Prevalence of bacterial pathogens in biofilms of drinking water distribution systems

S. M. September, F. A. Els, S. N. Venter and V. S. Brözel

ABSTRACT

Water for human consumption is required to be free from any bacteria that might pose a health risk. The presence of biofilms in the drinking water distribution system may play a role in the presence of potential pathogens in the drinking water supply. Ninety-five biofilm samples from various parts of South Africa were tested for the presence of *Escherichia coli, Aeromonas, Pseudomonas, Salmonella, Shigella* and *Vibrio* spp. Members of these genera were quantified by the three-tube most probable number (MPN) approach using enrichment broths and plating on selective agars. The heterotrophic culturable counts were determined for both the planktonic and biofilm phases of the samples. Biofilm density varied between 10 and 1.9×10^9 colony forming units cm^{-2}. The 16S rRNA identity of the putative pathogenic isolates revealed that high numbers of *Aeromonas, Pseudomonas, Klebsiella* and *Enterobacter* were present, but no putative *Salmonella* and *Shigella* could be confirmed. None of the *Pseudomonas* isolates belonged to the pathogenic *Pseudomonas aeruginosa* or *Pseudomonas mendocina* while the *Aeromonas* isolates showed relatedness to known pathogenic members of this group.

Key words | biofilm, distribution system, drinking water, pathogen

S. M. September
F. A. Els
S. N. Venter
Department of Microbiology and Plant Pathology,
University of Pretoria,
Pretoria, 0002,
South Africa

V. S. Brözel (corresponding author)
NPB 252B, Department of Biology and Microbiology,
Box 2140D, South Dakota State University,
Brookings, SD 57007,
USA
Tel: 605 688 6483
Fax: 605 688 5624
E-mail: *Volker.brozel@sdstate.edu*

INTRODUCTION

Water is the most common surface-exposed compound on Earth, but only *c.* 2.6% is available as potential drinking water (Szewzyk *et al.* 2000). According to the World Health Organization (WHO), drinking water should be free from any organism that might pose a health risk to the human population (WHO 2004). Water authorities throughout the world are thus dedicated to ensure that the water that reaches the consumer is safe for consumption and free from any substances that may be harmful to health. Water is normally disinfected before being distributed to the different end-point users and the microbial levels of the water, when leaving the treatment plant, have to be within limits set by water authorities. By the time it reaches the tap in the house of the consumer, water quality may differ dramatically from the quality at the time of treatment.

The decline in microbial water quality may be attributed to the recovery and subsequent growth of sub-lethally damaged bacteria, to system deficiencies such as cross-connections, broken water mains and contamination during bulk storage and repairs (Craun & Calderon 2001), and to the presence of biofilms within the distribution system from which cells may be released into the flow (Camper *et al.* 1999; Lee & Kim 2003). Drinking water distribution systems provide an oligotrophic environment, and post-treatment recovery and growth of bacteria is therefore a concern because of the effect the environment can have on public health (Assanta *et al.* 1998; Kerr *et al.* 1999). Many microorganisms are known to be transmitted by water, including *Aeromonas, Campylobacter, Escherichia coli, Helicobacter, Legionella, Pseudomonas, Salmonella, Vibrio, Cryptosporidium* and hepatitis E virus (Szewzyk *et al.* 2000; Hunter *et al.* 2001; Lee & Kim 2003; Payment *et al.* 2003). A number of these organisms are known to grow in distribution networks, with some only recently

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recognized as pathogenic to humans. They include members of the genera *Aeromonas, Legionella, Mycobacterium* and *Pseudomonas* (Szewzyk *et al.* 2000; September *et al.* 2004). Many of these organisms are also referred to as 'new emerging pathogens' since they have not previously been thought to be associated with water or could not be detected owing to a lack of proper detection methods (Szewzyk *et al.* 2000; Medema *et al.* 2003).

In many water systems, cells can attach and form biofilms on the surfaces of the piping material (Szewzyk *et al.* 2000). The majority of bacteria in drinking water systems occur in biofilms rather than in the water phase (Szewzyk *et al.* 2000). The presence of such biofilms within a water distribution system may act as a form of protection against extreme environmental conditions and predation for many of the above-mentioned pathogens (Costerton *et al.* 1995; Buswell *et al.* 1998; Thomas *et al.* 1999). Organisms in biofilms also tend to become more resistant to treatment or disinfection, and the biofilm can become a reservoir for the subsequent spread of pathogenic organisms (Camper *et al.* 1996). In addition, the organisms in the biofilm can influence the taste and odour of the water, and if biofilms develop on ferrous metal surfaces, they may cause corrosion of the pipes and also the release of iron particles into the water (Ridgway *et al.* 1981; Camper *et al.* 1999). Pathogens surviving treatment could colonize an existing biofilm where they could grow and later be released into the bulk flow. Pathogens released into the water would place consumers at risk. Little is currently known about the prevalence of pathogenic bacteria in biofilms of drinking water distribution systems.

The aim of this study was thus to determine the prevalence of *Aeromonas, E. coli, Pseudomonas, Salmonella, Shigella* and *Vibrio* in biofilms in drinking water distribution systems in both large and small towns and home storage systems in South Africa.

METHODS

Ninety-five samples were collected from two well-serviced urban areas (Pretoria (31 samples) and Pietermaritzburg (20 samples)), a semi-urban developing community (Botshabelo (five tap and 17 bucket samples)) as well as other towns with small distribution networks (22 samples) in South Africa. Water from the two urban areas had been treated by chlorination and chloramination while the rest were only chlorinated. Samples were collected during the period September 2001 to August 2002. Prior to collection, water was allowed to run to waste at a uniform rate for 2–3 min. Water samples were collected in sterile bottles containing sterile sodium thiosulphate to a final concentration of 0.01% (w/v) to neutralize any free or combined residual chlorine (Le Chevallier *et al.* 1988). Following removal of the tap, biofilm samples were taken from the inner surface

Table 1 | Pathogens to be isolated from the biofilms in a drinking water system and the respective enrichment broths, selective agars and incubation conditions employed

Pathogen	Enrichment	Incubation conditions	Selective medium	Incubation conditions
Aeromonas	Tryptone Soy Broth (Oxoid) + 1% Yeast Extract (Oxoid)	37°C, 24 h	*Aeromonas* Agar Base (Oxoid) + Ampicillin Selective Supplement (Oxoid)	37°C, 24 h
Escherichia coli	Nutrient Broth (Oxoid)	37°C, 24 h	Chromocult Coliform-Agar (Merck)	37°C, 24 h
Pseudomonas	Buffered Peptone Water (Oxoid)	37°C, 24 h	*Pseudomonas* Isolation Agar (Difco)	37°C, 24 h
Salmonella	Rappaport-Vassiliadis Enrichment Broth (Oxoid)	42°C, 24 h	XLD (Oxoid)	37°C, 24 h
Shigella	MacConkey Broth (Oxoid)	37°C, 24 h	Hektoen Enteric Agar (Oxoid)	37°C, 24 h
Vibrio	Alkaline Peptone Water	37°C, 24 h	Cholera Medium TCBS (Oxoid)	35–37°C, 24 h

of the service pipe using a sterile cotton-tipped swab moistened in 1 ml sterile $\frac{1}{4}$ strength Ringer's solution (Merck). The area of biofilm removed in this way was *c.* 1 cm^2. In the case of the buckets, a similar sized area (*c.* 1 cm^2) was swabbed. The swab was returned to the corresponding Ringer's solution tube. All the samples were transported to the laboratory on ice and analysed within 12 to 18 h.

Water from the systems was tested for the presence of faecal coliforms by filtering duplicate 100 ml volumes through 0.45 μm nitrocellulose filters (Millipore) and incubating on mFC agar (Merck) at 44.5°C for 22 to 24 h. Biofilm samples were dispersed by vigorous vortexing in sterile diluent. The heterotrophic culturable count was determined for both the water and biofilm phases of the samples by plating decimal dilutions (10^{-1} to 10^{-6} in $\frac{1}{4}$ strength Ringer's solution) onto R_2A agar (Oxoid) and incubating at 28°C for 5 days. Colonies were counted daily for 5 days.

Biofilm samples, as collected from various sites, were also analysed to determine the numbers of potential pathogens (*Aeromonas, E. coli, Pseudomonas, Salmonella, Shigella* and *Vibrio*). These pathogens were selected based on their reported association with drinking water supplies (Oragui *et al.* 1993; Assanta *et al.* 1998; Fernández *et al.* 2000; Gibbotti *et al.* 2000). For the enumeration of the different potential pathogenic bacteria, the three-tube MPN technique was employed. The 10^{-1} dilution of the biofilm samples was used to inoculate three replicate tubes of the relevant enrichment broths, as outlined in Table 1. From this triplicate set of tubes further serial dilutions were performed to obtain a dilution series of 10^{-1} to 10^{-4}. These were incubated for 24 h at the respective incubation conditions (Table 1). After 24 h, *c.* 10 μl from each tube was streaked onto the corresponding selective sub-culture medium (Table 1). The respective dilution was scored as positive where colony appearance corresponded to that as described in user manuals and the literature. Single presumptively positive colonies were picked from the highest dilution of each type of selective subculture medium and streaked onto nutrient agar (Oxoid) to obtain pure isolates.

A total of 74 of the isolates from the pathogen groups were selected at random from colonies appearing as those known to be pathogens for further identification. All putative *Salmonella, Shigell*a and *Vibrio* were picked for identification. These isolates were cultured on nutrient agar

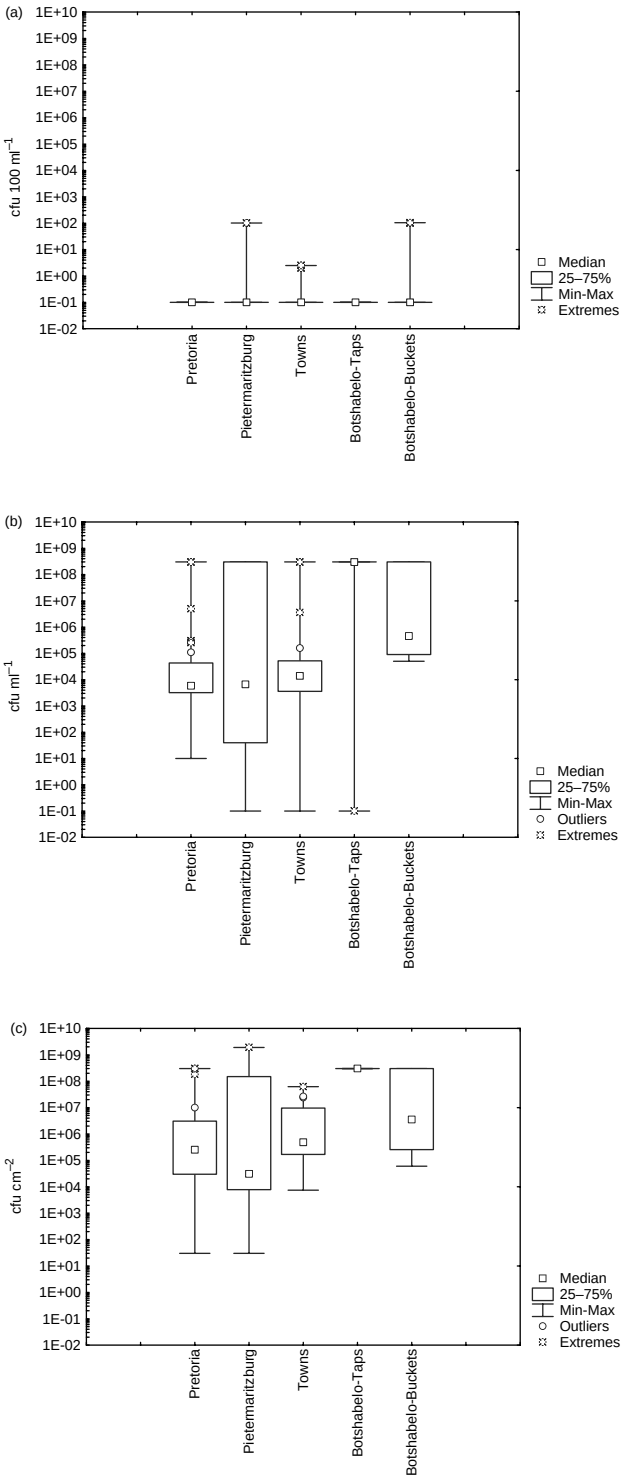

Figure 1 | Box and whisker plots showing the presence of (a) faecal coliforms (mFC agar) and the heterotrophic culturable counts (R2A, 5d) of (b) water and (c) biofilms of the different distribution networks sampled.

and genomic DNA was extracted using standard procedures (Lemanceau *et al.* 1995). The 16S rRNA genes were amplified from the genomic DNA extracts by PCR. PCR was performed in 50 μl reaction mixtures consisting of 50–100 ng of DNA template, 250 μM of each deoxynucleoside triphosphates, 25 pmol of each primer, 1 × reaction buffer, 1.5 mM $MgCl_2$ and 1 U of *Taq* polymerase (Southern Cross Biotechnologies). Primer sets fD1 (5′ AGA GTT TGA

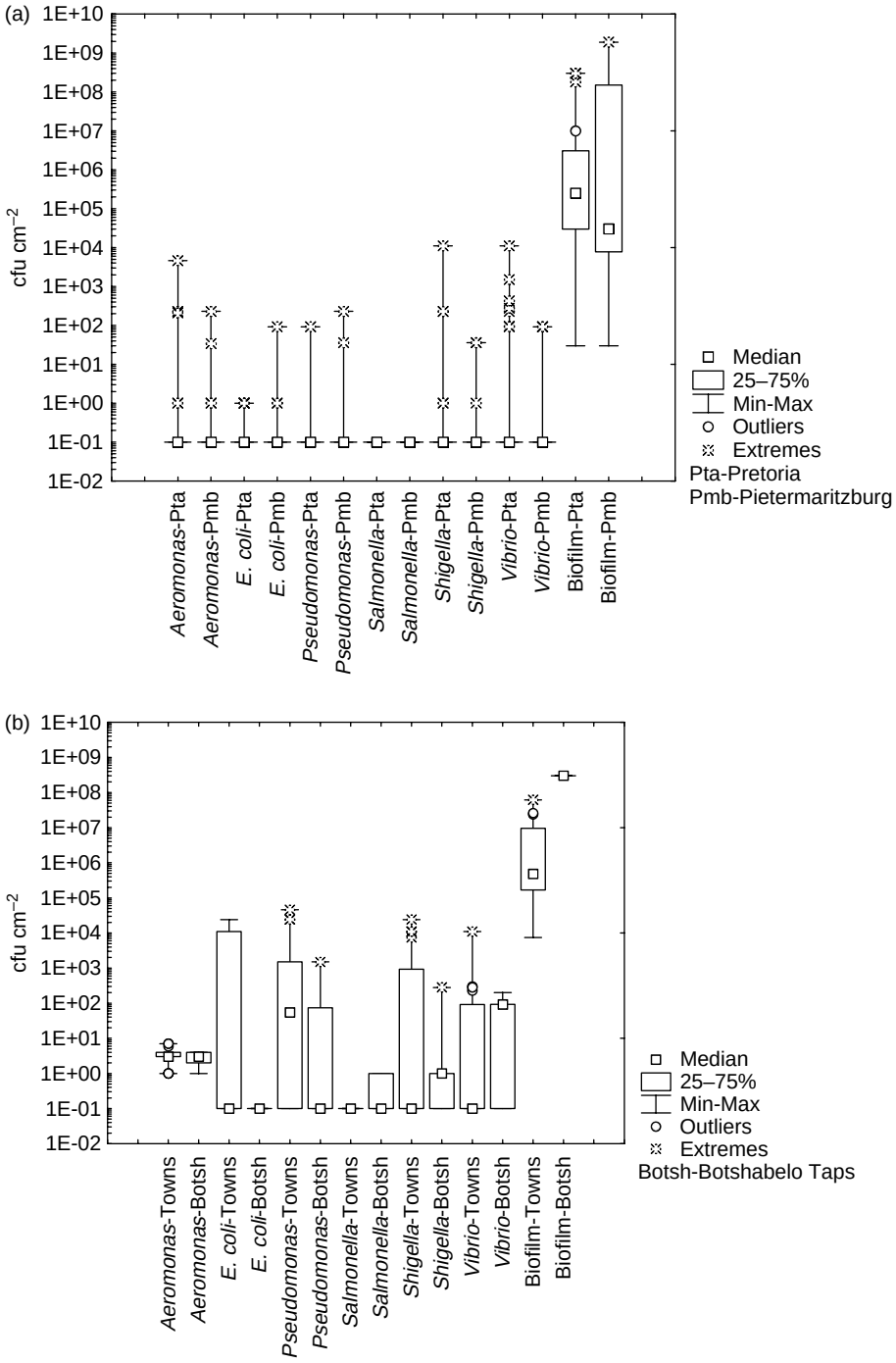

Figure 2 | Presence of the various presumptive pathogens as found in the biofilm samples from (a) Pretoria and Pietermaritzburg areas and (b) small towns and Botshabelo taps.

TCC TGG CTC AG 3′) and rP2 (5′ ACG GCT ACC TTG TTA CGA CTT 3′) (Weisburg *et al.* 1991) were used for the PCR amplification. In rare cases where PCR did not yield amplicons, the primers 63f (5′ CAG GCC TAA CAC ATG CAA GTC 3′) and 1387r (5′ GGG CGG WGT GTA CAA GGC 3′) (Marchesi *et al.* 1998) were used. The PCR conditions were 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and final extension at 72°C for 5 min (GeneAmp PCR system 2400; Perkin-Elmer).

The PCR products were analysed on 1.5% agarose gels containing 10 mg ml^{-1} ethidium bromide in 1 × TAE Buffer at 100 V for 30 min and photographed. PCR products were purified using a Geneclean™ Kit (Bio 101, Inc), but using silica instead of glassmilk (Boyle & Lew 1995), or the QIAquick PCR Purification Kit (Qiagen). These purification steps were performed using the standard protocols as supplied by the respective manufacturers. The nucleotide sequences of purified PCR products were determined using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) and an ABI PRISM 377 or ABI Prism 3100 Sequencer (Perkin Elmer). Sequences were edited by Sequence Navigator (PE Applied Biosystems) and the identities of isolates were determined by searching known sequences in GenBank using the basic BLAST search of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/BLAST/).

RESULTS AND DISCUSSION

Water for human consumption is usually disinfected before being distributed to the consumer to ensure that the level of any potentially harmful microbial agents falls under defined low levels, for example zero faecal coliforms per 100 ml. In many instances the quality of the water may have deteriorated by the time it reaches the consumer. This is often due to recontamination after treatment owing to the regrowth of sub-lethally damaged bacteria or contamination from bacteria harboured in biofilms (Camper *et al.* 1999; Lee & Kim 2003).

The heterotrophic culturable counts for the analysis of the water samples ranged from 1.0×10^{-1} to 1.9×10^{9} colony forming units (cfu) per ml (Figure 1(b)) and for the biofilm analysis of the same samples between 1.0×10^{1} and more than 1.9×10^{9} cfu cm^{-2} (Figure 1(c)). Bacterial biofilms were found on the walls of all the surfaces tested.

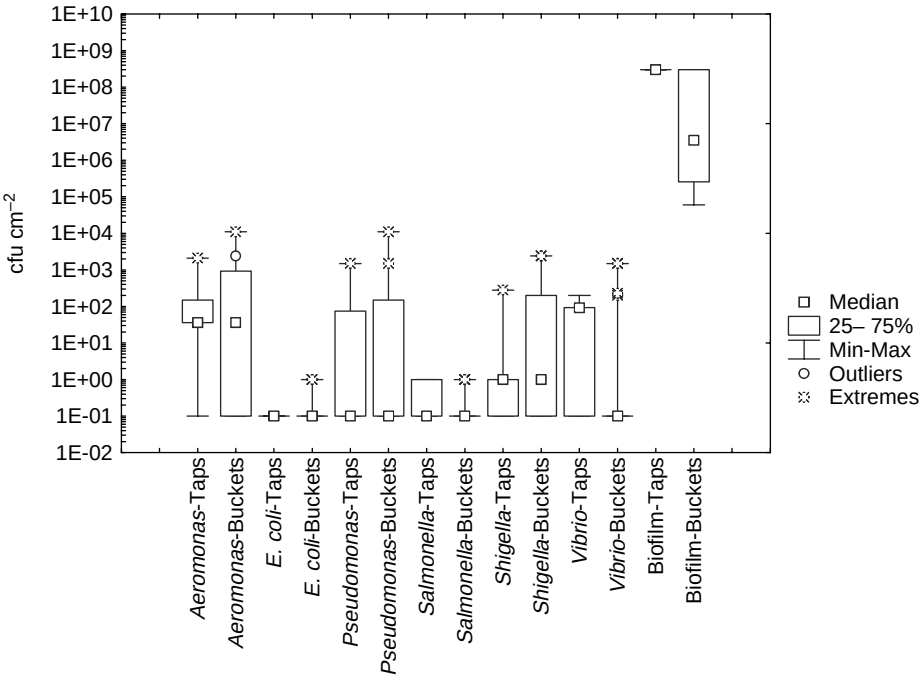

Figure 3 | Presence of the various presumptive pathogens as found in the tap and bucket samples from Botshabelo.

Table 2 | Partial 16S rDNA sequence identity of randomly selected isolates

Isolate	Isolation medium	16 s rDNA sequence identity
2 PTN E1	Chromocult Coliform-Agar	*Acinetobacter*
1 25B SH1	Hektoen Enteric Agar	*Acinetobacter*
1 25B A1	*Aeromonas* Agar Base	*Acinetobacter*
1 12B SH1	Hektoen Enteric Agar	*Acinetobacter*
1 ERC A1	*Aeromonas* Agar Base	*Acinetobacter*
2 127 PS1	*Pseudomonas* Isolation Agar	*Aeromonas*
2 241 SH1	Hektoen Enteric Agar	*Aeromonas*
2 241 PS1	*Pseudomonas* Isolation Agar	*Aeromonas*
1 24B V1	Cholera Medium TCBS	*Aeromonas*
1 24B V2	Cholera Medium TCBS	*Aeromonas*
1 24T PS1	*Pseudomonas* Isolation Agar	*Aeromonas*
1 25T SA1	XLD	*Aeromonas*
1 25T PS1	*Pseudomonas* Isolation Agar	*Aeromonas*
1 12B A1	*Aeromonas* Agar Base	*Aeromonas*
1 20T A1	*Aeromonas* Agar Base	*Aeromonas*
2 BGF A1	*Aeromonas* Agar Base	*Aeromonas*
2 SEA A1	*Aeromonas* Agar Base	*Aeromonas*
2 SEB E1	Chromocult Coliform-Agar	*Aeromonas*
1 24B A2	*Aeromonas* Agar Base	*Aeromonas*
1 24B PS1	*Pseudomonas* Isolation Agar	*Aeromonas*
2 BGB E1	Chromocult Coliform-Agar	*E. coli*
2 BGC E1	Chromocult Coliform-Agar	*E. coli*
1 WYL A1	*Aeromonas* Agar Base	*Enterobacter*
1 WYL V1	Cholera Medium TCBS	*Enterobacter*
2 111 A1	*Aeromonas* Agar Base	*Enterobacter*
1 11T V1	Cholera Medium TCBS	*Enterobacter*
1 VIA V2	Cholera Medium TCBS	*Enterococcus*
1 24B SA1	XLD	*Klebsiella*
2 111 SH1	Hektoen Enteric Agar	*Klebsiella*
1 12B SA1	XLD	*Klebsiella*
1 11T A1	*Aeromonas* Agar Base	*Klebsiella*
1 20B A1	*Aeromonas* Agar Base	*Klebsiella*
1 20T SA1	XLD	*Klebsiella*
2 127 E1	Chromocult Coliform-Agar	*Pantoea*
2 KHA E2	Chromocult Coliform-Agar	*Pantoea*
2 KHA A1	*Aeromonas* Agar Base	*Pantoea*
1 24B SA2	XLD	*Pantoea*
1 24T A2	*Aeromonas* Agar Base	*Pantoea*
1 WYL SH1	Hektoen Enteric Agar	*Pantoea*
1 WYL PS1	*Pseudomonas* Isolation Agar	*Pseudomonas*
1 REL PS1	*Pseudomonas* Isolation Agar	*Pseudomonas*
2 PC SH1	Hektoen Enteric Agar	*Pseudomonas*
2 CC PS1	*Pseudomonas* Isolation Agar	*Pseudomonas*
2 127 A1	*Aeromonas* Agar Base	*Pseudomonas*
2 ARW V1	Cholera Medium TCBS	*Pseudomonas*
2 ERC V1	Cholera Medium TCBS	*Pseudomonas*
2 PTN V1	Cholera Medium TCBS	*Pseudomonas*
2 ARW SH1	Hektoen Enteric Agar	*Pseudomonas*
1 24T SH2	Hektoen Enteric Agar	*Pseudomonas*
1 11B SA2	XLD	*Pseudomonas*
1 11B SH1	Hektoen Enteric Agar	*Pseudomonas*
1 11B V1	Cholera Medium TCBS	*Pseudomonas*
1 20B SA2	XLD	*Pseudomonas*
1 20B SH2	Hektoen Enteric Agar	*Pseudomonas*

Table 2 | (*continued*)

Isolate	Isolation medium	16 s rDNA sequence identity
1 HAW V1	Cholera Medium TCBS	*Pseudomonas*
1 HAW A1	*Aeromonas* Agar Base	*Pseudomonas*
1 HAW A2	*Aeromonas* Agar Base	*Pseudomonas*
1 VIA A1	*Aeromonas* Agar Base	*Pseudomonas*
1 VIA V1	Cholera Medium TCBS	*Pseudomonas*
1 HAW SH1	Hektoen Enteric Agar	*Pseudomonas*
1 FGA A1	*Aeromonas* Agar Base	*Pseudomonas*
1 FGA SH1	Hektoen Enteric Agar	*Pseudomonas*
1 FGA V1	Cholera Medium TCBS	*Pseudomonas*
1 FGB V2	Cholera Medium TCBS	*Pseudomonas*
1 HAW SH2	Hektoen Enteric Agar	*Pseudomonas*
1 VIA SH1	Hektoen Enteric Agar	*Pseudomonas*
1 VIA SH2	Hektoen Enteric Agar	*Pseudomonas*
2 BGE PS1	*Pseudomonas* Isolation Agar	*Pseudomonas*
2 TRA PS1	*Pseudomonas* Isolation Agar	*Pseudomonas*
2 SEA PS1	*Pseudomonas* Isolation Agar	*Pseudomonas*
2 SEG PS1	*Pseudomonas* Isolation Agar	*Pseudomonas*
2 SEB PS1	*Pseudomonas* Isolation Agar	*Pseudomonas*
2 BGA V1	Cholera Medium TCBS	*Pseudomonas*
2 BGE V1	Cholera Medium TCBS	*Vibrio*

These counts are minimum values, as not all cells were culturable and co-aggregating cells may have led to an underestimation of the count. Faecal coliforms were detected in 7.7% of the tap samples (two from Pietermaritzburg, four from small towns) and 23.5% of the Botshabelo buckets (Figure 1(a)). These waters did not comply with the South African National Standard (Anon 2000). There is no clear evidence that the disinfection methods used had an influence on the presence of faecal coliforms or the counts of both the planktonic and biofilm phase.

A variety of potential pathogens were isolated at various densities, up to 10^4 cfu cm^{-2} from some sampling points. These included *Aeromonas, E. coli, Salmonella, Shigella, Pseudomonas* and *Vibrio*. Samples from the larger distribution networks (Pretoria and Pietermaritzburg) in general had a much lower incidence of pathogens than those from the small towns and Botshabelo (Figure 2(a, b)). Comparison of the tap and container samples from Botshabelo showed that in general the containers harboured a higher number of pathogens, with the exception of presumptive *Salmonella* (Figure 3). This may be an indication that bacterial numbers in container-associated biofilms increased upon storage or that the containers were not properly cleaned (Jagals *et al.* 2003). In all of the samples, the number of pathogens detected was lower than the corresponding heterotrophic culturable counts of the biofilm samples analysed.

The partial sequences of the 16S rDNA genes of 74 randomly selected isolates were determined. All in all, 5 *Acinetobacter,* 15 *Aeromonas,* 4 *Enterobacter,* 6 *Klebsiella*, 6 *Pantoea* and 34 *Pseudomonas* were identified, together with one or two representatives of various other genera (Table 2). Notably, not one putative *Salmonella* or *Shigella* could be confirmed, indicating that none of these virulent pathogens could be detected in drinking water-associated biofilms tested. The genera listed all contain known pathogenic bacteria, some to a more prominent and others to a lesser degree. For example, the genus *Aeromonas* is known to contain species pathogenic to humans, notably *Aeromonas caviae* and *Aeromonas veronii* biotype *sobria*, associated, among other things, with travellers' diarrhoea (Vila *et al.* 2003). Within the genus *Pantoea,* normally associated with plant diseases, *Pantoea agglomerans* is linked with septic arthritis (De Champs *et al.* 2000; Kratz *et al.* 2003). The *Pseudomonas* isolates were all very similar to each other, but none could be assigned to the nosocomial pathogens *P. aeruginosa* or *P. mendocina*. None of the *Klebsiella* isolates were closely related to documented pathogens such as *Klebsiella pneumoniae*.

Most of the isolates chosen for further identification were gram negative rods. The partial 16S rDNA sequence identities of the isolates seldom corresponded to what was expected for the specific selective media. This showed that the different

selective media used are not as selective as reported when used for the analysis of non-medical samples. For example, species from *Acinetobacter* and *Pantoea*, for which we did not test, were isolated from media specific for *Aeromonas, E. coli*, *Salmonella* and *Shigella*. Cholera medium yielded several colonies appearing as *Vibrio cholera*, but which turned out to be *Pseudomonas*, *Aeromonas* and *Enterobacter*. XLD agar, for the growth of *Salmonella*, yielded *Aeromonas, Pantoea, Pseudomonas* and *Klebsiella*. Bacterial analyses of water based on selective isolation and culturing techniques should, therefore, be interpreted with caution.

CONCLUSIONS

- Biofilms occurred in all drinking water distribution systems at levels between 1.0×10^1 to more than 1.9×10^9 cfu cm^{-2}.
- Biofilms from the two large distribution networks (Pretoria and Pietermaritzburg) revealed a very low prevalence of putative pathogens, while the smaller networks (small towns and Botshabelo) harboured high numbers of a variety of potential gram negative pathogens.
- A large number of biofilm samples harboured presumptive *Aeromonas, Vibrio* and *Shigella* in high numbers, while some *Pseudomonas* and one presumptive *Salmonella* were detected.
- The selective culture media all proved unreliable, with a high incidence of false positives. Not one putative *Shigella* or *Salmonella* could be confirmed, indicating that none of these virulent pathogens could be obtained by culturing from the drinking water-associated biofilms tested.
- It is essential to put a comprehensive water safety plan in place to protect the water from the source to the tap. This plan should address multi-barrier treatment and integrity of the water distribution system to avoid the entrance of pathogens to the system. Such plans are described in detail by the World Health Organization (WHO 2004).

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