, 0.07; $MgCl_2{\cdot}6H_2O$, 0.14; $MgSO_4{\cdot}7H_2O$, 0.27; $FeCl_2{\cdot}4H_2O$, 0.07 and 0.7 mL of trace metal stock solution.[13] At the beginning of each feeding cycle, the initial dextrin/peptone concentration was 2200 mg/L, expressed as chemical oxygen demand (COD), and the BAC mixture concentration was 50 mg/L (equivalent to 140 mg COD/L). Upon feeding, the initial NH_4NO_3 concentration in the B community was 44 mg/L. The steady-state pH and volatile suspended solids (VSS) concentration of the DP, DPB, and B communities were 7.7/2500 mg/L, 8.1/1000 mg/L, and 6.9/138 mg/L, respectively. To characterize the feeding cycle of each community, liquid samples were taken at the start of each cycle and at appropriate time intervals to measure pH, soluble COD, and BAC concentration.

Microbial Community Analysis. A clone library-based molecular phylogenetic approach was used to decipher the bacterial community structure in the DP, DPB, and B communities. Samples were collected from each community in the middle of the feeding cycle, washed several times with a saline phosphate buffer, and then genomic DNA was extracted using the Microbial DNA isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's instructions. The 16S rRNA gene was amplified by PCR using the universal oligonucleotide primer pairs 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCARCCGCA-3′). The PCR amplification was carried out with the following conditions: single cycle denaturation at 94 °C for 5 min; 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 2 min at 72 °C, and final extension for 7 min at 72 °C. Cloning was performed using TOPO TA cloning kit (Invitrogen, Carlsbad, CA) as instructed by the manufacturer. The obtained clones were then grouped into different operational taxonomical units (OTUs) after being digested with restriction enzymes *Msp1/Taq1* based on the restriction patterns. Representative clones of each OTU were selected for the sequencing of nucleotides after PCR amplification and purification. The obtained 16S rRNA gene sequences were then queried against the NCBI database[14] using the MEGABLAST algorithm. The partial sequences were between 600 and 950 base-pair long. All 16S rRNA gene sequences were manually trimmed of the vector and primer sequence, and the alignment was performed with the program CLUSTALW. The sequence-based phylogenetic tree of the dominant bacteria was constructed by applying the neighbor-joining algorithm with Jukes Cantor correction using the program MEGA5. The tree topology was evaluated by bootstrap resampling analysis of 1000 resampling data sets. To define the community richness and diversity, Chao 1 richness index and Shannon−Wiener diversity index were calculated using FastGroup II.[15] Thirty-eight 16S rRNA gene nucleotide sequences (18 from DP, 11 from DPB, and 9 from B community) have been deposited in the National Center for Biotechnology Information (NCBI-GenBank) under accession numbers KC491136 to KC491173.

Assessment of Antimicrobial Susceptibility. The antimicrobial susceptibility and resistance of the three microbial communities were measured for the BAC mixture, penicillin G, tetracycline, and ciprofloxacin using a macrobroth dilution procedure adapted from the National Committee for Clinical Laboratory Standards using the Mueller-Hinton broth as the carbon and energy source.[16] Inocula were prepared by removing samples from each community and diluting in the Mueller-Hinton broth to an optical density of 0.1 (equivalent to 0.5 McFarland turbidity standard). Test dilution series were prepared using the broth and the test compounds at an initial concentration range from 0 to 500 μg/mL. Control series were identical to the test series without inoculum. All control and test series were prepared in triplicate 13 × 100 mm glass tubes, and then incubated overnight at room temperature (22−23 °C) while mixed using an orbital shaker at 190 rpm. After incubation, the optical density at 600 nm was measured using a HP 8453 UV−visible spectrophotometer (Hewlett-Packard, Palo Alto, CA) with the control tubes as blanks. The minimum inhibitory concentration (MIC) was recorded as the lowest antimicrobial concentration that prevented any measurable growth. The 90% and 50% inhibitory concentrations (IC_{90} and IC_{50}, respectively) were also recorded as the concentrations in which 90% and 50% growth inhibition was observed, respectively. At the end of the incubation, the concentration of BACs and the three antibiotics in all series was determined.

Contribution of Efflux Pump(s) to Antimicrobial Resistance. Thioridazine was used as an efflux pump inhibitor (EPI) in this study. Although thioridazine has been previously used to study the role of efflux pumps with a diverse group of both antimicrobials and bacteria,[17−19] thioridazine is also toxic. Therefore, we first determined the inhibitory effect of thioridazine to DP, DPB, and B microbial communities at an initial concentration range from 0 to 500 μg/mL using the above-described macrobroth dilution method. On the basis of this preliminary assay, a thioridazine concentration of 30 μg/

mL was chosen given that its effect on the three microbial communities varied from none to very low (Figure S1; Supporting Information). The MIC values of the BAC mixture, tetracycline, and ciprofloxacin were then measured again for the two BAC-exposed communities in the absence and presence of 30 μg/L thioridazine. On the basis of the change in the susceptibility of the two communities to the antimicrobials in the presence and absence of thioridazine, the contribution of efflux pump(s) to the BAC and antibiotic resistance was evaluated.

qPCR Assays. Several efflux pumps have been implicated to confer resistance to BACs and antibiotics, such as ATP-binding cassette (ABC) superfamily, the multidrug and toxic-compound extrusion (MATE) family, the major facilitator (MF) superfamily, the resistance nodulation division (RND) family, and the small multidrug resistance (SMR) family. Our previous metagenomic study of the same B community analyzed here revealed several efflux pump genes that were rarely found in the metagenome of the DP community.[20] Among these efflux genes, we selected representative genes of different efflux pump families for qPCR analysis and quantified their differential abundance in the three communities. The targeted genes included those encoding the membrane fusion (*mexA* and *mexE*) or inner membrane (*mexF*) protein of RND family, *pmpM* of MATE family, and *sugE* of SMR family. Primers (Table 1) were designed based on the complete gene sequences

Table 1. Primer Sets of Antibiotic Resistance Genes Used in this Study

target gene	5′ to 3′	length (bp)
PmpM-F	GCC GGT ATT GCG GGC GAT GA	421
PmpM-R	GGC GAA TAC CGC GAT GCC GA	
SugE-F	CAC CCG ACC GAT ACC GAC CAT	180
SugE-R	CGT ACC GGC GCC ATG GAC T	
MexA-F	ATG GCC ACT CTT GCC GGC TG	347
MexA-R	TGC TGC TTG CTC ACC GCC TC	
MexE-F	GTG AAC AGC GGC CAG AGC GT	607
MexE-R	TCG AAG CTC TCC AGC GCA CG	
MexF-F	TGT TCC CGC TCT GCG TGC TG	539
MexF-R	TGC CTG ATG GGC CTC GTG GT	

recovered from the B metagenome (accession number: SRR643892).[20] Mixed culture samples from the three communities were washed several times with a saline phosphate buffer before extracting genomic DNA using Soil DNA isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to manufacturer's instructions. The extracted DNA was quantified using a fluorospectrometer (Nanodrop 3300, Wilmington, DE). Cloning of the PCR amplicons of the target groups of genes was performed to prepare standards for qPCR. The PCR amplification was carried out with the following conditions: single cycle denaturation at 94 °C for 5 min; 30 cycles of 30 s at 94 °C, 30 s at corresponding annealing temperatures, 1 min 30 s at 72 °C, and final extension for 7 min at 72 °C. Cloning was performed using TOPO TA cloning kit (Invitrogen, Carlsbad, CA) as instructed by the manufacturer. The obtained clones were regrown in LB broth containing ampicillin. The cultures were then centrifuged at 10000 rpm for 1 min, and the supernatant was used as template for further PCR with the specific primer sets. The PCR amplicons were purified using PCR purification kit (QIAGEN Inc., Valencia, CA) before performing gel electrophoresis and quantification. The amplified and purified plasmid DNA from each clone was diluted 10-fold in PCR-grade water to create a dilution series for standards ranging from 10^7 to approximately 10 copies per microliter of extracted DNA.

Quantification of the DNA copies in the standard DNA templates and the sample DNA was performed using nonspecific fluorophore SYBR green I. The SYBR green I qPCR reaction mix of 20 μL included 10 μL of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 1 μL each of forward and reverse primers, 7 μL of PCR-grade water, and 1 μL of template DNA. Quantitative PCR was run on 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following protocol: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at the respective annealing temperatures, and 1 min 30 s at 72 °C. Quantification was performed during the extension step at 72 °C. Melt curve analysis to detect the presence of primer dimers or any unspecific binding was performed after the completion of 40 cycles by increasing the temperature from 60 to 95 °C at 0.5 °C increments every 10 s. All standards and samples were prepared in duplicate. Controls without any template DNA were also prepared for each primer set to check for any contamination during the qPCR reaction preparation and execution. For the standard curve, the threshold cycle (C_T) values were plotted against the logarithm of their initial template copy numbers. The mean value of the standard slope and the intercept were used to quantify the corresponding gene copies of the target microbial groups. Results are reported as gene copies per nanogram of extracted DNA.

Analytical Methods. Soluble COD was measured by the closed reflux colorimetric method.[21] BACs and expected biotransformation intermediates such as amines were measured using an HP 1100 series HPLC (Hewlett-Packard, Palo Alto, CA) as described by Tezel et al.[22] Analysis of the three antibiotics used in this study was performed using an HP 1100 series HPLC equipped with a diode array detector (DAD) and a 150 mm Cosmosil HILIC, 4.6 mm i.d. column. The mobile phase was 70% acetonitrile and 30% of a mixture of 10 mM ammonium acetate and 5 mM EDTA. The flow rate was 1.0 mL/min and the injection volume was 20 μL. The minimum detection limit was 1 mg/L. Culture samples were centrifuged for 10 min at 14 000 rpm and filtered using 0.2 μm PTFE syringe filters before the HPLC analysis.

RESULTS AND DISCUSSION

Feeding Cycle Characteristics. To understand the substrate degradation dynamics, characterization of a representative feeding cycle was performed for each microbial community by a time series analysis of substrate concentration, and results are shown in Figure 1. Rapid degradation of dextrin/peptone was observed in both the DP and DPB communities resulting in low residual soluble COD by the end of the 3-day feeding cycle (Figure 1A). However, degradation of the BAC mixture in the DPB community was relatively slow during the first 2 days, and then was completely degraded during the third day after most of the dextrin/peptone mixture was consumed (Figure 1B). Degradation of C_{12}BDMA-Cl commenced before that of C_{14}BDMA-Cl (Figure 1B). Zhang et al.[23] reported that BAC degradation was delayed in an aerobic, mixed activated sludge community when supplied with another, readily biodegradable substrate, such as glucose in their case. Our results indicate that BACs were utilized as secondary substrates in the DPB community. In contrast, degradation of

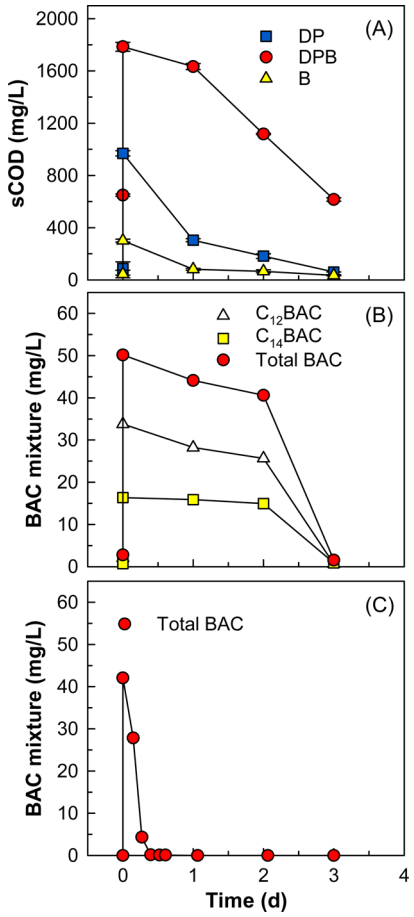

Figure 1. Time course of soluble chemical oxygen demand (sCOD) (A) in the DP, DPB, and B communities and BAC mixture in the DPB (B) and B (C) communities during a typical feeding cycle (Error bars represent one standard deviation of the mean; n = 3).

the BAC mixture was complete within 12 h in the B community (Figure 1C). Transient, low levels of benzyl dimethyl amine (BDMA) and its biotransformation products were detected in the DPB and B communities (data not shown). For the same BAC-degrading B community, Tezel et al.[22] reported that the biodegradation of BAC commenced with hydroxylation of the carbon of the alkyl group adjacent to nitrogen, cleavage of the C_{alkyl}–N bond, and formation of an aldehyde from the alkyl moiety and a tertiary amine such as BDMA. The resulting aldehyde is further degraded via β-oxidation, whereas debenzylation of BDMA results in the formation of dimethylamine and benzoic, which are further degraded and mineralized. Other proposed BAC degradation pathways include initiation by hydroxylation of the terminal carbon of the long alkyl chain or hydroxylation of the carbon of the methyl group.[24,25]

Effect of BACs on Microbial Community Composition. For the phylogenetic analysis of the DP, DPB, and B communities, 150, 130, and 112 clones were randomly selected, respectively. The phylotypes were named after their respective community followed by a numeral, which was assigned based on the clone number. Representative phylotypes of the three communities largely belonged to Proteobacteria, Bacteroidetes, Actinobacteria, Gemmatimonadetes, and Plactomycetes. Positions of phylotypes in the phylogenetic tree are presented in Figure 2. The clone library showed that the community diversity and richness decreased from DP to DPB to B community, resulting from the long-term exposure of the latter two communities to BAC. The Shannon–Wiener diversity index for the DP, DPB, and B community was 4.1, 3.7, and 2.7, respectively. Similarly, the Chao 1 richness index for the DP, DPB, and B community was 453.0, 295.5, and 46.2, respectively. Among the 20 phylotypes identified in the DP community, those belonging to Bacteroidetes (Class: Sphingobacteria, 63.4%) were the most abundant. The other phylotypes belonged to Proteobacteria (α-Proteobacteria, 3.7% and γ-Proteobacteria, 7.5%), Actinobacteria (6%), Gemmatimonadetes (3%), and Plactomycetes (1.5%). The DPB community, which had 11 identified phylotypes, was dominated by phylotypes belonging to Bacteroidetes (Class: Flavobacteriia, 50.4%) and the remaining phylotypes belonged to Proteobacteria (γ-Proteobacteria, 18.2%; α-Proteobacteria, 10.8%; and β-Proteobacteria, 1.7%). Nine phylotypes were identified in the B community, which was largely dominated by Proteobacteria (89.6%). The most abundant phylotype B8 (40.7%), as well as phylotype B102 (25.9%) are closely related to *Pseudomonas nitroreducens*, whereas phylotypes B4 (22.2%) and B104 (6.5%) are related to *Pseudomonas putida*. Besides the four dominant phylotypes (B4, B8, B102, and B104), the B clone library also consisted of a *Sedimintibacterium salmoneum* like phylotype, which belongs to phylum Bacteroidetes (Class: Sphingobacteria). Phylogenetic relationships of representative phylotypes identified in the three microbial communities are shown in Table S1 (Supporting Information).

On the basis of the above-discussed phylogenetic analysis of the three communities, the microbial diversity decreased as a result of the long-term exposure to the BAC mixture. The most drastic decrease in microbial diversity was observed in the B community, which was maintained by feeding only the BAC mixture. Exposure of the DPB and B communities to BACs confined their microbial diversity largely to the phyla of Proteobacteria and Bacteroidetes. Further, the majority of the phylotypes in the B community belonged to the genus *Pseudomonas* (γ-Proteobacteria), indicating that the *Pseudomonas* species are tolerant to BACs and largely responsible for the biodegradation of BACs. Members of the *Pseudomonas* genus have been frequently recovered as BAC-resistant organisms (e.g., *P. aeruginosa*) in previous studies.[5,26] Our results, therefore, demonstrate that long-term exposure to BACs substantially reduces community diversity and results in the enrichment of BAC-resistant/degrading species, predominantly *Pseudomonas* species. Tezel et al.[22] also reported the dominance of *Pseudomonas* species in the BAC-degrading B community characterized 2 years prior to the present study, and the dominant phylotypes had higher similarity to *Pseudomonas nitroreducens*. Several species of *Pseudomonas*, such as *P. aeruginosa* and *P. stutzeri*, are reported to be adaptive to exposure to antimicrobial compounds including BAC.[27,28] Our results suggest that microorganisms belonging to genus *Pseudomonas* are mostly responsible for BAC degradation and community resistance to BAC.

Community Susceptibility to BACs and Antibiotics. The antimicrobial susceptibility of the three microbial communities was evaluated using the BAC mixture and three clinically important antibiotics, penicillin G, tetracycline, and ciprofloxacin. The BAC mixture and the three antibiotics were stable in noninoculated abiotic, media controls (Figure 3A) under conditions otherwise similar to those of the test series inoculated with the three microbial communities.

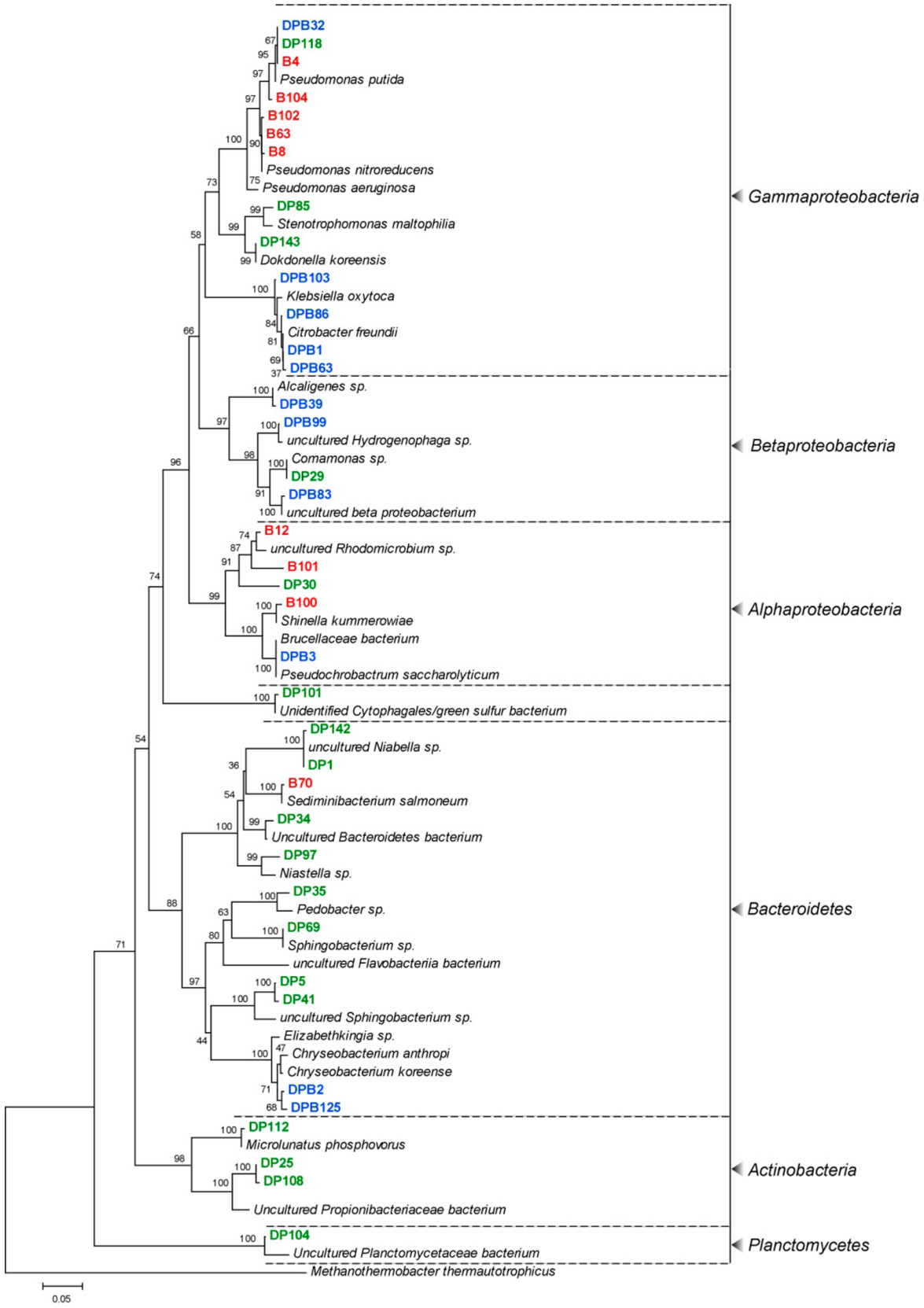

Figure 2. Phylogenetic tree of phylotypes identified in the DP, DPB, and B communities constructed by the neighbor-joining method based on the 16S rRNA gene sequences. The scale bar represents 0.05 substitutions per nucleotide position. *Methanothermobacter thermautotrophicus* was used as an outgroup.

DP Community. The DP community was not exposed to any of the four antimicrobials during the course of its development and maintenance, and therefore it was considered to be the most susceptible (least resistant) community. The

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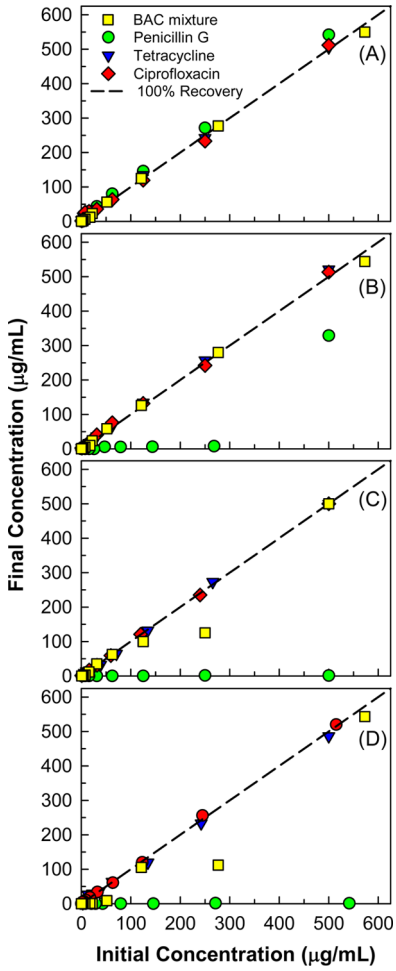

Figure 3. Penicillin G, tetracycline, ciprofloxacin, and BAC mixture concentrations recovered at the end of the macrodilution assay conducted with abiotic media control series (A) and biotic series inoculated with the DP (B), DPB (C), and B (D) communities.

IC_{50}, IC_{90}, and MIC values against BAC of the DP community were 50, 90, and 100 μg/mL, respectively. Previous studies have reported BAC MIC values between 20 and 30 μg/mL for BAC unexposed microorganisms.[5] However, most of the reported MIC values are for isolates. Degradation of BAC was not observed at any concentration during the susceptibility test (Figure 3B). The DP community did not exhibit any resistance to tetracycline or ciprofloxacin, and microbial growth was completely inhibited at all concentrations of these two antibiotics. Tetracycline and ciprofloxacin were completely recovered at the end of the assay at all test concentrations (Figure 3B). However, the DP community showed significant resistance to penicillin G with IC_{50}, IC_{90}, and MIC values of 100, 250, and 290 μg/mL, respectively, which are higher than that for BAC. At the end of the assay, penicillin G was not detected up to a test concentration of 250 μg/mL and only 70% was recovered at the test concentration of 500 μg/mL (Figure 3B). However, it is not clear if the observed decrease in penicillin G concentration is the result of complete biodegradation or partial biotransformation. The higher resistance of the DP community to penicillin G may be attributed to the inoculum used to develop this community, which was collected from a contaminated aquatic sediment. It is also possible that members of the DP community resistant to penicillin G helped to lower its concentration and protected more susceptible members in this community. Such a protective resistance mechanism in microbial communities was previously reported by Lee et al.[29]

DPB Community. The BAC IC_{50}, IC_{90}, and MIC values of the DPB community were 125, 220, and 250 μg/mL, respectively. The DPB community was almost three times more resistant to the BAC mixture than the DP community. Some BAC degradation by the DPB community was also observed during the MIC test (Figure 3C). Given the high concentration of degradable substrates in the Mueller–Hinton broth during the macrodilution test, the lack of degradation of the BACs during this test is consistent with the aforementioned preferential use of degradable substrates as opposed to BACs. The DPB community also had a higher resistance to the three antibiotics tested as compared to the DP community. The penicillin G IC_{50}, IC_{90}, and MIC values were all higher than 500 μg/mL, the highest concentration tested. Penicillin G was not recovered at any test concentration (Figure 3C). The tetracycline IC_{50}, IC_{90}, and MIC values were 120, 210, and 250 μg/mL, respectively. The tetracycline MIC value was significantly higher than previously reported values. Values less than 100 μg/mL have been reported for environmental and pathogenic bacteria isolates.[30] The ciprofloxacin IC_{50}, IC_{90}, and MIC values for the DPB community were 2, 8, and 16 μg/mL, respectively. Tetracycline and ciprofloxacin degradation was not observed at any test concentration (Figure 3C).

B Community. The inhibitory concentrations for the BAC-enriched, B community were as follows (IC_{50}, IC_{90}, and MIC values, respectively): against BAC, 200, 310, and 460 μg/mL; against tetracycline, 10, 32, and 95 μg/mL; against ciprofloxacin, 4, 15, and 18 μg/mL. The penicillin G IC_{50} was 400 μg/mL and the IC_{90} and MIC values were both higher than 500 μg/mL, the highest concentration tested. Compared to the DP community, the B community exhibited a higher resistance to all four antimicrobial agents, but it was less resistant to tetracycline compared to the DPB community. The high resistance of the BAC-enriched community is the result of long-term (>4 years) exposure to the BAC mixture. Significant BAC degradation was observed during the susceptibility assay (at corresponding initial concentrations): 100% (below 50 μg/mL) and 60% (250 μg/mL). BAC was not degraded at 500 μg/mL, the highest concentration tested (Figure 3D). Similar to the susceptibility results of the DPB community, at the end of the assay penicillin G was not detected at any test concentration, indicating degradation or transformation of this antibiotic. In contrast, both tetracycline and ciprofloxacin were not degraded during the susceptibility assay at any test concentration (Figure 3D).

These results demonstrate that exposure of microbial communities to QACs exerts selective pressure, which increases the resistance of the communities to both QACs and antibiotics. Several resistance mechanisms may have contributed to the observed increase in antibiotic resistance of the BAC-exposed microbial communities used in this study. The primary defense mechanism of microorganisms against QACs is the modification or fortification of the outer cell membrane. It has been reported that the lipopolysaccharides of the outer membrane of gram-negative bacteria act as a permeability barrier protecting the cell against biocides.[31] Sakagami et al.[26] demonstrated that *P. aeruginosa* defends itself against BAC by increasing the phospholipids and fatty-neutral lipids content of the cell wall. Other important mechanisms of QACs resistance are efflux pumps as well as QACs degradation. The

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predominant mechanism of the DPB and B communities resistance to BACs is believed to be BACs degradation. At BAC mixture concentrations less than the MIC value, significant BAC degradation was observed. Tezel et al.[22] reported that biotransformation products of BACs were significantly less toxic than BACs themselves; therefore, biotransformation is effective in reducing the antimicrobial properties of BACs. However, multiple resistance mechanisms may act simultaneously. For example, at relatively high QACs concentrations, a decrease of outer membrane permeability along with efflux pumps may act to lower the intracellular QACs concentration while QACs are being degraded, thus lowering their otherwise deleterious effects. The contribution of a group of *qac* genes encoding efflux pumps related to QACs resistance has been previously described,[32] and efflux of QACs in gram-negative and gram-positive bacteria has also been shown.[33]

In the present study, complete or partial biodegradation of penicillin G may have contributed to the observed resistance against penicillin G in all three communities. Penicillin G is a β-lactam antibiotic, which interferes with bacterial cell wall synthesis. Resistance mechanisms against penicillin G may include hydrolysis, efflux, membrane impermeability, altered target, and antibiotic modification.[34] The genetic determinants of penicillin resistance are diverse including the presence of *bla* and *oxa* genes which encode for β-lactamase and metallo-β-lactamase.[35] The observed resistance to penicillin G of the three communities used in the present study is likely the result of coresistance (i.e., an acquired ability of a microorganism to tolerate a certain antimicrobial agent by a mechanism developed against another antimicrobial agent with a totally distinct mode of action).[9]

The resistance of the microbial communities used in the present study against tetracycline and ciprofloxacin must be related to mechanisms other than degradation or alteration of these antibiotics. The antimicrobial mode of action of tetracycline is inhibition of translation by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site.[34] The mechanism of tetracycline resistance involves efflux pumps, ribosomal protection, and enzymatic modification of the antibiotic.[34,36] Genetic determinants of tetracycline resistance are widely distributed among gram-positive as well as gram-negative bacteria, and include a wide variety of *tet*, *otr*, and *tet*P genes encoding for efflux pumps and specialized proteins.[34] On the other hand, ciprofloxacin is a member of fluoroquinolone antibiotics and their bacteriocidal action involves inhibition of DNA gyrase and topiosomerase enzymes necessary for DNA replication.[36] Acetylation and multidrug resistant efflux pumps belonging to MATE and RND families have been shown to be responsible for resistance to fluoroquinolones.[34,36] Although the DPB and B communities exhibited significant resistance to tetracycline and ciprofloxacin, degradation or alteration of these antibiotics was not observed, indicating that the resistance to these antibiotics could not be attributed to enzyme-mediated degradation. Thus, in the case of the present study, the most likely resistance mechanism for these two antibiotics is the action of efflux pump(s). To confirm the role of efflux pumps, another susceptibility test was performed with the DPB and B communities for tetracycline and ciprofloxacin in the presence and absence of an efflux pump inhibitor (EPI), and results are discussed in the following section.

Role of Efflux Pump(s). A susceptibility test of the DPB and B communities against the BAC mixture, tetracycline, and ciprofloxacin was conducted in the presence and absence of thioridazine, acting as an EPI. The susceptibility of the DPB and B communities to BACs was not affected by the presence of thioridazine, suggesting that mechanisms other than efflux pump(s) contribute to the observed resistance against BACs. Further confirmation of this result can be achieved by the use of other EPIs, such as phenyl arginine β-naphthylamide, which is a broad spectrum inhibitor known to inhibit RND-type pumps. In contrast to the results relative to susceptibility of BACs, the susceptibility of the DPB and B communities to tetracycline and ciprofloxacin increased significantly in the presence of EPI. The tetracycline and ciprofloxacin MIC values of the DPB community decreased from 250 and 16 μg/mL to 150 and 10 μg/mL, respectively. Likewise, tetracycline and ciprofloxacin MIC values of the B community decreased from 95 and 18 μg/mL to 6.3 and 6.3 μg/mL, respectively. These results confirm that efflux pumps contribute to the observed increased resistance to tetracycline and ciprofloxacin of the two, BAC-exposed microbial communities. However, simultaneous action of multiple resistance mechanisms is possible, in particular a modified and impermeable outer membrane along with the efflux pumps.[37,38] The results of this test also indicate that the role of efflux pumps is not significant for BAC resistance. Thus, BAC degradation appears to be the most possible resistance mechanism in the DPB and B communities which adapted to BAC for over 4 years.

Quantification of Multidrug Efflux Pump Genes. After confirming the role of efflux pump in the observed resistance of the DPB and B communities against tetracycline and ciprofloxacin, it was also necessary to quantify the efflux pump genes present in the three microbial communities used in the present study. The gene concentrations in the three microbial communities are presented in Figure 4. From the five

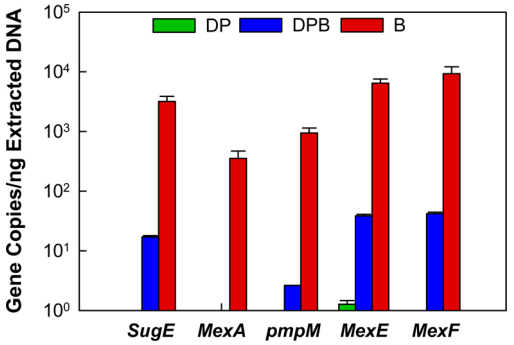

Figure 4. Efflux-related antibiotic resistance gene counts in the DP, DPB, and B communities (Error bars represent one standard deviation of the mean; n = 3).

genes found in both the DPB and B communities, only *mexE* was identifiable in the DP community and at a very low abundance. The B community was found to harbor the highest concentrations of the targeted antibiotic resistance genes. Although four out of the five genes were also detected in the DPB community, the gene counts were several orders of magnitude lower than in the B community. RND family efflux pumps are known to be related to resistance against aminoglycosides, fluoroquinolones, and cefepime.[39−41] Other target genes, *pmpM* and *sugE* belong to the MATE and SMR families. While *sugE* confers resistance to QACs,[42] a wide range of toxic compounds, such as benzalkonium chloride, fluoroquinolones, ethidium bromide, acriflavine, and tetraphe-

nylphosphonium chlorides, are substrates for *pmpM* efflux pumps.[43] Detection of a high concentration of multidrug resistance genes in the present study further supports the observed higher resistance of the DPB and B communities to the tested antibiotics as compared to the DP community. Analysis of the microbial community structure in the DP, DPB, and B communities revealed that *Pseudomonas* species are highly enriched in the B community, and these organisms are known to encode RND-family efflux pump genes such as *mexAB-oprM* and *mexEF-oprN*.[39,40] Thus, the qPCR results for the efflux pump genes are consistent with the enrichment of *Pseudomonas* species in the BACs-exposed communities. However, gene homologues of *sugE* are also conserved in phylogenetically diverse bacteria and occasionally encoded on mobile genetic elements such as integrons and plasmids. Thus, future studies will identify which organisms encode the efflux pump genes and whether these genes have been subjected to horizontal gene transfer during the operation of our bioreactors. Finally, it is important to note that the efflux pumps may not be directly selected by exposure to BACs but rather they were carried along in the genome of the organism(s) that degrade(s) BACs and thus were selected (enriched) in the B and DPB communities (e.g., *Pseudomonas*). Whether enrichment of efflux pump genes is directly or indirectly selected by exposure to BACs will be also part of future studies. In either case, however, exposure to BACs clearly resulted in increased microbial community antibiotic resistance based on the evidence presented here.

Environmental Relevance. The present study demonstrated that long-term exposure of microbial communities to subinhibitory concentrations of QACs increases coresistance to clinically relevant antibiotics. The primary resistance mechanisms of the microbial communities against BAC and antibiotics were degradation and extrusion of these compounds by a wide range of multidrug efflux pumps. The efflux pumps contributed to the increased resistance of the microbial communities, especially to tetracycline and ciprofloxacin. Thus, long-term exposure of microbial communities to biocides selects for more resistant microorganisms, giving rise to a multidrug resistant community equipped with diverse resistance mechanisms. The enrichment and spread of bacteria which harbor multidrug resistance genes, in particular those related to clinically relevant antibiotics, leads to the evolution of superbugs, which are a growing threat to both human and environmental health. Thus, efforts to minimize the use of antimicrobials, as well as develop biological systems to effectively degrade such agents, are urgently needed to avert environmental contamination by antimicrobials and the spread of superbugs.

■ ASSOCIATED CONTENT

Supporting Information

Table S1, phylogenetic relationships of representative phylotypes identified in the DP, DPB, and B microbial communities; and Figure S1, effect of the efflux pump inhibitor (EPI) thioridazine on the growth of the DP, DPB, and B microbial communities. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (404)-894-9367; fax (404) 894-8266; e mail: spyros.pavlostathis@ce.gatech.edu.

Notes

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The chemical name Benzalkonium was spelled incorrectly in the title of the version of this paper published August 19, 2013. The correct version published September 3, 2013.