Formulation of Biocides Increases Antimicrobial Potency and Mitigates the Enrichment of Non-Susceptible Bacteria in Multi-Species Biofilms

FORBES, Sarah <http://orcid.org/0000-0002-8361-6390>, COWLEY, Nicola, HUMPHREYS, Gavin <http://orcid.org/http://orcid.org/0000-0002-9138-0859>, MISTRY, Hitesh, AMÉZQUITA, Alejandro and MCBAIN, Andrew J <http://orcid.org/http://orcid.org/0000-0002-5255-5425>

zam-aem/zam00717/zam7741d17z | xppws | S=3 | 1/31/17 | 8:57 | 4/Color Fig: 2,3,4 | ArtID: 03054-16 | DOI:10.1128/AEM.03054-16 | CE: jtc

Editor: Drake | Section: Public and Environmental Health Microbiology | Designation: T

AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

PUBLIC AND ENVIRONMENTAL HEALTH MICROBIOLOGY

Formulation of Biocides Increases Antimicrobial Potency and Mitigates the Enrichment of Nonsusceptible Bacteria in Multispecies Biofilms

AQ: au Sarah Forbes,[a] Nicola Cowley,[b] Gavin Humphreys,[b] Hitesh Mistry,[a] Alejandro Amézquita,[c] Andrew J. McBain[b]

AQ: aff Biomolecular Sciences Research Centre, Sheffield Hallam University, Sheffield, United Kingdom[a]; Faculty of Biology, Medicine and Health, The University of Manchester, United Kingdom[b]; Unilever, Safety and Environmental Assurance Centre, Colworth Science Park, Sharnbrook, Bedfordshire, United Kingdom[c]

ABSTRACT The current investigation aimed to generate data to inform the development of risk assessments of biocide usage. Stabilized domestic drain biofilm microcosms were exposed daily over 6 months to increasing concentrations (0.01% to 1%) of the biocide benzalkonium chloride (BAC) in a simple aqueous solution (BAC-s) or in a complex formulation (BAC-f) representative of a domestic cleaning agent. Biofilms were analyzed by culture, differentiating by bacterial functional group and by BAC or antibiotic susceptibility. Bacterial isolates were identified by 16S rRNA sequencing, and changes in biofilm composition were assessed by high-throughput sequencing. Exposure to BAC-f resulted in significantly larger reductions in levels of viable bacteria than exposure to BAC-s, while bacterial diversity greatly decreased during exposure to both BAC-s and BAC-f, as evidenced by sequencing and viable counts. Increases in the abundance of bacteria exhibiting reduced antibiotic or BAC susceptibility following exposure to BAC at 0.1% were significantly greater for BAC-s than BAC-f. Bacteria with reduced BAC and antibiotic susceptibility were generally suppressed by higher BAC concentrations, and formulation significantly enhanced this effect. Significant decreases in the antimicrobial susceptibility of bacteria isolated from the systems before and after long-term BAC exposure were not detected. In summary, dose-dependent suppression of bacterial viability by BAC was enhanced by formulation. Biocide exposure decreased bacterial diversity and transiently enriched populations of organisms with lower antimicrobial susceptibility, and the effects were subsequently suppressed by exposure to 1% BAC-f, the concentration most closely reflecting deployment in formulated products.

IMPORTANCE Assessment of the risks of biocide use has been based mainly on the exposure of axenic cultures of bacteria to biocides in simple aqueous solutions. The current investigation aimed to assess the effects of formulation on the outcome of biocide exposure in multispecies biofilms. Formulation of the cationic biocide BAC significantly increased antimicrobial potency. Bacteria with lower antimicrobial susceptibility whose populations were enriched after low-level biocide exposure were more effectively suppressed by the biocide at in-use concentrations (1% [wt/vol]) in a formulation than in a simple aqueous solution. These observations underline the

Received 7 November 2016 **Accepted** 19 January 2017

Accepted manuscript posted online ●●●

Citation Forbes S, Cowley N, Humphreys G, Mistry H, Amézquita A, Mcbain AJ. 2017. Formulation of biocides increases antimicrobial potency and mitigates the enrichment of nonsusceptible bacteria in multispecies biofilms. Appl Environ Microbiol 83:e03054-16. https://doi.org/10.1128/AEM.03054-16.

Editor Harold L. Drake, University of Bayreuth

Address correspondence to Andrew J. McBain, andrew.mcbain@manchester.ac.uk

ance of simulating normal deployment conditions in considering the risks and benefits of biocide use.

KEYWORDS biocides, antibiotics, susceptibility, resistance, formulation, CDFF, microbicides

zam-aem/zam00717/zam7741d17z | xppws | S=3 | 1/31/17 | 8:57 | 4/Color Fig: 2,3,4 | ArtID: 03054-16 | DOI:10.1128/AEM.03054-16 | CE: jtc

AQ: A

The potential for the home environment to act as a reservoir for bacterial pathogens has long been considered by epidemiologists (1, 2). In particular, the domestic sink drain has been identified as a potential risk due to continual hydration and nutrient availability, which promotes microbial growth and the establishment of taxonomically diverse biofilms (3, 4). The open nature of the drain allows its continuous inoculation with a plethora of microorganisms originating both from food waste (5) and, potentially, in the case of organisms such as *Legionella* sp., from the tap water (6). While there are only a small number of reports in the literature on the microbial ecology of such environments, the persistence and recalcitrance of bacterial biofilms are widely acknowledged (7, 8) and often result in ineffective disinfection by chemical control agents (9). Furthermore, the fact that biocide-containing formulations are diluted from the point of application within a drain system to sublethal concentrations increases the risk of resistance selection. Bacterial biofilms are also recognized for the spread of antibiotic resistance determinants between species within the biofilm via horizontal gene transfer, further adding to concern over their role in the spread of antimicrobial resistance within the domestic environment (10).

The widespread use of products containing biocides has led to concern over the potential selection of bacteria exhibiting reduced susceptibility to the primary antimicrobial compound and potentially to third-party agents such as chemically unrelated biocides and antibiotics through the induction of cross-resistance (11–13). Quaternary ammonium compounds (QACs) are cationic biocides with broad-spectrum antimicrobial activity that are widely used as antiseptics, disinfectants, and preservatives (14). The antibacterial efficacy and surfactant-like properties of QACs such as benzalkonium chloride (BAC) have led to their use in a variety of domestic cleaning and personal care products (14, 15). Increased expression of multidrug efflux pumps in certain bacteria has been previously associated with reductions in susceptibility both to BAC and antibiotics (16–18).

While the generation of non-biocide-susceptible bacteria has been reported for certain combinations of bacterium and biocide (19, 20), such observations have been based mainly on data generated through the exposure of bacteria to biocides in simple aqueous solutions in the laboratory. In the real world, biocides are formulated into products containing various sequestrants and surfactants that may affect antimicrobial potency as well as having mitigating effects on the development of bacterial insusceptibility (11). Furthermore, data used to assess the risks and benefits of biocide use have mostly been generated using pure cultures of bacteria, while bacteria in the environment usually exist as complex mixed-species biofilm communities that are inherently recalcitrant to antimicrobial treatment (7, 21).

Assessments of the risks of biocide use have been based mainly on the exposure of axenic cultures of bacteria to biocides in simple aqueous solutions. The aim of the current study was therefore to investigate the effect of BAC delivered in a simple aqueous solution (BAC-s) or in a complex formulation (BAC-f), representative of a general-purpose cleaning product, on the bacteriological composition and antibiotic and BAC susceptibility properties of a multispecies biofilm community. Domestic drain biofilms were chosen because they are (i) taxonomically diverse, with high cell density, and (ii) commonly exposed to antibacterial compounds and because (iii) methods for their stable maintenance have previously been developed and validated (22–24).

Susceptibilities of bacteria isolated from constant-depth film fermenters (CDFFs) before and during BAC exposure to BAC and the antibiotics ampicillin (AMP), kanamycin (KAN), ciprofloxacin (CIP), cephalothin (CEF), and tetracycline (TET) were assessed via plating onto agars containing graded concentrations of BAC and set antibiotic concentrations. Bacterial composition in modeled drain biofilms was also evaluated by 16S rRNA gene sequencing of isolated culturable bacteria and through next-generation sequencing (NGS) analysis of communities using the Illumina MiSeq platform.

AQ: B

zam-aem/zam00717/zam7741d17z | xppws | S=3 | 1/31/17 | 8:57 | 4/Color Fig: 2,3,4 | ArtID: 03054-16 | DOI:10.1128/AEM.03054-16 | CE: jtc

RESULTS

Assessment of compositional changes in BAC-exposed biofilms by differential viable counting. Drain microcosm experiments were run for 8 weeks to stabilize bacterial populations prior to BAC exposure. Following 8 subsequent weeks of continuous exposure to BAC-s or BAC-f at 0.01% (wt/vol), the concentration was then increased to 0.1% (wt/vol) for a further 8 weeks and then to 1% (wt/vol) for a further 7 weeks.

With respect to statistically significant effects of BAC dosing, the major observations were as follows. (i) BAC dosing caused a statistically significant decrease in total bacterial counts ($P < 0.01$), and formulation of BAC increased antibacterial potency, resulting in significantly greater bacterial inactivation than that seen with BAC in a simple aqueous solution ($P < 0.001$). Immediately after exposure to 0.01% (wt/vol) BAC, total aerobic counts had reduced by 6.8 Log_{10} CFU/mm^2 for BAC-f versus 6.7 Log_{10} CFU/mm^2 for BAC-s. Counts of enteric bacteria decreased by 6.0 Log_{10} CFU/mm^2 for BAC-f versus 6.7 Log_{10} CFU/mm^2 for BAC-s, while counts of Gram-positive bacteria decreased by 6.2 Log_{10} CFU/mm^2 for both BAC-s and BAC-f. Bacterial viability subse- quently increased to numbers approaching the preexposure numbers over the follow- ing 8 weeks while the systems were under conditions of continuous exposure to 0.01% (wt/vol) BAC (Fig. 1A, panel i). In contrast, total anaerobe and pseudomonas counts initially increased regardless of formulation and then subsequently declined to the preexposure numbers by week 16. A similar pattern was observed when the dosing concentration was increased to 0.1% (wt/vol) BAC. At 0.1% (wt/vol) BAC, a decrease in viability was observed within all functional groups of bacteria, with the exception of the pseudomonads, which showed an elevation in numbers (Fig. 1A, panel ii). At week 26, the dosing concentration was increased to 1% (wt/vol) BAC, reflecting the BAC concentration commonly used in household products. At this concentration, a more substantial decrease in bacterial viability for all selected functional groups was observed in both the BAC-s- and BAC-f-exposed systems.

At the end of the antimicrobial exposure period, comparing the total effects of BAC-f and BAC-s, respectively, total viable counts were as follows: 3.9 and 6.1 Log_{10} CFU/mm^2 for total aerobes, 3.7 and 5.7 Log_{10} CFU/mm^2 for enteric bacteria, 3.9 and 5.5 Log_{10} CFU/mm^2 for Gram-positive species, 3.7 and 5.1 Log_{10} CFU/mm^2 for total anaerobes, and 3.2 and 4.3 Log_{10} CFU/mm^2 for pseudomonads. Significantly greater reductions in total levels of viable bacteria occurred after exposure to BAC-f at the in-use concentration (1%) than after exposure to BAC-s ($P < 0.001$). Statistically significant changes both in the abundance of different functional groups of culturable bacteria and in the abundance of bacteria capable of growing on the antibiotic- and BAC-containing agars ($P < 0.001$) occurred during the BAC exposure period (Fig. 1B and Table 1). While the overall number of viable microorganisms detected on BAC-containing agars decreased substantially as the BAC exposure concentration increased, the relative proportion of organisms exhibiting low susceptibility to BAC within the total viable population increased. Comparing the percentages of the culturable bacterial populations capable of growing on BAC-containing agars (Table 1 and Fig. 1) before BAC exposure to those measured after exposure, there was an increase in the proportion of bacteria capable of growing in the presence of BAC (0.1 to 1,000 $\mu g\ ml^{-1}$).

BAC-s and BAC-f caused similar increases in the relative abundances of bacteria selected on 0.1 $\mu g\ ml^{-1}$ and 1 $\mu g\ ml^{-1}$ BAC-containing agar. In contrast, the increase in the relative abundance of bacteria capable of growing in the presence of 10 $\mu g\ ml^{-1}$ and 100 $\mu g\ ml^{-1}$ BAC observed during exposure to BAC-s was significantly greater than that seen during exposure to BAC-f ($P < 0.001$). BAC-s and BAC-f caused similar increases in the relative abundances of bacteria capable of growing in the presence of 1,000 $\mu g\ ml^{-1}$ BAC (Tables 1 and 2 and Fig. 1). The growth of these bacteria was suppressed during exposure to BAC at 1% ($P < 0.001$), and formulation significantly increased this effect ($P < 0.001$).

With respect to the relative abundances of bacteria capable of growing on antibiotic-containing agars, increases were observed in both systems at low BAC

zam-aem/zam00717/zam7741d17z | xppws | S=3 | 1/31/17 | 8:57 | 4/Color Fig: 2,3,4 | ArtID: 03054-16 | DOI:10.1128/AEM.03054-16 | CE: jtc

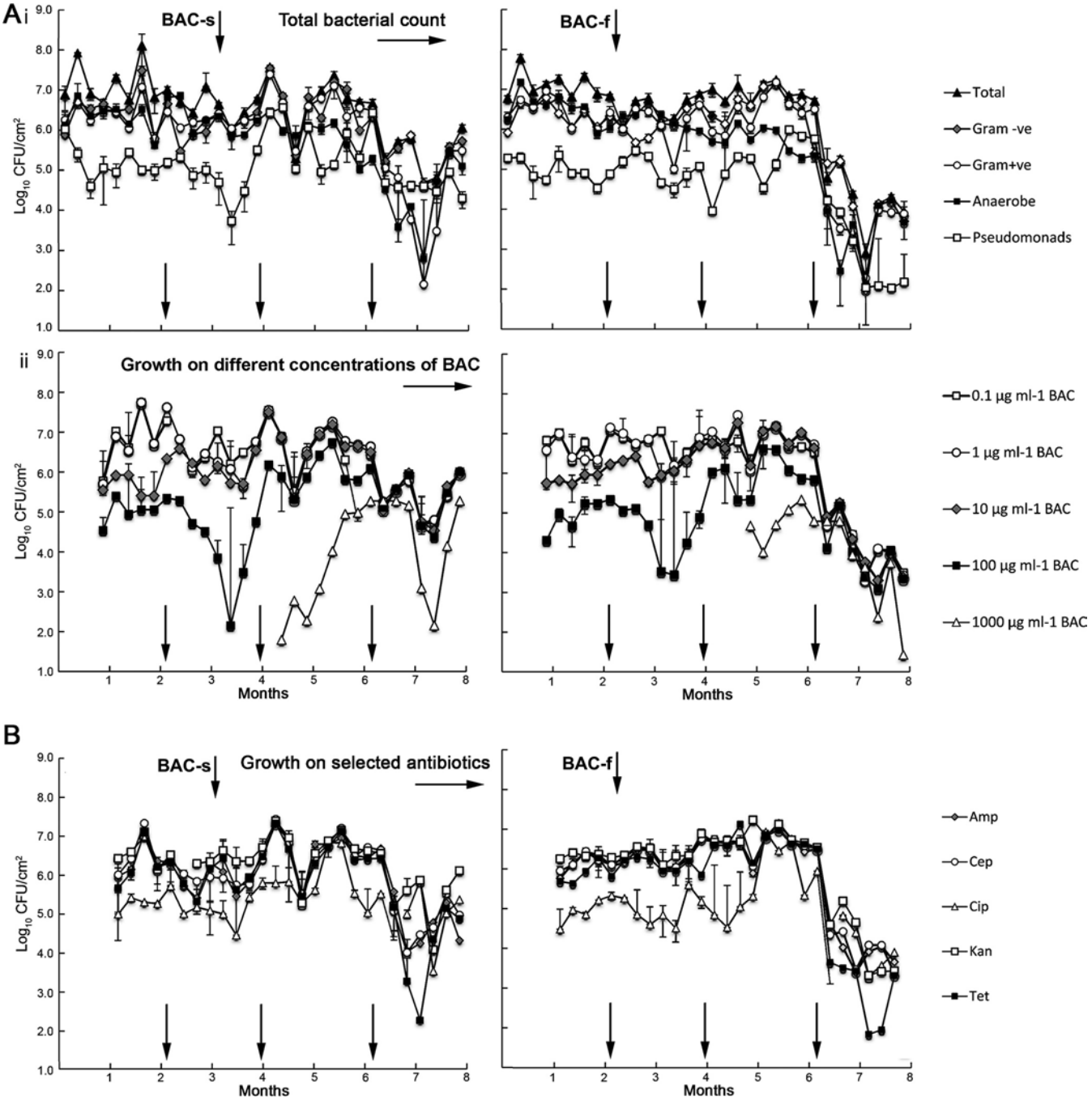


FIG 1 Differential viable counts of bacteria in microcosms under conditions of long-term exposure to benzalkonium chloride in simple solution (BAC-s) or formulation (BAC-f). (A) Bacteria isolated according to growth on media (i) selective for different functional groups of bacteria or (ii) containing various concentrations of BAC. (B) Bacteria isolated according to susceptibility to selected antibiotics. The first of the lower arrows indicates commencement of exposure to 0.01% BAC, the second arrow, commencement of exposure to 0.1% BAC (weeks 17 to 25), and the third arrow, commencement of exposure to 1% BAC. Amp, ampicillin; Cep, cephalothin; Cip, ciprofloxacin, Kan, kanamycin; Tet, tetracycline.

concentrations. However, at the in-use concentration (1%), the counts decreased for the bacteria regardless of the antibiotic. Statistical modeling suggests that effects on bacteria capable of growing on antibiotic selection plates could be grouped as follows: the bacteria growing on the CIP and KAN selection plates showed similar population decreases; those growing on AMP and CEP plates showed similar, larger decreases; and those growing on TET plates underwent the largest decrease.

The species of bacteria isolated on agars containing BAC or antibiotics before and after exposure to 1% (wt/vol) BAC-s and BAC-f were determined through sequencing of

zam-aem/zam00717/zam7741d17z | xppws | S=3 | 1/31/17 | 8:57 | 4/Color Fig: 2,3,4 | ArtID: 03054-16 | DOI:10.1128/AEM.03054-16 | CE: jtc

TABLE 1 Changes in the relative proportions of bacteria isolated on BAC- and antibiotic-containing agars before and after exposure to 1% (wt/vol) BAC in simple aqueous solution or in complex formulation with sequestrants and surfactants

Isolated on agar containing (μg ml^{-1}):	% change after exposure to[a]:	
	BAC-s	BAC-f
BAC (0.1)	31.2	31.1
BAC (1)	30.5	30.7
BAC (10)	89.9	21.3
BAC (100)	75.7	27.8
BAC (1,000)	7.0	9.0
Ampicillin	32.6	37.3
Cephalothin	36.9	42.7
Ciprofloxacin	18.7	42.5
Kanamycin	60.8	−7.4
Tetracycline	12.6	−3.7

[a]Data represent average percent changes in levels of bacteria isolated on agars containing BAC or antibiotics before (0% [wt/vol] BAC, weeks 6 to 8) and after (1% [wt/vol] BAC, weeks 30 to 32) exposure to formulated BAC (BAC-f) or unformulated BAC (BAC-s). Relative proportions of bacteria on selective agars were calculated as the percentages of total aerobic microorganisms present each week. Change data represent increases unless otherwise indicated.

the 16S rRNA gene (Table 2). Isolates varied in their abundance throughout the BAC exposure period, with insusceptible organisms that grew from potentially undetectable levels prior to BAC treatment becoming more prevalent. The biocide (Table 3) and antibiotic (Table 4) susceptibilities of numerically dominant culturable bacteria that could be isolated from the microcosms both before and during or after BAC exposure were determined. Data indicate that the increased abundance of bacteria selected on BAC- or antibiotic-containing agars was not reflected in reductions in susceptibility in these isolated bacteria (Tables 3 and 4). Determination of MIC and minimum bactericidal concentrations (MBC) for BAC in bacteria isolated before and after BAC-s or BAC-f exposure showed a <2-fold change in susceptibility for certain combinations of bacterium and exposure conditions. Minor increases and decreases in antibiotic susceptibility were observed in isolates after BAC-s or BAC-f exposure, although no bacterial isolate became resistant according to British Society for Antimicrobial Chemotherapy (BSAC) criteria (23).

TABLE 2 Bacteria isolated from the drain microcosms before and after exposure to BAC-s or BAC-f, corresponding to data presented in Fig. 1[a]

Wk	Bacterial species isolated before or after exposure to BAC ([μg ml^{-1}]):	
	BAC-s	BAC-f
4 (before BAC exposure)	*Bacillus cereus* (BAC 0.1, CEF, KAN), *Aeromonas hydrophilia* (BAC 0.1, AMP), *Elizabethkingia meningoseptica* (AMP, CEF, CIP, KAN), *Achromobacter spanius* (BAC 0.1), *Sphingobacterium spiritovorum* (CEF), *Chryseobacterium pallidium* (TET)	*Bacillus cereus* (BAC 0.1, CEF, KAN), *Aeromonas hydrophilia* (BAC 0.1, AMP), *Elizabethkingia meningoseptica* (AMP, CEF, CIP, KAN), *Delftia lacustris* (KAN), *Aeromonas* DH6 (KAN), *Chryseobacterium pallidium* (TET)
24 (during exposure to 0.1% [wt/vol] BAC)	*Bacillus cereus* (BAC 0.1; CEF, KAN), *Elizabethkingia meningoseptica* (AMP, CEF, CIP, KAN), *Aeromonas hydrophilia* (BAC 0.1, AMP), *Stenotrophas maltophilia* (BAC 0.1), *Pseudomonas geniculata* (BAC 0.1, AMP), *Enterobacter cloacae* (BAC 0.1)	*Bacillus cereus* (BAC 0.1, CEF, KAN), *Elizabethkingia meningoseptica* (AMP, CEF, CIP, KAN), *Aeromonas hydrophilia* (BAC 0.1, AMP), *Stenotrophomonas pavanii* (BAC 0.1), *Pseudomonas geniculata* (BAC 0.1, AMP), *Pseudomonas putida* (BAC 0.1, AMP)
32 (during exposure to 1% [wt/vol] BAC)	*Bacillus cereus* (BAC 0.1, BAC 1.0, CEF, TET), *Alcaligenes* sp. (BAC 0.1, CIP, KAN), *Pseudomonas* sp. (BAC 0.1, CEF), *Pseudomonas aeruginosa* (BAC 0.1, AMP), *Pseudomonas nitroreducens* (AMP)	*Bacillus cereus* (BAC 0.1, BAC 1.0, CEF), *Alcaligenes* sp. (BAC 0.1)

[a]Organisms represent numerically dominant culturable bacteria that were isolated on media containing BAC or antibiotics before (week 4) and after (weeks 24 and 32) microcosm exposure to formulated BAC (BAC-f) or unformulated BAC (BAC-s) (week 24, 0.1% [wt/vol] BAC; week 32, 1% [wt/vol] BAC). Isolates were identified by 16S rRNA gene sequencing. Acronyms refer to agar containing BAC or antibiotics as follows: BAC 0.1, BAC at 0.1 μg ml^{-1}; BAC 1.0, BAC at 1 μg ml^{-1}; AMP, ampicillin; CEF, cephalothin; CIP, ciprofloxacin; KAN, kanamycin; TET, tetracycline.

zam-aem/zam00717/zam7741d17z | xppws | S=3 | 1/31/17 | 8:57 | 4/Color Fig: 2,3,4 | ArtID: 03054-16 | DOI:10.1128/AEM.03054-16 | CE: jtc

TABLE 3 Susceptibility to benzalkonium chloride of bacteria isolated before, during, and after exposure of microcosms to BAC-s or BAC-f[a]

	MIC or MBC (mg ml^{-1}) under indicated conditions of exposure to BAC in microcosms ($n = 6$) as:							
	BAC-f				BAC-f			
	Before exposure		After exposure		Before exposure		After exposure	
Bacterium	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Aeromonas hydrophila[b]	31.3	31.3	31.3	62.5	31.3	31.3	31.3	31.3
Bacillus cereus[c]	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8
Elizabethkingia meningoseptica[b]	7.8	15.6	15.6	15.6	7.8	15.6	15.6	15.6

[a]MICs and minimum bactericidal concentrations of bacteria isolated from systems treated with BAC in simple solution (BAC-s) or in complex formulation with sequestrants and surfactants (BAC-f) before and after exposure to BAC. The indicated isolates were the only organisms isolated before and after the BAC exposure regimen.
[b]0.1% (wt/vol) BAC.
[c]1.0% (wt/vol) BAC.

AQ: D

Prior to exposure to BAC-s, *Bacillus cereus*, *Aeromonas hydrophila*, and *Achromobacter spanius* were detected on BAC-containing agars, while *Alcaligenes* sp., *Pseudomonas* spp., and *B. cereus* were detected after exposure to 1% (wt/vol) BAC (Table 2). In comparison, in the BAC-f-treated system, *B. cereus* and *A. hydrophila* were selected on BAC-containing agars prior to exposure whereas *B. cereus* and an *Alcaligenes* sp. were isolated after exposure. On agars containing the indicated antibiotics (Table 2), *Elizabethkingia meningoseptica* (AMP, CEF, CIP, and KAN), *B. cereus* (CEF and KAN), *A. hydrophila* (AMP), *Sphingobacterium spiritivorum* (CEF), and *Chryseobacterium pallidum* (TET) were isolated from the BAC-s system before exposure whereas *B. cereus* (CEF and TET), *Alcaligenes* sp. (CIP and KAN), and multiple *Pseudomonas* spp. (CEF and AMP) were isolated after exposure. In contrast, in the BAC-f-treated system, *Elizabethkingia menin-*

TABLE 4 Antibiotic susceptibility in bacteria isolated before, during, and after exposure of microcosms to BAC-s or BAC-f[a]

		Susceptibility (mm) under indicated conditions of exposure to BAC in microcosms, as:			
		BAC-s		BAC-f	
Bacterium	Antibiotic	Before exposure	After exposure	Before exposure	After exposure
Aeromonas hydrophila[b]	AMP	ns	ns	ns	ns
	CEF	ns	ns	ns	ns
	CIP	18	17.7 (1.1)	17.3 (0.6)	17.0
	KAN	ns	ns	ns	ns
	TET	8.3 (0.6)	8.0	9.0	9.3 (0.6)
B. cereus[c]	AMP	ns	ns	ns	ns
	CEF	21.0 (1.0)	22.0 (2.0)	23.0 (1.0)	25.3 (0.6)
	CIP	18.6 (0.6)	22.3 (1.5)	18.3 (0.6)	19.0
	KAN	10.7 (0.6)	8.3 (0.6)	11.0	10.3 (0.6)
	TET	10.7 (0.6)	20.3 (0.6)	10.3 (1.5)	20.3 (1.5)
E. meningoseptica[c]	AMP	6.0	6.0 (1.0)	6.0 (1.0)	5.7 (0.6)
	CEF	ns	ns	ns	ns
	CIP	18.0	17.7 (1.1)	17.3 (0.6)	17.0
	KAN	ns	ns	ns	ns
	TET	8.3 (0.6)	8.0	9.0	9.3 (0.6)

[a]Antibiotic susceptibility of bacteria isolated from systems treated with BAC in simple solution (BAC-s) or in complex formulation with sequestrants and surfactants (BAC-f) before and after exposure to BAC. Units are in mm ($n = 3$). Values in parentheses represent ●●●. ns, nonsusceptible (i.e., no inhibition observed). See footnotes to Tables 1 and 3.
[b]0.1% (wt/vol) BAC.
[c]1.0% (wt/vol) BAC.

zam-aem/zam00717/zam7741d17z | xppws | S=3 | 1/31/17 | 8:57 | 4/Color Fig: 2,3,4 | ArtID: 03054-16 | DOI:10.1128/AEM.03054-16 | CE: jtc

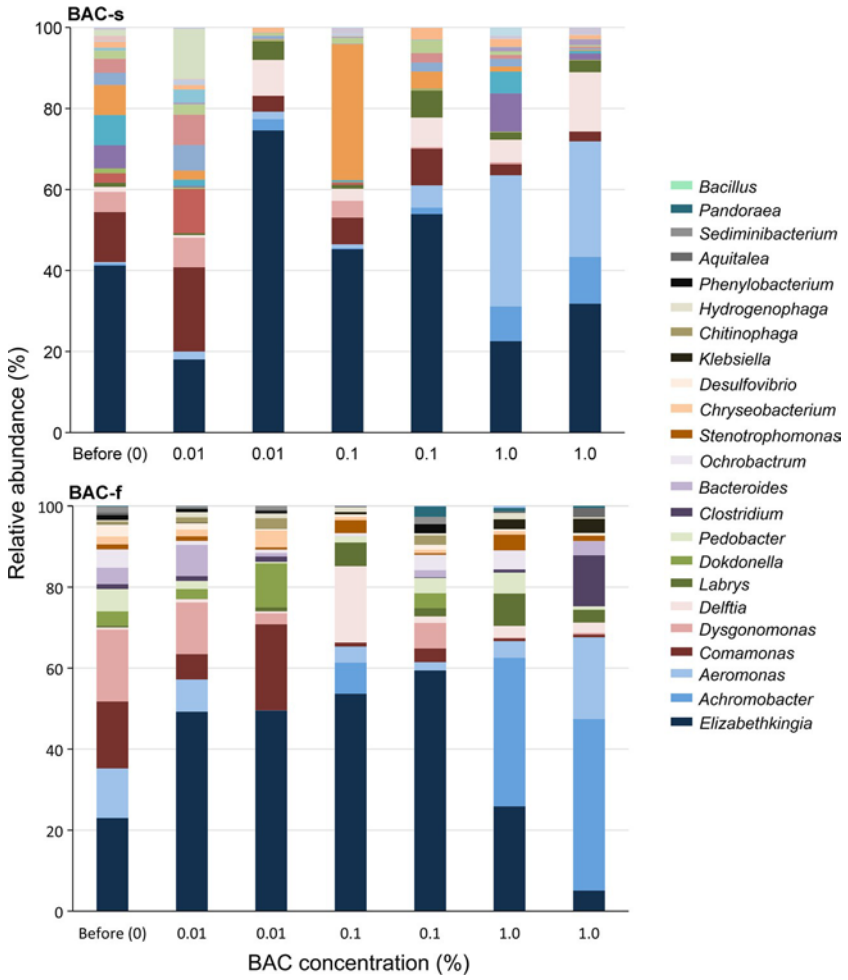


FIG 2 Relative abundances of bacterial genera detected at >1% relative abundance in drain microcosms before (weeks 1 to 8) and after exposure to 0.01% BAC (weeks 9 to 16), 0.1% BAC (weeks 17 to 25), and 1% BAC (weeks 26 to 32). (A) BAC-s. (B) BAC-f. See legend to Fig. 1.

goseptica (CIP, AMP, CEF, and KAN), *Delftia lacustris* (KAN), *Aeromonas* sp. strain DH6 (KAN), *A. hydrophila* (AMP), and *B. cereus* (CEF and KAN) were isolated prior to BAC exposure whereas only *B. cereus* (CEF) was isolated after exposure.

High-throughput sequence analysis of bacterial diversity. NGS analysis identified ca. 416 observable species from drain microcosms prior to biocide exposure. *Elizabethkingia* predominated, representing 20% and 35.6% of the bacterial relative abundance during exposure to BAC-f and BAC-s, respectively (Fig. 2). This bacterium increased in abundance following exposure to 0.01% (relative abundance, 41.03% ± 1.49%) and 0.1% (relative abundance, 47.35% ± 0.08%) BAC-s before decreasing in abundance to levels below the baseline following exposure to 1.0% BAC-s (relative abundance, 13.0% ± 12.48%). Reductions in the relative abundances of sequences associated with *Elizabethkingia* were marked, with concomitant increases in *Clostridium* sp., *Aeromonas* sp., and *Achromobacter* during exposure to 1.0% (wt/vol) BAC-f (Fig. 2, top panel). For exposure to BAC-f, despite fluctuations in the relative abundance of *Elizabethkingia* spp. following dosing with 0.01% BAC (40.69% ± 40.97%), this genus remained at levels comparable to baseline during microcosm exposure to 0.1% (37.8% ± 7.11%) and 1.0% (25.4% ± 6.52%) BAC-s (Fig. 2, bottom panel).

The results of ordination of unweighted UniFrac distances suggest that baseline microcosm samples exhibited a degree of homology in terms of the presence/absence

zam-aem/zam00717/zam7741d17z | xppws | S=3 | 1/31/17 | 8:57 | 4/Color Fig: 2,3,4 | ArtID: 03054-16 | DOI:10.1128/AEM.03054-16 | CE: jtc

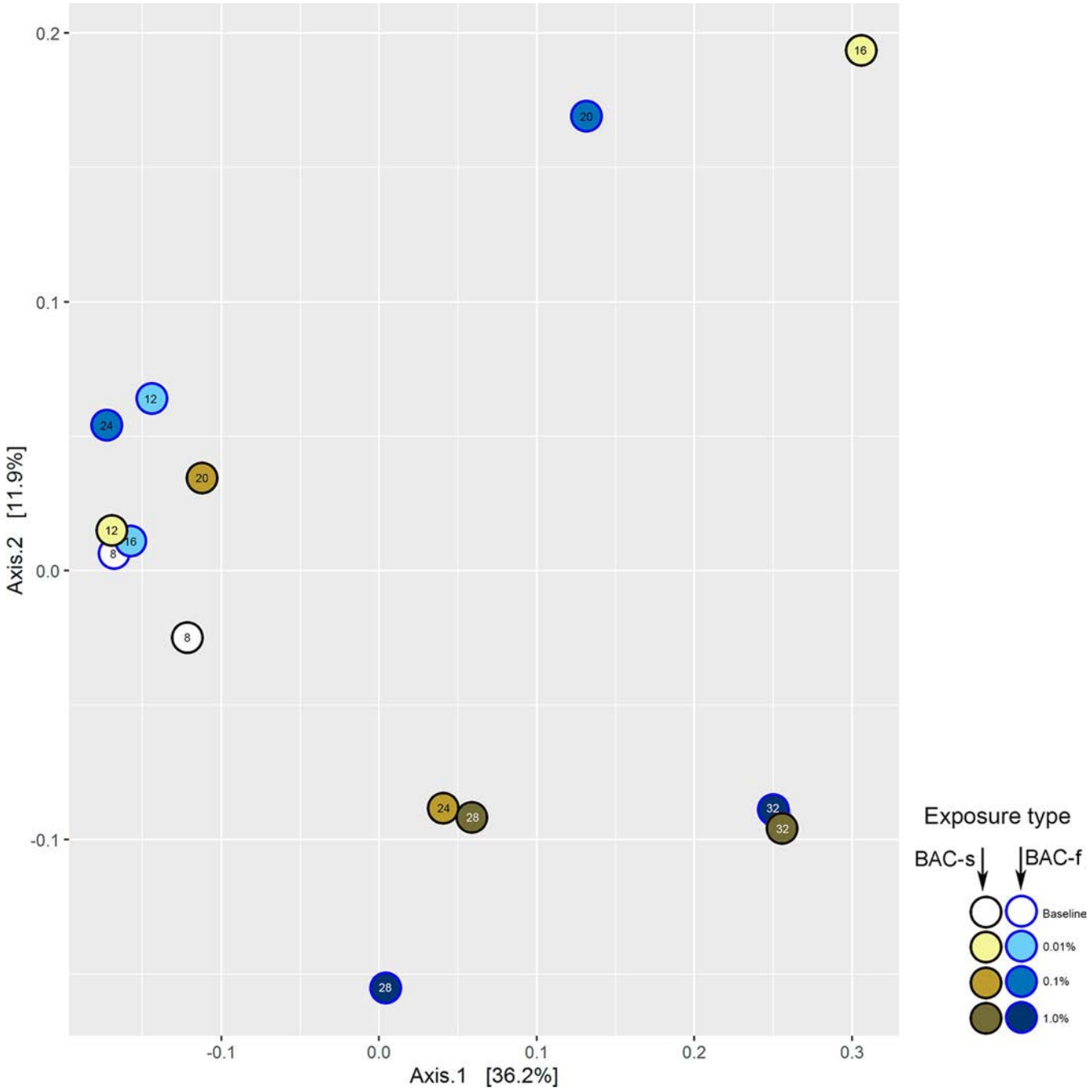
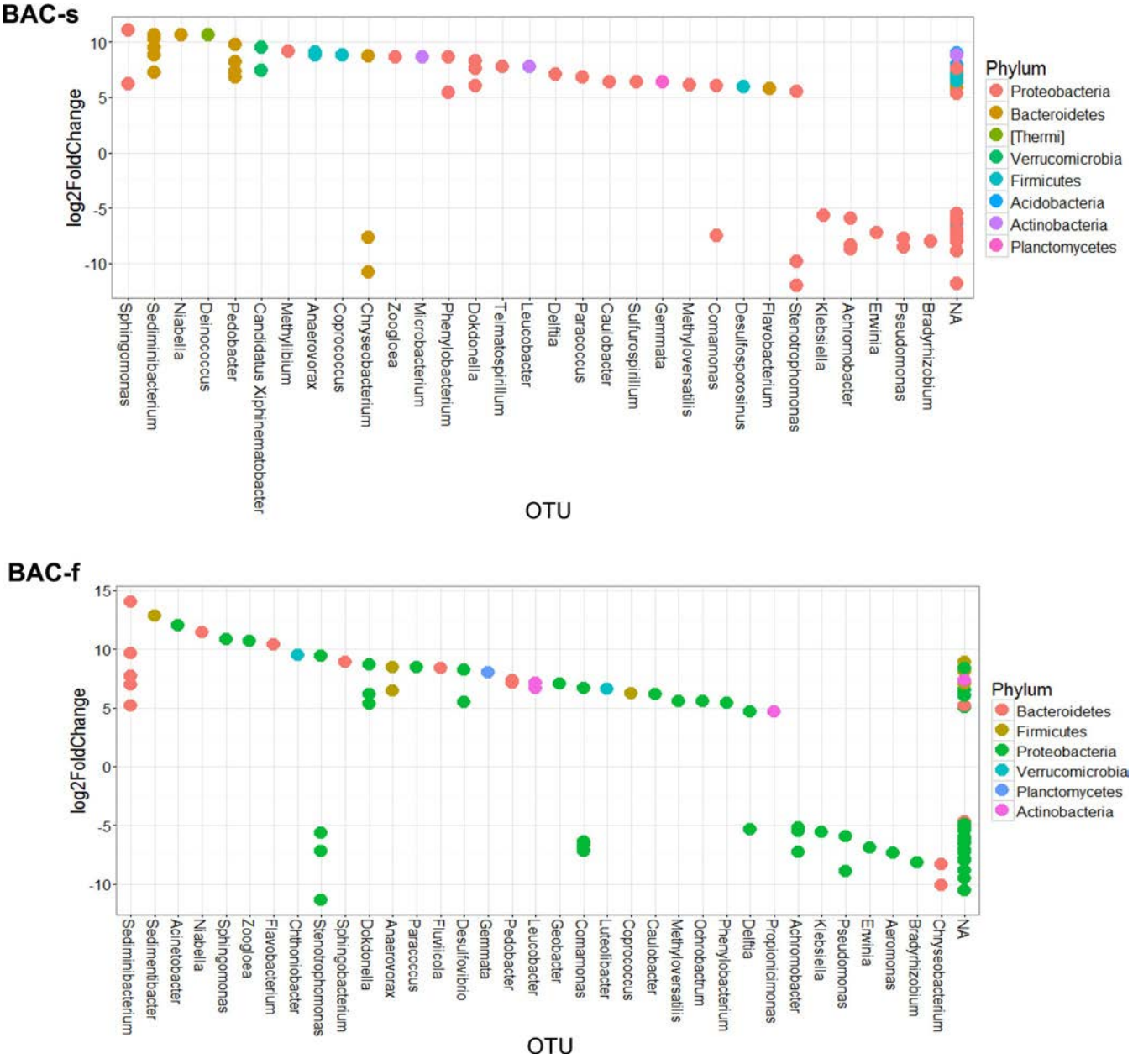


FIG 3 Changes in drain microcosm population dynamics in response to exposure to concentrations of benzalkonium chloride in aqueous solution (BAC-s) or in complex formulation (BAC-f). Ordinations were generated following principal-coordinate analyses of unweighted UniFrac distances. Numbers denote sampling time points (weeks). See legend to Fig. 1.

F3 F4 of identified taxa between independent model runs (Fig. 3). During week 32 (1.0% BAC-f and BAC-s), samples from CDFFs exposed to BAC-s and BAC-f clustered together, independently from other samples. The most significant changes in relative abundance of detected genera occurred at a 1% exposure concentration for both BAC-f and BAC-s (Fig. 4). NGS analysis revealed significant decreases and increases in the relative abundances of detectable genera after exposure to 1% (wt/vol) BAC-s or BAC-f, reflecting the in-use concentration. Data indicate larger numbers of significant changes (both increases and decreases) in detectable genera after treatment with BAC-f than after treatment with BAC-s. There were significant decreases in the relative abundances

zam-aem/zam00717/zam7741d17z | xppws | S=3 | 1/31/17 | 8:57 | 4/Color Fig: 2,3,4 | ArtID: 03054-16 | DOI:10.1128/AEM.03054-16 | CE: jtc



COLOR COLOR

FIG 4 Significant ($P < 0.01$) changes in relative abundances in individual OTUs between microcosms left unexposed or exposed to 1% BAC. (Top panel) BAC-s. (Bottom panel) BAC-f. See legend to Fig. 1.

of operational taxonomic units (OTUs) assigned to *Chryseobacterium*, *Stenotrophomonas*, *Comamonas*, *Klebsiella*, *Achromobacter*, *Erwinia*, *Pseudomonas*, and *Bradyrhizobium* for 1% (wt/vol) BAC-s and BAC-f exposure (Fig. 4). BAC-s was also associated with relative decreases in OTUs assigned to *Delftia* and *Luteolibacter* and *Aeromonas*. Increases in the relative abundances in detectable genera showed greater diversity between the systems, with the only consistent relative increases occurring for OTUs assigned to *Sphingobacterium*, *Sediminibacterium*, *Niabella*, *Pedobacter*, *Anaerovorax*, *Coprococcus*, *Dokdonella*, *Leucobacter*, *Delftia*, *Gemmata*, *Methyloversatilis*, *Comamonas*, *Flavobacterium*, and *Stenotrophomonas* during exposure to both BAC-s and BAC-f.

DISCUSSION

Investigations into potential risks of the development of biocide resistance have focused largely on the exposure of pure cultures of bacteria to active antimicrobials,

such as quaternary ammonium compounds, in simple aqueous solutions (25, 26). In the environment, however, bacteria form complex, multispecies biofilm communities, and biocides are deployed in formulation with various sequestrants and surfactants.

In the current investigation, the effects of the formulation of the quaternary ammonium compound benzalkonium chloride (BAC) on biocide and antibiotic susceptibility in bacteria maintained within a domestic drain biofilm microcosm were investigated. The approach to establishing domestic biofilm ecosystems has been previously validated for the maintenance of compositionally stable microcosms established using excised domestic drain biofilms. By utilizing this approach, the effects of long-term biocide exposure on a microbial community representative of a common biocide-exposed domestic environment can be determined (22).

While exposure to BAC both in a simple aqueous solution and in formulation caused substantial decreases in bacterial diversity, exposure to the biocide in a complex formulation resulted in greater overall reductions in total levels of viable bacteria (Fig. 1). Relative abundances of bacteria capable of growing on BAC- and antibiotic-containing agars increased transiently in both systems during exposure to lower concentrations of BAC, probably associated with the clonal expansion of *B. cereus*, *Alcaligenes* sp., and *Pseudomonas* spp. The relatively low intrinsic susceptibility of these bacteria to biocides and antibiotics has been widely documented in previous reports (17, 27–29). Increases in the abundance of such organisms may accompany reductions in the levels of the more BAC-susceptible bacteria. This may be due to the decreased competition for nutrients which has been previously reported in similar microcosm systems, and it further supports the reduction in overall bacterial viability observed in both of our systems after BAC treatment (22, 23). Bacteria isolated from the microcosms exhibited minor (<2-fold) reductions in BAC susceptibility after BAC exposure in testing in planktonic culture (MIC and minimum bactericidal concentration [MBC]). BAC concentrations that inhibited the growth of these bacteria were, however, considerably lower than the concentrations of selective agar on which the bacteria were initially isolated from the microcosms and ca. 100-fold lower than the BAC concentrations to which the microcosms were exposed. The former may have been due to differences in the methodologies, and the latter may be indicative of the well-documented recalcitrance associated with growth in the biofilm phenotype.

Low susceptibility to BAC in pseudomonads has been previously attributed to increases in the expression or activity of multidrug tolerance efflux pumps that can actively remove QACs, such as BAC, from the cytoplasmic membrane core, thereby reducing their antibacterial efficacy (20, 21). Since multidrug efflux pump expression has also been associated with changes in bacterial susceptibility to chemically unrelated third-party agents (22), it has been suggested that efflux-mediated decreases in QAC susceptibility can be accompanied by changes in the effectiveness of clinically important antibiotics against certain bacteria (30). *B. cereus* spores are insusceptible to BAC and therefore may germinate on agar plates, likely explaining the apparent prevalence of this bacterium after biocide exposure (31). While no documentation of comparatively low susceptibility to BAC in aeromonads has been previously reported, insusceptibility to ampicillin in this genus is common (32). Ampicillin insusceptibility in pseudomonads has been widely reported, and tetracycline resistance has also been documented for various chryseobacteria (33). Multidrug resistance has been reported in numerous clinical isolates of *E. meningoseptica*, an environmental bacterium (34, 35). While induced changes in bacterial susceptibility in various pure cultures of bacteria in response to BAC exposure have been reported (30, 36), data generated in the current investigation suggest that the compositional changes associated with altered antimicrobial susceptibility profiles within the biofilm were most likely attributable to the clonal expansion of bacterial species, such as *B. cereus* and pseudomonas, that are reportedly innately insusceptible to these antimicrobial agents. This suggestion was supported by further susceptibility testing of key isolates, where substantial decreased susceptibility was not observed.

A previous investigation into the effects of the formulation of biocides in reducing

zam-aem/zam00717/zam7741d17z | xppws | S=3 | 1/31/17 | 8:57 | 4/Color Fig: 2,3,4 | ArtID: 03054-16 | DOI:10.1128/AEM.03054-16 | CE: jtc

the development of insusceptibility in monospecies cultures of bacteria suggested that formulated biocides exhibited greater antibacterial potency than unformulated actives and that susceptibility decreases after repeated exposure were lower in frequency and extent (11, 37). This association among formulation, increased antibacterial potency, and the partial mitigation of antimicrobial insusceptibility therefore also appeared to hold true in mixed-species biofilms, as outlined in the current investigation.

Formulated biocides are likely to interact with bacteria in a manner that is distinct from that seen in simple aqueous solutions, since the excipients that are present, particularly surfactants, may associate with additional cellular targets, enhance bacterial permeability, or solubilize the active compound. This is likely to be partly responsible for increased overall antimicrobial potency of the formulation. For example, nonionic surfactants, such as alcohol ethoxylates, found in the formulation used in the current investigation, have previously shown bacteriostatic effects due to their destabilizing impact on the bacterial cell membrane, leading to an increase in membrane permeability and potentially facilitating the entry of other agents such as BAC into the bacterial cell (38). Additionally, the formulation included chelating agents such as sodium tripolyphosphate, which may, as is the case with many chelators, have antimicrobial activity due to its ability to disrupt the bacterial cell envelope (39). This may occur through the sequestering of stabilizing divalent cations, causing the release of lipopolysaccharides from the cell envelope and consequently impairing barrier function (40, 41). This provides a plausible explanation for the heightened antimicrobial activity observed with the BAC formulation compared to the simple aqueous BAC solution.

As expected, NGS detected considerably higher microbial diversity within the microcosms than could be detected by culture, highlighting the limitation of the use of culture-based analysis alone when investigating such complex bacterial communities. A proportion of the bacteria within the system was therefore not isolated, or, alternatively, extracellular bacterial DNA may have remained within the biofilm sampled after bacterial cell lysis from BAC treatment. However, this is unlikely due to the use of propidium monoazide (PMA). Interesting, while *B. cereus* was among the predominant organisms isolated through culture, *Bacillus* was not among the dominant genera detected by NGS. This may have been due to the presence of a high abundance of *Bacillus* spores within the biofilm that were not susceptible to DNA extraction techniques.

Conclusion. The formulation of BAC increased antimicrobial potency, which was reflected in significantly greater viability decreases in complex domestic drain biofilm microcosms. During exposure to BAC, clonal expansion of innately insusceptible bacteria occurred, mainly in *B. cereus* and *Pseudomonas* spp. This increase in the abundance of the less antimicrobial-susceptible bacteria was more pronounced after exposure to BAC-s than after exposure to BAC-f. The BAC formulation suppressed bacteria with reduced levels of BAC and antibiotic susceptibility significantly more effectively than BAC in a simple aqueous solution. Formulation of BAC therefore mitigated the development of BAC and antibiotic insusceptibility within the mixed-species biofilm microcosms. Significant decreases in antibiotic and biocide susceptibility were not detected in monospecies cultures of bacteria isolated from the microcosms before and after biocide exposure.

MATERIALS AND METHODS

Chemicals and growth media. Bacteriological growth media were obtained from Oxoid (Basingstoke, UK). All other chemical reagents were obtained from Sigma-Aldrich (Dorset, UK). Growth media were sterilized at 121°C and 15 lb/in^2 for 15 min prior to use.

Antimicrobials. Benzalkonium chloride (BAC) was obtained from Sigma-Aldrich (Dorset, UK). CIP (1 μg), CEF (20 μg), AMP (10 μg), KAN (5 μg), and TET (10 μg) antibiotic discs were obtained from Oxoid (Basingstoke, UK). BAC was tested both in a simple aqueous solution and in a formulation representative of a general-purpose cleaner at 1% (wt/vol), reflective of its normal use in consumer products, and containing sodium tripolyphosphate (a chelator) and alcohol ethoxylates (nonionic surfactants) as previously described (11).

Domestic drain biofilm microcosms. Biofilm samples (1 g) were removed using a sterile scraper and a collection tube from the horizontal pipe section of a polyvinyl chloride (PVC) kitchen drain outlet in a

zam-aem/zam00717/zam7741d17z	xppws	S=3	1/31/17	8:57	4/Color Fig: 2,3,4	ArtID: 03054-16	DOI:10.1128/AEM.03054-16	CE: jtc

household that did not use biocidal products apart from bleach. The samples were transported to the laboratory for processing within 2 h. The drain biofilm was macerated by vortex mixing using glass beads in 20 ml of phosphate-buffered saline (PBS) (0.01 M, pH 7.4) for 5 min to produce a slurry which was subsequently used to inoculate paired sterile constant-depth film fermenter (CDFF) systems. After initial CDFF inoculation, the drain medium was run continuously through the system and consisted of the following (values represent milligrams per milliliter in tap water): starch, 1.0; peptone, 0.5; tryptone, 0.5; yeast extract, 0.5; NaCl, 1.0; margarine (Flora; Unilever, London, UK), 0.05; domestic detergent (Fairy Original; Procter and Gamble, Newcastle Upon Tyne, United Kingdom), 0.05; hemin, 0.001; and tomato ketchup (Heinz, Uxbridge, United Kingdom). For the initial 8 weeks, the CDFF systems remained antimicrobial free. Subsequently, one system was dosed with BAC incorporated into a formulation representative of a domestic general-purpose cleaning agent containing 3% nonionic surfactant and 0.5% sequestrant (BAC-f) and a second system was exposed to BAC in a simple aqueous solution (BAC-s). Both systems were exposed to solutions containing increasing concentrations (0.01% to 1%) of BAC for the remaining 28 weeks.

Differential counting of viable bacteria. To evaluate the microbial composition of the CDFF systems, biofilm samples were removed for culture-based analysis and were resuspended in 1 ml of sterile PBS. Aliquots (0.1 ml) of appropriate dilutions of biofilm slurry were plated in triplicate onto the following selective agars: tryptone soya agar (TSA) (for total counts), Wilkins-Chalgren agar (WC) (for total strict and facultative anaerobes), MacConkey agar (MK) (for enteric bacteria), *Pseudomonas* selective agar (C-F-C) (for pseudomonads), and Columbia colisitin naladixic acid agar (CCNA) (for Gram-positive species). For biofilm community antimicrobial susceptibility profiling, bacteria were plated onto TSA containing 0.1, 1, 10, 100, or 1,000 μg ml^{-1} BAC or KAN (10 μg ml^{-1}), CIP (1 μg ml^{-1}), CEF (25 μg ml^{-1}), TET (10 μg ml^{-1}), or AMP (25 μg ml^{-1}) antibiotic at concentrations previously used in an investigation into the effects of biocide use on antibiotic susceptibility in bacteria within the domestic environment (42). The plates were incubated for up to 5 days either aerobically or in an anaerobic chamber (Don Whitley Scientific, Shipley, United Kingdom) (atmosphere, H_2 [10%], CO_2 [10%], and N_2 [80%]) prior to enumeration of bacteria.

16S rRNA gene sequencing of bacterial isolates. Single bacterial colonies were aseptically transferred to a microcentrifuge tube containing 100 μl of NANOpure water, subjected to vortex mixing for 30 s, and boiled at 100°C for 15 min to lyse cells. Microcentrifuge tubes were centrifuged at 16,000 × *g* using a bench-top centrifuge for 10 min to remove cellular debris. The supernatant was retained as the DNA template for the PCR. PCR was performed using primers 8FLP (5′-GAG TTT GAT CCT GGS TCA G-3′) and 806R (5′-GGA CTA CCA GGG TAT CTA AT-3′) at 5 μM per reaction. PCR was conducted using a TGradient PCR machine (Biometra Göttingen, Germany) and run for 35 thermal cycles consisting of 94°C (1 min), 53°C (1 min), and 72°C (1 min). A 15-min elongation step was included in the final cycle. PCR products were purified using a QIAquick PCR purification kit (Qiagen, West Sussex, UK) per the manufacturer's instructions, and the resulting DNA yield was quantified using a NanoDrop 2000c UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A reaction mixture containing 4 pM forward or reverse primer and 40 to 50 ng of DNA in a 10-μl total volume was used for DNA sequencing. DNA sequencing was performed using the Applied Biosystems 3730 DNA analysis system at the DNA Sequencing Facility within The University of Manchester.

Determination of biocide and antibiotic susceptibility in microcosm isolates. For BAC susceptibility, MICs were determined using the microdilution method as described previously (25). Briefly, overnight bacterial cultures were adjusted to an optical density at 600 nm (OD_{600}) of 0.8 and further diluted 1:100 in tryptone soya broth in a 96-well microtiter plate containing doubling dilutions of BAC. Plates were incubated at 37°C (24 h) with agitation (100 rpm). The MIC was defined as the lowest concentration at which bacterial growth did not occur. Growth was viewed as turbidity (600 nm) in comparison to an uninoculated well (negative control) and was detected using a microtiter plate reader (PowerWave XS; BioTek, Bedfordshire, United Kingdom). Minimum bactericidal concentrations (MBC) were determined as stated previously (23, 25, 43). Briefly, aliquots (10 μl) from wells exhibiting no turbidity were spot plated onto TSA prior to 4 days of incubation at 37°C (42). The MBC was defined as the lowest concentration of biocide at which no growth occurred after 4 days of incubation. Antibiotic susceptibilities were determined for CIP (1 μg), CEF (20 μg), AMP (10 μg), KAN (5 μg), and TET (10 μg). Disc diffusion assays were performed according to the British Society for Antimicrobial Chemotherapy (BSAC) disc diffusion method for antimicrobial susceptibility testing (44).

DNA extraction from biofilms. Biofilm samples were washed three times in 5 ml of PBS (0.01 M, pH 7.4) before being resuspended in 500 μl PBS containing 50 μM propidium monoazide (PMA), to remove extracellular DNA. Samples were incubated in PMA solution for 5 min at room temperature prior to inactivation via exposure to a halogen light source for 5 min on ice. Bacterial cells were pelleted by centrifugation at 5,000 × *g* for 10 min, and DNA was extracted using a Qiagen DNeasy blood and tissue extraction kit (Qiagen Ltd., West Sussex, UK) per the manufacturer's instructions.

High-throughput sequencing of 16S rRNA gene amplicons. Amplification of the 16S rRNA gene was performed using primer pair BAKT_341_F/BAKT_805_R (45) with additional Illumina adaptor overhang nucleotide sequences. PCRs were performed using MyTaq red mix (Bioline, UK) and comprised 35 cycles of 94°C (1 min), 52.6°C (1 min), and 72°C (1 min). Amplicon purification was achieved using a Qiagen PCR purification kit (Qiagen, UK) per the manufacturer's protocol. Next-generation sequencing (2× 300 bp) was performed using the Ilumina MiSeq platform at the Genomic Technologies Core Facility, The University of Manchester.

Data processing was performed using QIIME 1.8.0 (46). Mating of paired-end sequences and removal of reads containing ambiguous bases were performed using PandaSeq (47). OTU picking was performed

zam-aem/zam00717/zam7741d17z	xppws	S=3	1/31/17	8:57	4/Color Fig: 2,3,4	ArtID: 03054-16	DOI:10.1128/AEM.03054-16	CE: jtc

using a closed reference strategy at a sequence similarity level of 97% against release 13_8 of the Greengenes database (48, 49). Singleton OTUs were discarded from the final BIOM table. Experimental data (sample data, phylogenetic tree, taxonomy table, OTU table) generated in QIIME were subsequently AQ: E imported into R 3.2.2 and analyzed using Phyloseq (50) following rarefaction to equal sample depths (E value, 250,000). Ordinations were performed on unweighted UniFrac distances using Phyloseq 1.13.2. Final OTU and taxonomy tables were exported as comma-delimited data tables, and values were graphically rendered using Prism version 6.05 (GraphPad Software Inc., La Jolla, CA, USA). Statistically significant variations in OTUs were determined following $\log_2$ transformation of abundance data in DESeq2 (51). Dispersions were estimated using a parametric fit type, and the Wald significance test was used as the test argument. Corrections for multiple testing were performed using the Benjamini-Hochberg procedure, with significance denoted as an adjusted *P* value of <0.01.

Statistical modeling. The series of nested questions of interest in this study were as follows. (i) (*a*) Does the total bacterial count decline from the pre-1% BAC treatment time point to the post-1% BAC treatment time point? (*b*) If so, do the amounts of count reduction differ under formulated versus unformulated conditions? (ii) (*a*) Do pseudomonad counts decline during BAC treatment? (ii) (*b*) Does BAC formulation influence the antipseudomonad effect? (iii) (*a*) Does the count of BAC-insusceptible bacteria decline from the pre-BAC treatment time point to the post-BAC treatment time point? (*b*) If so, do the amounts of this decline differ under formulated versus unformulated conditions? (iv) Does the BAC concentration in plate count agars (10 versus 1,000 mg/liter) affect how much of a decline is observed? (v) (*a*) Does the count of bacteria capable of growing on media containing antibiotics decline AQ: F from the pre-1% BAC exposure time point to the post-1% BAC exposure time point under different? (*b*) If so, do the amounts of decline differ under formulated versus unformulated conditions? (vi) Does the type of antibiotic affect how much of a decline is observed?

The nested nature of the questions stated above supports the use of the stepwise modeling AQ: G approach (data not shown), where, at each step, starting with the outer question in the series and working inward, the sum of squared residuals (SSR [lower values indicate better fit]) was recorded. An F-test was applied to assess the significance of the reduction in SSR after each step, with the corresponding *P* value reported. A constant model was used to analyze the data for simplicity. In doing so, we were assessing how the average bacterial count over time is affected in the groups created by the questions above. Below, we provide details of how the constant model was used within the stepwise modeling approach.

In the first step, we fitted a constant model to the data of interest. In the second step, we allowed the constant value to differ before and after 1% BAC treatment. In the third step (the final step for question series i and ii), we allowed the constant values corresponding to the post-1% BAC treatment time point to differ between the formulated and unformulated conditions. For series iii, a fourth (final) step was conducted in which we allowed the constant values post-1% BAC treatment determined under the formulated and unformulated conditions to differ with respect to the amounts of BAC agar (10 versus 1,000 mg/liter). For series iv, the final question involved 5 different antibiotic treatments, and so we first assumed that all antibiotics had different effects after accounting for pre- versus post-1% BAC treatment and formulated versus unformulated conditions. We then analyzed the model parameters and assessed whether the 95% confidence intervals overlapped for certain antibiotics. If they did, we grouped those bacteria together in the next model iteration and assessed via the F-test whether this grouping made a difference to overall model fit. All statistical modeling was conducted in Rv3.1.1.

ACKNOWLEDGMENTS

A.A. is an employee of Unilever. The rest of us have no conflicts of interest to declare.

This project was funded by Unilever's Safety & Environmental Assurance Centre (SEAC).

S.F., A.A., and A.J.M. conceived and designed the experiments. S.F. and N.C. performed the experiments. S.F., N.C., A.A., and A.J.M. analyzed the data. H.M. performed the statistical modelling. S.F. and A.J.M. wrote the paper.

This project was funded by Unilever's Safety and Environmental Assurance Centre (SEAC).

REFERENCES

1. Hutchinson GR, Parker S, Pryor JA, Duncan-Skingle F, Hoffman PN, Hodson ME, Kaufmann ME, Pitt TL. 1996. Home-use nebulizers: a potential primary source of *Burkholderia cepacia* and other colistin-resistant, gram-negative bacteria in patients with cystic fibrosis. J Clin Microbiol 34:584–587.
2. Finch J, Prince J, Hawksworth M. 1978. A bacteriological survey of the domestic environment. J Appl Bacteriol 45:357–364. https://doi.org/10.1111/j.1365-2672.1978.tb04236.x.
3. Levin MH, Olson B, Nathan C, Kabins S, Weinstein R. 1984. *Pseudomonas* in the sinks in an intensive care unit: relation to patients. J Clin Pathol 37:424–427. https://doi.org/10.1136/jcp.37.4.424.
4. Perryman FA, Flournoy D. 1980. Prevalence of gentamicin-and amikacin-resistant bacteria in sink drains. J Clin Microbiol 12:79–83.
5. Francis GA, Thomas C, O'beirne D. 1999. The microbiological safety of minimally processed vegetables. Int J Food Sci Technol 34:1–22. https://doi.org/10.1046/j.1365-2621.1999.00253.x.
6. Yoder J, Roberts V, Craun GF, Hill V, Hicks L, Alexander NT, Radke V, Calderon RL, Hlavsa MC, Beach MJ. 2008. Surveillance for waterborne disease and outbreaks associated with drinking water and water not intended for drinking–United States, 2005–2006. MMWR Surveill Summ 57:39–62.
7. Anderl JN, Franklin MJ, Stewart PS. 2000. Role of antibiotic penetration

limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antimicrob Agents Chemother 44:1818 –1824. https://doi.org/10.1128/AAC.44.7.1818-1824.2000.
8. Anderl JN, Zahller J, Roe F, Stewart PS. 2003. Role of nutrient limitation and stationary-phase existence in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antimicrob Agents Chemother 47:1251–1256. https://doi.org/10.1128/AAC.47.4.1251-1256.2003.
9. Gilbert P, Maira-Litran T, McBain AJ, Rickard AH, Whyte FW. 2002. The physiology and collective recalcitrance of microbial biofilm communities. Adv Microb Physiol 46:203–256. https://doi.org/10.1016/S0065-2911(02)46005-5.
10. Davison J. 1999. Genetic exchange between bacteria in the environment. Plasmid 42:73–91. https://doi.org/10.1006/plas.1999.1421.
11. Cowley N, Forbes S, Amézquita A, McClure P, Humphreys G, McBain AJ. 7 August 2015. The effect of formulation on microbicide potency and mitigation of the development of bacterial insusceptibility. Appl Environ Microbiol https://doi.org/10.1128/AEM.01985-15.
12. Chuanchuen R, Beinlich K, Hoang TT, Becher A, Karkhoff-Schweizer RR, Schweizer HP. 2001. Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects nfxB mutants overexpressing MexCD-OprJ. Antimicrob Agents Chemother 45:428–432. https://doi.org/10.1128/AAC.45.2.428-432.2001.
13. Walsh SE, Maillard J-Y, Russell A, Catrenich C, Charbonneau D, Bartolo R. 2003. Development of bacterial resistance to several biocides and effects on antibiotic susceptibility. J Hosp Infect 55:98 –107. https://doi.org/10.1016/S0195-6701(03)00240-8.
14. Ioannou CJ, Hanlon GW, Denyer SP. 2007. Action of disinfectant quaternary ammonium compounds against *Staphylococcus aureus*. Antimicrob Agents Chemother 51:296 –306. https://doi.org/10.1128/AAC.00375-06.
15. Bloomfield SF. 2002. Significance of biocide usage and antimicrobial resistance in domiciliary environments. J Appl Microbiol 92:144S–157S. https://doi.org/10.1046/j.1365-2672.92.5s1.15.x.
16. Jones M, Herd T, Christie H. 1989. Resistance of *Pseudomonas aeruginosa* to amphoteric and quaternary ammonium biocides. Microbios 58:49–61.
17. Nagai K, Murata T, Ohta S, Zenda H, Ohnishi M, Hayashi T. 2003. Two different mechanisms are involved in the extremely high-level benzalkonium chloride resistance of a *Pseudomonas fluorescens* strain. Microbiol Immunol 47:709 –715. https://doi.org/10.1111/j.1348-0421.2003.tb03440.x.
18. Levy SB. 2002. Active efflux, a common mechanism for biocide and antibiotic resistance. J Appl Microbiol 92:65S–71S. https://doi.org/10.1046/j.1365-2672.92.5s1.4.x.
19. Tattawasart U. 2000. Cytological changes in chlorhexidine-resistant isolates of P*seudomonas stutzeri*. J Antimicrob Chemother 45:145. https://doi.org/10.1093/jac/45.2.145.
20. Braoudaki M, Hilton AC. 2004. Adaptive resistance to biocides in *Salmonella enterica* and *Escherichia coli* O157 and cross-resistance to antimicrobial agents. J Clin Microbiol 42:73–78. https://doi.org/10.1128/JCM.42.1.73-78.2004.
21. Bjarnsholt T, Kirketerp-Moller K, Kristiansen S, Phipps R, Nielsen AK, Jensen PO, Hoiby N, Givskov M. 2007. Silver against *Pseudomonas aeruginosa* biofilms. APMIS 115:921–928. https://doi.org/10.1111/j.1600-0463.2007.apm_646.x.
22. McBain AJ, Bartolo RG, Catrenich CE, Charbonneau D, Ledder RG, Price BB, Gilbert P. 2003. Exposure of sink drain microcosms to triclosan: population dynamics and antimicrobial susceptibility. Appl Environ Microbiol 69:5433–5442. https://doi.org/10.1128/AEM.69.9.5433-5442.2003.
23. McBain AJ, Bartolo RG, Catrenich CE, Charbonneau D, Ledder RG, Rickard AH, Gilbert P. 2003. Microbial characterization of biofilms in domestic drains and the establishment of stable biofilm microcosms. Appl Environ Microbiol 69:177–185. https://doi.org/10.1128/AEM.69.1.177-185.2003.
24. Moore LE, Ledder RG, Gilbert P, McBain AJ. 2008. *In vitro* study of the effect of cationic biocides on bacterial population dynamics and susceptibility. Appl Environ Microbiol 74:4825– 4834. https://doi.org/10.1128/AEM.00573-08.
25. Forbes S, Dobson CB, Humphreys GJ, McBain AJ. 2014. Transient and sustained bacterial adaptation following repeated sublethal exposure to microbicides and a novel human antimicrobial peptide. Antimicrob Agents Chemother 58:5809 –5817. https://doi.org/10.1128/AAC.03364-14.
26. Méchin L, Dubois-Brissonnet F, Heyd B, Leveau J. 1999. Adaptation of *Pseudomonas aeruginosa* ATCC 15442 to didecyldimethylammonium bromide induces changes in membrane fatty acid composition and in resistance of cells. J Appl Microbiol 86:859 – 866. https://doi.org/10.1046/j.1365-2672.1999.00770.x.
27. Poole K, Krebes K, McNally C, Neshat S. 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J Bacteriol 175:7363–7372. https://doi.org/10.1128/jb.175.22.7363-7372.1993.
28. Jensen LB, Baloda S, Boye M, Aarestrup FM. 2001. Antimicrobial resistance among *Pseudomonas* spp. and the *Bacillus cereus* group isolated from Danish agricultural soil. Environ Int 26:581–587. https://doi.org/10.1016/S0160-4120(01)00045-9.
29. Agersø Y, Sandvang D. 2005. Class 1 integrons and tetracycline resistance genes in *Alcaligenes*, *Arthrobacter*, and *Pseudomonas* spp. isolated from pigsties and manured soil. Appl Environ Microbiol 71:7941–7947.
30. Langsrud S, Sundheim G, Holck A. 2004. Cross-resistance to antibiotics of *Escherichia coli* adapted to benzalkonium chloride or exposed to stress-inducers. J Appl Microbiol 96:201–208. https://doi.org/10.1046/j.1365-2672.2003.02140.x.
31. Bloomfield SF, Arthur M. 1994. Mechanisms of inactivation and resistance of spores to chemical biocides. Soc Appl Bacteriol Symp Ser 23:91S–104S.
32. Rahim Z, Sanyal S, Aziz K, Huq M, Chowdhury A. 1984. Isolation of enterotoxigenic, hemolytic, and antibiotic-resistant *Aeromonas hydrophila* strains from infected fish in Bangladesh. Appl Environ Microbiol 48:865–867.
33. Popowska M, Rzeczycka M, Miernik A, Krawczyk-Balska A, Walsh F, Duffy B. 2012. Influence of soil use on prevalence of tetracycline, streptomycin, and erythromycin resistance and associated resistance genes. Antimicrob Agents Chemother 56:1434 –1443. https://doi.org/10.1128/AAC.05766-11.
34. Hsu MS, Liao CH, Huang YT, Liu CY, Yang CJ, Kao KL, Hsueh PR. 2011. Clinical features, antimicrobial susceptibilities, and outcomes of *Elizabethkingia meningoseptica* (*Chryseobacterium meningosepticum*) bacteremia at a medical center in Taiwan, 1999 –2006. Eur J Clin Microbiol Infect Dis 30:1271–1278. https://doi.org/10.1007/s10096-011-1223-0.
35. Hsu CH. 2005. Structural and DNA-binding studies on the bovine antimicrobial peptide, indolicidin: evidence for multiple conformations involved in binding to membranes and DNA. Nucleic Acids Res 33:4053. https://doi.org/10.1093/nar/gki725.
36. Romanova N, Wolffs P, Brovko L, Griffiths M. 2006. Role of efflux pumps in adaptation and resistance of *Listeria monocytogenes* to benzalkonium chloride. Appl Environ Microbiol 72:3498 –3503. https://doi.org/10.1128/AEM.72.5.3498-3503.2006.
37. Forbes S, Knight CG, Cowley NL, Amézquita A, McClure P, Humphreys G, McBain AJ. 2016. Variable effects of exposure to formulated microbicides on antibiotic susceptibility in firmicutes and proteobacteria. Appl Environ Microbiol 82:3591–3598. https://doi.org/10.1128/AEM.00701-16.
38. Moore SL, Denyer SP, Hanlon GW, Olliff CJ, Lansley AB, Rabone K, Jones M. 2006. Alcohol ethoxylates mediate their bacteriostatic effect by altering the cell membrane of *Escherichia coli* NCTC. 8196. Int J Antimicrob Agents 28:503–513. https://doi.org/10.1016/j.ijantimicag.2006.08.023.
39. Cutter C, Willett J, Siragusa G. 2001. Improved antimicrobial activity of nisin-incorporated polymer films by formulation change and addition of food grade chelator. Lett Appl Microbiol 33:325–328. https://doi.org/10.1046/j.1472-765X.2001.01005.x.
40. Vaara M. 1992. Agents that increase the permeability of the outer membrane. Microbiol Rev 56:395.
41. Haque H, Russell A. 1974. Effect of ethylenediaminetetraacetic acid and related chelating agents on whole cells of gram-negative bacteria. Antimicrob Agents Chemother 5:447– 452. https://doi.org/10.1128/AAC.5.5.447.
42. Marshall BM, Robleto E, Dumont T, Levy SB. 2012. The frequency of antibiotic-resistant bacteria in homes differing in their use of surface antibacterial agents. Curr Microbiol 65:407– 415. https://doi.org/10.1007/s00284-012-0172-x.
43. Andrews JM. 2001. Determination of minimum inhibitory concentrations. J Antimicrob Chemother 48:5–16. https://doi.org/10.1093/jac/48.suppl_1.5.
44. Andrews JM. 2001. The development of the BSAC standardized method of disc diffusion testing. J Antimicrob Chemother 48(Suppl 1):29 – 42. https://doi.org/10.1093/jac/48.suppl_1.29.
45. Herlemann DP, Labrenz M, Jurgens K, Bertilsson S, Waniek JJ, Andersson AF. 2011. Transitions in bacterial communities along the 2000 km salinity

zam-aem/zam00717/zam7741d17z	xppws	S=3	1/31/17	8:57	4/Color Fig: 2,3,4	ArtID: 03054-16	DOI:10.1128/AEM.03054-16	CE: jtc

gradient of the Baltic Sea. ISME J 5:1571–1579. https://doi.org/10.1038/ismej.2011.41.
46. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336. https://doi.org/10.1038/nmeth.f.303.
47. Masella AP, Bartram AK, Truszkowski JM, Brown DG, Neufeld JD. 2012. PANDAseq: paired-end assembler for Illumina sequences. BMC Bioinformatics 13:31. https://doi.org/10.1186/1471-2105-13-31.
48. Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R. 2010. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26:266 –267. https://doi.org/10.1093/bioinformatics/btp636.
49. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 72:5069 –5072. https://doi.org/10.1128/AEM.03006-05.
50. McMurdie PJ, Holmes S. 2015. Shiny-phyloseq: Web application for interactive microbiome analysis with provenance tracking. Bioinformatics 31:282–283. https://doi.org/10.1093/bioinformatics/btu616.
51. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550. https://doi.org/10.1186/s13059-014-0550-8.

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES 1

AQau—Please confirm the given-names and surnames are identified properly by the colors. = Given-Name, = Surname

AQau—An ORCID ID was provided for at least one author during submission. Please click the name associated with the ORCID ID icon in the byline to verify that the link is working and that it links to the correct author.

AQaff—Please confirm the following full affiliations or correct here as necessary. This is what will appear in the online HTML version:

[a]Biomedical Research Centre, Sheffield Hallam University, Sheffield, United Kingdom
[b]Faculty of Biology, Medicine and Health, The University of Manchester, United Kingdom
[c]Unilever, Safety and Environmental Assurance Centre, Colworth Science Park, Sharnbrook, Bedfordshire, United Kingdom

AQaff—This affiliation line will appear in the PDF version of the article and matches that on page 1 of the proof; corrections to this affiliation line may be made here **or** on page 1 of the proof:

Biomedical Research Centre, Sheffield Hallam University, Sheffield, United Kingdom[a]; Faculty of Biology, Medicine and Health, The University of Manchester, United Kingdom[b]; Unilever, Safety and Environmental Assurance Centre, Colworth Science Park, Sharnbrook, Bedfordshire, United Kingdom[c]

AQfund—The Funding Information below includes information that you provided on the submission form when you submitted the manuscript. This funding data will not appear in the manuscript, but it will be provided to CrossRef in order to make the data publicly available. Therefore, please check it carefully for accuracy and mark any necessary corrections. Statements acknowledging financial support may also appear within the manuscript itself (in Acknowledgments); any such statements should also be checked for accuracy, but will have no bearing on funding data deposited with CrossRef.

Funder	Grant(s)	Author(s)	Funder ID

AQA—The Materials and Methods section has been moved to the end of the text per ASM style. To ensure sequential order, references have been renumbered in the text and References. Please check and correct the renumbering if necessary. If any reference should be deleted from the References list, please mark "Reference deleted" in the margin next to that entry; do not renumber subsequent references.

AQB—Please check introductions of abbreviations throughout.

AQC—Please explain in footnote a of Table 4 what the numbers in parentheses in the cell entries represent.

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES **2**

AQD—Please check corrected genus and species names throughout. AQE—

If "E value, 250,000" is not as meant for "e 250,000," please clarify.

AQF—Please clarify what is meant by "under different?" Also, if "(vi)" is not as meant for "(xi)" in the last sentence in the paragraph, please clarify.

AQG—"Table S1" has been changed to "data not shown" here (the supplemental material was not accepted by the ASM editor).