



Rapid selection of antimicrobial-resistant bacteria in complex water systems by chlorine and pipe materials

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Abstract

Antimicrobial resistance is a major health issue induced by the overuse of antibiotics and disinfection reagents, e.g. chlorine. Resistant bacteria thus occur in water supply systems, and they transfer genes to other microbial populations, including pathogens. Treatment and inactivation of resistant bacteria are difficult in complex systems because the behaviour of resistant bacteria in such systems is poorly known, as most previous investigations are commonly performed in pure media. Therefore, we tested here the effect of 0.5 mg/mL chlorine and pipe materials made of polyvinyl chloride (PVC), copper and cement, on microbial populations in biofilms, during 5 days. Bacterial survival was monitored by viable counts, and resistant genes were analysed by quantitative polymerase chain reaction (qPCR). Results show that, in 56% of the cases, resistant bacteria became immediately enriched into biofilms due to chlorine exposure. Higher proportion of resistant bacteria were found in biofilms on PVC and copper pipes. Our findings imply that resistant microbial strains are very rapidly selected and that the pipe material has an influence on microbial selection.

Keywords Biofilm · Chlorine · Disinfectant · Water supply · Antibiotic-resistant bacteria

Introduction

Antimicrobial resistance has emerged globally as an environmental problem, but it can cross into being a food/water issue (Rusu et al. 2015; Daghrir and Drogui 2013). Water treatment plants and distribution systems use filtration and disinfection to control micro-organisms and maintain the potability of water (Morente et al. 2013). While generally effective, bacteria (including those resistant) invade water systems via line disruptions or compromised cisterns, which can introduce additional bacterial hazards. One cannot fully eradicate antimicrobial-resistant bacteria at normal treatment dosages; rather, much higher treatment dosages often become required. Unfortunately, higher concentrations produce odour and taste problems and are difficult to achieve

and maintain in water. While disinfectants help attenuate bacteria, nonetheless, they can selectively cause robust bacteria to proliferate (e.g. *Aeromonas*; McBain et al. 2004) and enrich antimicrobial-resistant bacteria.

The emergence, enrichment and transfer of resistance traits among bacteria depend on the microbial conditions in water distribution system. For example, biofilm-forming bacteria facilitate the attachment of micro-organisms along the distribution system and create community structures with greater complexity in terms of bacterial species than those in finished water (Chu et al. 2003), enhancing the capability to retain and protect pathogens. Furthermore, biofilm structures, with increased cell densities, facilitate the horizontal gene transfer of resistance genes. Moreover, bacterial physiology and metabolic rates vary along biofilm depth and reflect the gradients of nutrients versus chemical toxicants; the consequential reduced growth rates impact biocide efficacy (Boe-Hansen et al. 2002). Protective layers of biofilm bacteria provide better tolerance to metal ions and other toxic substances and improve survivability. Ultimately, biofilms in distribution systems become a generator and repository of resistance traits.

Conditions within water systems impact micro-organisms and further contribute to the formation, composition

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and proliferation of biofilms, e.g. source water (Zeng et al. 2013), excretion of extracellular polymeric substances (Fish et al. 2015), physico-chemical conditions of water, hydraulic operations and flow rates (Douterelo et al. 2016) and composition of pipe materials (Nguyen et al. 2012).

Pipes comprise of various metals: galvanized iron, lead, copper, cast iron, steel (Nguyen et al. 2012); plastic materials: chlorinated polyvinyl chloride, polypropylene, polyethylene, polyvinyl chloride and other materials: cementitious composites, such as concrete (Niquette et al. 2000); and elasto-materials. Pipe materials and biofilms differentially affect each other; e.g. the presence of slime-forming bacteria facilitates metal tolerance, and biofilm bacteria produce organic acids, which contribute to the dissolution of metals and cement. Consequently, exposure to leached materials from pipes can enhance antimicrobial resistance development, as frequently observed in contaminated environments in nature (e.g. Rodgers et al. 2018; Abdu et al. 2017). Ultimately, disinfection strategies and surface exposures may effectively attenuate most bacteria in distribution networks (e.g. Dong et al. 2018; Li et al. 2017), but could the stressors selectively contribute to resistance problem?

Here, we aimed to determine whether disinfectants and pipe material differentially affect bacteria with antimicrobial resistance. We employed experimental microcosms to examine how factors selectively influenced bacterial populations in a municipal water supply system, which included the formation of biofilms. These microcosms represented miniaturized analogues of actual distribution systems, and the bacteria were harvested from tap water; we hypothesized that different pipe material and disinfectant residuals impact microbial communities to select resistant bacteria and biofilm formation.

Materials and methods

Microcosm set-up

Experimental design involved laboratory microcosms with autoclaved phosphate buffer saline in Millipore water (10 mM, pH 7) having polyvinyl chloride, copper and cement pipes (internal volume 102 cm³, surface area 240 cm²) versus presence/absence of free chlorine (0.5 mg/mL), and as such six independent experiments were conducted. Peristaltic pumps (MP model M312) provided continuous recirculation (2 mL/min). Before each experiment, the microcosms, pipes and tubing were disinfected twice by 500 mg/L of sodium hypochlorite for 2 h.

Bacterial strains

Bacillus, *Paenibacillus* and *Micrococcus* were isolated from drinking water, except *Bacillus subtilis* R2 (the National Collection of Type Cultures, NCTC 10400, UK). The presence of antibiotic-resistant genes (*sul1* and *sul2*), mobile genetic elements (*intI1*) and chlorine susceptibility were determined as described previously (Khan et al. 2016a, b). The paired microcosm assays (chlorinated and chlorine free) were run with two populations of each genus having different levels of resistance: *sul1* (*Bacillus* and *Paenibacillus*), *sul2* and *intI1* (*Micrococcus*).

Experimental operation

Overnight culture in 50 mL Luria–Bertani broth was centrifuged 3 × (3000 rpm, 10 min), washed with phosphate buffer saline, added (final concentration 1 × 10⁵ cell/mL) to microcosms and recirculated for 5 days at 20 °C (± 2), with 1 mL harvested from each pipe on days 0, 3 and 5 for viable counts (Nutrient agar). Biofilm samples were taken on day 5 from 4 cm² area using sterile swabs, and 50 mL water was filtered through 0.22-µm membrane filter (Millipore, UK) on days 0 and 5. Filters and swabs were stored at – 20 °C. An additional 30–50 mL was collected on days 3 and 5 to measure temperature, pH (Model S40 SevenMulti™, Mettler Toledo™), dissolved oxygen (DO200 meter, VWR), total organic carbon (Teledyne Tekmar Apollo 9000 Combustion TOC Analyzer) and free chlorine (*N,N*-diethyl-*p*-phenylenediamine colorimetric method, Hach).

DNA extraction and quantitative polymerase chain reaction

DNA was extracted using the ISOLATE II Genomic DNA kit (Bioline, UK) as per manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was used to enumerate *Bacillus* (DeClerck et al. 2004), *Paenibacillus* (Pettersson et al. 1999) and *Micrococcus* species (Walcott and Gitaitis 2000). qPCR involves the amplification (copying) of specific DNA sequences in a manner with timing of fluorescent detection to count the number of genes in a sample. Strains were discerned with primers associated with their antibiotic resistance genes (*sul1*, *sul2*; Pei et al. 2006) or mobile genetic element (*intI1*; Luo et al. 2010); 16S-rRNA genes were quantified as a surrogate measure of 'total bacteria'. Serially diluted DNA standards (10⁸–10² genes/mL) and a control without DNA template were run with each qPCR assay.

Data analyses

From qPCR data, we calculated per cent resistance [resistance genes]/[total genus] and bacterial abundances on pipe surfaces and water. Fold increases in total bacteria and %resistance were calculated by comparing abundances between days 0 and 5. Abundances were log-transformed for presentation and statistical (e.g. normality) purposes. Data were analysed by Excel (Microsoft Office 2010) and Minitab version 17.

Results and discussion

Abundances of bacteria in biofilm

Microbial populations responded almost immediately by attaching to surfaces, with a greater percentage of those having resistant traits—suggesting that conditions in drinking water can select for bacterial contaminants with antimicrobial resistance. Surface swabs on day 5 for biofilm formation indicated that in two third of the cases bacteria adhered themselves onto surfaces (Fig. 1). Bacterial viability declines under stressful conditions, which resulted in differences in plate (viability) and genetic counts; however, the likely cause for declining gene counts in the water is the surface attachment of cells, which would escape detection in the water. One exception was *Bacillus*, where only 35% of

total bacteria and 65% of the resistant strain were found in biofilms in the polyvinyl chloride microcosms. Further, only the resistant strains of *Micrococcus* and *Paenibacillus* were found on copper surfaces. Percentages of total and resistant populations were equivalent for other treatments.

Here, up to $10^{6.5}$ cfu/cm² was found on surfaces in 5 days, more than reported previously (i.e. 10^5 cfu/cm² Frias et al. 2001). Bacteria showed different biofilm potentials: *Paenibacillus* > *Bacillus* > *Micrococcus* (Fig. 1) depending on pipe materials. Reported trend is as follows: polyvinyl chloride < copper < steel < iron < cement < plastic (e.g. polyethylene) < elastomeric surfaces (Yu et al. 2010), with some variation in adhesion potentials (Niquette et al. 2000). Conversely in this study, polyvinyl chloride and copper pipes allowed more bacterial attachment with chlorine. Biofilm formation was faster on plastic pipes than on copper pipes, due to copper toxicity (Yu et al. 2010); however, in this experiment, phosphate levels may have confounded effects of copper toxicity.

Waterborne resistant and total populations

Each pair of bacteria was added (totalling 2×10^5 cfu/mL, log 5.3) into the microcosms; heterotrophic plate counts determined viabilities (Table 1). In unchlorinated systems, viable cell counts declined by various orders of magnitude, except *Micrococcus*, which showed greater viability between days 3 and 5 in polyvinyl chloride and copper pipes.

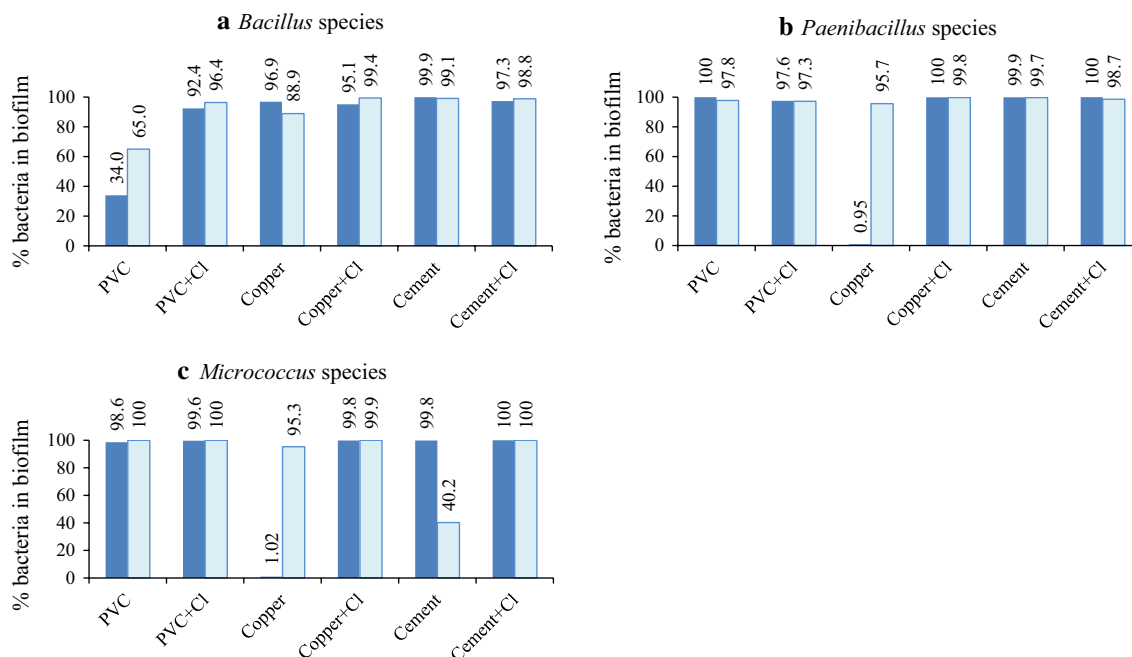


Fig. 1 Percentage of bacteria attached to surfaces after 5 days. Chlorination selection was found in resistant bacteria (*Bacillus* R1 and *Paenibacillus* R), which were higher in chlorinated pipes than non-

chlorinated in all cases. *Cl* chlorinated, *PVC* polyvinyl chloride. ■ Total bacteria. ■ Resistant bacteria

Table 1 Heterotrophic plate count (log cfu/mL) in the different pipe material in the absence and presence of 0.5 mg/L free chlorine; each species was added on day 0 at a concentration of 10^5 cfu/mL (log total bacteria = 5.3)

Treatment	<i>Bacillus</i>		<i>Paenibacillus</i>		<i>Micrococcus</i>	
	Day 3	Day 5	Day 3	Day 5	Day 3	Day 5
Polyvinyl chloride	2.9	2.0	3.2	1.5	1.6	2.8
Polyvinyl chloride and chlorine	ND	ND	1.2	0.5	ND	1.9
Copper	2.8	1.2	3.3	2.0	0.9	2.8
Copper and chlorine	0.3	ND	1.2	1.7	ND	ND
Cement	2.9	2.6	3.5	2.4	3.5	3.0
Cement and chlorine	ND	ND	1.4	0.7	ND	ND

Micrococcus showed greater viability between days 3 and 5 in polyvinyl chloride and copper pipes
cfu colony forming units, ND not detected

Chlorination compromised viabilities by 1–2 orders greater reduction than untreated systems (Table 1). No viable *Micrococcus* were recovered from any chlorinated microcosm on day 3, although *Paenibacillus* and *Micrococcus* showed some recovery by day 5 in copper and polyvinyl chloride pipes, respectively.

Both the susceptible and resistant strains were simultaneously challenged in the microcosms and monitored by quantitative PCR to differentiate strains. Their presence in water declined across all treatments (Table 2), except for *Paenibacillus* and *Micrococcus* spp. in the polyvinyl chloride and cement treatments. In 44.4% of the cases, the resistant strains also declined, but at a lesser rate—resulting in mixed populations skewed towards a greater proportion of resistant bacteria.

Viability counts (Table 1) and gene detection (non-viable) are not analogous. Genetic (resistance and 16S rRNA) traits indicate the presence of bacteria in the system, whereas plate counts indicate whether bacteria are capable of growing. Although not growing, their presence in the water (even with chlorine) could increase the prevalence of resistant genes if conditions change.

Low concentrations of disinfectants exert selective pressure on resistant populations, but another driving force, i.e. pipe materials, showed differential selection patterns in *Micrococcus* species (Table 1). Further, pipe materials impacted bacterial concentrations; e.g. polyvinyl chloride pipes had greatest abundances of bacteria, and copper, the least, which may be attributable to its antimicrobial effects.

Depending on the bacteria, resistant populations also proliferated in circulating water, e.g. *Micrococcus* and *Paenibacillus* (Table 2).

Water chemistry

Water conditions remained similar: 3.3 mg/L dissolved oxygen (± 0.7 , 95% confidence intervals); 87.4 ± 58.4 mg/L total organic carbon; and initial chlorine 0.50 mg/L, but declined to 0.15 (± 0.06) and 0.06 (± 0.03) on days 3 and 5, respectively. Initial pHs were 7.6 (± 0.1); however, elevated pHs were observed in copper (9.4 ± 1.8) and cement microcosms (9.0 ± 1.3). Neither pH nor dissolved oxygen was impacted by inoculations or chlorination.

The dissolved oxygen and total organic carbon were within World Health Organization guidelines for drinking water. Dissolved oxygen influences corrosion in water pipes and bacterial activity, while the organic carbon increases chlorine demand and microbial growth. Here, any residual organic carbon likely reduced the free chlorine concentration as observed.

Relationship between physico-chemical properties, abundances of genes and bacteria

Comparison of population abundances and viabilities to the physico-chemical conditions (Table 3) indicated that dissolved oxygen increased bacterial abundances and might have an inverse impact on relative abundance of resistant

Table 2 Changes in total and resistant bacterial abundances after 5 days in microcosms as determined by polymerase chain reaction

Assay	Organism type	PVC	PVC + Cl	Copper	Copper + Cl	Cement	Cement + Cl
<i>Bacillus</i>	Total <i>Bacillus</i>	–	–	–	–	–	–
	% Resistant	–	–	+	–	–	–
<i>Paenibacillus</i>	Total <i>Paenibacillus</i>	ND	+	–	–	–	ND
	% Resistant	+	+	0	–	–	0
<i>Micrococcus</i>	Total <i>Micrococcus</i>	–	–	–	–	ND	ND
	% Resistant	+	–	+	+	+	–

+ increase, – decrease, 0 unchanged, ND not detected, Cl chlorine, PVC polyvinyl chloride

Table 3 Pearson correlation analysis between physico-chemical properties and bacterial abundances; significant correlations ($p < 0.010$) highlighted in bold

Assay	Genes	Pearson correlation						Biofilm									
		Water			Biofilm			Water			Biofilm						
		DO	TOC	Cl	pH	HPC	DO	TOC	Cl	pH	HPC	DO	TOC	Cl	pH	HPC	
<i>Bacillus</i>	<i>Bacillus</i> R1/total bacteria	r value	-0.17	0.03	-0.60	0.54	0.28	0.43	-0.50	-0.59	0.95	0.28	0.43	-0.50	-0.59	0.95	-0.23
		p value	0.69	0.95	0.12	0.17	0.51	0.39	0.31	0.91	0.00	0.66	0.39	0.31	0.91	0.00	0.66
	Total bacteria	r value	0.63	-0.23	0.32	-0.55	0.20	-0.39	-0.00	0.29	-0.67	0.20	-0.39	-0.00	0.29	-0.67	0.38
		p value	0.10	0.59	0.44	0.15	0.63	0.45	0.99	0.58	0.14	0.46	0.45	0.99	0.58	0.14	0.46
<i>Paenibacillus</i>	<i>Paenibacillus</i> R/total bacteria	r value	-0.89	0.78	0.01	0.82	-0.10	-0.10	0.15	0.34	0.43	-0.10	-0.10	0.15	0.34	0.43	0.33
		p value	0.02	0.07	0.95	0.05	0.33	0.87	0.81	0.57	0.47	0.59	0.87	0.81	0.57	0.47	0.59
	Total bacteria	r value	0.88	-0.78	-0.04	-0.81	0.06	-0.09	-0.12	-0.51	-0.35	0.06	-0.09	-0.12	-0.51	-0.35	-0.19
		p value	0.02	0.07	0.94	0.05	0.92	0.89	0.84	0.38	0.57	0.76	0.89	0.84	0.38	0.57	0.76
<i>Micrococcus</i>	<i>Micrococcus</i> S/total bacteria	r value	-0.31	0.33	-0.17	0.85	-0.23	0.63	0.31	0.01	-0.32	-0.23	0.63	0.31	0.01	-0.32	-0.42
		p value	0.50	0.43	0.72	0.02	0.62	0.18	0.55	0.99	0.54	0.71	0.18	0.55	0.99	0.54	0.71
	Total bacteria	r value	0.46	-0.30	0.20	-0.88	0.12	0.05	-0.82	0.72	-0.47	0.12	0.05	-0.82	0.72	-0.47	-0.53
		p value	0.29	0.51	0.67	0.01	0.80	0.93	0.04	0.11	0.35	0.28	0.93	0.04	0.11	0.35	0.28

Dissolved oxygen increased bacterial abundances and might have an inverse impact on relative abundance of resistant strains (though significant in *Paenibacillus*). Elevated pH reduced total bacteria, which were observed by inverse correlations in absolute abundances and positive correlations in relative numbers
 DO dissolved oxygen, TOC total organic carbon, Cl chlorine, HPC heterotrophic plate count (log transformed)
 Significant limit was $p < 0.10$

strains (though significant in *Paenibacillus* assays only). Occasional correlations occurred between organic carbon and bacterial abundances. Further, elevated pH reduced total bacteria, which were observed by inverse correlations in absolute abundances, and positive correlations in relative numbers—suggesting that presence of resistant strains were less impacted. There were no significant correlations between viable counts and resistant gene abundances, nor chlorine and resistance genes; while interesting, this is not surprising as gene presence does not represent viability, as discussed previously.

Conclusion

This study demonstrated the factors affecting the fate of antimicrobial resistance in water distribution systems; pipe material, residual disinfectant and water age shift community dynamics. Resistant bacteria developed biofilm on polyvinyl chloride and copper pipes (66.7%) more than on cement pipes (16.7%). Biofilm-forming bacteria behave differently than planktonic cells, and due to physiological adaptations, they exhibit greater resistance to disinfectants as 55.6% cases showed more resistant population in biofilm in the presence of chlorine. In terms of total population, more bacteria were in biofilms. Further, increased population densities and selective pressures by sub-inhibitory concentrations of disinfectants and pipes create conditions, under which micro-organisms acquire new traits and become more resistant.

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