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Effects of Quaternary-Ammonium-Based Formulations on Bacterial Community Dynamics and Antimicrobial Susceptibility

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Quaternary ammonium compounds (QACs) are widely used as adjuncts to hygiene in domestic cleaning products. Current concern that the increased use of such biocides in consumer products might contribute to the emergence of antibiotic resistance has led us to examine the effects of a QAC-containing domestic cleaning fluid on the population dynamics and antimicrobial susceptibility of domestic sink drain biofilm communities. QAC susceptibilities of numerically dominant, culturable drain bacteria (15 genera, 17 species) were determined in vitro before and after repeated QAC exposure (14 passages). A fully characterized drain microcosm was then exposed to short-term (12 days) and long-term (3 months) dosing with a QAC-containing domestic detergent (QD). QAC exposure of isolated cultures caused both increases (three species) and circa twofold decreases (six species) in QAC susceptibility. The susceptibility of *Ralstonia* sp. was considerably decreased following 14 consecutive QAC passages. Control drain microcosm biofilms maintained dynamic stability, as evidenced by culture and denaturing gradient gel electrophoresis (DGGE) analysis. Bacterial population densities were largely unaffected during short-term exposure to use levels of QD, although 50% QD caused circa 10-fold viability reductions. DGGE analysis supported these observations; identified the major microcosm genera as *Pseudomonas*, *Pseudoalteromonas*, *Erwinia*, and *Enterobacter*, and showed that aeromonads increased in abundance under 10 to 50% QD. Long-term exposure of the microcosms to QD did not significantly alter the pattern of antimicrobial susceptibility. These data demonstrate the recalcitrance of domestic drain biofilms toward QAC and that although repeated QAC exposure of drain isolates in pure culture results in susceptibility change in some test bacteria, such changes do not necessarily occur within complex communities.

Quaternary ammonium compounds (QACs) are amphoteric surfactants that are widely used for the control of bacterial growth in clinical and industrial environments (6). Broad-spectrum antimicrobial activity (43) and surfactant properties have made QACs such as benzalkonium chloride the favored hygienic adjuncts in disinfectant cleansing formulations, and they have been increasingly deployed in domestic cleaning products over the last decade (5, 16).

The antimicrobial action of QACs involves perturbation of cytoplasmic and outer membrane lipid bilayers through association of the positively charged quaternary nitrogen with the polar head groups of acidic phospholipids (17). The hydrophobic tail subsequently interacts with the hydrophobic membrane core. At concentrations normally used for application to inanimate surfaces, QACs form mixed-micelle aggregates with hydrophobic membrane components that solubilize membrane and lyse the cells (41). Lethality occurs through generalized and progressive leakage of cytoplasmic materials (21).

Reports that the hydroxydiphenylether biocide triclosan (TCS) can select for mutants of *Escherichia coli* that exhibit TCS tolerance (32, 42) have raised the possibility that other biocides might contribute to the emergence of resistance (16, 40). QACs have been actively deployed since the 1930s with no apparent reduction in their effectiveness (12). Numerous pure-culture laboratory studies have, however, reported apparent decreases in the susceptibility of repeatedly exposed bacteria (1, 3, 35, 38, 44). The reported changes in susceptibility are generally attributable to modification of the acidic phospholipid content of the membrane (13, 51) or the acquisition or hyperexpression of certain multidrug efflux pumps (24). The genetic determinants of insusceptibility are in some cases mobile. For example, Bjorland et al. (3) have shown that a novel plasmid-born gene, *qacJ*, mediates QAC resistance in various equine staphylococci.

Efflux pumps can actively remove QAC from the membrane core, thereby reducing effectiveness at sub-MICs. The multidrug efflux pumps QacA to -G, for example, contribute to biocide nonsusceptibility in *Staphylococcus aureus* (39), while many pseudomonads are nonsusceptible to a range of biocides by virtue of expression of efflux pumps (9, 23).

Since multidrug efflux pump expression has been associated with changes in the MICs of therapeutically important antibiotics, there is the theoretical potential for decreases in QAC susceptibility to be accompanied by potentially more serious changes in the effectiveness of clinically important antimicrobials (4, 15, 23). A number of studies, however, have reported that no such associations exist. These studies include quantification of antibiotic resistance of bacteria with acquired QAC resistance (22, 25) and surveys of large numbers of bacteria

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isolated from homes that use or abstain from household antibacterial products (12). Loughlin et al. (25), for example, reported that *P. aeruginosa*, when adapted to benzalkonium chloride, showed decreased susceptibility to other membrane-active agents but not to clinically relevant antibiotics.

The use of QACs in the home will expose a wide range of environmental and potentially pathogenic bacteria to these biocides, often at sublethal concentrations, making the home a potentially high-risk environment for resistance selection.

The aim of the present study was to investigate the impact of QAC-containing detergent (QD) on the microbial ecology and antibiotic and biocide susceptibility properties of domestic kitchen drain biofilms. Kitchen sink drain outlets are a site of considerable bacterial colonization in the home (27) and also an environment that is commonly exposed to biocides at sublethal concentrations (28).

The effect of repeated QAC exposure on the susceptibility of numerically important culturable drain bacteria was studied in pure culture. Effects of QAC exposure on biofilm communities were then investigated with a drain biofilm microcosm previously characterized and validated for stability and utility (27). These microcosms have previously been used to model the effects of TCS exposure in the home (28) and of biocide exposure of dental plaque communities (29, 30). Differential plate counts and culture-independent methods (denaturing gradient gel electrophoresis [DGGE]), in conjunction with sequencing (31), were used to characterize the developed communities and the effects of QD exposure therein. MICs of the biocides cetrimide; chloro-3,5-dimethylphenol, chlorhexidine, and TCS and the antibiotics chlortetracycline, ciprofloxacin, erythromycin, fusidic acid, penicillin, and vancomycin were determined against the numerically dominant aerobic and facultative clones isolated both from control microcosms and following 3 months of continuous QD exposure.

MATERIALS AND METHODS

Exposure of test bacteria to QACs. A stock solution (4 mg/ml) of a proprietary QAC, Bardac (Lonza Group, Fair Lawn, N.J.) was prepared as recommended by the manufacturer, in deionized, distilled water; sterilized by filtration through cellulose acetate filters (0.2-μm pose size; Millipore); and then stored at −70°C. A model CU spiral plater (Spiral Systems) was used to prepare gradient plates of Bardac. This apparatus can deposit accurate volumes of fluids onto the surface of an agar plate such that either a uniform deposition density or a continuously varying dilution is established across the radius of the plate with a very high degree of reproducibility (50). Petri dishes (10-cm diameter) were filled with 27.5 ± 1.0 ml of R2A agar to produce a mean agar depth of approximately 3.5 mm. The plates were kept for 2 days at room temperature prior to use to ensure dryness of the agar surface. Bardac (50 μl) was then deposited onto the agar surface with the variable cam of the spiral plater. This establishes a concentration gradient covering a circa 1,000-fold dilution series.

The following bacteria, isolated from a domestic kitchen sink drain biofilm by methods previously described (27), were used: *Aeromonas hydrophila* MBRG 4.3, *Aeromonas* sp. strain MBRG 4.2, *Ralstonia* sp. strain MBRG 4.13, *Aranicola proteolyticus* MBRG 20.1, *Stenotrophomonas maltophilia* MBRG 4.17, *Pseudomonas* sp. strain MBRG 4.7, *Eubacterium* sp. strain MBRG 4.14, *Chryseobacterium* sp. strain MBRG 4.29, *Achromobacter xylosoxidans* MBRG 4.31, *Pseudomonas nitroreducens* MBRG 4.6, *Enterococcus saccharolyticus* MBRG 20.4, *Chryseobacterium* sp. strain MBRG 4.28, *Bacillus cereus* MBRG 4.21, *Citrobacter* sp. strain MBRG 20.9, *Sphingobacterium multivorum* MBRG 30.1, *Pseudoxanthomonas* sp. strain MBRG 40.1, and *Microbacterium phyllosphaerae* MBRG 4.30. Overnight batch cultures of these isolates (20 μl) were deposited in radial lines across the plates.

After incubation, growth observed near the endpoint in the transition area was aseptically removed and transferred to the next QAC gradient plate at the same concentration. This process was continued until 14 passages had been completed. After 7 (P7) and 14 (P14) QAC passages, the bacteria were archived at −70°C for subsequent MIC and minimal bactericidal concentration (MBC) testing.

Domestic drain microcosms. Domestic drain microcosms were established as described previously (27, 28). Briefly, drain biofilm (2.5 g) was macerated with a sterile mortar and pestle and diluted 1:10 in sodium phosphate buffer (22.5 ml, 0.1 M, pH 6.5) containing 0.45% (wt/vol) NaCl which had been prereduced (boiled for 5 min and cooled under a constant stream of anaerobic gas [5:95 CO_2/N_2 ratio]). The samples were homogenized for 1 min in a flask shaker (Griffin, London, England) in the presence of approximately five glass beads (3.5 to 5.5 mm; BDH, Poole, United Kingdom). The diluted material from samples was used to inoculate two constant-depth film fermentors (CDFFs). Initially, short-term microcosms were established to study the lethality of QD. For these experiments, growth medium was continuously added to the fermentors by a peristaltic pump (Gilson) at a rate of 5 ml/h. The components of the medium intended to simulate dish washing water were as follows (grams per liter): starch, 1.0; peptone, 0.5; tryptone, 0.5; yeast extract, 0.5; NaCl, 1.0; margarine (Flora; Unilever, Crawley, United Kingdom), 0.05; hemin, 0.001; tomato ketchup (Heinz, Uxbridge, United Kingdom), 0.05. For long-term microcosm studies, the fermentors were inoculated a total of twice 7 days apart with additional resampled, homogenized, but undiluted drain biofilm. Anaerobiosis was maintained for the first 48 h by continuous gassing with oxygen-free gas (5:95 CO_2/N_2 ratio) at 1 liter/h, and the temperature was uncontrolled (the ambient laboratory temperature ranged from 18 to 24°C). Biofilms were shielded from light by covering the CDFFs with aluminum foil shrouds. Throughout, the microcosms were maintained on a feast-famine regimen (four times daily 20-min perfusion, 0.5 ml/min, of synthetic dishwater) as described above but supplemented with domestic detergent (Fairy Original; Procter & Gamble, Newcastle Upon Tyne, United Kingdom) at 0.05 g/liter.

Discontinuous feeding regimens were controlled with programmable electronic timers (Micromark, London, United Kingdom). In order to model more accurately the open nature of a domestic sink drain, the fermentor pans were continuously wetted with untreated tap water (1 ml/h). CDFFs enable the continuous culture of biofilms at accurately set depth (26–31, 37). In all cases, the biofilm (Teflon plug) depth was set at 5.0 mm. Developed communities were characterized periodically over the course of the investigation, immediately by culture, and samples were archived for subsequent DGGE.

Addition of QD to microcosms. For short-term lethality studies, dilutions of a QAC-containing detergent (Lysol all-purpose cleaning liquid [Reckitt and Colman Inc., Wayne, N.J.]) were added to established drain microcosms by a peristaltic pump for 10 min at 6-h intervals at a flow rate of 55.2 ml/h. For long-term studies, duplicate microcosms were stabilized for 6 months, after which the Lysol liquid was substituted for the domestic detergent in the artificial dishwater supplied to one at 0.2% (wt/vol) for 3 months.

Bacterial characterization by culture. Drain biofilm material (1.0 g) or CDFF plugs (two) were macerated with a sterile mortar and pestle, homogenized, and diluted 1:10 (as described above). For enumeration, dilutions of macerated drain or model biofilm (1:10) were serially diluted with prereduced half-strength peptone-water (7.5 g/liter). During long-term experiments, in order to minimize variation due to sampling of immature biofilms, only those CDFF pans that had been in situ for at least 1 month were removed for analysis. Aliquots (0.1 ml) of appropriate dilutions were plated in triplicate onto a variety of selective and nonselective media (Oxoid, Basingstoke, United Kingdom) as follows: Wilkins-Chalgren agar (anaerobic and facultative heterotrophic counts), R2A (aerobic and facultative heterotrophic counts), pseudomonas isolation agar with C-N selective supplements (*Pseudomonas aeruginosa*), and MacConkey agar no. 3 (enteric organisms).

Plates were incubated for up to 5 days, both aerobically and in an anaerobic cabinet (atmosphere, 10:10:80 H_2-CO_2-N_2). Criteria used for selecting bacterial populations for use as markers of population change in the biofilm communities included numerical dominance, ease of selective cultivation, and visual recognition. Morphologically distinct colonies were subcultured and archived (−60°C) for subsequent identification on the basis of morphology, oxidase test, Gram reaction, and rRNA gene sequencing.

Direct bacterial cell counts. The proportion of the viable bacterial communities that could be cultured by the methods used was estimated by comparison with vital staining and direct microscopy. A subsample (100 μl) of the 10^{-2} or 10^{-3} dilution (prepared for viable-count determination) was stained with a live-dead bacterial viability kit (BacLight; Molecular Probes, Leiden, The Netherlands) and counted with an improved Neubauer counting chamber in conjunction with fluorescence microscopy with a 100-W mercury vapor lamp. Live (green fluorescent) and dead (red fluorescent) cells were visualized separately with fluorescein and Texas red band-pass filters, respectively, in accordance with the manufacturer's instructions.

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TABLE 1. MICs and MBCs for selected domestic drain isolates before and after repeated exposure to a commercial QAC[a]

Isolate[b]	MIC (mg/liter)			MBC (mg/liter)		
	Before	P7	P14	Before	P7	P14
Aeromonas hydrophila M 4.3	15.6	15.6	15.6	31.2	41.6 (18)	31.2
Aeromonas jandei M 4.2	15.6	15.6	15.6	52 (18)	31.2	83.3 (36)
Ralstonia sp. strain M 4.13	15.6	31.2	125	125	104.1 (36)	187 (108.2)
Aranicola proteolyticus M 20.1	7.8	31.2	31.2	48.4 (23.8)	83.3 (36)	83.3 (36)
Stenotrophomonas maltophilia M 4.17.	7.8	7.8	7.8	31.2	31.2	31.2
Pseudomonas sp. strain M 4.7	15.6	15.6	31.2	52 (63.1)	88.5 (63.1)	72.9 (47.7)
Eubacterium sp. strain M 4.14	15.6	15.6	31.2	20.8 (9)	31.2	31.2
Chryseobacterium indologenes M 4.29	15.6	15.6	15.6	31.2	26 (9)	31.2 (27)
Achromobacter xylosoxidans M 4.31	3.9	3.9	3.9	3.9	3.9	7.8
Pseudomonas nitroreducens M 4.6	15.6	7.8	7.8	57.2 (59.1)	31.2 (27)	57.2 (59.1)
Enterococcus saccharolyticus M 20.4	31.2	7.8	7.8	25.0	62.5	62.5
Chryseobacterium sp. strain M 4.28	3.9	3.9	15.6	3.9	3.9	41.6 (18)
Bacillus cereus M 4.21	3.9	3.9	3.9	31.2	31.2	62.5
Citrobacter sp. strain M 20.9	7.8	7.8	7.8	10.4 (4.5)	44.2 (31.5)	7.8
Sphingobacterium multivorum M 30.1	3.9	1.9	7.8	3.9	1.9	62.5
Pseudoxanthomonas sp. strain M. 40.1	7.8	3.9	3.9	15.6	3.9	3.9
Microbacterium phyllosphaerae M 4.30	3.9	3.9	3.9	14.3 (14.7)	15.6 (13.5)	7.8

[a] Data were determined by broth dilution endpoint. Data show means from duplicate experiments. Where data varied between replicates, standard deviations are given in parentheses.
[b] M, MBRG.

Antimicrobial susceptibility of isolated drain bacteria. Stock solutions (4 mg/ml) of the following compounds were prepared as indicated by the manufacturers, in deionized, distilled water: penicillin V, erythromycin, chlorhexidine, ciprofloxacin, fusidic acid, vancomycin, and DMP. TCS and chlortetracycline were dissolved in 25% ethanol. All solutions were sterilized by filtration through cellulose acetate filters (0.2-μm pore size; Millipore) and stored at −70°C. A model CU spiral plater (Spiral Systems) was used for spiral gradient MIC endpoint determinations. Stock solutions (50 μl) of the antimicrobial compounds were then deposited onto the agar surface with the variable cam of the spiral plater, as described above, to establish a concentration gradient covering a 1,000-fold dilution series (50). Control plates comprised identical agar plates with no added antimicrobial. Plates were dried for up to 1 h at room temperature prior to deposition of inoculate along the spiral track with the uniform cam of the spiral plater, After further drying (1 h), plates were inverted and incubated aerobically overnight at 37°C. The radial location of the growth endpoint from the center of the spiral was measured with calibrated calipers. MICs were determined on the basis of inhibition zones with the equation $P = (RP \times DF)/(h \times SR)$, where P (potency) is the effective antimicrobial concentration at the interface between growth and inhibition (micrograms per milliliter), RP is the original antimicrobial concentration deposited (micrograms per milliliter), DF is the deposition factor, h is the agar height (millimeters), and SR is the surface ratio.

Community DNA extraction. Archived biofilm material (0.2 to 0.5 g) was mixed with 1 ml of Tris buffer (0.12 M, pH 8.0), vortex mixed, and subjected to two cycles of freezing and heating (−60°C for 10 min, 60°C for 2 min). Samples were then transferred to a bead beater vial containing 0.3 g of sterile zirconia beads (0.1-mm diameter). Tris-equilibrated phenol (pH 8.0; 150 μl) was added, and the suspension was shaken three times for 80 s at maximum speed (Mini-Bead-Beater; Biospec Products, Bartlesville, Okla.). After 10 min of centrifugation at 13, 000 × *g*, the supernatant was extracted three times with an equal volume of phenol-chloroform and once with chloroform-isoamyl alcohol (24:1 [vol/vol]). The DNA was precipitated from the aqueous phase with 3 volumes of ethanol, air dried, and resuspended in 100 μl of deionized water. The amount and quality of DNA extracted were estimated by electrophoresis of 5-μl aliquots on a 0.8% agarose gel and by comparison to a molecular weight standard (stained with ethidium bromide). DNA extracts were stored at −60°C prior to analysis.

PCR amplification for DGGE analysis. The V2-V3 region of the 16S rRNA gene (corresponding to positions 339 to 539 of *Escherichia coli*) was amplified with the eubacterium-specific primers HDA1-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3′) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′) as previously described (49). The reactions were performed in 0.2-ml tubes with a DNA thermal cycler (model 480; Perkin-Elmer, Cambridge, United Kingdom). In all cases, reactions were carried out with Red *Taq* DNA polymerase ready mix (25 μl; Sigma, Poole, Dorset, United Kingdom), HDA primers (2 μl of each, 5 μM), nanopure water (16 μl), and extracted community DNA (5 μl). Optimization studies, as described by Muyzer et al. (34), showed that extracted community DNA required a minimum of a 1:10 dilution to ensure reliable PCRs. Quantification and standardization of extracted DNA were achieved with a fluorescence assay (DNA Quantitation Kit; Sigma), in accordance with the manufacturer's instructions. The thermal program was as follows: 94°C (4 min), followed by 30 thermal cycles of 94°C (30 s), 56°C (30 s), and 68°C (60 s). The final cycle incorporated a 7-min chain elongation step (68°C).

DGGE analysis. Biofilm samples were analyzed by DGGE with a D-Code universal mutation detection system (Bio-Rad, Hemel Hempstead, United Kingdom). Polyacrylamide (8%) gels (16 by 16-cm, 1 mm deep) were run with 1× TAE buffer diluted from 50× TAE buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA). Initially, separation parameters were optimized by running PCR products from selected pure cultures of drain bacteria and PCR amplicons from extracted drain DNA on gels with a 0 to 100% denaturation gradient, perpendicular to the direction of electrophoresis (a 100% denaturing solution contained 40% [vol/vol] formamide and 7.0 M urea). Denaturing gradients were formed with two 10% acrylamide (acrylamide/bisacrylamide ratio, 37.5:1) stock solutions (Sigma). On this basis, a denaturation gradient for parallel DGGE analysis ranging from 30 to 60% was selected for community analyses. DNA for loading onto gels was quantified and, when necessary, standardized between samples, with a fluorescence assay (see above). Electrophoresis was carried out at 150 V and 60°C for approximately 4.5 h. All gels were stained with SYBR Gold stain (diluted to 10^{-4} in 1× TAE buffer; Molecular Probes, Leiden, The Netherlands) for 30 min. Gels were viewed and images were documented with a BioDocit system (UVP).

Sequencing of bacterial isolates and excised gel bands. For analysis of the major resolved DGGE amplicons, selected resolved bands were cut out of the polyacrylamide gels with a sterile scalpel under UV illumination and incubated at 4°C for 20 h together with 20 μl of nanopure water in nuclease-free universal bottles. Portions (5 μl) were removed and used as a template for a PCR identical to that outlined for DGGE analysis. PCR products were purified with QIAquick PCR purification kits (Qiagen Ltd., West Sussex, United Kingdom) and sequenced with the reverse (non-GC clamp) primer (HDA2). The sequencing was 94°C (4 min) followed by 25 cycles of 96°C (30 s), 50°C (15 s), and 60°C (4 min). Once chain termination was complete, sequencing was done in a Perkin-Elmer ABI 377 sequencer. DNA sequences were compiled with GENETOOL LITE 1.0 (DoubleTwist.com) to obtain consensus sequences, or to check and edit unidirectional sequences. For excised DGGE band PCRs, the presence of a GC clamp upon sequence analyses confirmed that the correct target had been reamplified, rather than a contaminant.

Phylogenetic identification of DGGE amplicons. BLAST searches were performed, with each compiled sequence, against those in the European Molecular Biology Laboratories (EMBL) prokaryote database. Closest relative species were assigned on the basis of compiled partial 16S rRNA gene sequence comparisons with BLAST against sequences in the EMBL database.

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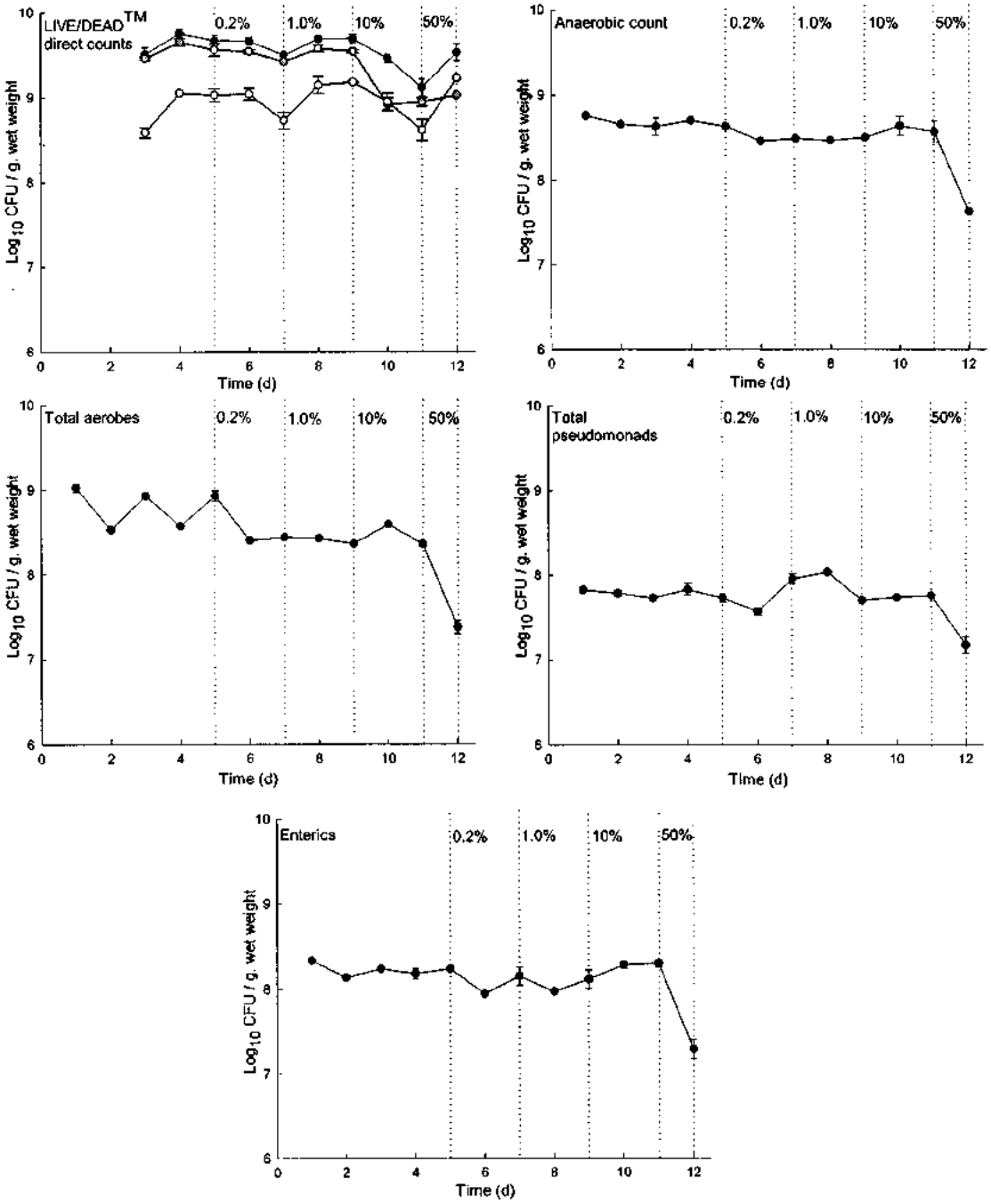

FIG. 1. Bioburdens, direct counts, and viable counts of selected groups of drain bacteria in the simulator before and during product addition. Data are means ± standard deviations from two separate sample pans. The schedule of QD addition is shown. Graphs show 5 days prior to dosing and then successive 2-day exposures to 0.2, 1.0, 10, and 50% QD. Symbols for LIVE/DEAD counts: ●, total counts; ◍, live-cell counts; ○, dead-cell counts.

Chemicals. Bardac (a commercial twin-chain dimethyl ammonium chloride) was obtained from Lonza Group. Lysol disinfectant, whose active ingredients (at 2.7% [vol/vol]) are dimethyl benzyl ammonium chlorides with chain lengths of C_{14} (50%), C_{12} (40%), and C_{16} (10%), is manufactured by Reckitt and Colman. Formulated bacteriological media were purchased from Oxoid. TCS (Irgasan DP300) was obtained from Oils and Soaps Ltd., Bradford, United Kingdom. Unless otherwise stated, all other chemicals and antimicrobial agents were obtained from Sigma.

Statistical analyses. Individual MIC measurements for randomly isolated bacteria before and after 3 months of QD exposure were grouped as total bacteria and arranged in distribution tables. These data were then subjected to χ^2 analysis with a Microsoft Excel macro.

RESULTS AND DISCUSSION

Most research into biocide resistance development has used pure cultures. Few have studied the effects of biocides on the resistance properties and population dynamics of bacterial

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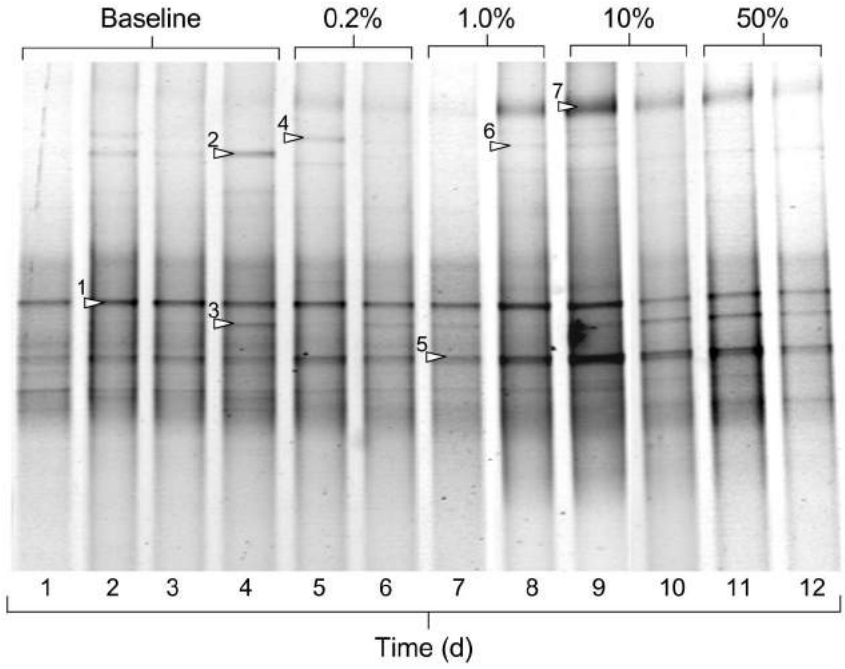

FIG. 2. Negative image of a parallel DGGE gel showing community fingerprints for drain microcosm samples before QD addition and after 2-day exposures to QD at 0.2, 1.0, 10, and 50% (vol/vol).

communities, where competition and other community phenomena might dictate against susceptibility changes with associated fitness costs (19, 26). In this study, we have investigated the effects of QACs on domestic sink drain bacteria with microcosms and pure cultures derived from them. By these approaches, it is possible to more accurately characterize the theoretical risks, derived from pure-culture resistance experiments, by using microcosms that more accurately simulate environmental conditions.

Effect of repeated QAC exposure of isolated drain bacteria. Susceptibility data (MICs and MBCs) for 17 drain biofilm isolates, determined before and after 7 and 14 QAC passages, are shown in Table 1. On the basis of MIC determinations, marked (more-than-twofold) decreases in QAC susceptibility occurred for *Ralstonia* sp., *A. proteolyticus*, and *Chryseobacterium* sp. Similar MBC increases occurred for *Chryseobacterium* sp. and *S. multivorum*. Interestingly, QAC exposure caused marked increases in susceptibility for *E. saccharolyticus*, *Citrobacter* sp., *Pseudoxanthomonas* sp., and *M. phyllosphaerae*. This probably relates to cellular injury. Previous pure-culture laboratory investigations have reported similar alterations in susceptibility for a variety of biocidal compounds and many types of test bacteria (10, 25, 33, 38). Few papers, however, have reported an increased frequency of QAC resistance in organisms isolated from highly exposed environments. For example, a recent study examined 60 households, half of which were defined as heavy biocides users and half of which were not, and no association was found between QAC use and resistance (12).

Effect of QD on the viability and vitality of bacterial drain microcosms. The microcosm detailed here has previously been validated as a stable laboratory model of domestic drain biofilm. It provides for the long-term maintenance and laboratory analysis of this ecosystem, which is a major site of bacterial colonization in the home (7, 11, 14, 27). We used differential culture to characterize community effects of QD, together with vital direct live-dead counts. CFU counts were circa 10-fold lower than direct counts (Fig. 1). This could indicate either failure to culture portions of bacterial diversity present within the microcosms or variable plating efficiencies of culturable

TABLE 2. Characterization of dynamic changes in microcosms on the basis of sequences of dominant PCR amplicons derived from DGGE gels at baseline and during escalating QD exposure

DGGE amplicon	Size (bp)	Ambiguity %	Closest relative (% sequence similarity)[a]
1	180	5.6	*Pseudoalteromonas* sp. strain AF172987 (89)
2	173	12.7	*Acinetobacter baumannii* AY269254 (97)
3	177	15.1	*Erwinia alni* AJ233409 (78)
4	173	4.0	*Enterobacter aerogenes* AY186054 (90)
5	179	1.1	*Enterobacter* sp. strain AY191239 (99)
6	169	4.1	*Enterobacter* sp. strain AY191239 (99)
7	187	3.7	*Aeromonas* sp. strain AY379975 (91)

[a] Identities based on BLAST database.

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TABLE 3. Distribution of bacterial drug susceptibilities in the microcosm before and after 3 months of QD exposure[a]

Antimicrobial	No. of clones tested		No. (%) of clones for which MIC (mg/liter) was:									
			0.01–0.1		0.11–1.0		1.1–10.0		10.1–100		(>101–nonsusceptible)	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
CM	48	13	3 (6)	1 (8)	1 (2)	2 (15)	29 (60)	8 (61)	3 (6)	0 (0)	12 (25)	2 (15)
E	48	13	8 (17)	3 (23)	0 (0)	0 (0)	20 (44)	1 (8)	0 (0)	1 (8)	20 (44)	8 (61)
CHX	48	13	3 (6)	1 (8)	4 (8)	6 (46)	28 (58)	3 (23)	0 (0)	0 (0)	13 (27)	3 (23)
CP	48	13	38 (79)	11 (83)	2 (4)	0 (0)	3 (6)	1 (8)	0 (0)	0 (0)	5 (10)	1 (8)
CT	48	13	22 (49)	7 (54)	5 (10)	1 (2)	11 (23)	5 (38)	4 (8)	0 (0)	6 (13)	0 (0)
DMP	48	13	3 (6)	2 (15)	5 (10)	2 (15)	15 (31)	1 (8)	1 (2)	0 (0)	24 (50)	8 (61)
FA	48	13	5 (10)	6 (46)	3 (6)	0 (0)	18 (38)	6 (46)	3 (6)	0 (0)	19 (40)	1 (8)
PV	48	13	5 (10)	1 (8)	0 (0)	1 (8)	18 (38)	0 (0)	0 (0)	0 (0)	25 (52)	11 (84)
TCS	48	13	6 (13)	8 (17)	1 (2)	2 (15)	16 (33)	3 (6)	1 (2)	0 (0)	24 (50)	0 (0)
V	48	13	4 (8)	2 (15)	0 (0)	2 (15)	25 (52)	3 (23)	3 (6)	0 (0)	16 (33)	6 (46)

[a] Susceptibilities were determined by agar dilution endpoint (duplicate determinations for each isolate) for randomly isolated cell clones at baseline and following 3 months of QD exposure. CM, cetrimide; E, erythromycin; CHX, chlorhexidine; CP, ciprofloxacin; CT, chlortetracycline; DMP, chloro-3,5-dimethylphenol; FA, fusidic acid; PV, penicilin V; V, vancomycin. No significant increase in susceptibility occurred for any agent with respect to distributions of susceptibility, as determined by χ^2 analysis.

bacteria. Previous work with this microcosm has confirmed the latter scenario (27, 28). Figure 1 shows viable and direct counts for short-term (12 days) exposure of the microcosms to QAC-containing detergent (QD). Direct vital and differential viable counts showed that the microcosms achieved dynamic stability prior to QD addition. Total vital counts showed that QD concentrations above 10% lowered bacterial viability circa 1 order of magnitude, although by culture, this effect was only apparent at 50% QD, where 100-fold reductions in strict anaerobes, facultative anaerobes, pseudomonads, and enteric species were apparent. The failure of normal-use levels of QD to display marked antibacterial activity may be attributed to several factors. (i) Many numerically important drain bacteria were nonsusceptible to QAC. QAC tolerance has been described previously (22) and relates to the physiology of the bacteria (48), particularly the expression of efflux pumps (8, 9). (ii) Another possibility is the acknowledged nonsusceptibility of biofilms to antimicrobials (2, 18, 28, 45). (iii) Furthermore, microbial communities such as those established in the microcosms might physically bind or chemically degrade QAC, thereby ameliorating or considerably reducing lethality. In these respects, the degradation of the QAC benzyldimethylalkylammonium chloride has been reported for *Aeromonas hydrophila* (36), and since *A. hydrophila* was a major species within the drain microcosms, QAC degradation is a possibility.

DGGE analysis of short-term microcosms. In an attempt to characterize the composition of the microcosms, including potentially nonculturable bacteria, and to better understand dynamic changes that may occur following QAC dosing, microcosm biofilm samples were analyzed by PCR-DGGE. Figure 2 shows DGGE fingerprints from extracted community DNA. PCR-DGGE was done with eubacterium-specific primers (V2-V3 region of the 16S rRNA gene). This analysis indicated that microcosms maintained dynamic stability prior to addition of QD and that biocide exposure, increasing from 0.2 to 50% over 8 days, did not markedly affect DGGE fingerprints. Data in Table 2 show closest relatives on the basis of BLAST searches with DNA sequences obtained from major DGGE gel bands. The dominant phylotypes before and during QD addition were related to *Pseudoalteromonas* sp. (band 1), *Acinetobacter baumannii* (band 2), *Erwinia alni* (band 3), *Enterobacter aerogenes* (band 4), and *Enterobacter* sp. (bands 5 and 6). QD exposure at 10% caused putative clonal expansion of a bacterium with homology to *Aeromonas* sp. (Fig. 2; Table 2), a genus known to be relatively nonsusceptible to a range of antimicrobials (28). These data highlight the lack of general antibacterial lethality of the formulation on the microcosm community, since very few changes occurred that could be unambiguously attributed to the action of QD, despite the fact that biocide exposure was substantial. An issue of concern when using DGGE to monitor dynamic changes in microbial ecosystems is the detection of nonviable organisms. In this respect, meaningful real-time monitoring depends on the rapid turnover of dead cells and the degradation of associated DNA within the test community. We have, however, previously demonstrated that PCR template turnover within the microcosm is faster than 12 h (27, 28). An advantage of DGGE over techniques such as quantitative Northern hybridization (20) is that the technique will identify any amplifiable target sequence above the detection threshold (34). DGGE is therefore not biased toward those organisms for which probes can be designed, which by definition will normally be limited to those expected to be important in the target community. It should be remembered, however, that PCR-based methods are biased in that the relative concentrations of PCR products do not necessarily properly reflect the composition of the in situ community (47).

Effect of QD on microcosm drug susceptibilities. Kitchen sink drains are arguably one of the most highly biocide-exposed environments, owing to the widespread use of antimicrobial-containing domestic detergents (46). An important part of this study involved determining the effects of QD on bacterial susceptibilities to QAC and selected chemically unrelated antibiotics and biocides. Data in Table 3 show distributions of MICs for total randomly selected bacterial isolates, expressed as frequencies and percent frequencies of MIC ranges, for the microcosm communities before and after 3 months of sustained QD exposure. The concentration of QD used (0.2%) is consistent with normal household use (D. Charbonneau, Procter & Gamble Inc., personal communication). Data show that there was considerable variation in suscepti-

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bility to most of the agents. χ^2 analysis demonstrated that no statistically significant shifts in susceptibility distributions occurred for total bacteria toward any of the antimicrobials tested. This contrasts with the pure-culture data, where repeated QAC exposure caused some changes in QAC susceptibility in certain test bacteria (Table 1). There are several possible reasons for this apparent anomaly. Data in Table 3 demonstrate that up to 25% of the bacteria within unexposed microcosms were nonsusceptible to QAC (cetrimide). Bacteria that are innately nonsusceptible will not decrease in susceptibility under any circumstances. In terms of hitherto susceptible bacteria, it is possible that protection from QAC lethality conferred by the biofilm phenotype (18), as evidenced by data in Fig. 1, reduced the selective pressure. The level of QAC exposure used in these investigations, and also in most domestic kitchens, is also likely to have been below the MICs for many of the susceptible test bacteria. The possibility of biocide binding or inactivation will further reduce these effects. With respect to extrapolation from the pure-culture susceptibility changes to in situ community effects, environmental bacteria may rarely encounter selection pressures as strong as those that can be artificially created in the laboratory. In addition, the level of QD exposure in these studies, although similar to that used in the home, may have been below the normal MICs for many of the susceptible species. Another possibility is that where susceptibility changes did occur, fitness costs may have caused these lineages to be outcompeted, although there is little evidence to support this theory (26).

In conclusion, QD was poorly effective against sink drain biofilms, even at high concentrations. While repeated exposure of drain isolates in pure culture caused alterations in QAC susceptibility, long-term exposure of domestic drain biofilms to sublethal levels of QD did not significantly alter the distribution of susceptibility to QAC or to a range of other biocides and antibiotics. We therefore conclude that QD exposure of domestic microbial communities does not necessarily result in increased antimicrobial resistance.

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