Relationship between antibiotic- and disinfectant-resistance profiles in bacteria harvested from tap water

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Accepted: 19 February 2016

Chemosphere 0045-6535

ABSTRACT:

Chlorination is commonly used to control levels of bacteria in drinking water; however, viable bacteria may remain due to chlorine resistance. What may be concerning is that surviving bacteria, due to co-selection factors, may also have increased resistance to common antibiotics. This would pose a public health risk as it could link resistant bacteria in the natural environment to human population. Here, we investigated the relationship between chlorine- and antibiotic-resistances by harvesting 148 surviving bacteria from chlorinated drinking-water systems and compared their susceptibilities against chlorine disinfectants and antibiotics. Twenty-two genera were isolated, including members of *Paenibacillus*, *Burkholderia*, *Escherichia*, *Sphingomonas* and *Dermacoccus* species. Weak (but significant) correlations were found between chlorine-tolerance and minimum inhibitory concentrations against the antibiotics tetracycline, sulfamethoxazole and amoxicillin, but not against ciprofloxacin; this suggest that chlorine-tolerant bacteria are more likely to also be antibiotic resistant. Further, antibiotic-resistant bacteria survived longer than antibiotic-sensitive organisms when exposed to free chlorine in a contact-time assay; however, there were little differences in susceptibility when exposed to monochloramine. Irrespective of antibiotic-resistance, spore-forming bacteria had higher tolerance against disinfection compounds. The presence of chlorine-resistant bacteria surviving in drinking-water systems may also carry additional risk of antibiotic resistance.

Key words: susceptibility, antimicrobial-resistant bacteria, disinfectant-resistance, drinking-water

INTRODUCTION

Antibiotic-resistant bacteria (ARB) and their genes (ARG) are considered emerging environmental contaminants with a widespread distribution (Pruden et al., 2006, Diehl and Lapara, 2010, Dodd, 2012, Chen et al., 2015) with natural and anthropogenic activities contributing to its development and dispersion in the environment (Allen et al., 2010, Gaze et al., 2011, Wellington et al., 2013) and water bodies (Pruden et al., 2012, Su et al., 2012). As the demand for safe drinking-water increases around the world (Brettar and Hofle, 2008), these compromised natural-water resources could more increasingly become considered as sources of either drinking-water or contamination to the system.

Drinking-water treatment plants use a number of treatment methods to improve water quality: e.g., flocculation, sedimentation, filtration, and disinfection. Among the processes, chemical disinfection contributes greatly to the control of microorganisms from treatment plant to point of use (Berry et al., 2006). However, it has been known that chemical disinfection has limitations in its immediate and prolonged effectiveness, and multiple factors reduce the effectiveness of disinfectants against bacterial populations (Scully et al., 1999, Cherchi and Gu, 2011, Jaglic et al., 2012, Bessa et al., 2014), including the presence of organic matter having amino nitrogen compounds (Scully and Hartman, 1996), bacterial growth phase (Cherchi and Gu, 2011) and the presence of extracellular polymeric matrix (Bridier et al., 2011, Wong et al., 2010).

It has increasingly been discovered that resistance traits horizontally transfer in microbial communities due to either cross-resistance (e.g., efflux mechanisms capable of detoxifying multiple stressors) or co-resistance (e.g., closely linked genetic traits on a mobile genetic element) factors. For example, Templeton et al. (2009) found greater frequency of chlorine tolerance among antibiotic-resistant *E. coli* as compared to antibiotic-sensitive *E. coli* grown in the presence of chlorine (Templeton et al., 2009). Genetic factors, such as class

1 and class 2 integrons that transfer multiple resistance genes could be responsible for such traits (Gillings et al., 2009, Ozgumus et al., 2009, Koczura et al., 2012, Mokracka et al., 2012, Su et al., 2012, Hsu et al., 2014, Chen et al., 2015).

Wastewater treatment studies (Diehl and Lapara, 2010, Burch et al., 2013) have reported decrease in total bacteria, but increased ratio of resistant bacteria (Galvin et al., 2010; Guo et al., 2014; Al-Jassim et al., 2015) following treatment; a similar trend may occur in drinking-water systems (Bergeron et al., 2015). There have been reports of drinking-water treatment plants (DWTP) (Armstrong et al., 1981, Armstrong et al., 1982, Xi et al., 2009, Farkas et al., 2013, Pruden et al., 2006) and water distribution systems (DWDS) (Laroche et al., 2010, Talukdar et al., 2013, Xi et al., 2009) influencing the emergence and spread of antibiotic-resistance. For example, relative abundance of sulfonamide resistance genes increased from 3.5% to 33% in DWTP (Chao et al., 2013) and a broader range of ARGs (Fahrenfeld et al., 2013). Stressful environments such as extreme pH, high salinity, nutrient deprivation (Bessa et al., 2014), oxidation (Scully et al., 1999), or chlorine exposure (Ridgway and Olson, 1982) promote populations with greater resistance. Sub-inhibitory concentrations, not only select resistant populations, but could invoke a stress response which may include genetic exchange.

Bacteria opportunistically colonise water distribution systems (Wang et al., 2013), and water meters (Hong et al., 2010). Additionally, localised disruptions in the distribution mains (e.g., in building cisterns and plumbing) also introduce bacterial populations, which may include agents of waterborne disease and increased health risks and maintenance costs to the system (Falkinham et al., 2015).

This study compares the susceptibilities of bacteria harvested from drinking-water taps to chlorine disinfectants and four antibiotics: tetracycline (TET), sulfamethoxazole (SMX), ciprofloxacin (CIP) and amoxicillin (AMX). We hypothesized that bacteria isolated

from water taps would have similar disinfectant- and antibiotic-resistance profiles. Further, we determine whether disruptions to service lines provide a source of contamination and increase the risk of ARB and ARG.

METHODS

Sampling and bacteria isolation

In UK, most drinking-water is sourced from surface water (Scottish-Water, 2012a, Scottish-Water, 2012b) and does not deviate from many conventional water-treatment works: screening, coagulation, flocculation, sedimentation or clarification, filtration (rapid gravity, slow sand, or membrane), and pH adjustment. Both chlorination and chloramination used for disinfection in Scotland, UK to provide good quality water for human use. Monochloramine is used in the distribution system as it has a longer residence time than chlorine and produces fewer by-products.

To compare tolerances between disinfection and antibiotics, bacteria were harvested from 52 water samples, collected from flushed (5 min) taps in Glasgow, Scotland, UK. Samples were collected in pre-sterile screw capped bottles and brought to the laboratory for processing within two hours to minimise changes in the samples. Thirty-eight samples were collected from buildings that had tank cisterns for drinking-water storage, with tank capacities ranged from 16,000 to 27,000 L; the remaining 14 samples were from closed systems.

A vacuum-filtration method, with 0.22 μm pore-size cellulose-nitrate gridded membrane filters (Millipore, UK) was used to harvest cells from 100 mL of each water sample; the filter was placed on a Standard Plate Count Agar plate APHA (Oxoid, UK) and incubated for 48 h at 35 ± 2 °C for the development of colonies. The plastic lid was retained to minimise aerosol contamination; sterilised distilled water was used as controls. Isolated

bacterial strains were preserved by using a bacterial bead preservation kit (Cryo vials TS/71-MX, Technical Service Consultants Ltd. UK) and stored at -80 °C throughout the study period. For each set of experiments, one bead was taken out from the cryovials, grown in LB broth overnight, and streaked on a Nutrient Agar (Oxoid, UK) plate to obtain isolated colonies.

Identification of bacteria isolates

Representative colonies were selected for phylogenetically characterisation by sequencing the V4 region of each 16S-rRNA gene. The DNA of bacterial isolates was extracted by a thermal freeze thaw method (Knapp et al., 2012), alternating between -80 °C and 70 °C in 100 µL PBS (phosphate buffer solution; pH 7.4). PCR reaction was performed with a Bio-Rad iQ5 Real-Time PCR Detection System. Forward and reverse primers (Sigma-Aldrich, Life Sciences, UK) were V4-16S-515F (5′-TGTGCCAGCMGCCGCGGTAA) and V4-16S-806R (5′-GGCTACHVGGGTWTCTAAT) (Caporaso et al., 2011). Each PCR reaction contained 10 µL of Universal Supermix (Bio-Rad, UK), 500 ηM of each primer, 0.1 µL SYBR green, 6 µL of nuclease free water and 3 µL of DNA template. A PCR run consisted of initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s and then a 10 min final extension at 72 °C. PCR product length was verified on 2% agarose gel (Bio-Rad, UK) with ethidium bromide (Sigma-Aldrich, UK) and a 50-bp DNA ladder.

A QIAquick PCR Purification Kit (Qiagen, UK) was used to purify PCR products. DNA concentrations were determined by the EPOCH^{TM} Microplate spectrophotometric system (BioTek, UK). Five µL of purified DNA was mixed with the same volume of 5 µM forward primer solution in total volume of 10 µL. Sequencing for the identification of bacteria was performed by LightRun Sequencing Service (GACT Biotech Ltd, London, UK). Bacteria were identified up to genus by sequences comparison using the BLAST program

100 through the National Center for Biotechnology Information (NCBI)
101 (http://blast.ncbi.nlm.nih.gov).

102 **Disinfectant susceptibility testing**

103 Testing was performed using the Kirby-Bauer disc diffusion method, as
104 recommended by the Clinical and Laboratory Standards Institute (Clinical And Laboratory
105 Standards Institute, 2012a), against 127 bacterial isolates with disinfectant solutions of
106 commercial bleach (4.5% sodium hypochlorite, Domestos™, UniLever, UK), 14.5% standard
107 sodium hypochlorite (Alfa Aesar, UK), and a control (tap water) (Sassone et al., 2008,
108 Poggio et al., 2010, Luddin and Ahmed, 2013). Experiments were performed in duplicate and
109 mean zone of inhibition was determined for each isolate. We arbitrarily considered bacteria
110 having zone $\leq$ 20 mm to be chlorine tolerant (or resistant), as high concentration of standard
111 sodium chlorite (14.5%) was also used.

112 **Antibiotic susceptibility testing for MIC**

113 Bacterial isolates were also tested for antibiotic susceptibility against tetracycline
114 hydrochloride ('TET'; Sigma-Aldrich, UK), sulfamethoxazole ('SMX'; Molekula, UK),
115 amoxicillin trihydrate ('AMX'; Alfa Aesar, UK) and ciprofloxacin ('CIP'; Fluka, UK) by
116 Agar Dilution Method recommended previously by the Clinical and Laboratory Standards
117 Institute (Clinical And Laboratory Standards Institute, 2012b). A master replica plate,
118 containing 20-24 bacterial isolates, was freshly prepared for each experiment. The isolates
119 were tested against a series of concentrations, 0.002–512 µg mL^{-1}, of each antibiotic in
120 Mueller-Hinton Agar (Oxoid, UK) (Armstrong et al., 1981). All plates were incubated at 35 ±
121 2 °C for 24 h. Minimum inhibitory concentrations (MIC) were calculated for each antibiotic
122 (µg mL^{-1}) against all isolates. *E. coli* ATCC 25922 (NCTC 12241) was used as a control, and
123 the maximum MIC values of antibiotics against the organisms reported by CLSI were used as
124 reference for the interpretation (Clinical And Laboratory Standards Insitute, 2011, Guo et al.,

2013, Yuan et al., 2015). Any bacterium forming colonies above maximum MIC values mentioned by CLSI (TET $\geq$ 16 μg mL^{-1}, SMX $\geq$ 512 μg mL^{-1}, CIP $\geq$ 4 μg mL^{-1}, and AMX $\geq$ 32 μg mL^{-1}) were considered "resistant" to that antibiotic; those inhibited at lower concentrations were considered 'susceptible'.

Disinfectant suspension tests for chlorine resistance

Six isolates were selected for chlorine and monochloramine suspension tests to verify Kirby-Bauer results at fixed concentrations and exposure time. Suspension tests were performed in 200 mL of 10 mM PBS at pH 7.0. All glassware was treated with 10% nitric acid overnight, soaked in bleach (5% sodium hypochlorite, Alfa Aesar), rinsed with nano-pure water, air-dried and autoclaved (Chiao et al., 2014). A stock solution of 14.5% sodium hypochlorite was used to prepare 0.5, 1.0, 2.0, 4.0 and 8.0 mg L^{-1} free chlorine solutions. Bacteria were grown overnight with continuous shaking in Tryptic Soya Broth (Fluka, UK), centrifuged at 3500 rpm for 15 min, washed 3 times with PBS, pH 7.0, and suspended in PBS to prepare the stock culture of $1x10^8$ cfu mL^{-1}. This stock culture was added to free-chlorine solution to achieve a final bacterial count of $1x10^5$ cfu mL^{-1} and mixed well to ensure bacterial exposure to the disinfectant. At 0, 15 and 60 min contact times, 10 mL samples were taken out, dechlorinated with 100 μL of 1 M sodium thiosulfate (Fisher Scientific, UK) (Ridgway and Olson, 1982), and 100 μL aliquots from disinfectant quenched samples were plated on Standard Plate Count Agar APHA (Oxoid, UK) plates after making dilutions in PBS, whenever required. Plates were incubated for 48 h at 35 ± 2 °C for heterotrophic plate count (HPC). Each experiment was reproduced three times, and the mean was calculated from three individual experiments.

Temperature and pH were recorded with a Multi 7 Mettler-Toledo meter (Mettler-Toledo International Inc., Columbus, OH, USA) at each time point of exposure. Free chlorine and total chlorine concentrations were determined using the N,N-diethyl-p-phenylenediamine

(DPD) colorimetric method (APHA, 1999) with HACH DPD reagent and pocket colorimetric analysis system (HACH, USA) at 0, 15, and 60 min contact times. Two controls of PBS with bacteria without disinfectant and PBS with disinfectant and without bacteria were used for each set of experiments.

Disinfectant suspension test for monochloramine

Monochloramine suspension tests were performed similarly as described for the chlorine suspension test except PBS pH 8.0 was used for the experiments (Howard and Inglis, 2005, Chiao et al., 2014). The monochloramine solution (10 mg L^{-1}) was prepared by mixing 68.9 µL of 14.5% NaOCl (Alfa Aesar, UK) and 2 mL of 1.91% NH_4Cl solutions (Sigma-Aldrich, UK) in a volumetric flask and making up the volume to 1 L with PBS, pH 8.0 (Driedger et al., 2001, Chiao et al., 2014). Five solutions of monochloramine were prepared similarly having concentrations of 0.5, 1.0, 2.0, 4.0 and 8.0 mg L^{-1}. Monochloramine concentration was determined using the Indophenol method with MonochlorF reagent (HACH, USA, Method 10172) and HACH Pocket colorimeter analysis system (Lee et al., 2007). The remaining protocol was the same as used for the chlorine suspension test.

Data collection and statistical analysis

Chlorine and monochloramine disinfectant suspension tests were performed against six identified bacterial isolates and mean cfu mL^{-1} ± SD were calculated for each contact time and concentration. Cell counts were $\log_{10}$ transformed before plotting. Statistical analysis was performed using Minitab version 17. MIC data was compared against zones of inhibition of hypochlorite assays using the non-parametric Spearman correlation test.

RESULTS

Water Conditions

Minimum free chlorine and total chlorine concentrations were found to be 0.01 mg L^{-1} and 0.1 mg L^{-1}, respectively at the time of collection of samples. Thirty-eight samples were collected from buildings having a cold-water storage tank, or cistern, within the building, while 14 samples were collected from the buildings with completely closed supply lines (Table 1). Water storage tanks are inspected once in six months and disinfected generally on annual basis in these buildings. All reported drinking-water quality values were within permissible concentrations at time of sampling; however, disinfection conditions declined at point of use.

Bacterial communities in drinking water

Approximately 80% of water samples tested positively for at least one bacterium (per 100 mL water). The frequency of positive detections was similar between building types; however, cistern-related samples had greater abundances of bacteria: averaging 3.4 colony forming units (CFU) from cistern-systems, versus 1.4 CFU in buildings without cisterns.

Bacteria identified in this study included members from the phyla of Alphaproteobacteria (*Blastomonas* and *Sphingomonas*), Betaproteobacteria (*Acidovorax*, *Burkholderia*, *Comamonas*, *Cupriavidus*, *Ralstonia*, and *Variovorax*), Gammaproteobacteria (*Enhydrobacter*, *Escherichia*, and *Pantoea*), Actinobacteria (*Arthrobacter*, *Dermacoccus*, *Dietzia*, *Janibacter*, *Kocuria*, and *Micrococcus*), and Firmicutes (*Bacillus*, *Paenibacillus*, *Brevibacillus*, and *Staphylococcus*) (Table S1).

Twenty different genera were found in water samples collected from buildings having cisterns, and eight genera were found in samples from buildings with closed systems (Table 1). There are differences in bacterial communities found in drinking-water system when the water has been stored before use. *Bacillus*, *Burkholderia*, *Kocuria*, *Micrococcus*, *Paenibacillus*, and *Staphylococcus* were present in both types of buildings at relatively similar proportions. Fourteen groups were found only in the drinking-water samples taken

198 from the buildings with storage tank or cistern: *Cupriavidus*, *Blastomonas*, *Acidovorax*,
199 *Variovorax*, *Arthrobacter*, *Escherichia*, *Enhydrobacter*, *Pantoea*, *Comamonas*,
200 *Sphingomonas*, *Dietzia*, and an unrecognised Epsilonproteobacteria (Table 1), while
201 *Janibacter* and *Brevibacillus* were present only in those samples taken from buildings
202 without a drinking-water storage tank.

203 **Disinfection susceptibility test by disk diffusion method**

204 This test assayed bacteria to determine their susceptibilities to sodium hypochlorite,
205 either as 14.5% standard sodium hypochlorite solution or 4.5% commercial bleach on the
206 same agar plate. Bacteria showed a broad range of susceptibility patterns producing zones of
207 inhibition between 7 mm and 65 mm in diameter against the two disinfectants. We arbitrarily
208 classified results to facilitate analysis (there are no known standard metrics to define
209 'resistance'), and 13 (8.8%) bacteria showed zones of inhibition $\leq$ 20 mm in diameter; 96
210 (64.9%) isolates showed zones of inhibition between 21-40 mm, while 18 (12.2%) isolates
211 produced zones of inhibition of $\geq$ 41 mm (Table 2). In case of 4.5% commercial bleach, 98
212 (66.2%) isolates showed zone of inhibition $\leq$ 20 mm, 29 (19.6%) isolates showed between
213 21-40 mm, while no isolate showed any zone of inhibition $\geq$ 41 mm (Table 2).

214 Comparing the means of size of zone of inhibition by two disinfectants indicated that
215 (as expected) the standard sodium hypochlorite was more effective against isolated bacteria
216 (Table S2), but interestingly 10 (6.8%) cultures (4 *Bacillus* spp., 2 *Acidovorax* spp., 1
217 *Burkholderia* sp., 1 *Paenibacillus* sp. and 2 unidentified bacteria) were more sensitive to
218 commercial bleach (Table S1); this may be due to the presence of other antimicrobial agents,
219 e.g., non-ionic and cationic surfactants, or pH, of the commercial bleach solution. Twenty-
220 one isolates were not tested as they did not form a proper lawn on the agar plate as required
221 for agar diffusion method; at least three attempts to create a lawn were made for each
222 bacteria.

There were no differences in zones of inhibition to chlorine among bacteria collected from each building type (Mann Whitney, W = 7086, $p = 0.747$). There is no treatment-related bias to chlorine resistance based on the presence or absence of a cistern.

Antibiotic susceptibility test for MICs

To confirm the presence of ARB in tap water, antibiotic susceptibility testing was performed against four antibiotics to determine their MIC profiles: tetracycline (TET), sulfamethoxazole (SMX), ciprofloxacin (CIP) and amoxicillin (AMX). These antibiotics belong to different antimicrobial classes and involve different mechanisms for resistance as they inhibit protein synthesis, folic-acid cycle, DNA gyrase (involved in DNA replication), and synthesis of cell walls, respectively (Kohanski et al., 2010).

Among the 148 isolates, 115 (77.7%) showed resistance against at least one antibiotic (Table 3), based on maximum values of MICs for organisms described by CLSI (Clinical And Laboratory Standards Insitute, 2011, Guo et al., 2013, Yuan et al., 2015). Amoxicillin resistance was most prevalent, found in 96 (64.9%) isolates which were grown in AMX concentrations $\geq$ 32 μg mL^{-1} (Table 3), while sulfamethoxazole resistance was also widely distributed (45.9%, n = 68). Twenty bacteria (13.5%) were resistant to tetracycline, and thirteen (8.8%) possessed resistance against ciprofloxacin.

The presence of resistance traits against two or more antibiotics indicates that these organisms could have multidrug resistances. Multi-drug resistant bacteria were found in the drinking-water samples; six (4.1%) bacteria were resistant to all four antibiotics tested (TET, SMX, CIP, and AMX). Ten (6.8%) bacteria showed resistance against three antibiotics: 7 to TET, SMX, and AMX and 3 to SMX, CIP, and AMX. Out of 148 bacteria, 44 (29.7%) showed double resistance; further details can be found in Table 3.

Among building types, there were no differences between MIC for TET and SUL (Mann Whitney test: $p = 0.424$ and $p = 0.296$, respectively). Bacteria from cistern-systems

had higher MIC for AMX (Mann Whitney test, $p < 0.001$) with median value of 64 μg mL^{-1} in cisterns, versus 0.125 in closed systems. Conversely, bacteria in closed systems had higher MIC for CIP than those from cisterns (Mann Whitney, $p < 0.001$): 0.063 μg mL^{-1} versus 0.016 μg mL^{-1}, respectively.

Bacteria show similar resistance patterns against antibiotics and disinfectants (Table S1). Spearman correlation tests ($p = 0.05$) indicate an inverse relationship between zones of inhibition against 14.5% standard sodium hypochlorite and antibiotic MICs. This suggests that bacteria with chlorine tolerance also tended to have greater tolerance to antibiotics. Correlations were weak but significant; AMX ($r = -0.303$; $p = 0.001$), SMX ($r = -0.278$; $p = 0.002$), and TET ($r = -0.219$; $p = 0.014$) (Table 5). There were no patterns between ciprofloxacin-resistance and chlorine tolerance ($r = -0.002$; $p = 0.981$).

Disinfection suspension test for chlorine

Six bacteria were selected for the disinfectant suspension test on the basis of the number of antibiotics to which they were resistant: *Arthrobacter* (TET, SMX, CIP, and AMX), *Bacillus* (SMX and AMX), *Cupriavidus* (TET, SMX, CIP, and AMX), *Burkholderia* (type M: TET, SMX, and AMX), *Burkholderia* (type S: AMX) and *Paenibacillus* (No resistance) (Table 4). *Burkholderia* were represented with 'M' (multiple resistant) and 'S' (single resistant) to differentiate the two strains.

The chlorine suspension test was performed to evaluate contact time (0, 15 and 60 min) and disinfectant concentrations (0-8 mg L^{-1}) on inactivation of the bacteria at pH 7.0 and 20 °C (Table S3, S4). *Burkholderia* sp. (M) showed greatest resistance to chlorine than other bacteria at 15 and 60 min contact times (Figure 1, a-f). A decrease of 2-3 log-units of cfu mL^{-1} was observed at concentrations 0.5-2 mg L^{-1} of free chlorine as compared to the control for all time durations (versus log cfu = 5). However, to reduce viable counts further, it required longer exposures and higher concentrations (4-8 mg L^{-1} free chlorine), while complete

inhibition did not occur at any concentration or contact time against *Burkholderia* (M) (Figure 1, d). *Bacillus* sp. had the second highest survival rates at concentrations of 4.0 and 8.0 mg L^{-1}; however, viabilities were greater for *Bacillus* sp. than *Burkholderia* sp. (M) at quick exposures (0 min) at lower concentrations of 0.5-2 mg L^{-1} (Figure 1, c-d). These bacteria were resistant to three (TET, SMX and AMX) and two (SMX and AMX) antibiotics, respectively, and had small zones of inhibition, 15 and 7 mm respectively, against standard sodium hypochlorite (Table 4).

Cupriavidus sp. and *Arthrobacter* sp. had resistances against all antibiotics (TET, SMX, CIP, and AMX); both had initial resistance to immediate exposure (0 min) to chlorine at 0.5 and 1.0 mg L^{-1}, but were inhibited with increased concentrations and contact times (Figure 1 a-b). They produced zone of inhibition of 35 and 40 mm in disk diffusion method (Table 4).

Paenibacillus sp. and *Burkholderia* (S) sp. showed a decrease of 3-4 log-units at small doses of 0.5 and 1.0 mg L^{-1} at immediate contact (0 min) (Figure 1, e-f). *Paenibacillus* sp. was susceptible to all antibiotics tested in this study, while the *Burkholderia* sp. (S) had resistance against AMX only (Table 4), and they produced large zones of inhibition, 54 and 65 mm respectively, in the disinfectant susceptibility testing.

The results show that the six bacteria demonstrated similar patterns of resistances and susceptibilities in the agar diffusion test and the suspension test for disinfectants. Those that produced small zones of inhibition had greater survival in the suspension tests. Additionally, all four bacteria having double, triple and quadruple antibiotic-resistances survived better than the single antibiotic-resistant and susceptible bacteria when exposed to free chlorine.

Disinfection suspension test for monochloramine

The monochloramine suspension test was performed at pH 8.0 and 20 °C (Table S5, S6). The inhibitory effect of monochloramine was not as immediate as for free-chlorine

298 exposure; rates of decrease in survival count were less than one-order of magnitude (Figure 2,
299 a-f), as compared to free-chlorine where declines of 2-3 orders of magnitudes were observed.
300 Among the six bacteria, *Burkholderia* sp. (M) showed the highest survival rates and was the
301 only test microorganism that showed resistance to all concentrations even after 60 min
302 contact time with both chlorine and monochloramine (Figure 1 d and 2 d). *Bacillus* sp. was
303 inactivated at 4.0 mg L^{-1} at 15 min contact time, while showed growth at 8.0 mg L^{-1} at the
304 same contact time (Figure 2c). *Bacillus* sp. showed greater survival than the quadruple
305 antibiotic-resistant species *Cupriavidus* and *Arthrobacter* at higher doses of 2-8 mg L^{-1} at 15
306 and 60 min contact time, but it showed less survival at immediate contact (0 min) (Figure 2,
307 a-c). *Paenibacillus* sp., which was antibiotic sensitive showed greater survival rates than
308 antibiotic-resistant *Cupriavidus* sp. *Arthrobacter* sp. and *Bacillus* sp. at brief (0 min) and 15-
309 min exposures (Figure 2e). The resistance of *Paenibacillus* sp. against monochloramine
310 might also be due to the presence of spores, which allowed them to tolerate the high
311 concentration of disinfectant. For all bacteria, declines in the viability count (cfu mL^{-1}) by
312 monochloramine were less than the chlorine exposure, irrespective of their antibiotic-
313 resistances (Figure 2, a-f). Inhibition did not occur at low doses, as compared to chlorine
314 where inhibition occurred even at 0.5 mg L^{-1} of free chlorine after 60 minutes, indicating that
315 free chlorine has more inhibitory activity for bacteria of DWDS than monochloramine.

316 ## DISCUSSION

317 Drinking-water samples had diverse genera; some could be potentially pathogenic.
318 For example, species of *Burkholderia* (Falkinham, 2015), *Kocuria* (Purty et al., 2013),
319 *Paenibacillus* (Ouyang et al., 2008), and *Dermacoccus* (Takahashi et al., 2015) can impact
320 immune-compromised patients and have been transmitted via drinking water (Hunter, 1997,
321 Godoy et al., 2003). Many of these bacteria demonstrate antimicrobial-resistance, e.g.,

members of *Burkholderia cepacia* complex (Desai et al., 1998, Coenye et al., 2001) and *Cupriavidus*' resistance to metal (Vandamme and Coenye, 2004). Moreover, the presence of *Pantoea* sp. (Pindi et al., 2013) and *Sphingomonas* sp. (Koskinen et al., 2000) are undesirable.

Different factors contribute to the introduction of bacteria into water distribution systems. In this study, most bacteria were from buildings with storage tanks, or cisterns, for drinking water. The building's plumbing represents an ideal place for opportunistic bacteria (Wang et al., 2012) by providing them low organic carbon level, high surface to volume ratio, and periods of stagnation (Falkinham, 2015, Falkinham et al., 2015). During periods stagnation or increased water-age residual chlorine levels decline, and the efficacy of bacterial growth inhibition becomes reduced (EPA, 2002). The bacterial community structure in a distribution system becomes influenced (Wang et al., 2014), including those with antimicrobial resistance (Falkinham, 2015, Falkinham et al., 2015) .

The response of ARBs to chlorine widely varies (Shi et al., 2013), and it becomes very difficult to ascertain specific mechanisms from these observations. Disinfection efficiency does not remain the same throughout the supply system, and gradients of exposure concentrations develop. Responses range from lethality/complete inhibition at high concentrations, selective survivability of resistant populations at sub-inhibiting concentrations, to triggering biochemical stress responses at much lower (sub-inhibitory) concentrations.

Surviving bacteria may innately have increased resistance. Spore-forming bacteria tend to be more resistant, and Gram-negative bacteria are less susceptible than Gram-positive bacteria (Russell, 1998). This might be a reason that in our study, the *Bacillus* species having spores and antibiotic-resistance against two antibiotics showed more tolerance to chlorine, as compared to multiple-antibiotic resistant *Cupriavidus* and *Arthrobacter* which do not form

spores. Increases in the abundance of antibiotic-resistant *Pseudomonas*, *Acidovorax* and *Pleamonas* and ARGs have been observed after chlorine treatment (Jia et al., 2015).

One mechanisms by which sub-inhibitory levels increase the risk of selection of ARB is by chemical stress (Huang et al., 2013). Chlorine has been shown to increase the abundance of antibiotic-resistance bacteria and genes in opportunistic bacteria (Shrivastava et al., 2004, Shi et al., 2013). This is often attributed to the enrichment of bacteria with plasmids and integrons, which are involved in the transfer and enrichment of resistant markers among bacteria (Shi et al., 2013), as part of their stress-response mechanism. While not tested here, it remains a possibility in our systems; further examines are required.

Inactivation of antibiotic-resistant and -sensitive bacteria diminishes when previously exposed to chlorine disinfectant. Bacterial strains with antibiotic resistance have shown to be more tolerant to chlorination (Templeton et al., 2009; Huang et al., 2013). Bacteria show a biphasic mode of inactivation during chlorine disinfection for drinking-water production. A sharp decline of 2-4 $\log_{10}$ in viable cells is not unusual and occurs within 15 min of exposure of 0.1-3 mg L^{-1} of free chlorine, indicating that chlorine does not require a long exposure time for effectiveness (Lee and Nam, 2002). A 100-fold decrease in viability of bacteria after 60-minute exposure to 1 mg L^{-1} free chlorine, with bacteria viability decreasing quickly between 10-20 min of exposure to 1 mg L^{-1} of chlorine concentration (Howard and Inglis, 2003). These authors also found that *E. coli* and *Ps. aeruginosa* growth decreased more than other bacteria, e.g. *Burkholderia* sp., during an initial five minutes contact with 1 mg L^{-1} chlorine. In our study, we observed the same phenomenon, and most bacteria inactivation occurring in the initial 15 minutes.

In many water distribution systems, residual disinfectant is present which could select for disinfectant-resistant cells by allowing these bacteria to grow, and decreasing the growth of other disinfectant-sensitive competitors (Falkinham et al., 2015). Populations might have

had previous exposure to chlorine, which increased their resistance to chlorine. This might be a reason that in our study, some isolated bacteria showed resistance against concentrated standard sodium hypochlorite and produce smaller zones of inhibition (< 20 mm).

In this study, we found greater numbers of bacteria in post-cistern systems; in areas where chlorine efficacy could be reduced. These bacteria likely have, or develop, disinfectant resistance, which could also carry higher risks of possessing resistance to antibiotics. More detailed investigation is required to properly conclude chlorination efficacy as part of drinking-water treatment protocols, including other possible disinfection methods which could remove bacteria from these systems. Also, the mechanisms for co-selection must be determined. Overall, the results provide additional evidence as to why care should be taken to minimise the introduction of bacteria into drinking-water distribution systems as these bacteria may cause public health risk with increased exposure and greater chances of antibiotic resistance.

■ ASSOCIATED CONTENT

Supporting Information

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Author Contributions

All authors contributed to the research. CK conceptualised the research topic; SK performed the experiments and wrote the paper. All reviewed and edited the paper.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGEMENTS:

We are thankful to Dr. Ameet Pinto, University of Glasgow, Scotland, UK for his guidance in monochloramine experiments. The research is supported by Schlumberger Foundation, Faculty for the Future grant in the form of tuition fee and stipend for the SK's PhD.

■ ABBREVIATIONS

ARB Antibiotic-resistant bacteria

ARG Antibiotic resistance genes

PBS Phosphate buffer saline

DPD N,N-diethyl-p-phenylenediamine

PCR Polymerase chain reaction

DNA Deoxyribonucleic acid

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Table 1. Bacteria found in buildings with cistern or storage tank and without cistern, or storage tank

Building type	Total samples collected	Positive	Bacteria selected	Bacteria submitted for identification	Not identified, no sequence found, no similarity found	Bacteria identified	Bacteria Identified in samples
Cistern	38	31	128	84	12	72	*Cupriavidus*=14, *Blastomonas*=9, *Acidovorax*=8, *Ralstonia*=6, *Burkholderia*=4, *Dermacoccus*=4, *Variovorax*=4, *Bacillus*=3, *Staphylococcus*=3, *Arthrobacter*=2, *Escherichia*=2, *Enhydrobacter*=2, *Kocuria*=2, *Micrococcus*=2, *Paenibacillus*=2, *Pantoea*=1, Epsilonproteobacteria=1, *Comamonas*=1, *Sphingomonas*=1, *Dietzia*=1
No Cistern	14	11	20	16	1	15	*Paenibacillus*=4, *Bacillus*=4, *Micrococcus*=2, *Burkholderia*=1, *Brevibacillus*=1, *Janibacter*=1, *Kocuria*=1, *Staphylococcus*=1
Total	52	42	148	100	13	87	

Table 2. Disinfectant susceptibility of isolates (zone of inhibition in mm) by Disk Diffusion Method

Disinfectant	Size of Zone of inhibition	No. of Organisms (%)	Organisms
Standard Sodium hypochlorite (14.5%)	≤ 20 mm	13 (8.8)	5 *Bacillus* species, 1 *Burkholderia* specie, 1 *Paenibacillus* specie, 2 *Acidovorax* specie, 4 uncharacterised bacteria
	21-40 mm	96 (64.9)	14 *Cupriavidus* species, 6 *Blastomonas* species, 4 *Acidovorax* species, 4 *Staphylococcus* species, 4 *Variovorax* species, 2 *Paenibacillus* species, 2 *Arthrobacter* species, 2 *Bacillus* species, 2 *Dermacoccus* species, 2 *Enhydrobacter* species, 2 *Kocuria* species, 2 *Micrococcus* species, 2 *Ralstonia* species, 1 *Brevibacillus* specie, 1 *Comamonas* specie, 1 Epsilonproteobacteria, 1 *Pantoea* specie, 1 *Sphingomonas* specie, 43 uncharacterised bacteria
	≥ 41 mm	18 (12.2)	2 *Micrococcus* species, 2 *Paenibacillus* species, 1 *Acidovorax* specie, 1 *Blastomonas* specie, 1 *Escherichia* specie, 1 *Ralstonia* specie, 1 *Dietzia* specie, 1 *Burkholderia* specie, 8 uncharacterised bacteria
Commercial bleach (4.5% sodium hypochlorite	≤ 20 mm	98 (66.2)	13 *Cupriavidus* species, 6 *Blastomonas* species, 4 *Acidovorax* species, 3 *Staphylococcus* species, 4 *Bacillus* species, 4 *Variovorax* species, 4 *Paenibacillus* species, 3 *Dermacoccus* species, 2 *Arthrobacter* species,, 2 *Enhydrobacter* species, 2 *Ralstonia* species, 1 *Kocuria* species, 1 *Micrococcus* species, 1 *Burkholderia* specie, 1 *Comamonas* specie, 1 Epsilonproteobacteria, 1 *Pantoea* specie, 1 *Sphingomonas* specie, 44 uncharacterised bacteria
	21-40 mm	29 (19.6)	3 *Acidovorax* species, 3 *Bacillus* species, 2 *Micrococcus* species, 1 *Cupriavidus* species, 1 *Blastomonas* specie, 1 *Staphylococcus* specie, 1 *Paenibacillus* specie, 1 *Brevibacillus* specie, 1*Dietzia* specie, 1 *Kocuria* specie, 1 *Ralstonia* specie,1 *Burkholderia* specie, 12 uncharacterised bacteria
	≥ 41 mm	0	No organism
	Not tested	21 (14.2)	3 *Ralstonia* species, 3 *Burkholderia* species, 2 *Dermacoccus* species, 1 *Kocuria* specie, 1 *Blastomonas* specie, 1 *Acidovorax* specie, 1 *Janibacter* specie, 1 *Paenibacillus* specie, 1 *Escherichia* specie, 7 uncharacterised bacteria

Table 3. Single and multiple antibiotic-resistances of bacteria isolated from drinking-water distribution system

Resistant traits	Combinations	No. of Organisms (%)	Isolates
Quadruple	TET, SMX, CIP, and AMX	6 (4.1)	1 *Cupriavidus* specie, 1 *Arthrobacter* specie, 1 Epsilonproteobacteria, 1 *Kocuria* specie, 2 uncharacterised bacteria
Triple	TET, SMX, and AMX	7 (4.7)	1 *Cupriavidus* specie, 4 *Burkholderia* species, 2 uncharacterised bacteria
	SMX, CIP, and AMX	3 (2.0)	1 *Micrococcus* specie, 1 *Acidovorax* specie, 1 *Dermacoccus* specie
Double	SMX and AMX	34 (23.0)	9 *Cupriavidus* species, 1 *Comamonas* specie, 16 uncharacterised bacteria, 1 *Blastomonas* specie, 2 *Bacillus* specie, 1 *Acidovorax* specie, 2 *Staphylococcus* specie, 1 *Sphingomonas* specie, 1 *Kocuria* specie
	TET and AMX	5 (3.4)	1 *Cupriavidus* specie, 1*Dietzia* specie, 3 uncharacterised bacterium
	SMX and CIP	4 (2.7)	1 *Micrococcus* specie, 1*Kocuria* specie, 1 *Bacillus* specie, 1 *Dermacoccus* specie
	TET and SMX	1 (0.7)	1 *Staphylococcus* specie
Single	TET	1 (0.7)	1 Uncharacterised bacteria
	SMX	13 (8.8)	2 *Enhydrobacter* species, 1*Bacillus* specie, 1 *Arthrobacter* specie, 4 Uncharacterised specie, 1 *Brevibacillus* specie, 1*Dermacoccus* specie, 1 *Staphylococcus* specie, 2 *Micrococcus* species
	AMX	41 (27.7)	6 *Acidovorax* species, 18 uncharacterised bacteria, 1 *Bacillus* specie, 4 *Variovorax* species, 2 *Paenibacillus* species, 2 *Cupriavidus* species, 1 *Dermacoccus* specie, 5 *Ralstonia* species, 1 *Escherichia* specie, 1 *Burkholderia* specie
No Resistant	No Resistance	33 (22.3)	2 *Bacillus* species, 15 uncharacterised species, 4 *Paenibacillus* species, 8 *Blastomonas* species, 1 *Escherichia* specie, 1 *Pantoea* specie, 1 *Ralstonia* specie, 1 *Janibacter* specie

Resistance organisms: Tetracycline (TET) = 16 μg mL^{-1}, Sulfamethoxazole (SMX) = 512 μg mL^{-1}, Ciprofloxacin (CIP) = 4 μg mL^{-1} and Amoxicillin (AMX) = 32 μg mL^{-1}

Table 4. Antibiotic and disinfectant resistance of six test bacteria

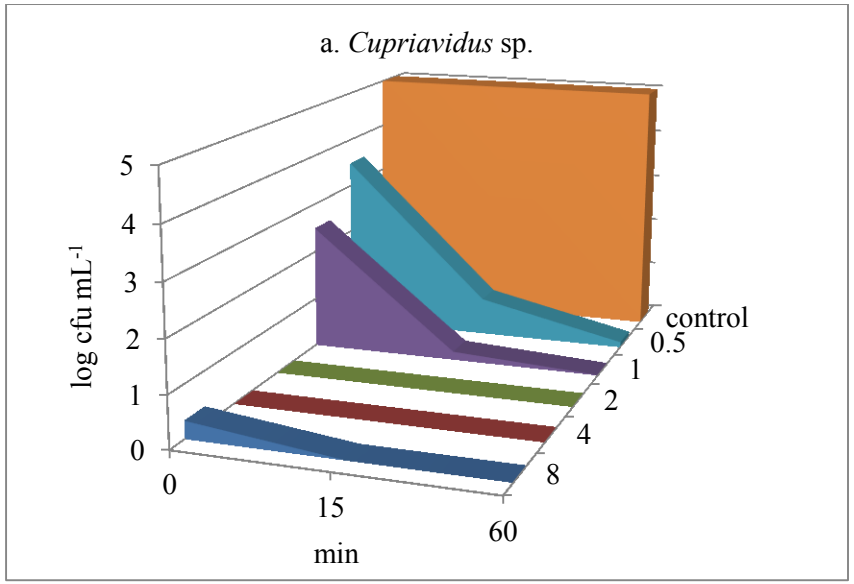
Code	Identification by 16S-rRNA	Antibiotic MICs (μg mL^{-1})				Resistant Traits for antibiotics	Size of zone of inhibition (mm ± SD) against NaOCl
		TET	SMX	CIP	AMX		
515	*Cupriavidus sp.*	515	512	16	512	TET, SMX, CIP, and AMX	35 ± 2.8
518	*Arthrobacter sp.*	512	512	512	512	TET, SMX,CIP, and AMX	40 ± 0.7
527	*Bacillus sp.*	1	512	0.064	512	SMX and AMX	7 ± 0.0
530	*Burkholderia sp.* (M)	64	512	0.064	512	TET, SMX, and AMX	15 ± 1.4
641	*Paenibacillus sp.*	0.016	16	0.008	0.064	Susceptible	54 ± 2.1
643	*Burkholderia sp*. (S)	8	8	0.032	512	AMX	65 ± 4.2

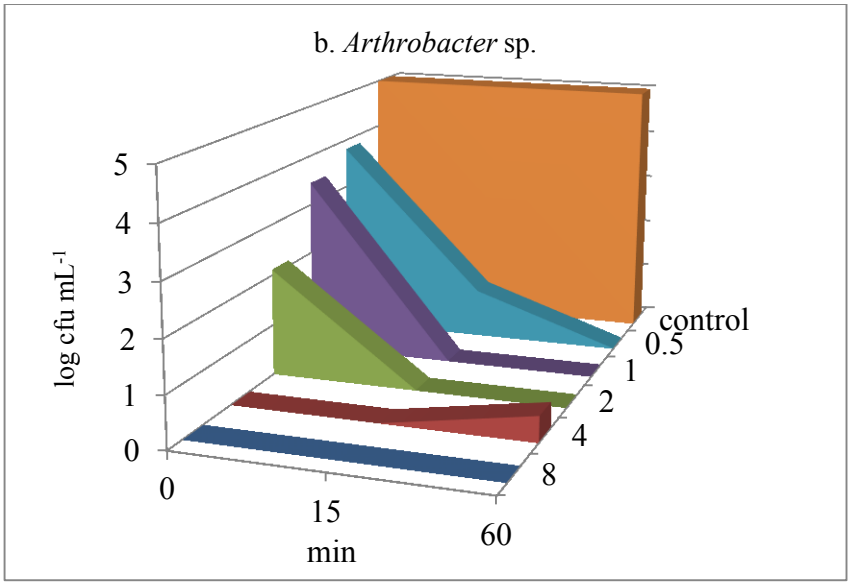
Resistant organisms: Tetracycline (TET) = 16 μg mL^{-1}, Sulfamethoxazole (SMX) = 512 μg mL^{-1}, Ciprofloxacin (CIP) = 4 μg mL^{-1} and Amoxicillin (AMX) = 32 μg mL^{-1}

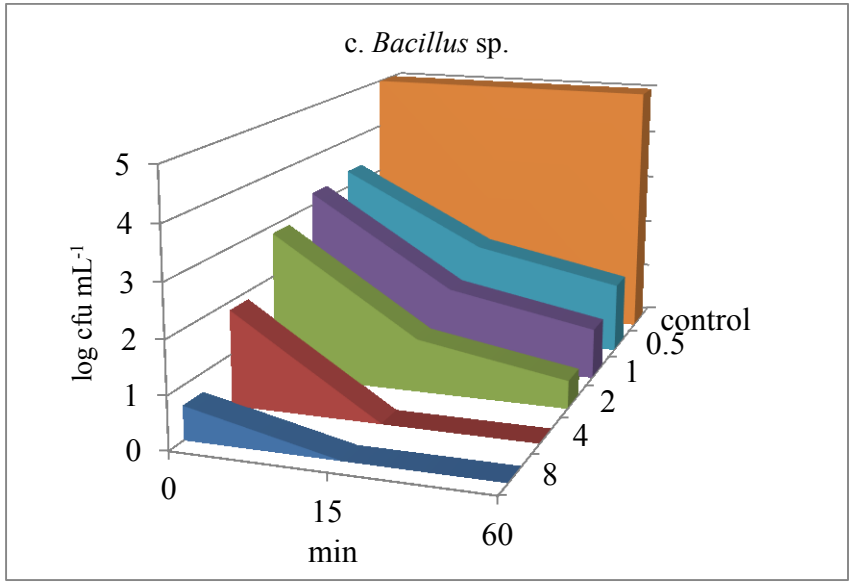
Table 5: Spearman correlation analysis for size of zone of inhibition by 14.5% standard NaOCl and minimum inhibitory concentrations (MIC) by four antibiotics (n=127). Significant level was $p< 0.05$.

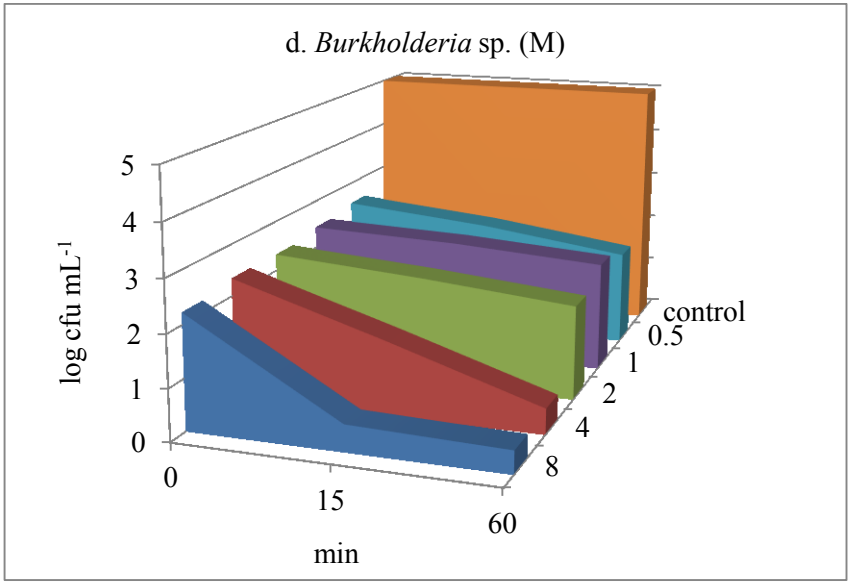
		TET	SMX	CIP	AMX
Standard NaOCl 14.5%	Spearman Correlation	-0.219	-0.278	-0.002	-0.303
	P value	0.014	0.002	0.981	0.001

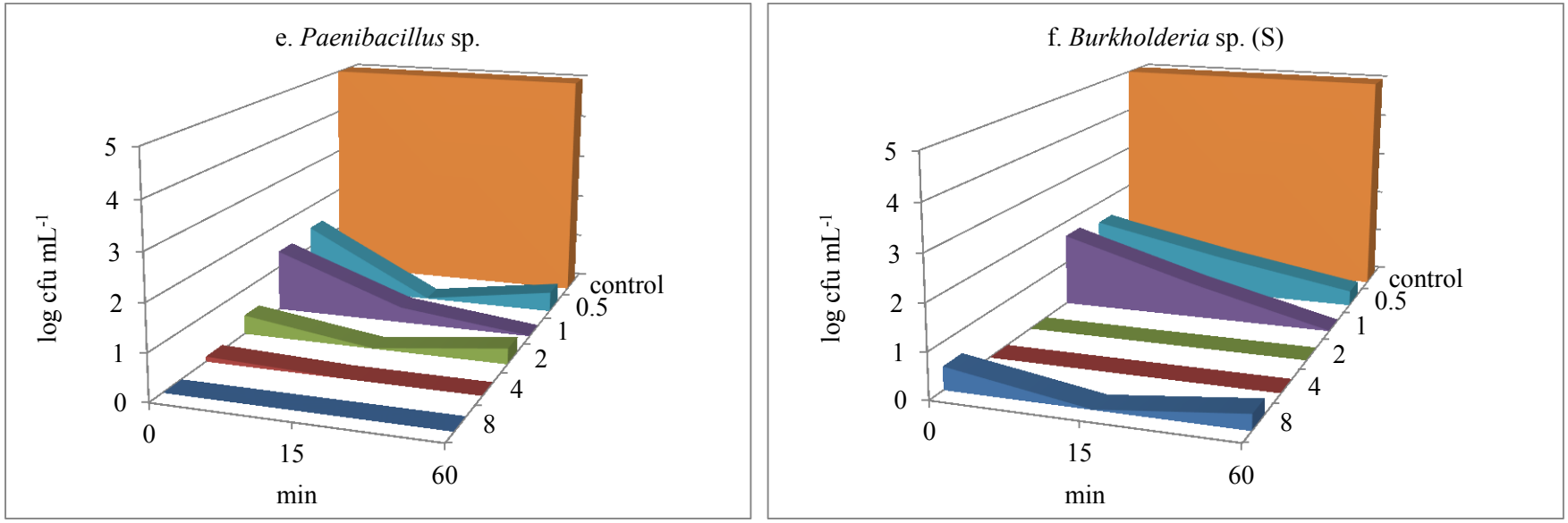
Tetracycline (TET), Sulfamethoxazole (SMX), Ciprofloxacin (CIP), Amoxicillin (AMX)


a. *Cupriavidus* sp.
log cfu mL-1
0
1
2
3
4
5
0
15
60
min
control
0.5
1
2
4
8


b. *Arthrobacter* sp.
log cfu mL-1
0
1
2
3
4
5
0
15
60
min
control
0.5
1
2
4
8

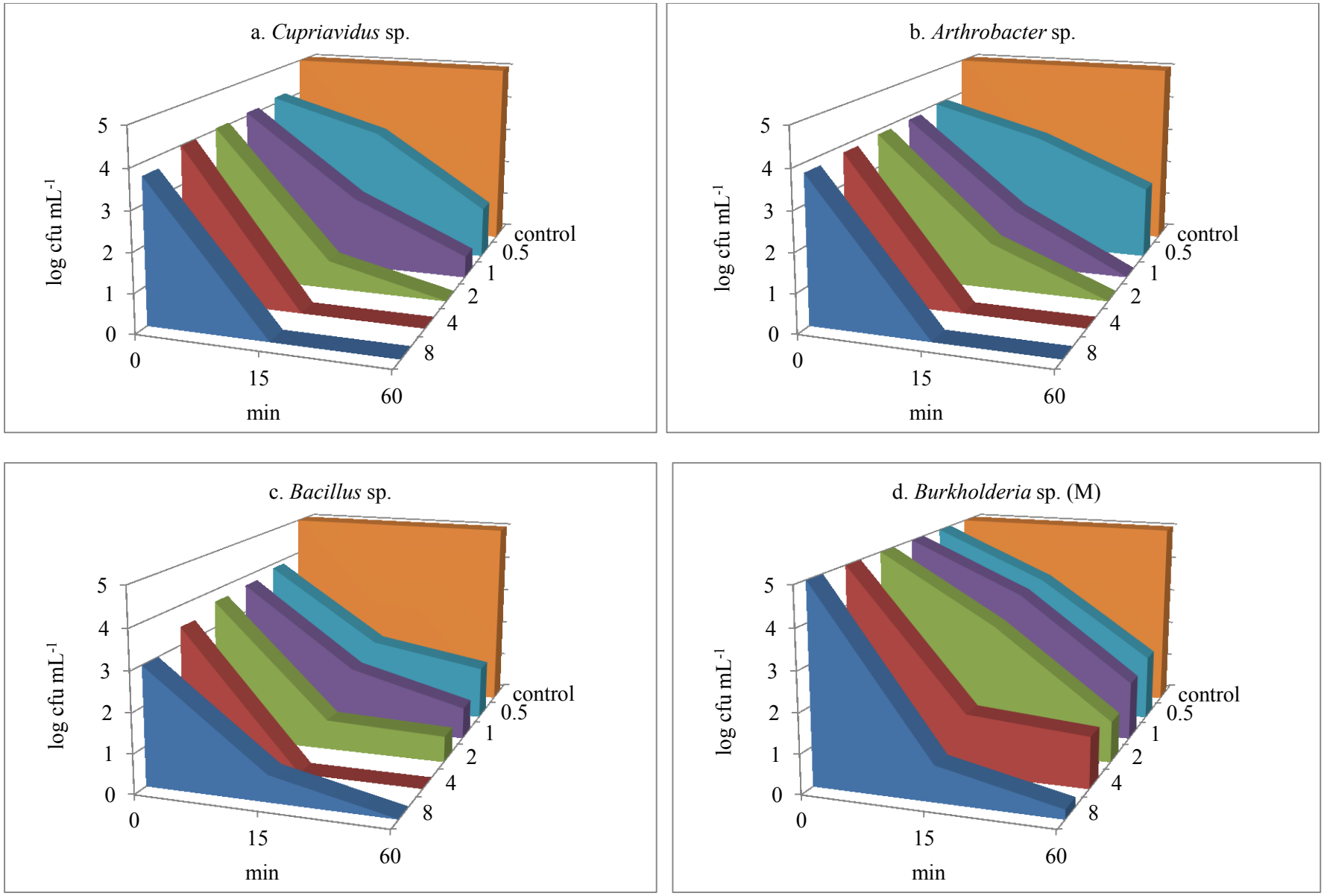

c. *Bacillus* sp.
log cfu mL-1
0
1
2
3
4
5
0
15
60
min
control
0.5
1
2
4
8

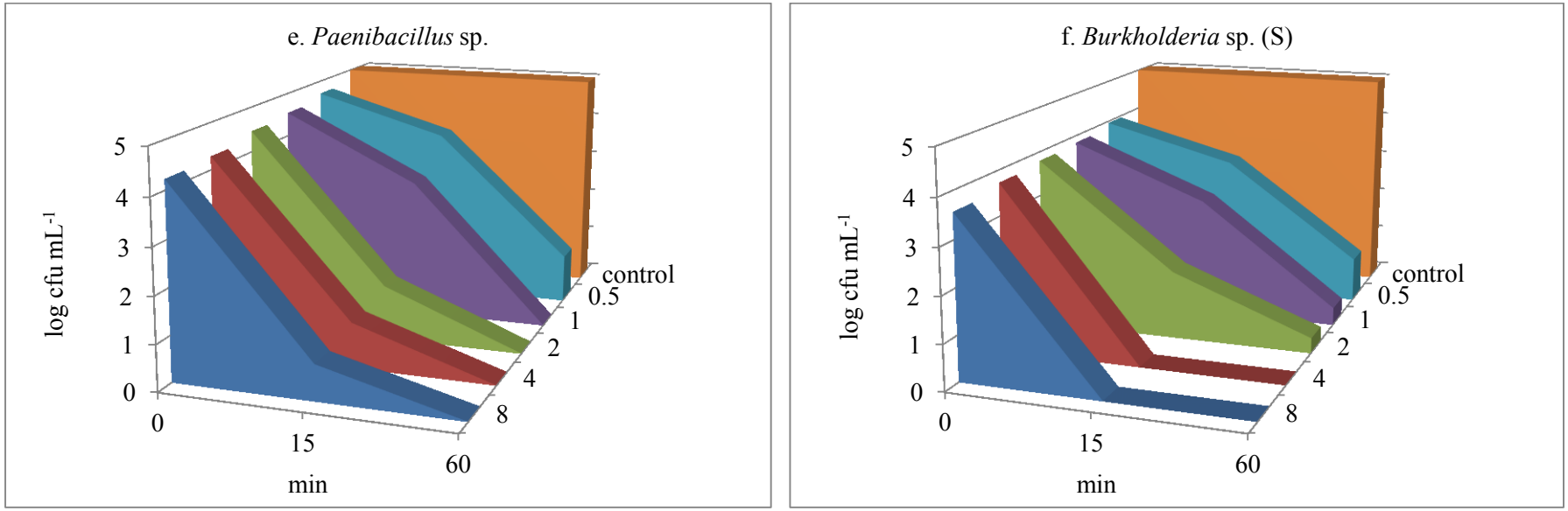

d. *Burkholderia* sp. (M)
log cfu mL-1
0
1
2
3
4
5
0
15
60
min
control
0.5
1
2
4
8



0.5 mg L^{-1}, 1.0 mg L^{-1}, 2.0 mg L^{-1}, 4.0 mg L^{-1}, 8.0 mg L^{-1}, C = control

Figure 1(a-f). Effect of different concentrations of free chlorine on survival of bacteria (mean log cfu mL^{-1}) at different contact time (n = 3).


a. *Cupriavidus* sp.
b. *Arthrobacter* sp.
c. *Bacillus* sp.
d. *Burkholderia* sp. (M)
log cfu mL-1
0
1
2
3
4
5
0
15
60
min
control
0.5
1
2
4
8



■ 0.5 mg L^{-1}, ■ 1.0 mg L^{-1}, ■ 2.0 mg L^{-1}, ■ 4.0 mg L^{-1}, ■ 8.0 mg L^{-1}, ■ control

Figure 2(a-f). Effect of different concentrations of monochloramine on survival of bacteria (mean log cfu mL^{-1}) at different contact time (n = 3).

Figure Legend:

Figure 1 (a-f). Effect of different concentrations of free chlorine on survival of bacteria (mean log cfu mL^{-1}) at different contact time (n = 3).

Figure 2 (a-f). Effect of different concentrations of monochloramine on survival of bacteria (mean log cfu mL^{-1}) at different contact time (n = 3).