Int. J. Environ. Res., 9(1):263-272,Winter 2015
ISSN: 1735-6865

Microbiological Assessment of the Biofilter Matrix Within a Biofiltration system Treating Borehole water in KwaZulu-Natal (South Africa)

Beukes, L.S. and Schmidt, S.*

Discipline of Microbiology, School of Life Sciences, University of KwaZulu-Natal, Private Bag X01, Pietermaritzburg 3209, South Africa

Received 3 June 2014; Revised 31 Oct. 2014; Accepted 6 Nov. 2014

ABSTRACT: To assess the microbiological status of a biofiltration system used to treat borehole water, filter matrix samples were analyzed after staining for the presence of active biofilms using confocal laser scanning microscopy (CLSM). CLSM revealed the presence of biofilms on the filter matrix with actively metabolizing microbial cells present. Thereafter, heterotrophs, manganese oxidizing bacteria (MOB) and iron oxidizing bacteria (IOB) present in the biofilms were quantified. For heterotrophs a count of 2.9×10^7 cfu/g was established using R2A agar while counts for presumptive MOB and IOB were established as 2.4×10^7 cfu/g and 3.1×10^7 cfu/g respectively. In addition, a clone library was established using DNA extracted from a pooled filter matrix sample to assess the diversity of bacteria present within the biofilter matrix. A total of 100 randomly selected clones were separated into 14 unique operational taxonomic unit (OTU's) based upon restriction patterns of amplified partial 16S rRNA genes. Overall, 38% of the clones were assigned to the phylum *Proteobacteria*, 13% to the phylum *Actinobacteria*, 24% to the phylum *Firmicutes*, 21% to the phylum *Nitrospirae* and 4% to the phylum *Verrucomicrobia*.

Key words: Biofiltration system/ biofilm / CLSM/microbial counts/16S rRNA gene clone library

INTRODUCTION

The provision of safe and healthy drinking water is considered a global top priority (WHO/UNICEF JMP, 2012; UNEP, 2013; WaterAid, 2013). Although iron and manganese are natural constituents in the earth's crust and are present in varying concentrations in surface and groundwater, they remain problematic from an aesthetic, health, technical and economic point of view (Bouchard *et al*., 2011; Hamilton, 2003; Lovley, 2000). Biological filtration of groundwater is employed successfully to remove these metals from water (Gage *et al*., 2001). This process involves passing chlorine-free aerated water through a column of filter sand, thereby allowing planktonic microorganisms to form biofilms on interfaces within the column (Gage *et al*., 2001). The vast majority of microorganisms in aquatic environments grow in the form of biofilms which are considered a prominent mode of microbial colonization and survival in metal-rich environments (Harrison *et al*., 2007). Biofilms typically consist of a large variety of microorganisms which exist in a highly organized community were nutrients are continuously recycled (Harrison *et al*., 2007). Bacteria in these biofilms produce capsular material which contributes to the stability of the biofilm (Sheng *et al*., 2010) and acts as a chemical buffer at the cell's surface were essential ions can accumulate and toxic substances can be immobilized when critical levels are reached within the cell (Sheng *et al*., 2010; Trevors, 1989). The microorganisms commonly involved in iron and manganese sequestration belong to genera such as *Leptothrix*, *Crenothrix*, *Hyphomicrobium*, *Metallogenium* and *Siderocapsa* (Takeda *et al*., 2012; Hedrich *et al*., 2011; Mouchet, 1992). Previous studies on a water distribution system in the Southeastern USA revealed the presence of over fourteen separate MOB (manganese oxidizing bacteria) species, indicating that multiple organisms are involved in the oxidation of this metal within water distribution systems (Cerrato *et al*., 2006). Similarly, large numbers of bacteria such as *Gallionella ferruginea* and *Leptothrix orchracea* - responsible for oxidizing iron in water distribution systems - were found in backwash sludge in a filtration system in Canada (Gage *et al*., 2001). Monitoring and assessing microbial communities in water purification systems is essential (Kormas *et al*., 2010). However, culture based techniques used to analyze microorganisms present in such systems typically target only a

*Corresponding author E-mail:schmidts@ukzn.ac.za

specific selection of relevant hygiene indicator organisms (Kormas *et al*., 2010). To overcome this limitation, an approach based on the amplification of suitable target genes can assist in detecting waterborne microorganisms which are not usually detected by commonly employed culture based techniques targeting fecal indicator bacteria (Colwell *et al*., 1985). As RNA is rapidly degraded in stressed cells and is unstable outside of the cell due to the abundance and stability of RNases in the environment (Keinänen-Toivola *et al*., 2006), approaches targeting DNA are more useful in characterizing active bacterial communities (Spiegelman *et al*., 2005; Morgan *et al*., 2002).

This study assessed the microbial status of the biofilter matrix within the manganese and iron filters of a biofiltration system, by initially analyzing the filter matrix for the presence of active biofilms in both filters, followed by quantifying viable heterotrophs, MOB and iron oxidizing bacteria (IOB) present therein. Finally, a snapshot of the bacterial diversity of the biofilter matrix was determined via the establishment of a 16S rRNA gene based clone library from a pooled filter matrix sample.

MATERIALS & METHODS

The biofilter system analyzed in this study is located on the premises of the Nottingham Road combined School in KwaZulu-Natal (S29°20'13.33", E29°59'35.318"), employing two sequential filters for the elimination of iron and manganese from borehole water (Fig. 1). It can treat up to about 2500 L of borehole water per hour and is running at ambient temperature (average annual temperature on site ~18.3°C). The borehole water treated typically contained 4-8 mg/L of iron and 0.3-0.5 mg/L of manganese with the concentration of iron reduced to <0.02 mg/L and manganese to <0.01 mg/L after treatment. Filter matrix samples (particle size typically in the range of 3-5 mm) consisting of so called manganese greensand (Casale *et al*., 2002) were collected from 5, 20 and 35 cm (top, middle and bottom layers) within the manganese and iron filters of the biofiltration system and thereafter pooled. Staining of these filter matrix samples was initially carried out using acridine orange (AO) according to procedures suggested by Kepner and Pratt (1994) with the following modifications. A pooled filter matrix sample of 5 g wet weight was gently rinsed three times with 20 mL sterile distilled water. Thereafter, the sample was stained using a 2 ng/mL solution of acridine orange prepared in distilled water containing 1% DMSO (dimethyl sulfoxide). The sample was stained for one minute in the dark in a 90 mm petri dish covered with 20 mL stain solution. Thereafter it was directly analyzed via confocal laser scanning microscopy (Zeiss LSM 710, Germany). Green fluorescence was detected after excitation at 488 nm with a long pass filter between 520-560 nm and red fluorescence was detected after excitation at 568 nm with a long pass filter at 590 nm.

Fig. 1. The biofilter system analyzed in this study. Label A indicates the iron filter, label B the manganese filter

A second staining procedure using 5-cyano-2,3-ditolyltetrazolium chloride (CTC) was carried out according to Bartosch *et al*. (2003), with the following modifications. The washed and pooled filter matrix sample of 5 g wet weight was stained with a 15 mM CTC solution (20 mL) in a sterile 90 mm petri dish at room temperature for 24 hours in the dark. Samples were then rinsed with sterile distilled water to remove any unbound stain followed by CLSM examination. The formazan (CTF) formed upon enzymic reduction of CTC was detected via its red fluorescence after excitation at 568 nm using a long pass filter at 590 nm. A sample of the filter matrix before the biofiltration plant started operation was used as a control to verify that AO and CTC were not staining any material other than the biofilm established during filtration. An additional non stained filter matrix sample (before the biofiltration plant started operation) served to determine whether the surface of the filter matrix emitted any background fluorescence during CLSM analyses.

A pooled filter matrix sample from both filters was used to quantify MOB, IOB and heterotrophs. Heterotrophs and manganese oxidizing bacteria were quantified using R2A and MSVP plus manganese sulfate according to Beukes and Schmidt (2012). Iron oxidizing bacteria were quantified using MSVP (minimal salts vitamins pyruvate) medium but with 2 mg/L iron sulfate instead of manganese sulfate. Five grams wet weight of the pooled filter matrix sample

was added to 45 mL of sterile MSVP medium followed by 3 minutes vortexing at maximum speed to displace the biofilm from the filter matrix. Thereafter decimal dilutions of samples were prepared using R2A medium or MSVP medium plus added manganese sulfate or iron sulfate as a diluent. One hundred microliters of appropriate decimal dilutions (typically in a range from 10^{-2} to 10^{-6}) were spread plated in triplicate using R2A or MSVP agar with added manganese sulfate or iron sulfate. Plates were incubated at 25°C for seven days in the dark.

A pooled and washed filter matrix sample from the manganese and iron filters was used for the establishment of a clone library. DNA was extracted from biofilm material attached to the filter matrix following the procedure by Zhou *et al*. (1996) except that a filter sand sample was used instead of soil. The pellet of crude nucleic acids obtained after extraction and centrifugation (14,000 × g for 5 minutes) was washed with cold 70% ethanol, and suspended in sterile distilled water to a final volume of 500 µl. Amplification of the isolated DNA was carried out using Hot Start PCR in a Labnet Multi GENE II Cycler with the following quantities of reagents per 25 µl reaction: 2 µl $MgCl_2$ (25mM), 3 µl PCR Buffer (Fermentas Hot Start PCR Buffer), 3 µl of a 2 mM dNTP mix, 0.25 µl forward (Eub 338, 5´-GCTGCCTCCCGTAGGAGT-3´, (Amann *et al*., 1990)) and reverse (Eub 907, 5´-CCGTCAATTCCTTTRAGTTT-3´, (Muyzer *et al.*, 1995)) primers (100 µmol), 0.2 µl Maxima Taq polymerase (5 u/µl), 1µl DNA template and 15.3 µl nuclease free water. Parameters used for PCR were as follows: an initial denaturing cycle was carried out at 95°C for 3 minutes, followed by a total of 35 cycles (1 min denaturing at 95°C, 1 min annealing at 65°C, 3 min extension at 72°C). A final extension cycle was carried out at 72°C for 7 minutes and holding at 4°C for ". 16S rRNA gene amplicons were analyzed by gel electrophoresis of 5 µl amplification mix in a 2% agarose gel (1x TBE (Tris-borate EDTA) buffer (10 mM, pH 8)) after post-staining with ethidium bromide and by using a 100-1000 bp DNA ladder as size marker (Fermentas). *E. coli* (ATCC 8739) served as a positive and sterile MilliQ water as a negative control for the PCR.

16S rRNA amplicons were cloned using a CloneJET™ PCR cloning kit (Fermentas) and transformed into *E.cloni*® 10G chemically competent cells (Stratagene) according to the manufacturer's specifications. Transformation was followed by spread plating onto LB agar containing ampicillin (50 mg/mL) and overnight incubation at 37°C. One hundred clones were randomly selected from the established clone library and checked for the correct insert via colony PCR. For this purpose one colony was placed in 100 µl of sterile distilled water, treated by five freeze-thaw cycles (i.e. 5 minutes at 95°C followed by 10 minutes in liquid nitrogen). Samples were then centrifuged at 14,000 × g for 5 minutes and a 2 µl sample was either directly used for PCR or samples were stored at -20°C for further use. The presence and correct size of inserts was determined using the supplied primer pair (pJET 1.2 forward and reverse primer) flanking the cloning site on the vector. PCR products of the correct size were further analyzed via ARDRA (amplified "rDNA" restriction analysis) to select clones representing phylotypes for subsequent sequence analysis. A double digestion was done at 37°C for 5 minutes using the following restriction enzyme reagents per 30 µl reaction: water, 16 µl; 10x Green Buffer, 3 µl; FastDigest® (Fermentas) Hin P1l, 0.5 µl; FastDigest® (Fermentas) Hae lll, 0.5 µl; PCR product, 10 µl. Restriction fragments were then separated on a 2% agarose gel (1x TBE buffer). After staining with ethidium bromide band patterns were analyzed using GeneSnap version 7.09.06 (SynGene, Cambridge, United Kingdom).

For each OTU - using a 97% similarity threshold for operational taxonomic unit assignment as suggested by Revetta *et al*. (2010) - one representative clone containing an insert of the correct size was analyzed by sequencing (Inqaba Biotec, Pretoria, South Africa). The partial 16S rRNA gene sequences obtained were deposited in GenBank (accession numbers KJ863381-KJ863394) and compared to 16S rRNA gene sequences deposited in the same database (NCBI GenBank, National Center for Biotechnology Information, www.ncbi.nlm.nih.gov). A phylogenetic tree using sequences deposited within GenBank for the closest related cultured genus representative - based on the similarity values - was generated based on a sequence alignment established with Muscle and the tree was constructed using the maximum likelihood method in MEGA 5.2 (Tamura *et al*., 2011) with resampling for 1000 bootstrap replicates. Rarefaction analysis was carried out using Estimate S (Version 9.1, R. K. Colwell, http://viceroy.eeb.uconn.edu/estimates) as a means to assess the species diversity covered by the clone library established. In addition, the ACE and Chao-1 values, which are estimators for the total species richness expected for the clone library, were calculated using Estimate S 9.1.Acridine orange and CTC were obtained from Merck (South Africa). Unless otherwise stated, all other chemicals used were of the highest purity commercially available.

RESULTS & DISCUSSION

Filter matrix samples were analyzed for biofilm formation approximately three weeks after

the biofiltration system started operating to safeguard that a biofilm had developed. It was found that the filter matrix provided a suitable support matrix enabling biofilm formation within the biofilters. CLSM (confocal laser scanning microscopy) proved to be a useful tool in the detection of biofilms on the matrix. According to Bartosch *et al*. (1996), CLSM allows for the *in situ* detection and quantification of microorganisms within the pores of mineral materials. The results obtained from AO and CTC staining clearly demonstrated the presence of active biofilms on the filter matrix. The unused biofilter matrix showed some degree of green fluorescence (Fig. 2a) after staining with AO while no red fluorescence was detected (Fig. 2b) after staining with CTC. This indicates that a metabolically active biofilm was not present on the filter matrix before operation started and the biofilm only developed during biofiltration of the borehole water, confirmed by the visible difference in fluorescence intensities between Fig. 2a and 3 and between Fig. 2b and 4.

Apparently AO is not entirely specific in staining biofilm material, as slight green fluorescence on the washed and autoclaved filter matrix was detected after staining even before biofiltration in the absence of DNA containing cells (Fig. 2a). This could be due to an interaction of the rough surface of the mineral material with AO leading to green fluorescence emission after excitation. However, no fluorescence was emitted from unused matrix particles without staining. The interaction of acridine orange with double stranded DNA (usually predominant in inactive bacterial cells) leads to green fluorescence at a wavelength of 530 nm (Pettipher *et al*., 1980) while the interaction of acridine orange with single-stranded RNA (usually predominant in active bacterial cells) results in red fluorescence at a wavelength of 640 nm (Hobbie *et al*., 1977; Darzynkiewicz *et al*., 1975). Thus AO staining apparently indicated the presence of inactive (areas with green fluorescence) and active (areas with red fluorescence) microbial cells (Fig. 3).

Acridine orange is known to emit orange-red fluorescence when bound to RNA and green fluorescence when bound to DNA (Kasten, 1981). Similarly, Pettipher *et al*. (1980) found that active microorganisms emitted orange fluorescence which is believed to be due to high dye/nucleotide ratios

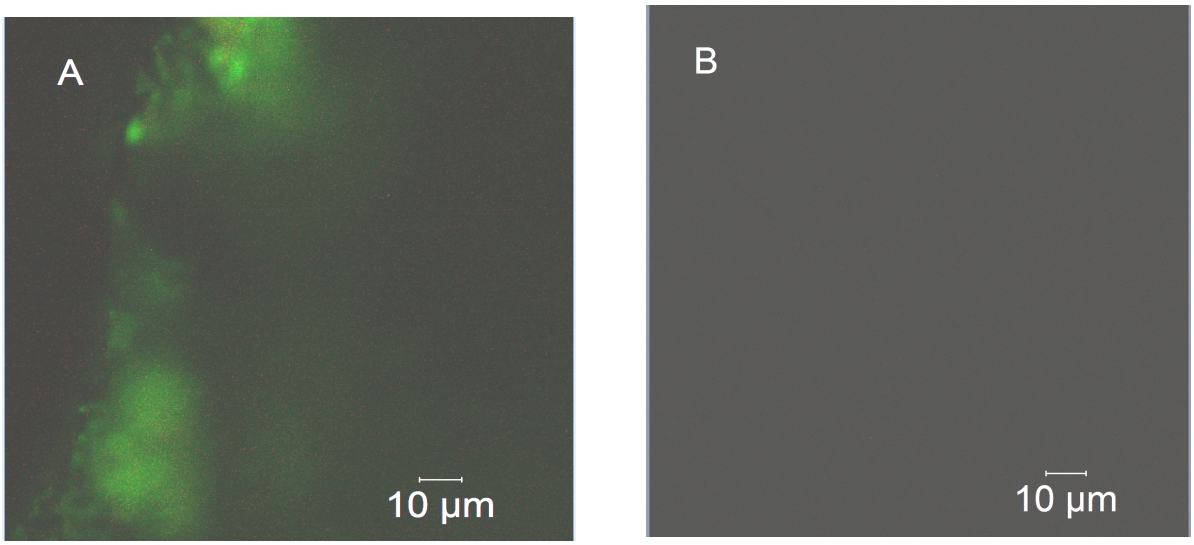

Fig. 2. Confocal laser scanning micrographs of a filter matrix particle before use in the biofiltration system after staining with acridine orange (a) and CTC (b)

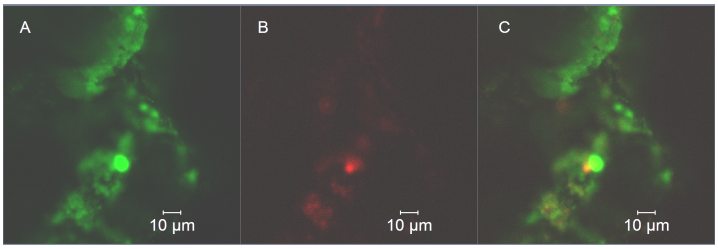

Fig. 3. Confocal laser scanning split micrograph of the filter matrix biofilm after 3 weeks biofiltration and staining with acridine orange. a. Presence of apparently inactive cells. b. Presence of apparently active cells. c. Combination of A and B

Table 1. Phylogenetic affiliation of clones representing OTU's from the biofilm material on the filter matrix based on partial 16S rRNA gene sequences

Phylotypes	No. of clones	Size (bp)	Closest related genus (cultured representative)	Genbank accession number*	Putative phylogenetic affiliation	Similarity score %
BFS-1	5	423	*Methylomonas*	FR798956.1	*γ-Proteobacteria*	97
BFS-2	4	396	*Alterococcus*	NR036763.1	*Verrucomicrobia*	82
BFS-3	10	443	*Enterobacter*	KF891344.1	*γ-Proteobacteria*	100
BFS-4	20	450	*Lactococcus*	KF826024.1	*Firmicutes*	99
BFS-5	3	444	*Ochrobactrum*	HQ222290.1	*α-Proteobacteria*	97
BFS-6	21	447	*Nitrospira*	AF155153.1	*Nitrospirae*	91
BFS-7	3	457	*Bacteriovorax*	AY294218.1	*δ-Proteobacteria*	93
BFS-8	3	457	*Myxobacterium*	AF482687.1	*δ-Proteobacteria*	97
BFS-9	2	457	*Myxococcus*	EU158306.1	*δ-Proteobacteria*	89
BFS-10	4	455	*Legionella*	JN380983.1	*γ-Proteobacteria*	99
BFS-11	13	456	*Tsukamurella*	AB907635.1	*Actinobacteria*	96
BFS-12	4	463	*Bradyrhizobium*	FJ459986.1	*α-Proteobacteria*	99
BFS-13	4	456	*Proteinimicrobium*	AM746627.1	*β-Proteobacteria*	96
BFS-14	4	456	*Staphylococcus*	AF269844.1	*Firmicutes*	100

(Back and Kroll, 1991). Furthermore, AO staining falls short in reliably distinguishing metabolically active from metabolically inactive microorganisms as both can in fact emit green fluorescence (Bartosch *et al*., 2003). Interestingly, Mason and Lloyd (1997) found that in bacterial cell suspensions exceeding 10^6 cells per ml, the differential staining of nucleic acids with acridine orange did not occur.

Given the shortcomings of AO staining to distinguish metabolically active from metabolically inactive cells, the alternate approach using CTC in combination with CLSM proved useful in detecting actively metabolizing bacteria within the biofilm. CTC acts as an artificial electron acceptor within functional electron transport systems or for certain active dehydrogenases, making it possible to visualize and quantify actively metabolizing microorganisms *in situ* as the reduction product CTF emits red fluorescence upon excitation (Bartosch *et al*., 2003). While CTC was frequently used to quantify active bacteria in aquatic environments (Servais *et al*., 2001; del Giorgio *et al*., 1997), only limited data are available using CTC on filter sand material. However, Fig. 4 clearly demonstrates that CTC is suitable for the detection of active microorganisms on the filter matrix using CLSM. Unused matrix particles without any stain (data not shown) as well as CTC-stained unused particles (Fig. 2b) did not emit red fluorescence indicating that only actively respiring microorganisms on the filter matrix reduce CTC to CTF.

Counts for presumptive iron oxidizers were established as 3.1×10^7 cfu/g, for manganese oxidizers as 2.4×10^7 cfu/g and for heterotrophs as 2.9×10^7 cfu/g. The values determined are in a range reported for other biofiltration systems used to eliminate manganese from groundwater. Burger *et al.* (2008) established heterotroph counts of 10^6-10^8 cfu/g for biofilter sand/matrix samples which is similar to the heterotrophic counts reported in this study. Using PYM agar - and not MSVP as in this study - supplemented with manganese sulfate, Vandenabeele *et al*. (1992) reported somewhat lower counts of 10^3-10^4 cfu/g for MOB and for heterotrophs for sand material used in groundwater treating biofilters.

More recently, Cerrato *et al*. (2010) reported counts for manganese oxidizers in biofilms isolated from drinking water systems in a range of 5×10^1 to 5×10^4 cfu/g of biofilm material. In a study conducted on groundwater seeps at neutral pH, counts for iron oxidizing microorganisms were 10^3-10^5 cells/ml (Blçthe and Roden, 2009) while Hirsch and Rades-Rohkohl (1988) established counts of 7.3×10^3 cfu/g for iron oxidizers and 1.5×10^3 cfu/g for manganese oxidizers in groundwater sediment.

The clone library was established from filter matrix samples with the assumption that most of the bacteria found in the water flowing through the system would colonize the filter matrix during biofiltration, forming active biofilms as confirmed by CLSM analyses (Fig. 3-4). One hundred randomly selected clones were divided into 14 OTU's based on ARDRA analysis (Tab. 1). Based on the analysis of sequences representing the 14 OTU's, 70% of clones analyzed had sequence similarity scores of e" 94% -which was suggested previously by De Santis *et al*. (2007) as criterion for assignment of clones at subfamily level - when compared to sequences deposited in NCBI.

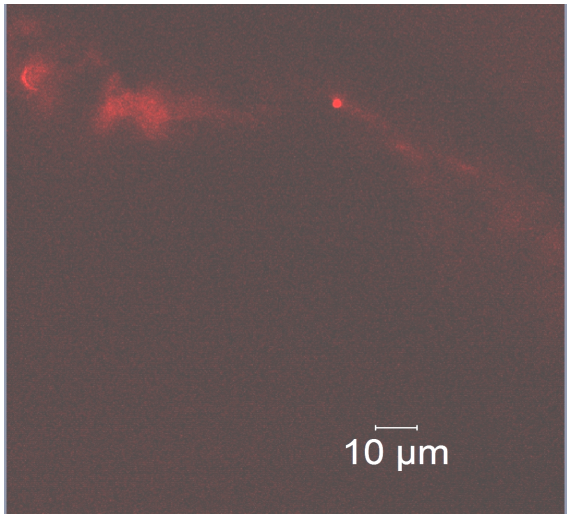

Fig. 4. Confocal laser scanning micrograph of the filter matrix biofilm after 3 weeks biofiltration and staining with CTC indicating the presence of metabolically active cells

Based on sequence similarities of e" 97%, 53 clones were provisionally assigned to the genera *Methylomonas* (5), *Enterobacter* (10), *Lactococcus* (20), *Ochrobactrum* (3) *Myxobacterium* (3), *Legionella* (4), *Bradyrhizobium* (4) and *Staphylococcus* (4). The phylogenetic tree established (Fig. 5) illustrates these relationships. While OTU BFS-6 represented the highest number of clones (21, genus *Nitrospira*), OTU BFS-9 was represented by only two clones most closely related to the genus *Myxococcus*.

Members of the genera *Nitrospira* and *Nitrobacter* were identified as the dominant nitrite oxidizing bacteria (NOB) in wastewater treatment effluents, rivers and sediments (Cébron and Garnier, 2005; Dionisi *et al*., 2002). The level of nitrite in the borehole water was typically < 50 µg/L (personal communication, Dudu Gwebu, Umgeni Water, Durban) thereby meeting the level of 3 mg/L (as nitrite ion) or 0.9 mg/l (as a nitrite-nitrogen couple) recommended by the WHO (2011). Cébron and Garnier (2005) found that large amounts of nitrogen present in river water were due to contamination from agricultural activities and from urban effluents. Thus the high proportion of *Nitrospira* clones might be due to contamination of the borehole water with urine, as a pit latrine was situated upstream from the biofiltration system. Urea present in urine can be transformed to ammonia which in turn can be oxidized to nitrite which is the substrate used by nitrite oxidizing bacteria (Bock *et al*., 1992).

Some of the other identified taxa in the biofilter matrix clone library are typically associated with humans, some are even pathogenic. The pit latrine situated upstream might be a possible explanation for the presence of potentially pathogenic bacteria such as *Legionella* spp. and fecal indicators such as *Enterobacter* spp. In the current study, MOB representatives like the previously isolated manganese oxidizing proteobacterium *Acinetobacter* sp. LB1 (Beukes and Schmidt, 2012) were not detected via the clone library. This could be due to the fact that *Acinetobacter* sp. LB1 was originally isolated from water samples of the manganese filter while the clone library was established from DNA extracted from pooled filter matrix samples combined from the manganese as well as the iron filter.

Krakat *et al*. (2010) determined the detection limit of ARDRA as approximately 10^5 cells/mL; a value similar to the detection limit of epifluorescence microscopy thus demonstrating the sensitivity of this method. In order for a reliable detection of individual manganese oxidizers such as *Acinetobacter* sp. LB1 given the established detection limit for ARDRA, these would need to be present at > 1% of the total manganese oxidizing population which was established as about 10^7 cfu/g in this study. Thus individual manganese oxidizers might not be detected via ARDRA if they are close to the reported detection limit of this method. This is further complicated by the fact that DNA extractions might not quantitatively capture the DNA of the less abundant groups present in biofilter material.

The phylum *Proteobacteria* has been shown to be the most dominant group of microorganisms in water distribution systems around the world (Schmeisser *et al*., 2003). Other studies based on sequences obtained from both biofilm and bulk water indicated that alpha-, beta-, and gamma-*Proteobacteria* were the most abundant members of the bacterial community inhabiting water distribution systems (Santo Domingo *et al*., 2003; Williams *et al*., 2004). Similarly, a study conducted on biofilm communities on copper pipes revealed that dominant members of the clone library were closely related to the classes beta- and gamma-*Proteobacteria* comprising *Acinetobacter* spp. and *Pseudomonas* spp. while less abundant groups were closely related to the classes alpha- and delta-*Proteobacteria*, *Flavobacteria*, *Sphingobacteria*, and uncultured bacteria (Pavissich *et al*., 2010). A recent study analyzing the bacterial community established in an activated carbon based biofilter system treating drinking water again demonstrated a clear dominance of members of the *Proteobacteria* within the population (Liao *et al*., 2013). These results are in agreement with this study as the dominant group of

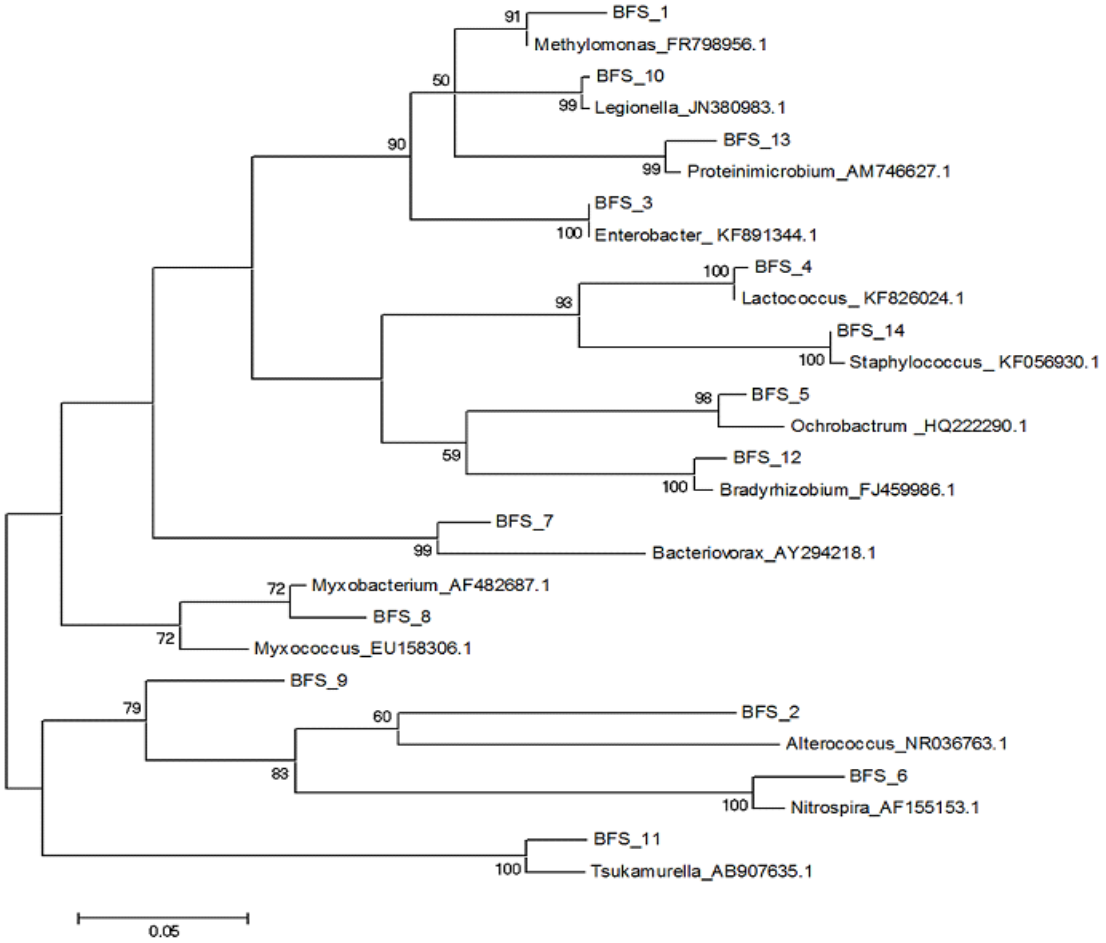

Fig. 5. Phylogenetic affiliation of 14 OTU's based on partial 16S rRNA gene sequences of representative clones in comparison to sequences of most closely related cultured representatives at genus level deposited in GenBank. Numbers shown at nodes indicate calculated bootstrap values (only values >50% are shown). The scale bar indicates five estimated changes per 100 nucleotides

bacteria was most closely related to the phylum *Proteobacteria* which comprised 38% of the clone library. The rest of the clone library was comprised of the phyla *Actinobacteria* (13%), *Firmicutes* (24%), *Nitrospirae* (21%) and *Verrucomicrobia* (4%) (Fig. 6).

Typical clone libraries of 16S rRNA genes contain less than 1000 sequences and would therefore display only a partial view of the microbial diversity in such samples (Dunbar *et al*., 2002). Both ACE and Chao-1 calculations for the clone library analyzed in this study matched the established number of OTU's. In addition, the rarefaction curve indicated that the number of clones analyzed adequately exposed the diversity of the bacterial biofilm population present on the filter matrix (Fig. 7). Advantages of this culture independent approach include a high phylogenetic resolution and the determination of the closest phylogenetic neighbor within samples (Singleton *et al*., 2001; Tyson *et al*., 2004); which is the reason why clone libraries are still considered the "gold standard" for preliminary microbial diversity assessments and surveys (Spiegelman *et al*., 2005). The usefulness of this approach for the microbiological analysis of biotechnological systems was recently demonstrated by Ramos *et al*. (2010) who successfully assessed the microbial diversity in a UASB reactor via a similar clone library based analysis.

CONCLUSION

The results obtained indicate that about 10^7 MOB, IOB and heterotrophs were present per gram of filter matrix. As confirmed by CLSM, biofilms containing actively metabolizing cells were formed on the filter matrix, demonstrating that the filter sand is a suitable support matrix enabling biofilm formation. The use of CTC was clearly more reliable for the detection of metabolically active cells than the use of AO. The clone library analysis demonstrated that the majority of OTU's belonged to the phylum *Proteobacteria*

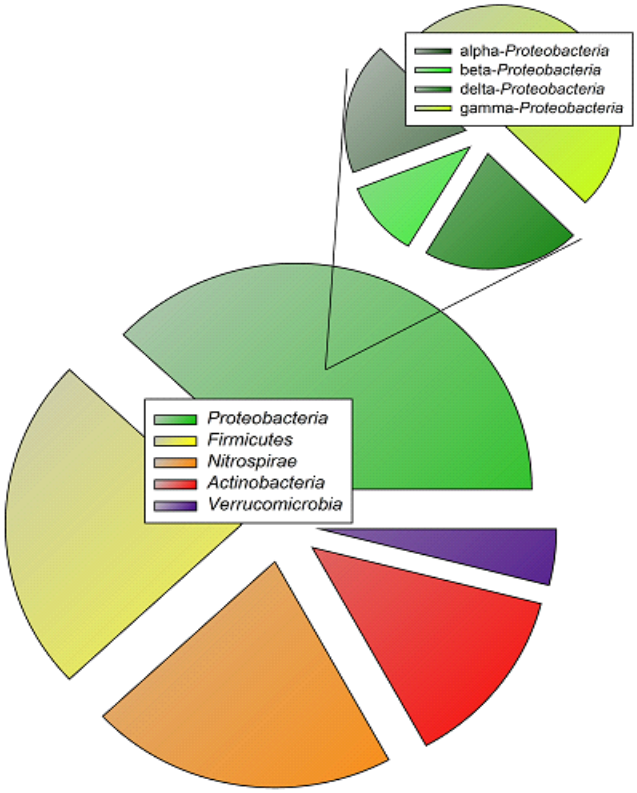

Fig. 6. Relative abundances of phylogenetic groups of *Bacteria* within the clone library analyzed

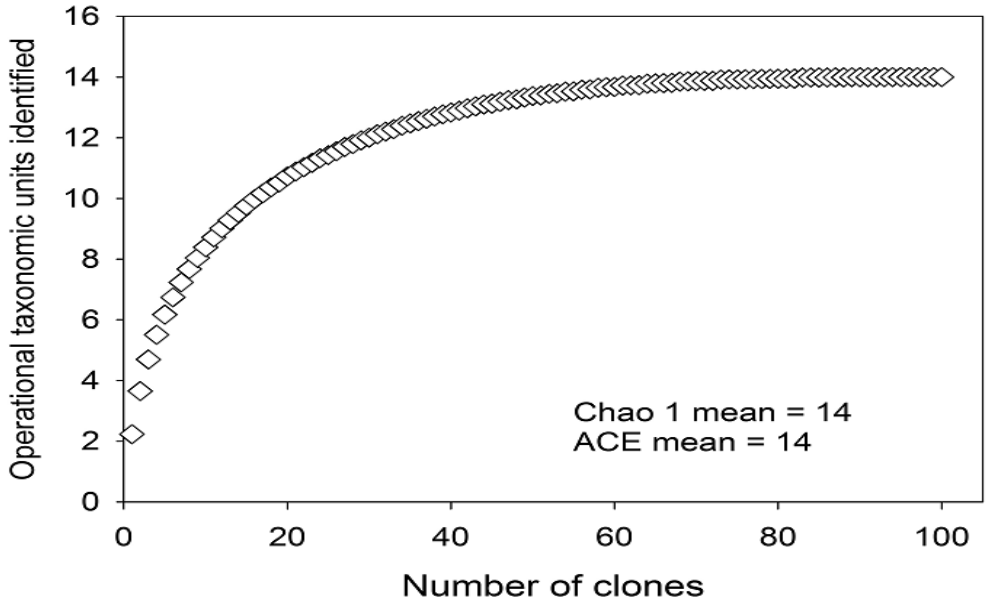

Fig. 7. Rarefaction curve depicting the relationship between the number of clones analyzed and the number of OTU's (phylotypes) identified. The Chao-1 and ACE values shown are calculated estimators for the total species richness expected for the clone library

which is in agreement with other studies for similar environments.

ACKNOWLEDGMENTS

This research was funded by Umgeni Water. The authors would like to thank Dudu Gwebu, Collin Van Der Merwe and Peter Thompson at Umgeni Water – Processing in Durban as well as the supporting staff at Umgeni Water Pietermaritzburg.

REFERENCES

Amann, R. I., Binder, B.J., Olson, R. J., Chisholm, S. W., Devereux, R. and Stahl, D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry

for analyzing mixed microbial populations. Appl. Environ. Microbiol., **56,** 1919-1925.

Back, J.P. and Kroll, R.G. (1991). The differential fluorescence of bacteria stained with acridine orange and the effects of heat. J. Appl. Bacteriol., **71,** 51-58.

Bartosch, S., Quader, H and Bock, E. (1996). Confocal laser scanning microscopy: a new method for detecting microorganisms in natural stone. Dechema Monographs, **133,** 37-43.

Bartosch, S., Mansch, R., Knötzsch, K. and Bock, E. (2003). CTC staining and counting of actively respiring bacteria in natural stone using confocal laser scanning microscopy. J. Microbiol. Methods, **52,** 75-84.

Beukes, L. S. and Schmidt, S. (2012). Isolation and characterization of a manganese oxidizing bacterium from a biofiltration system for the treatment of borehole water in KwaZulu-Natal (South Africa). Eng. Life Sci., **12,** 544-552.

Blçthe, M. and Roden, E. E. (2009). Microbial iron redox cycling in a circumneutral pH groundwater seep. Appl. Environ. Microbiol., **75,** 468-473.

Bock, E., Koops, H –P., Ahlers, B. and Harms, H. (1992). Oxidation of inorganic nitrogen compounds as energy source. (In A. Balows, H.G. Truper, M. Dworkin, W. Harder, & K.H. Scleifer (Eds.), The Prokaryotes (pp. 414-430). Springer-Verlag, New York.

Bouchard, M. F., Sauvé, S., Barbeau, B., Legrand, M., Brodeur, M –E., Bouffard, T., Limoges, E., Bellinger, D. C. and Mergler, D. (2011). Intellectual impairment in school-age children exposed to manganese from drinking water. Env. Health Persp., **119,** 138-143.

Burger, M. S., Krentz, C. A., Mercer, S.S. and Gagnon, G. A. (2008). Manganese removal and occurrence of manganese oxidizing bacteria in full-scale biofilters. J. Wat. Supply, Res Technol-AQUA, **57,** 351-359.

Casale, R. J., Le Chevallier, M.W. and Pontius, F. W. (2002). Manganese control and related issues. Denver, CO: AWWA Res Found Am Water Works Assoc.

Cébron, A. and Garnier, J. (2005). *Nitrobacter* and *Nitrospira* genera as representatives of nitrite-oxidizing bacteria: Detection, quantification and growth along the lower Seine River (France). Wat. Res., **39,** 4979-4992.

Cerrato, J., Reyes, L. P., Alvarado, C. N. and Dietrich, A. M. (2006). Effect of PVC and iron materials on Mn (II) deposition in drinking water distribution systems. Wat. Res., **40,** 2720-2726.

Cerrato, J. M., Falkinham, J. O., Dietrich, A. M., Knocke, W. R., McKinney, C. W. and Pruden, A. (2010). Manganese-oxidizing and -reducing microorganisms isolated from biofilms in chlorinated drinking water systems. Wat. Res., **44,** 3935-3945.

Colwell, R. R., Brayton, P. B., Grimes, D. J. and Roszak, D. B. (1985). Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: Implications for release of genetically engineered microorganisms. Nat. Biotech., **3,** 817-820.

Darzynkiewicz, Z., Traganos, F., Sharpless, T. and Melamed, M. R. (1975). Conformation of RNA in situ as studied by AO staining and automated cytofluometry. Exp. Cell. Res., **95,** 143-153.

del Giorgio, P. A., Prairie, Y. T. and Bird, D. F. (1997). Coupling between rates of bacterial production and the abundance of metabolically active bacteria in lakes, enumerated using CTC reduction and flow cytometry. Microb. Ecol., **34,** 144-154.

De Santis, T. Z., Brodie, E. L., Moberg, J. P., Zubieta, I. X., Piceno, Y.M. and Andersen, G.L. (2007). High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microb. Ecol., **53,** 371-383.

Dionisi, H. M., Layton, A.C., Harms, G., Gregory, I. R., Robinson, K. G. and Sayler, G. S. (2002). Quantification of *Nitrosomonas oligotropha*-like ammonia-oxidizing bacteria and *Nitrospira* spp. from full-scale wastewater treatment plants by competitive PCR. Appl. Environ. Microbiol., **68,** 245-253.

Dunbar, J., Barns, S. M., Ticknor, L. O. and Kuske, C. R. (2002). Empirical and theoretical bacterial diversity in four Arizona soils. Appl. Environ. Microbiol., **68,** 3035-3045.

Gage, B., O'Dowd, D.H. and Williams, P. (2001). Biological iron and manganese removal, pilot and full scale applications. In: 2001 Ontario Water Works Association Conference, Toronto, Ontario, Canada 17 pp.

Hamilton, W. A. (2003). Microbially influenced corrosion as a model system for the study of metal microbe interactions: A unifying electron transfer hypothesis. Biofouling, **19,** 65-76.

Harrison, J. J., Ceri, H. and Turner, R. (2007). Multimetal resistance and tolerance in microbial biofilms. Nat. Rev. Microbiol., **5,** 928-938.

Hedrich, S., Schlömann, M. and Johnson, D.B. (2011). The iron-oxidizing proteobacteria. Microbiology, **57,** 1551-1564.

Hirsch, P. and Rades-Rohkohl, E. (1988). Some special problems in the determination of viable counts of groundwater microorganisms. Microb. Ecol., **16,** 99-113.

Hobbie, J. E., Daley, R. J. and Jasper, S. (1977). Use of nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol., **33,** 1225-1228.

Kasten, F. H. (1981). Methods for fluorescence microscopy. In G. Clark (Ed.), Staining procedures, 4th ed. (pp 39-103). The Williams & Wilkins Co., Baltimore.

Keinänen-Toivola, M. M., Revetta, R. P. and Santo Domingo, J. W. (2006). Identification of active bacterial communities in a model drinking water biofilm system using 16S rRNA-based clone libraries. FEMS Microbiol. Lett., **257,** 182-188.

Kepner, R. L. Jr. and Pratt, J. R. (1994). Use of fluorochromes for direct enumeration of total bacteria in environmental samples: Past and present. Microbiol. Mol. Biol. Rev., **58,** 603-615.

Kormas, K. A., Neofitou, C., Pachiadaki, M. and Koufostathi, E. (2010). Changes of the bacterial assemblages throughout an urban drinking water distribution system. Environ. Monit. Assess., **165,** 27-38.

Krakat, N., Westphal, A., Schmidt, S. and Scherer, P. (2010). Anaerobic digestion of renewable biomass: Thermophilic temperature governs methanogen population dynamics. Appl. Environ. Microbiol., **76,** 1842-1850.

Liao, X., Chen, C., Wang, Z., Wan, R., Chang, C. H., Zhang, X. and Xie, S. (2013). Changes of biomass and bacterial communities in biological activated carbon ûlters for drinking water treatment. Proc. Biochem., **48**, 312-316.

Lovley, D.R. (2000). Environmental microbe-metal interactions. Washington D.C, ASM Press.

Mason, D. J. and Lloyd, D. (1997). Acridine orange as an indicator of bacterial susceptibility to gentamicin. FEMS Microbiol. Lett., **53,** 199-204.

Morgan, C. A., Hudson, A., Konopka, A. and Nakatsu, C. H. (2002). Analyses of microbial activity in biomass-recycle reactors using denaturing gradient gel electrophoresis of 16S rDNA and 16S rRNA PCR products. Can. J. Microbiol., **48,** 333-341.

Mouchet, P. (1992). From conventional to biological removal of iron and manganese in France. J. American Wat. Works Assoc. (AWWA), **84,** 158-167.

Muyzer, G., Teske, A., Wirsen, C.O. and Jannasch, H. W. (1995). Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol., **164,** 165-172.

Pavissich, J. P., Vargas, I. T., González, B., Pastén, P. A. and Pizarro, G. E. (2010). Culture dependent and independent analyses of bacterial communities involved in copper plumbing corrosion. J. Appl. Microbiol., **109,** 771-782.

Pettipher, G. L., Mansell, R., McKinnon, C. H. and Cousins, C. M. (1980). Rapid membrane filtration-epifluorescent microscopy technique for direct enumeration of bacteria in raw milk. Appl. Environ. Microbiol., **39,** 423-429.

Ramos, C. G., Grilo, A. M., Sousa, S. A., Barbosa, M. L., Nadais, H. and Leitão, J. H. (2010). A new methodology combining PCR, cloning, and sequencing of clones discriminated by RFLP for the study of microbial populations: Application to an UASB reactor sample. Appl. Microbiol. Biotechnol., **85,** 801-806.

Revetta, R. P., Pemberton, A., Lamendella, R., Iker, B. and Santo Domingo, J. W. (2010). Identification of bacterial populations in drinking water using 16S rRNA-based sequence analyses. Wat. Res., **44,** 1353-1360.

Santo Domingo, J. W., Meckes, M. C., Simpson, J. M., Sloss, B. and Reasoner, D. J. (2003). Molecular characterization of bacteria inhabiting a water distribution system simulator. Wat. Sci. Technol., **47,** 149-154.

Schmeisser, C., Stockigt, C., Raasch, C., Wingender, J., Timmis, K. N., Wenderoth, D. F., Flemming, H-C., Liesegang, H., Schmitz, R. A., Jaeger, K- E. and Streit, W. R. (2003). Metagenome survey of biofilms in drinking-water networks. Appl. Environ. Microbiol., **69,** 7298-7309.

Servais, P., Agogue, H., Courties, C., Joux, F. and Lebaron, P. (2001). Are the actively respiring cells (CTC positive) those responsible for bacterial production in aquatic environments? FEMS Microbiol. Ecol., **35,** 171-179.

Sheng, G. P., Yu, H. Q. and Li, X. Y. (2010). Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems. Biotechnol. Adv., **28,** 882-894.

Singleton, D. R., Furlong, M. A., Rathbun, S. L. and Whitman, W. B. (2001). Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Appl. Environ. Microbiol., **67,** 4374-4376.

Spiegelman, D., Whissell, G. and Greer, C. W. (2005). A survey of the methods for the characterization of microbial consortia and communities. Can. J. Microbiol., **51,** 355-386.

Takeda, M., Kawasaki, Y., Umezu, T., Shimura, S., Hasegawa, M. and Koizumi, J. (2012). Patterns of sheath elongation, cell proliferation, and manganese (II) oxidation in *Leptothrix cholodnii*. Arch. Microbiol., **194,** 667-673.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol., **28,** 2731-2739.

Trevors, J. T. (1989). The role of microbial metal resistance and detoxification mechanisms in environmental bioassay research. Hydrobiologia, **188/189,** 143-147.

Tyson, G. W., Chapman, J., Hugenholtz, P., Allen, E. E., Ram, R. J., Richardson, P. M., Solovyev, V. V., Rubin, E. M., Rokhsar, D. S. and Banfield, J. F. (2004). Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature, **428,** 37-43.

UNEP, (2013). Africa environment outlook 3: Summary for policy makers. United Nations Environment Programme (UNEP), Nairobi, Kenya.

Vandenabeele, J., de Beer, D., Germonpre, R. and Verstraete, W. (1992). Manganese oxidation by microbial consortia from sand filters. Microb. Ecol., **24,** 91-108.

WaterAid, (2013). Everyone, everywhere: A vision for water, sanitation and hygiene post-2015. WaterAid, London, UK.

WHO, (2011). Guidelines for drinking water quality, 4th edition. World Health Organisation (WHO), Geneva, Switzerland.

WHO/UNICEF JMP, (2012). A snapshot of drinking water and sanitation in Africa – 2012 update. A regional perspective based on new data from the WHO/UNICEF joint monitoring programme for water supply and sanitation. Prepared for AMCOW as a contribution to fourth Africa water week Cairo, Egypt.

Williams, M. M., Santo Domingo, J. W., Meckes, M. C., Kelty, C. A. and Rochon, H. S. (2004). Phylogenetic diversity of drinking water bacteria in a distribution system simulator. J. Appl. Microbiol., **96,** 954-964.

Zhou, J., Bruns, M. A. and Tiedje, M. J. (1996). DNA recovery from soils of diverse composition. Appl. Environ. Microbiol., **62,** 316-322.