Comparison of biofilm formation and water quality when water from different sources was stored in large commercial water storage tanks

Venessa van der Merwe, Stacey Collignon and Lise Korsten

Department of Microbiology and Plant Pathology

University of Pretoria

Main Campus

Hatfield

Pretoria

0002

South Africa

Corresponding author

Lise Korsten

lise.korsten@up.ac.za

Tel: +27 12 420 3295

Fax: +27 12 420 4588

Abstract

Rain-, ground- and municipal potable water were stored in low density polyethylene storage tanks for a period of 90 days to determine the effects of long term storage on the deterioration in the microbial quality of the water. Total viable bacteria present in the stored water and the resultant biofilms, were enumerated using heterotrophic plate counts. PCR and Colilert-18® tests were performed to determine if the faecal indicator

bacteria, *Escherichia coli*, might be present in the water and in the biofilm samples collected throughout the study. The municipal potable water at the start of the study was the only water source that conformed to the *South African water quality guidelines for domestic use.* After 15 days of storage, this water source had microbiologically deteriorated to levels considered unfit for human consumption. *E. coli* was detected in the ground- and potable- water and ground- and potable biofilms periodically; whereas, it was detected in the rain water and associated biofilms at every sampling point. Imperfections in the UV resistant inner lining of the tanks revealed to be ecological niches for microbial colonisation and biofilm development. The results from the current study confirmed that long term storage can influence water quality and increase the number of microbial cells associated with biofilms on the interior surfaces of water storage tanks.

Keywords: water storage, biofilm, rain water harvesting, water borne pathogens

Introduction

A great proportion of rural communities in South Africa lack access to clean potable water (Momba and Notshe 2003; DAFF 2010). Due to the lack of efficient potable water delivery systems communities have to travel vast distances to collect water, making use of small plastic based water transport devices (Momba and Kaleni, 2002; Jagals *et al.,* 2003; Momba and Notshe, 2003). Water storage is mainly achieved through rain water harvesting or collecting surface- or ground water which is either used directly or retained in small volumes (Momba and Notshe, 2003; WHO, 2008). Water contained within

water storage tanks can be contaminated via: storm water run-off; faulty septic systems; contaminated soil; run-off from manure in the nearby vicinity; or livestock/ wildlife faeces (Beuchat, 2002; Cessford and Burke, 2005). In some cases, communities have access to street taps installed by the municipality to provide potable water, however, families still have to collect and temporarily store the water (Nala *et al.,* 2000).

The conditions under which the water is stored often affects the quality of the water, as stored water is more susceptible to environmental influences and contamination than if the water were still in its natural habitat (Jagals *et al.,* 2003). It is therefore a concern that the collection and storage of untreated water supplies such as, roof catchments (rainwater harvesting), surface- and ground water, which may be contaminated with pathogens, can provide an ideal environment for microbial proliferation. Numerous studies have been done to monitor the microbial quality of water that is transported and stored in small household containers (Momba and Mnqumevu, 2000; Jagals *et al.,* 2003; Momba and Kaleni, 2003; Momba and Notshe, 2003; Maraj *et al.,* 2006). Many of the studies have shown that the transport and storage of water after collection from the source, results in microbial deterioration of the water which often leads to levels of heterotrophic bacteria that are unsuitable for human consumption.

Studies have shown that water storage containers made of plastic based materials, such as polyethylene, are able to support more bacterial incorporation into biofilms on their interior surfaces than those made of metal based materials (Momba and Kaleni, 2002; Momba and Notshe, 2003). In addition, studies have shown that plastic based

water storage containers have a greater affinity to support the incorporation of faecal coliforms into biofilm structures (Momba and Kaleni, 2002; Momba and Notshe, 2003). This is concerning as these biofilms can act as reservoirs for pathogenic microorganisms, that can, through growth and detachment, be responsible for the majority of the planktonic cells found in the aqueous environment (Van der Wende *et al.,* 1989; Percival *et al.,* 1998; Chang *et al.,* 2003).

In the current study, a comparison of the water quality of three different water sources (rain-, ground- and potable water) was conducted to determine the effect of storage on water quality as well as the resultant development of biofilms. Water quality and biofilm biomass changes were followed through heterotrophic plate counts and scanning electron microscopy. The detection of *E. coli* and total coliforms was also performed for all samples through PCR and Colilert-18® analysis.

Materials and methods

Water storage tank design- Three 750 litre water storage tanks were set up in the same vicinity at the University of Pretoria's Experimental farm (S25° 45' 10" E28º 14' 46") (Pretoria, South Africa) after being washed and sterilised with 70% ethanol. The tanks were made from food grade low density polyethylene and the interior was lined with UV resistant carbon black lining to prevent algal growth. All of the tanks had a green exterior and were filled with water from different sources i.e. ground- , rain- and municipal potable water. The rain water that was harvested was the first rain of the season; a first-flush apparatus was not utilised. The tank containing the municipal

potable water served as the control as the water is municipally treated compared to the untreated ground- and rain- water.

The water storage tanks used in this study were specifically modified for the monitoring of biofilm formation and the collection of water from different levels within the tanks (Figure 1). The tanks were horizontally divided into three un-partitioned layers: the top level which represented the most aerobic environment; the bottom level which was considered the most anaerobic and which had the most sedimentation; and the middle level which shared the above two properties. When positioned in the field, the tanks were all orientated in the same manner so as to ensure that the one side received the morning sunlight and the other the afternoon sunlight. Taps were placed on the 'afternoon sun' side of the tanks to enable water collection from the different horizontal layers at the various testing intervals.

The top of the tank was also modified to allow the suspension of biofilm collectors inside the tanks (Figure 1). The biofilm collectors were cut-outs of a tank not used in the current study. Ninety biofilm collectors with a surface area of ± 140mm² were suspended in each tank. Three collectors were attached to a sterile fishing line at different heights and suspended from the top of the tanks so that each collector was placed within a specific region (Figure 1). The experiment was repeated twice. Repeats were separated by seven days. Day 0 was the start of the experiment when water was added to the tanks.

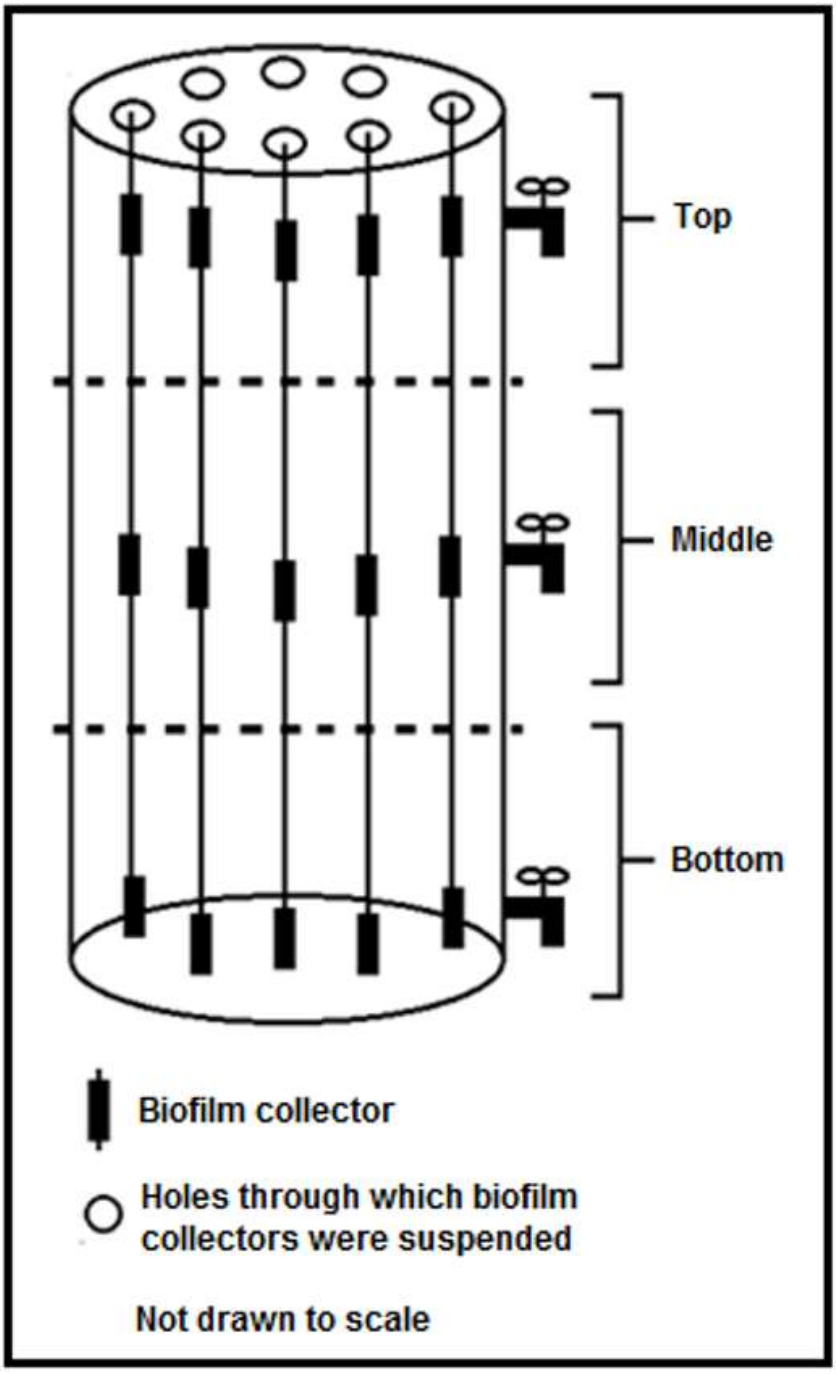

Figure 1. Schematic presentation of the experimental set up of water storage tanks used in the current study. Tanks were horizontally divided into three un-partitioned layers: top, middle, and bottom. Biofilm collectors were positioned so that collectors attached to a single fishing line were suspended in each of the horizontal layers. Water- and biofilm samples were collected from each layer at various time intervals.

Water analyses- One liter water samples were collected in triplicate from each horizontal level of the tank at day: 0, 15, 30, 60 and 90. The samples collected at day 0 were not from the tanks themselves but from the source water that was used to fill the tanks on that same day. Water analysis of day 0 samples could therefore be used to determine the background heterotrophic bacteria and *E. coli* in all the water sources at the start of the study. The water samples were filtered through a 0.45µm pore size cellulose nitrate filter (Satorious, Johannesburg, South Africa). The contents on the filters were dislodged in 9ml 0.1% Peptone Buffered Water (Merck, Pretoria), serially diluted and used to perform viable plate counts on Standard 1 Nutrient Agar (Merck) supplemented with 0.1% cyclohexamide (Sigma Aldrich, Johannesburg). Samples were incubated at 25°C for 48 hr after which colonies were recorded and transformed to $Log_{10}(x + 1)$ CFU ml^{-1}.

Biofilm analyses- Biofilm collectors were installed at different positions within the tanks (Figure 1). Three 'strings' of collectors where removed from the tanks at each sampling point so that a total of nine replicates were obtained. Biofilm formation on biofilm collectors was followed for 90 days with collectors being retrieved from the tanks at the following intervals: Day 1, 3, 5, 7, 9, 11, 20, 30, 60 and 90. Samples were transported to the laboratory in sterile Petri-dishes for analysis.

As the biofilm collectors were cut-outs of an existing tank, the one side consisted of low density polyethylene whilst the other consisted of UV resistant carbon black lining. The side of the biofilm collector that was made of the low density polyethylene was swab sterilised with 70% ethanol to remove all biofilm formation to allow for quantification of biofilm biomass that developed on the UV resistant carbon black lining only as this represented the inside of the tank. Cells not associated with the surface were removed by rinsing the collectors with double distilled water before biofilm cells were removed. Attached cells were removed from biofilm collectors in a modified version of the Lehtola *et al.* (2006) protocol; mechanical shaking with 5g 4mm glass beads in 1ml 0.1% Peptone Buffered Water (Merck) for 10 min at 12Hz was used to detach biofilm cells. The cells were then suspended and diluted by the addition of 8ml 0.1% Peptone Buffered Water (Merck). The bacterial content of the biofilms was then analysed through heterotrophic plate counts on Standard 1 Nutrient Agar (Merck) supplemented with 0.1% cyclohexamide (Sigma Aldrich). Samples were incubated at 25°C for 48 hr after which colonies were recorded and transformed to Log_{10} (x + 1) CFU cm^{-2}.

Nucleic acid extraction- Filters from the water samples (see water analysis) and, biofilm biomass removed from collectors (see biofilm analysis), were enriched in Tryptone Soy Broth (Merck) for 24 hr at 37°C. DNA was extracted from each sample using an optimised version of the Triton-X100 method (Wang and Slavik 2005). One millilitre of the samples was centrifuged for 5 min at 6000g respectively. Once large enough pellets were obtained they were re-suspended in double distilled water and centrifuged for 5 min at 16 000g; this was performed three times for each sample. The pellets were then

re-suspended in 50µl 1% (v/v) Triton X-100 (Sigma Aldrich) and boiled for 10 min followed by a 10 min incubation on ice. The solution was then centrifuged at 16 000g for 5 min and the supernatant removed. Three microlitres RNase (Roche, Johannesburg) was then added to the supernant which was subsequently incubated at 37°C for 2 hr. The DNA extracted was used as a template for PCR.

Polymerase Chain Reaction for Escherichia coli detection- A polymerase chain reaction for the detection of *Escherichia coli* was performed using the primers: Eco1 5'-GACCTCGGTTTAGTTCACAGA-3', Eco2 5'-CACACGCTGACGCTGACCA-3' (585 bp) (Schippa *et al.* 2010). The PCR amplification was carried out in a GeneAmp 2400 PCR system (Applied Biosystems, Foster City, USA) with a PCR reaction mixture containing: 16.1 µl sterilised Sabax water (Adcock Ingram, Johannesburg), 0.3 µl primer Eco1 (10pM), 0.3 µl primer Eco2 (10pM) (Whitehead Scientific, Cape Town, South Africa), 1.5 µl template DNA (~25ng/µl), 2.5 µl PCR buffer, 1.5 µl $MgCl_2$ (10x), 1.25 µl DMSO, 0.75 µl BSA, 0.5 µl dNTP's (10 mM of each) and 0.3 µl Taq polymerase (5U/µl) (all from Celtic Molecular diagnostics, Cape Town). The samples were initially incubated for 2 min at 95˚C to denature the template DNA. This was followed by 35 cycles under the following conditions: 30 sec at 94˚C, 45 sec at 61˚C and 1.5 min at 72˚C with an additional extension at 72˚C for 7 min. The products of the amplification were then analysed by electrophoresis in a 2% (w/v) agarose gel containing 0.01% Ethidium bromide (Merck).

Enumeration of total coliforms and E. coli by Colilert-18®- 100ml triplicate water samples were collected from each horizontal division of each of the water storage tanks

at Day: 0, 45 and 90. The samples collected at day 0 were not from the tanks themselves but from the source water that was used to fill the tanks on that same day. Colilert-18® tests (Dehteq, Johannesburg) were performed on each sample according the manufacturer's instructions. Positive (*E. coli* inoculated sterile water) and negative (sterile water) controls were also included. All Quanti-Tray ®/2000 trays were then incubated at 37˚C for 18 hr. MPN/100ml values were recorded according to a tabulation of 95% confidence intervals provided by the manufacturer (IDEXX, Maine, USA).

Scanning electron microscope examination of biofilm collectors- The formation of biofilms within the water storage tanks was followed throughout the 90 days that the study ran via scanning electron microscopy. Samples were collected in triplicate from each region of the tank (Figure 1) at day: 15, 30, 60, and 90. The biofilm collectors were fixed in 2.5% Glutaraldehyde in 0.075M phosphate buffer (pH 7) from being harvested till the completion of the field study. The fixed samples were then rinsed three times in 0.075M phosphate buffer for 10 min each followed by three rinses in distilled water. Samples were then dehydrated in a graded ethanol series of 30%, 50%, 70% 90%, 100%, 100% and 100% for 10 min each. This was followed by critical point drying with liquid CO_2 and spluttering with gold before being viewed with a Jeol JSM-840 Scanning Electron Microscope (Jeol, Tokyo, Japan) at 5KV.

Statistical analysis- Data obtained from water (Log_{10} (x + 1) CFU ml^{-1}) and biofilm (Log_{10} (x + 1) CFU cm^{-2}) samples, were analysed using an analysis of variance (ANOVA) with SAS-9.2 software (SAS Institute Inc., Cary, USA). Means obtained were compared by

the Fishers protected least significant difference (LSD) test at a 5% (P = 0.05) level of significance. Repeats were considered as blocks. A significant difference was observed between the blocks and this was accounted for when the two repeats were averaged for data analysis.

Table 1. Number of heterotrophic bacterial plate counts (Log_{10} (x + 1) CFU ml^{-1}) in different waters stored in low density polyethylene water storage tanks

Time interval (Days)	Water source		
	Rain	**Ground**	**Potable**
0	5.33 (0.75) ^{A}	2.26 (0.11) ^{F}	0.22 (0.15) ^{H}
15	5.11 (0.29) ^{A}	3.13 (0.75) ^{DE}	3.61 (0.41) ^{BC}
30	5.17 (0.22) ^{A}	1.88 (0.73) ^{G}	3.73 (0.29) ^{B}
60	5.29 (0.66) ^{A}	2.00 (0.32) ^{FG}	3.30 (0.38) ^{CD}
90	3.98 (0.34) ^{B}	1.79 (0.55) ^{G}	2.81 (0.39) ^{E}

All means obtained from eighteen replicates with standard deviations shown in parentheses. All means followed by the same letter are not significantly different (P < 0.05). An analysis of variance indicated a highly significant difference between the water sources (F=664.03; p<0.0001) as well as over time (F= 51.12; p<0.0001). As the interactions between the two variables were also highly significantly different (F= 13.06; p<0.0001), this relationship was used to analyse data.

Results

Water analysis

The heterotrophic bacterial deterioration of the different water sources is presented in Table 1. Significant interactions occurred between the different water sources and time (F= 13.06; p<0.0001) and therefore this was considered for data analysis. No significant

difference was observed between water samples that were collected from different positions within the tanks (data not shown).

All the water sources tested contained viable heterotrophic cells throughout the study ranging from 3.98 to 5.33 Log_{10} (x + 1) CFU ml^{-1}; 1.79 to 3.13 Log_{10} (x + 1) CFU ml^{-1}; and 0.22 to 3.73 Log_{10} (x + 1) CFU ml^{-1} for the rain-, ground- and potable water respectively (Table 1). The rain water showed significantly higher heterotrophic plate count (HPC) values throughout the study with significantly similar values found only for the potable water at day 15 and 30. The rain water HPC values decreased gradually over the 90 day period, however, the only significant decrease was observed between day 60 and day 90. The ground water HPC values increased significantly between the source water and day 15. The ground water also showed an overall decrease in HPC values although the only significant decrease was observed between day 15 and day 30. The potable water showed the most significant increase in HPC values between the source water (day 0) and the water that was stored over the 90 day period. The potable water HPC values remained steady between day 15 and 30 after which significant decreases occurred.

Biofilm analysis

All of the biofilm collectors analysed showed the association of heterotrophic bacterial cells with the surface as early as day 1; biofilm heterotrophic bacterial numbers are presented in Table 2. Significant interactions were observed between the biofilms that developed from the different water sources over time ($F= 7.13$; $p<0.0001$) and therefore

Table 2. Heterotrophic plate counts (Log_{10} (x + 1) CFU cm^{-2}) of bacteria that were incorporated into biofilms on the surfaces of storage tanks containing rain-, ground- and potable water

Time interval (Days)	Water Source		
	Rain	Ground	Potable
1	5.16 (0.86) ^{d}	3.72 (0.82) ^{efgh}	3.66 (0.90) ^{efgh}
3	5.49 (0.71) ^{bcd}	4.16 (0.19) ^{e}	3.70 (0.23) ^{efgh}
5	5.77 (0.38) ^{b}	4.16 (0.51) ^{e}	3.25 (0.49) ^{h}
7	5.85 (0.72) ^{b}	3.92 (1.08) ^{efg}	2.14 (1.80) ^{i}
9	5.98 (0.42) ^{b}	4.02 (1.50) ^{ef}	3.89 (0.55) ^{efg}
11	5.77 (0.40) ^{b}	2.56 (2.12) ^{i}	3.77 (1.00) ^{efgh}
20	5.86 (0.51) ^{b}	3.49 (0.59) ^{fgh}	3.67 (0.29) ^{efgh}
30	5.69 (0.72) ^{bc}	3.42 (0.61) ^{gh}	3.64 (0.53) ^{efgh}
60	6.54 (0.74) ^{a}	3.29 (0.32) ^{h}	3.97 (0.71) ^{efg}
90	5.12 (1.00) ^{d}	3.67 (0.64) ^{efgh}	3.98 (0.45) ^{efg}

All means obtained from eighteen replicates with standard deviations shown in parentheses. All means followed by the same letter are not significantly different ($P < 0.05$). An analysis of variance indicated a highly significant difference between the water sources ($F=381.54$; $p<0.0001$) as well as over time ($F= 3.79$; $p=0.0001$). As the interactions between the two variables were also highly significantly different ($F= 7.13$; $p<0.0001$), this relationship was used to analyse data.

this was considered for data analysis. An analysis of variance between the positions within the tanks from which the collectors were collected (Figure 1), showed a significant difference between the various un-partitioned sectors ($F= 12.78$; $p<0.0001$).

The bottom sector of the tank showed significantly less bacterial incorporation into biofilm structures than the top and middle sectors (data not shown).

The HPC values obtained from biofilms that developed from the rain water where significantly higher than the biofilms that developed from the other water sources for the entire duration of the study. The HPC values of the rain-water biofilm increased non-significantly till day 30 where there was a significant increase in the amount of heterotrophic bacterial incorporation into the biofilm till day 60. The HPC values then decreased significantly again till the end of the study where a final HPC value of 5.12 $Log_{10}(x + 1)$ CFU cm^{-2} was obtained. The final HPC value recorded for the rain-water biofilm was non-significantly different from the HPC value obtained at day 1. The ground- water- and potable-water biofilms showed very similar heterotrophic bacterial incorporation patterns. The HPC values for the two biofilms were not significantly different for the biggest part of the study. Significant differences were observed on day 5, 7, 11 and 60. Between day 9 and 11, the potable-water biofilm reached HPC values that where higher than the ground-water biofilms which had had more bacterial incorporation into biofilms till this point.

Prevalence of total coliforms and E. coli in water and biofilm samples

E. coli was detected through PCR in all the water sources (Table 3) and biofilm samples (Table 4) at some point during the study. The rain water showed the highest prevalence of *E. coli* as it was detected on all water sampling days throughout the study. The rain-water biofilms also showed high prevalence of *E. coli* as it was also detected on every

Table 3. Presence (+) / absence (-) of *E. coli* in stored rain, ground and potable water detected via PCR

Water source	Repeat	Time (days)				
		0	15	30	60	90
Rain water	1	+	+	+	+	+
	2	+	+	+	+	+
Ground water	1	-	+	+	-	-
	2	+	+	+	+	-
Potable water	1	-	+	-	-	-
	2	-	-	-	-	+

sampling day except for day 90 of the second repeat. The ground water showed the second highest prevalence of *E. coli* in both the water samples and biofilms with the least *E. coli* being detected in potable- water and biofilm samples.

Table 4. Presence (+) / absence (-) of *E. coli* in biofilms that developed from stored rain, ground and potable water detected via PCR

Water source	Repeat	Time (days)									
		1	3	5	7	9	11	20	30	60	90
Rain water	1	+	+	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+	+	-
Ground water	1	+	+	+	+	-	-	-	+	-	+
	2	-	+	-	-	-	+	+	-	-	+
Potable water	1	-	+	+	-	-	+	+	-	-	+
	2	-	+	-	-	-	-	-	-	-	-

Colilert-18® analysis of water samples revealed the presence of total coliforms in the potable-, ground- and rain source water (Table 5). Total coliform levels in the rain source water were so high that they could not be counted through Colilert-18® analysis; this pattern prevailed throughout the duration of the study with countable levels only being detected at day 90 of the first repeat. Total coliforms were periodically detected over the duration of the study in both repeats of the potable water with faecal coliforms only being detected at day 45 in the second repeat despite no *E. coli* being detected in the source water. As was observed with PCR analysis, the ground water showed the second highest prevalence of total coliforms in both repeats throughout the duration of the study; however, E. coli was only detected in the second repeat (Table 5).

Scanning electron microscope examination of biofilm collectors

Scanning electron micrographs of the different biofilms correspond with the HPC values (Figure 2 A and B). The rain-water biofilms which had the highest heterotrophic bacterial incorporation also appeared to be larger in size than the other biofilms (Data not shown). The ground-water biofilms appeared to be more built-up than the potable-water biofilms although the ground-water biofilm had predominantly more fungal incorporation compared to the predominant appearance of bacteria in the potable-water biofilms. Microscopic imperfections in the UV resistant carbon black lining were commonly encountered. Biofilm formation within the imperfections was also commonly observed (Figure 2 C and D).

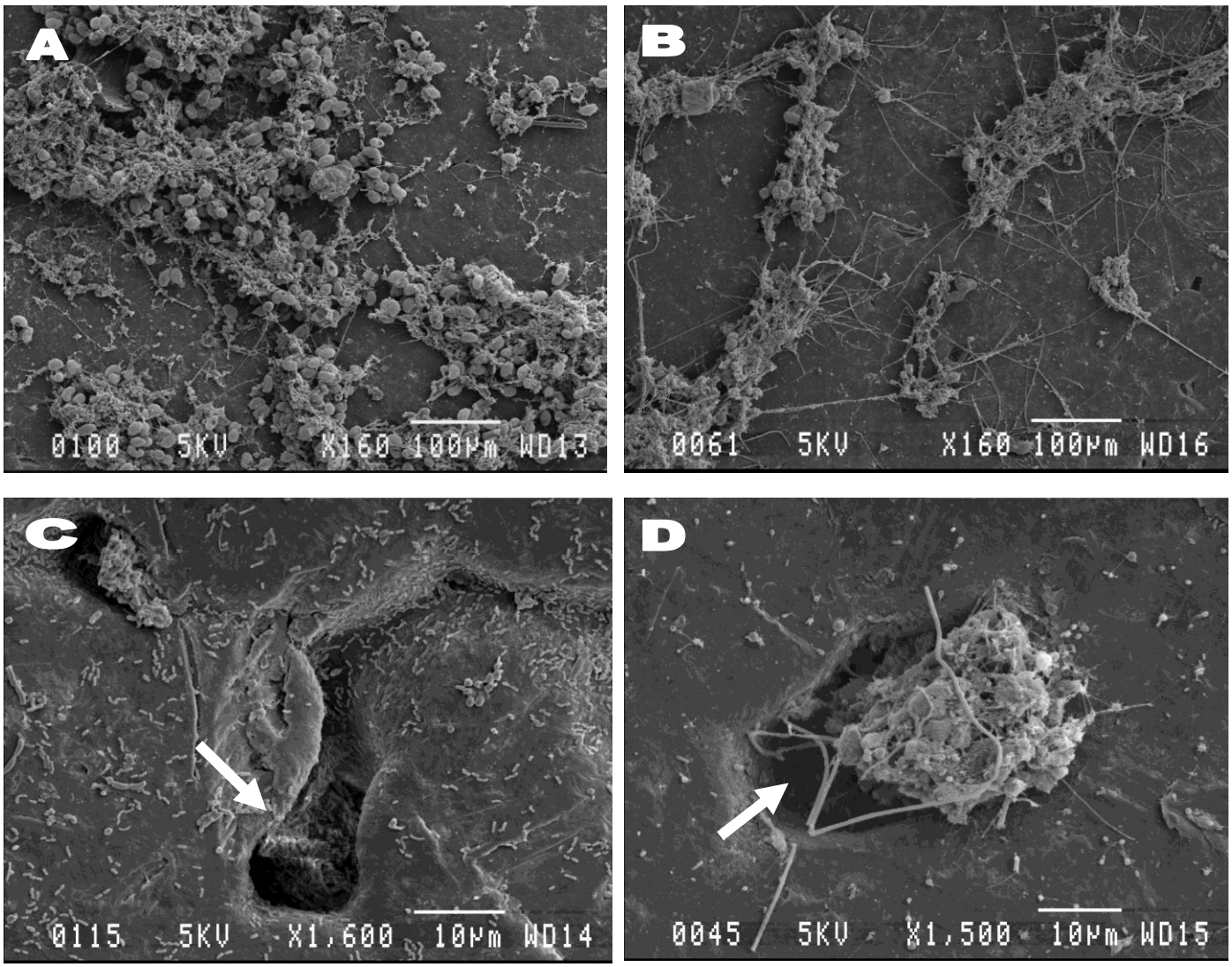

Figure 2. Scanning electron microscopy (SEM) images of microorganisms associated with A-B) the interior surfaces of water storage containers C-D) imperfections in the UV resistant carbon black lining

Table 5. Total- and faecal coliform detection in water samples from the potable-, ground- and rain water storage tanks through Colilert-18® analysis

Water Source	Repeat	Time (days)		
		0	45	90
Potable water	1	0.00 (0)	2.31 (0.44)	45.06 (0)
	2	0.67 (0)	0.00 (0)	76.41 (0)
Ground water	1	3.43 (0)	0.00 (0)	45.06 (0)
	2	28.50 (3.47)	139.53 (23)	164.38 (1.26)
Rain water	1	>2419.6 (1624.65)	>2419.6 (>2419.6)	41.61 (7.19)
	2	>2419.6 (>2419.6)	>2419.6 (126.16)	>2419.6 (24.13)

Tabulation of total coliforms recorded with faecal coliforms shown in parenthesis

Discussion

The presence of coliforms and *E. coli* has been reported in stored rain water (Zhu *et al.* 2004; Evans *et al.* 2006; Ahmed *et al.* 2008; Ahmed *et al.* 2010), ground water (Momba and Mnqumevu 2000; Momba and Notshe 2003) and potable water (Momba and Kaleni 2002). Direct PCR analysis of the water samples in the current study showed the presence of *E. coli* in the bulk liquid phase of all the stored water sources tested; this was confirmed through the Colilert-18® test. In addition to direct PCR analysis, alternate PCR technologies such as qPCR have been used before as a diagnostic test to identify *E. coli* in stored water (Ahmed *et al.* 2010; Ahmed *et al.* 2012). Colilert-18® analysis used in the current study has also been efficiently used in the past (Juhna *et al.* 2007; Fremaux *et al.* 2009). To our knowledge, no studies have used PCR analysis coupled with Colilert-18® tests to study the microbial quality of stored water.

Direct PCR analysis and Colilert-18® tests of the rain water samples in the current study showed the presence of *E. coli* in all rain water samples tested throughout the study period. As a result, the rain water failed to meet water quality guideline standards (DWAF 1996; SABS 2006). According to SANS 241, the permissible number per 1% of samples for total coliforms and *E.* coli is 10 per 100ml and 1 per 100ml respectively (SABS 2006). HPC bacteria are also used as indicators of the general microbial quality of water (DWAF 1996; Nala *et al.* 2003). The permissible target range for HPC values in water for drinking purposes is between 0-100 counts ml^{-1} and was only compliant in the case of the potable water before it was stored.

Harvested rain water is generally considered of good quality but is dependent on atmospheric microbial levels as well as the surface from which the water is collected (Zhu *et al.* 2004; Helmreich and Horn 2010; Ahmed *et al.* 2012). Handia *et al*. (2003) found that the collection of rain water with the use of a first flush device yielded water that was safe for human consumption without prior treatment. However, the majority of studies have found that water collected through rainwater harvesting is in fact not fit for human consumption due to levels of faecal coliform contamination (Zhu *et al.* 2004; Ahmed *et al.* 2008; Ahmed *et al.* 2010). In the current study, the building from which rain was harvested was situated next to cattle pens which contributed towards dust generation and deposition on building roofs. The dry deposition on the building from which the rain was harvested contained large amounts of heterotrophic bacteria, in particular *E. coli*, since the resultant water that was collected showed the highest HPC and *E. coli* values. The overall microbial quality of the rain water was not considered ideal and although there were no drastic increases in the HPC values as was seen with the potable water, the water was still considered to have deteriorated.

The potable water had the lowest HPC value at the start of the study compared to the ground- and rain water respectively. However, the quality of the potable water deteriorated so rapidly that by day 15 it displayed HPC values above the acceptable limits for potable water and were significantly higher than the ground water HPC values. Water that is stored often stagnates and as a result, disinfectant residuals in potable water may dissipate to levels favourable enough to lead to increases in microbial growth (Maraj *et al.* 2006). Although the potable water did not have the highest HPC values, it

showed the greatest increase in heterotrophic bacterial growth out of all the stored water sources.

The stored ground water did also not conform to drinking water standards (DWAF 1996; SABS 2006) due to the high HPC values obtained and the presence of *E. coli*. In a study by Momba and Notshe (2003), the authors found that the quality of stored ground water within plastic based containers could deteriorate within 24 hr after storage and gradually deteriorate over the next 72 hr period when the water was tested. In the current study, the HPC values showed a significant increase in the first 15 days of storage; however the HPC values decreased from this point to a value lower than the starting HPC value. The decrease in HPC values could be attributed to unfavourable conditions, such as nutrient depletion (Momba and Notshe 2003) or that many of the planktonic cells became incorporated into the biofilm on the surface.

The majority (99.9%) of microorganisms present in water related environments are attached to surfaces exposed to water (Donlan and Costerton 2002; Juhna *et al.* 2007; Huq *et al.* 2008). Despite this, heterotrophic plate counts of routine water samples, and not biofilm samples, are still highly regarded in determining the microbial safety of different water sources (DWAF 1996). This underestimation of the amount of microorganisms present in the water and its surrounding environment can often be misleading and result in water quality being miscalculated. In the current study, a comparison of biofilms that developed in the different storage tanks to the water

contained within them, confirmed that there was just as much, if not more bacterial cells attached to the surface compared to planktonic cells.

E. coli was found to have been incorporated into biofilm structures in this study within 24h for the rain- and ground-water biofilms and after three days for the potable-water biofilms. This was also observed by Momba and Kaleni (2002) who showed that biofilm formation from ground- and potable water on polyethylene material could occur within 24 hr after initial exposure and that the indicator organisms had already adhered to the surfaces within that time frame. The occurrence and survival of *E. coli* in the bulk liquid phase of stored water, facilitates the incorporation of the pathogen into biofilms that develop on the interior surfaces of the water storage tanks (Momba and Kaleni 2002).

Escherichia coli incorporation into biofilms that develop from rain water has not been demonstrated as frequently as incorporation into biofilms supported by other untreated water sources (Momba and Mnqumevu 2000; Momba and Kaleni 2002; Banning *et al.* 2003; Momba and Notshe 2003). The detection of *E. coli* in the ground- and potable water samples of the current study through PCR analysis was more sporadic and no relationship between the appearance of *E. coli* in the water and in the biofilms could be deduced. As the water storage tanks used in the current study were sealed to prevent unnecessary introduction of contamination (Maraj *et al.* 2006); the presence of *E. coli* therefore indicates contamination prior to storage. This was evident in the water samples taken at day 0 direct from the water sources (Table 3 and 5). Other studies have reported that contamination of collected water can be as a result of dust deposits,

leaves from trees, or bird droppings (Zhu *et al.* 2004; Kahinda *et al.* 2007; Ahmed *et al.* 2012).

In addition to microbial colonisation and biofilm formation on the interior surface of water storage tanks, the current study also revealed microbial association with microscopic imperfections in the UV resistant carbon black lining of the tanks. Scanning electron micrographs revealed whole microcolonies developing within these imperfections. The protection afforded to the biofilms within these imperfections in the current study, prevented complete removal of surface associated microorganisms with the removal method employed. This would have therefore resulted in an underestimation of the number of heterotrophic bacteria associated with the surface. Microbial growth within imperfections, such as those found in the current study, may act as a mode of survival for microorganisms as they would not be removed during routine cleaning of the tanks. As biofilms naturally protect the cells from antimicrobial agents such as antibiotics, disinfectants or germicides (Webb *et al.* 2003), growth within the storage tank imperfections can further decrease the efficiency of antimicrobial agents in biofilm control.

In light of the findings of the current study, future research should investigate the ability of microbial biofilm formation within water storage container imperfections, to protect the cells from removal and disinfection activities thereby providing more information on how to combat their formation. Methods should also be devised to attempt to remove these

microbial growths from the surface. Future research should also focus on the mechanism of *E. coli* survival in water storage tanks.

Conclusions

The findings of the current study revealed that both untreated and municipally treated water sources were able to support biofilm formation on the interior of low density polyethylene water storage tanks as early as one day after collection. It was also found that the storage period and the microbial quality of the source water could influence water quality deterioration in terms of water HPC values and the rate of biofilm formation. Imperfections in the interior surface of storage tanks were also found to provide an ecological niche for biofilm formation and persistence. To our knowledge, this has not been shown before. Due to the widespread use of water storage tanks, similar to those employed in the current study, suitable information should be given to the public about the potential risks associated with the storage of water (especially first seasonal rain water) and the potential for water deterioration in the absence of disinfectant applications or periodic cleaning of the water storage containers/ tanks.

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