

## Antibiotic-resistant heterotrophic plate count bacteria and amoeba-resistant bacteria in aquifers of the Mooi River, North West province, South Africa

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### ABSTRACT

Groundwater in the Mooi River catchment is prone to mining, agricultural, municipal and septic tank pollution. In this study physico-chemical and microbiological parameters were determined using appropriate methods. Bacterial isolates were identified by 16S rRNA sequencing (heterotrophic plate count (HPC) bacteria and amoeba-resistant bacteria (ARB)) and multiplex polymerase chain reaction (*Escherichia coli*). Antibiotic resistance tests were also performed. Physico-chemical parameters were generally within target water quality ranges for drinking water. HPC bacteria ranged between  $10^5$  and  $10^7$  colony-forming units (cfu)/ml. *E. coli* were enumerated from Trimpark, School and Cemetery. The Blaauwbank borehole was negative for faecal streptococci. *Pseudomonas* spp. were most abundant in the bulk water. Opportunistic pathogens isolated included *Pseudomonas aeruginosa*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Flavobacterium*, *Bacillus cereus* and *Mycobacterium* spp. Varying patterns of antibiotic resistance were observed. Most HPC bacterial isolates were resistant to cephalothin and/or amoxicillin and a few were resistant to erythromycin and streptomycin. *Pseudomonas* spp. was also the most abundant ARB. Other ARBs included *Alcaligenes faecalis*, *Ochrobactrum* sp. and *Achromobacter* sp. ARBs were resistant to streptomycin, chloramphenicol, cephalothin, and/or amoxicillin compared to HPCs. The presence of *E. coli* and ARB in these groundwater sources indicates potential human health risks. These risks should be further investigated and quantified, and groundwater should be treated before use.

**Key words** | antibiotic resistance, ARB, groundwater, HPC bacteria, opportunistic pathogens

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### INTRODUCTION

People living in rural areas in the Northern provinces of South Africa are largely dependent on groundwater resources to meet the demand for water for domestic purposes. In the North West province, this amounts to 65% of 3.5 million people (Department of Water Affairs (DWA) 2009). As groundwater is generally considered to be of good physico-chemical quality, this resource is usually supplied to communities without prior treatment (Momba *et al.* 2006). However, the microbiological quality may be impaired (Mpenyana-Monyatsi & Momba 2012). Testing for this aspect is generally neglected by authorities as well as individual households.

Ingestion of contaminated water may lead to infection by pathogens causing illnesses such as cholera, typhoid fever, paratyphoid fever and bacillary dysentery (Fourie & van Ryneveld 1994; Lawrence *et al.* 2001). Microbiological tests, based on faecal indicator bacteria, such as faecal coliforms, specifically *Escherichia coli*, are used to indicate the possibility that pathogenic species of faecal origin could be present (Department of Water Affairs & Forestry (DWA) 1996; Atlas & Bartha 2002; World Health Organization (WHO) 2011). Heterotrophic plate count (HPC) bacteria include all naturally occurring bacteria that utilise organic nutrients at low concentrations

(Edberg *et al.* 1997; Edberg & Allen 2004). These bacterial counts give an indication of the general microbial quality of water and do not indicate faecal pollution (DWAF 1996).

Free-living amoebae (FLA) are unicellular eukaryotes that are natural predators of bacteria in the environment (Moliner *et al.* 2010). The diversity and abundance of amoebae in water sources are dependent on certain environmental factors. These include temperature, moisture content in non-water sources, pH and nutrient availability (including the presence of bacterial species) (Greub & Raoult 2004). FLA feed on bacteria through phagocytosis (Greub & Raoult 2004). For bacteria to be able to survive in the phagolytic environment of FLA, some bacterial species have developed certain mechanisms of resistance. These amoeba-resistant bacteria (ARB) are able to resist digestion by the FLA, thus the latter may become a reservoir for these bacteria (Thomas *et al.* 2006; Pagnier *et al.* 2008). FLA can resist harsh environments by forming cysts. These cyst forms would then protect intracellular ARB against different forms of disinfection that would normally kill planktonic bacteria. Previously identified ARB genera include *Acinetobacter*, *Aeromonas*, *Bacillus*, *Pseudomonas*, *Alcaligenes*, *Brevundimonas*, *Chryseobacterium*, *Comamonas*, *Delftia*,

*Flavobacterium* and *Ochrobactrum* (Barker & Brown 1994; Pagnier *et al.* 2008).

As the mechanism of phagocytosis used by FLA is similar to that of macrophages in multicellular organisms (Greub & Raoult 2004; Thomas *et al.* 2006; Cosson & Soldati 2008), intracellular ARB may also be able to resist phagocytosis and destruction by human macrophages. All these factors may contribute to the distribution of inherently, or adopted, pathogenic ARB. ARB can therefore be considered as emerging human pathogens (Pagnier *et al.* 2008).

The aim of this study was to investigate the occurrence of antibiotic-resistant HPC bacteria and ARB in selected aquifers of the Mooi River catchment of the North West province. A secondary goal was to determine the general physical-chemical and microbiological parameters and whether these are potentially faecally polluted.

## MATERIALS AND METHODS

### Study area

The study area was the upper reaches of the Mooi River catchment (Figure 1). Five boreholes in this catchment

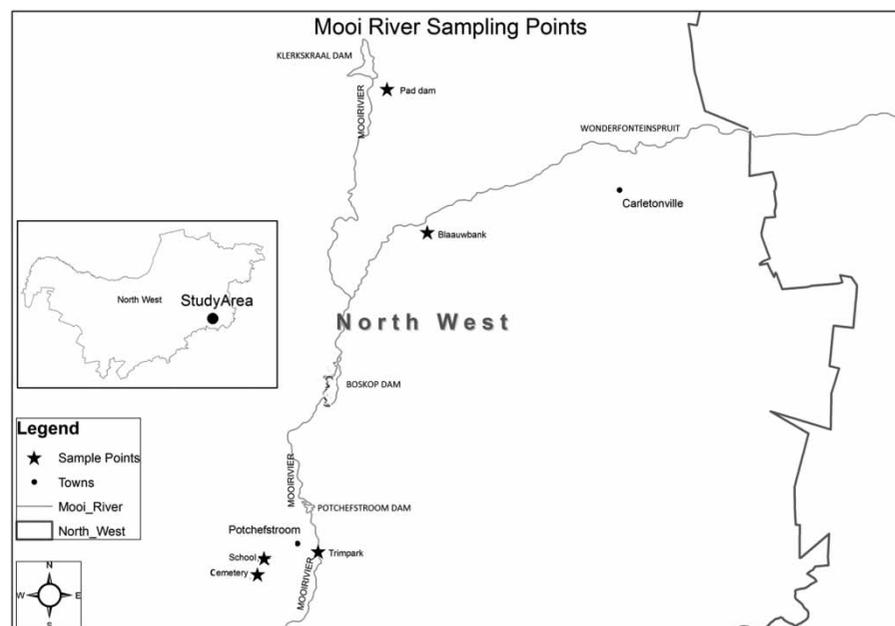


Figure 1 | A map of the sampling points in the Mooi River catchment.

were sampled during a warm-wet season. Three boreholes are located in the municipal district of Potchefstroom. One of the boreholes is situated in a public park (Trimpark) on the banks of the Mooi River. Two boreholes are situated in the Ikageng township – one in a cemetery (Cemetery), and the other just outside the perimeter of a primary school (School). One of the sampled boreholes is situated near the Klerkskraal dam (Pad-dam), approximately 45 kilometres north of Potchefstroom. Another borehole is situated on a farm (Blaauwbank) also in a northern direction approximately 40 kilometres from Potchefstroom. All these are capped boreholes.

### Physical-chemical parameters and sample collection

Boreholes were purged for 15 min. Before a water sample was retrieved, a multimeter (Hydralab DS5, Stevens Water Monitoring Systems, USA) was lowered into each borehole to measure certain physical and chemical parameters of the water *in situ*. Parameters included pH, temperature, electrical conductivity (EC), dissolved oxygen (DO) and salinity. A sterilised electrical water sampler was lowered into each borehole to collect water. The sampler was opened when lowered to allow water to fill the empty cavity. It was closed using a remote system. The water samples were poured into autoclave-sterilised one-litre Schott bottles. A sterilised plastic funnel was used to facilitate this process. The bottles containing the water samples were put onto ice, transported to the laboratory and analysed within six hours of collection. Chemical analysis (chemical oxygen demand, sulphate, nitrate) was conducted in the laboratory using reagents, protocols and a spectrophotometer (DR2800) from Hach (Germany).

### Enumeration of HPC bacteria

A dilution series (up to  $10^{-5}$ ) of the water samples was made and spread plated onto Difco R2A (Becton, Dickinson and Company, France) agar. Plates were incubated at room temperature ( $\pm 25^{\circ}\text{C}$ ) for 5 days. The total number of colony-forming units (cfu) on the plates was determined and converted to cfu/ml. Representatives of various morphotypes (morphologically distinct colonies, based on shape, pigmentation, etc.) were also recorded and purified

by successive streak plating on R2 agar (Oxoid, Merck, Germany).

### Isolation of FLA and ARB

The water samples were tested for the presence of FLA and ARB by amoebal enrichment on non-nutrient agar containing heat-killed *E. coli* as a nutrient source. The agar plates were incubated aerobically at  $32^{\circ}\text{C}$ , and viewed microscopically at regular intervals for the presence of trophozoites and/or cysts. FLA present on the plates were then purified by using a standard agar plug transfer method (Greub & Raoult 2004) until sufficient numbers were obtained to be transferred to 24-well tissue culture plates containing Page's amoebal saline, and were incubated again at  $32^{\circ}\text{C}$ . The wells were viewed microscopically at regular intervals for the presence of trophozoites and/or cysts, and the presence and motility of intra- and extracellular bacteria and nematodes. Gram, acid fast and Giemsa staining were performed to differentiate between Gram-positive, Gram-negative and acid-fast bacteria, as well as to visualise amoebal trophozoites and/or cysts. Samples containing intra-amoebal bacteria were confirmed as described below.

### Enumeration of faecal coliforms (*E. coli*), total coliforms and faecal streptococci

Hundred-millilitre aliquots of water were filtered through  $0.45\ \mu\text{m}$ -pore size membrane filters (GN-6 Metricel, Pall Corporation, USA) using the membrane filtration technique. Individually filtered membranes were placed onto membrane-lactose-glucose (MLG) agar, cephalothin (KF)-streptococcus agar and membrane faecal coliform (mFC) agar. Analyses were conducted in triplicate and media were from Merck (Germany).

Preliminary investigations indicated that the water samples had to be diluted ( $10^{-2}$ ) to obtain countable cfu values. MLG agar was incubated at  $35^{\circ}\text{C}$  for 24 h, KF-streptococcus agar at  $35^{\circ}\text{C}$  for 48 h and mFC agar at  $45^{\circ}\text{C}$  for 24 h. Green (possible *E. coli*) and yellow (total coliforms) colonies were counted on the MLG agar, and pink colonies (faecal streptococci) on the KF-streptococcus agar. The number of blue colonies (faecal coliforms) on the mFC agar was used for comparison with the MLG agar results.

Only the MLG data are presented. Levels of the various bacteria were expressed (converted where applicable) as cfu/100 ml. Selected putative *E. coli* isolates (green colonies) representing the sites that were positive were purified by successive streak plating on mFC agar and further identified by multiplex polymerase chain reaction (PCR).

### Identification of *E. coli*

DNA isolation of putative *E. coli* was performed by using a Nucleospin tissue DNA isolation kit according to the instructions of the manufacturer (Macherey-Nagel, Germany). PCR was used to detect the *mdh*, *lacZ* genes using the primer pairs (*mdh*F 5'-GGTATGGATCGTTCCGACCT-3'; *mdh*R 5'-GGCAGAATGGTAACACCAGAGT-3'; Omar *et al.* 2010) and (*lacZ*F 5'-CTGGCGTAATAGCGAAGAGG-3'; *lacZ*R 5'-GGATTGACCGTAATGGGATATG-3'; Ram & Shanker 2005) in a multiplex PCR (mPCR) (Omar *et al.* 2010). Fermentas (US) PCR master mix was used in the PCR process and subsequently electrophoresis was used to determine whether amplification was successful.

### Identification of HPC bacteria and ARB

Pure colonies of HPC and ARB were grown in nutrient broth and the DNA isolated by a microwave method. Briefly 20 µl of culture was pelleted, the broth discarded and cells resuspended in TE buffer (10 mM Tris-HCL, 1 mM EDTA [pH 8.0]). The tubes were microwaved for two minutes at 700 W to rupture cells. These tubes were then centrifuged for one minute at 13,000 rpm and 1 µl of the various supernatants was used for PCR reactions.

For the amplification of 16S rRNA genes the primer pair 27F and 1492R and conditions described by Lane (1991) were used. The Fermentas (USA) master mix was also used for PCR and electrophoresis to determine whether the PCRs worked. Amplification products were purified by Macherey-Nagel, (Germany) and then sequenced using the BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) according to the instructions from the manufacturers. An ABI 3130 Genetic Analyser (Applied Biosystems, UK) was used to sequence the amplicons. Chromatograms were viewed in Geospiza Finch TV (version 1.4) software and BLAST searches ([http://www.](http://www.ncbi.nlm.nih.gov/BLAST)

[ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)) were used to determine the identity of the amplified sequences. Sequences were deposited in Genebank under accession numbers KF260970-KF261054.

### Antibiotics resistance testing of isolated *E. coli*, HPC bacteria and ARB

Pure colonies were transferred to nutrient broth and incubated at ±25 °C for 5 days (HPC bacterial & ARB isolates) and at ±37 °C for 24 h (*E. coli* isolates). The nutrient broth was used to make spread plates onto Mueller–Hinton (Merck, Germany) agar plates. Antibiotics included amoxicillin 10 µg (β-lactam), cephalothin 30 µg (β-lactam), ciprofloxacin 5 µg (fluoroquinolone), streptomycin 30 µg (aminoglycoside), erythromycin 15 µg (macrolide) and chloramphenicol 30 µg (chloramphenicol) (Mastdiagnostics, UK). The Mueller–Hinton plates were incubated at 35 °C for 24 h and inhibition zones recorded in millimetres.

### Statistics

Where appropriate, Microsoft Excel was used to calculate averages and standard deviations.

## RESULTS

### Physico-chemical and bacteriological

Physico-chemical data for all of the boreholes are summarised in Table 1. pH ranged between 6.6 and 7.5. The maximum temperature recorded was at the Blaauwbank borehole (23.6 °C). High electrical conductivity (EC) values were measured at the Trimpark (467 mS/m), School (125 mS/m) and Cemetery (255 mS/m) boreholes. Levels of nitrates at the School borehole was high (10.7 mg/l) but not at health injuring levels. Chemical oxygen demand (COD) levels were low and ranged between 0 and 3.5 mg/l. Sulphate levels at Trimpark were also high but four-fold lower than the maximum total water quality range (TWQR) for drinking water (DWAf 1996).

Bacterial levels for all of the boreholes are summarised in Table 2. Faecal coliforms (potentially *E. coli*) were enumerated from the Trimpark (13 cfu/100 ml), School

**Table 1** | Physico-chemical results recorded at the different boreholes

| Borehole           | Temp | pH  | EC mS/m | Salinity ppm | SO <sub>4</sub> mg/l | Nitrate mg/l | DO mg/l | COD mg/l |
|--------------------|------|-----|---------|--------------|----------------------|--------------|---------|----------|
| TWQR* domestic use | N/A  | 6–9 | <70     | N/A          | <200                 | <10          | N/A     | N/A      |
| <b>Blaauwbank</b>  | 23.6 | 7.5 | 40      | 198          | 0                    | 0            | 1.2     | 3.5      |
| <b>Pad-dam</b>     | 21.2 | 6.6 | 31      | 157          | 0                    | 0            | 2.3     | 2.4      |
| <b>Trimpark</b>    | 21.9 | 7.3 | 467     | 233          | 50                   | 0.6          | 2.3     | 2.3      |
| <b>School</b>      | 23.4 | 7   | 125     | 643          | 21                   | 10.7         | 3.1     | 2.2      |
| <b>Cemetery</b>    | 23.2 | 6.8 | 255     | 127          | 7                    | 0            | 2.3     | 0        |

\*TWQR is based on the South African Department of Water Affairs (DWAF 2006) values.

**Table 2** | Bacteriological results for all of the boreholes sampled. Target water quality range (TWQR\*) is based on the South African Department of Water Affairs (DWAF 2006) values

| Borehole          |                          | Faecal coliforms ( <i>E. coli</i> )<br>cfu/100 ml | Total coliforms<br>cfu/100 ml | HPC bacteria<br>cfu/ml | Faecal streptococci<br>cfu/100 ml |
|-------------------|--------------------------|---|-------------------------------|------------------------|-----------------------------------|
|                   | <b>TWQR domestic use</b> | 0   | <10                           | <1,000                 | N/A                               |
| <b>Blaauwbank</b> | <b>Ave</b>               | 0   | 0                             | $1.9 \times 10^6$      | 0                                 |
|                   | <b>SD</b>                | ± 0   | ± 0                           | ± $1.6 \times 10^6$    | ± 0                               |
| <b>Pad-dam</b>    | <b>Ave</b>               | 0   | 3,360                         | $5.0 \times 10^7$      | $2.4 \times 10^4$                 |
|                   | <b>SD</b>                | ± 0   | ± 0                           | ± 0                    | ± $1.5 \times 10^4$               |
| <b>Trimpark</b>   | <b>Ave</b>               | 13  | 19,040                        | $1.0 \times 10^5$      | $2.5 \times 10^2$                 |
|                   | <b>SD</b>                | ± 1   | ± 0                           | ± 0                    | ± 96                              |
| <b>School</b>     | <b>Ave</b>               | 3   | $9.7 \times 10^3$             | $1.8 \times 10^6$      | $1.6 \times 10^3$                 |
|                   | <b>SD</b>                | ± 1   | ± $9.9 \times 10^3$           | ± $2.2 \times 10^6$    | ± $4.0 \times 10^2$               |
| <b>Cemetery</b>   | <b>Ave</b>               | 276   | $2.6 \times 10^3$             | $2.5 \times 10^5$      | $1.5 \times 10^2$                 |
|                   | <b>SD</b>                | ± 5   | ± $8.5 \times 10^3$           | ± $1.9 \times 10^4$    | ± 64                              |

Ave – average, N/A – not applicable, SD – standard deviation.

(3 cfu/100 ml) and Cemetery (276 cfu/100 ml) boreholes. All HPC bacterial, total coliform and faecal streptococci counts were high, except for the Blaauwbank borehole that was not positive for total coliforms and faecal streptococci. The Pad-dam borehole had the highest HPC bacterial ( $5.0 \times 10^7$  cfu/ml) and faecal streptococci ( $2.4 \times 10^4$  cfu/ml) levels. However, the highest total coliform levels were at Trimpark, School and Cemetery and were all in the 3-log cfu/100 ml range.

### ***E. coli* identification and antibiotic resistance**

A mPCR protocol was followed for positive identification of selected presumptive *E. coli* isolates representing the various sites. For this identification, fragments of the malate dehydrogenase (*mdh*) and lactose promoter (*lacZ*) were amplified. Only if both gene fragments were amplified was

the result accepted as positive for *E. coli*. All of the selected isolates were positive.

These confirmed *E. coli* isolates were used in the antibiotic resistance analysis. All these *E. coli* were resistant to amoxicillin (A), KF and erythromycin (E) but were susceptible to ciprofloxacin (CIP). Except for a Trimpark isolate, all the others were resistant to kanamycin (K). This Trimpark isolate, however, had intermediate resistance to kanamycin. What was evident was that all the *E. coli* isolates, from various geographically separated boreholes, shared the same antibiotic resistance phenotype (A-E-KF).

### **HPC bacteria identification and antibiotic resistance**

The initial selection of HPC bacterial isolates was based on morphologically different colonies on the original plates. After 16S rRNA sequencing analysis on representative

colonies, a total of fifteen different genera were identified. Results are summarised in Table 3. The most abundant genera identified were *Pseudomonas* (7 isolates), *Bacillus* (5 isolates) and *Comamonas* (4 isolates). The least abundant were *Fermicutes*, *Delftia*, *Massilia*, *Siphonobacter*, *Dechlorosoma*, *Brevundimonas*, *Lysinibacillus*, *Aeromonas*

and *Chitinophaga* with only one isolate each. The greatest diversity of genera was obtained at the School borehole (7 genera). The Trimpark borehole had the least diversity (2 genera).

Species that were resistant to four different antibiotic groups (S-C-E- $\beta$ -lactams, antibiotic resistance profile v in

**Table 3** | A list of bulk water isolated HPC bacteria and ARB that were isolated

| Species isolated                   | Blaauwbank |     | Pad-dam |        | Trimpark |     | Cemetery |        | School    |          |
|------------------------------------|------------|-----|---------|--------|----------|-----|----------|--------|-----------|----------|
|                                    | HPC        | ARB | HPC     | ARB    | HPC      | ARB | HPC      | ARB    | HPC       | ARB      |
| <i>Achromobacter piechaudii</i>    |            |     |         |        |          |     |          |        |           | √ (xi)   |
| <i>Aeromonas hydrophila</i>        |            |     |         |        |          |     |          |        | √ (iv)    |          |
| <i>Alcaligenes faecalis</i>        |            |     |         | √ (ix) |          |     |          |        |           | √ (xii)  |
| <i>Bacillus cereus</i>             | √ (i)      |     | √ (i)   |        |          |     |          |        | √ (3) (i) |          |
| <i>Brevundimonas diminuta</i>      |            |     |         |        |          |     | √ (viii) |        |           |          |
| <i>Chitinophaga</i>                |            |     |         |        |          |     |          |        | √ (v)     |          |
| <i>Chryseobacterium</i>            |            |     | √ (iv)  |        | √ (iv)   |     |          |        |           |          |
| <i>Comamonas odontotermitis</i>    | √ (iv)     |     |         |        |          |     | √ (v)    |        |           |          |
| <i>Comamonas thiooxydans</i>       |            |     | √ (ii)  |        |          |     |          |        |           |          |
| <i>Dechlorosoma suillum</i>        | √ (i)      |     |         |        |          |     |          |        |           |          |
| <i>Delftia tsuruhatensis</i>       |            |     | √ (iii) |        |          |     |          |        |           |          |
| <i>Flavobacterium cucumis</i>      |            |     |         |        |          |     |          |        | √ (i)     |          |
| <i>Leifsonia xyli</i>              | √ (-)      |     |         |        |          |     | √ (i)    |        |           |          |
| <i>Lysinibacillus sphaericus</i>   |            |     |         |        |          |     |          |        | √ (iv)    |          |
| <i>Massilia timonae</i>            | √ (i)      |     |         |        |          |     |          |        |           |          |
| <i>Ochrobactrum</i>                |            |     |         |        |          |     |          | √ (ix) |           | √ (2)(v) |
| <i>Pseudomonas aeruginosa</i>      |            |     |         | √ (v)  |          |     |          |        |           | √ (v)    |
| <i>Pseudomonas anguilliseptica</i> |            |     |         |        |          |     |          |        | √ (viii)  |          |
| <i>Pseudomonas fluorescens</i>     |            |     |         |        | √ (v)    |     | √ (v)    |        |           | √ (v)    |
| <i>Pseudomonas nitroreductans</i>  |            |     |         |        |          |     |          |        |           | √ (x)    |
| <i>Pseudomonas plecoglossicida</i> |            |     |         |        |          |     | √ (x)    | √ (x)  |           |          |
| <i>Pseudomonas putida</i>          |            |     |         |        | √ (v)    |     |          | √ (x)  |           |          |
| <i>Pseudomonas stutzeri</i>        |            |     |         |        |          |     | √ (vi)   |        |           |          |
| <i>Siphonobacter aquaeclarae</i>   | √ (i)      |     |         |        |          |     |          |        |           |          |
| Uncultured <i>Fermicutes</i>       |            |     | √ (i)   |        |          |     |          |        |           |          |
| <i>Pseudomonas</i> sp              |            |     |         | √ (x)  |          |     | √ (i)    |        |           |          |
| <i>Comamonas</i> sp                |            |     |         |        |          |     |          |        | √ (viii)  |          |
| <i>Achromobacter</i> sp            |            |     |         |        |          |     |          |        |           | √ (x)    |

√ – presence.

Antibiotic resistance profile: (i) A; (ii) S-A; (iii) S-E-A; (iv) S-KF-A; (v) S-C-KF-E-A; (vi) KF; (vii) CIP-A; (viii) KF-E-A; (ix) S-C-KF-A; (x) C-KF-E-A; (xi) E; (xii) S-C-A.

Antibiotics: A – Amoxicillin; C – Chloramphenicol; CIP – Ciprofloxacin; E – Erythromycin; KF – Cephalothin; S – Streptomycin.

The sign (√) indicates that the species was detected (either as a HPC bacterium or as an ARB and in one case both as HPC bacteria and ARB). The Roman numerals (i to xii) indicate the antibiotic resistance profile of the species.

Table 3) are: *Pseudomonas putida* (Trimpark), *Pseudomonas fluorescens* (Trimpark & Cemetery), *Pseudomonas plecoglossicida* (Cemetery), *Comamonas* sp. (Cemetery) and *Chitinophaga* sp. (School). *Leifsonia xyli* from the Blaauwbank borehole was susceptible to all antibiotics and *Pseudomonas stutzeri* from the Cemetery borehole was resistant to only one (KF –  $\beta$ -lactam) antibiotic.

Resistance to  $\beta$ -lactam antibiotics was a general phenotype observed among the *E. coli* and HPC bacterial isolates. *Comamonas odontotermitis*, *Lysinibacillus sphaericus* and *Aeromonas hydrophila* displayed the same antibiotic resistance phenotype (A-KF-S). Resistance to erythromycin was also common.

### ARB identification and antibiotic resistance

Five different ARB genera were isolated. *Pseudomonas* (7 isolates) was the most abundant genus followed by *Ochrobactrum* (3 isolates). The least abundant genera were *Alcaligenes* and *Achromobacter* with only two isolates each. Six different bacterial species were isolated and identified from the School borehole. Only two different genera were identified from the Cemetery borehole. *Pseudomonas aeruginosa* was identified in the water from the School and Pad-dam boreholes.

All bacteria identified were resistant to three or more antibiotic groups, with the exception of *Achromobacter piechaudii* (School) that was resistant to only one antibiotic group. *P. aeruginosa* (Pad-dam & School), *Ochrobactrum* (School) and *P. fluorescens* (School) were resistant to all antibiotic groups. Again, the antibiotic group to which most individuals were resistant was the  $\beta$ -lactam group. Erythromycin and chloramphenicol resistance was also common. ARBs isolated were Gram-negative bacteria and the HPC bacteria isolated directly from the bulk water were either Gram-negative or Gram-positive. The ARBs were resistant to a greater number of antibiotics compared to the HPC bacteria isolated from the bulk water. The ARBs from the Pad-dam borehole were all resistant to chloramphenicol and cephalothin. The Gram-negative HPC bacteria from the Pad-dam bulk water were all resistant to amoxicillin and to a lesser degree, streptomycin. In this case the antibiotic resistance phenotype appears to be compartmentalised.

The ARBs from the Cemetery borehole had the same antibiotic resistance pattern (A-C-E-KF) except *Ochrobactrum* sp. (A-C-KF-S). The only bacterium isolated from both bulk water and amoeba was *P. plecoglossicida*. This species was isolated from the cemetery borehole. Both isolates were resistant to the same antibiotics (A-C-E-KF).

Amongst the bacteria that were isolated and identified from the amoebae, five belonging to two different Gram-negative genera had the same antibiotic resistance profile (S-C-KF-E-A). This pattern was also observed in three different genera from the bulk water (*Chitinophaga*, *Pseudomonas* and *Comamonas*). Two of the ARB isolates (*P. nitroreductans* and *Achromobacter* sp. [School]) were resistant to the same antibiotics (C-KF-E-A). The other isolates from the bulk water were all resistant to  $\beta$ -lactam antibiotics and in two cases to either streptomycin or erythromycin. The ARBs were all resistant to erythromycin and some were also resistant to streptomycin, chloramphenicol and amoxicillin. There was thus considerable similarity in the antibiotic resistance profiles between the isolates from various boreholes. These similarities were observed for isolates from the same boreholes but that were isolated from different compartments. These included the bulk water on the one hand and those from the amoebae on the other.

## DISCUSSION

Due to the unequal distribution of surface water resources in the province, people are reliant on groundwater resources to fulfil the demand for water for domestic use. Groundwater is perceived as being inherently of pristine quality since it percolates through several layers of soil and rock (Mackintosh & Colvin 2003; Momba et al. 2006; Ferguson et al. 2012; Mpenyana-Monyatsi & Momba 2012). For this reason, many of the rural areas in South Africa are supplied with groundwater for domestic use without prior treatment (Lehloesa & Muyima 2000; Mackintosh & Colvin 2003).

### Physico-chemical quality

Physico-chemical parameters of the borehole water were generally in compliance with target water quality ranges

for drinking water (DWAF 1996), except for high EC and salinity levels at the Trimpark, School and Cemetery boreholes. The School borehole also had a high nitrate level. The School and Cemetery boreholes are situated roughly two kilometres away from a fertiliser by-products waste heap. This may explain the high salt and EC levels observed at these boreholes.

Dolomites form the main geological structures of the Mooi River catchment area. Inherent qualities of dolomites (highly transmissive, rapid recharge; DWAF 2006), would allow the leaching of specific ions into the groundwater. This may account for the high EC and salinity levels in the aquifer water.

### Microbiological

HPC bacteria utilise low concentrations of organic nutrients for growth (Edberg *et al.* 1997; Edberg & Allen 2004). These bacterial counts give an indication of the general microbial quality of water and do not indicate faecal pollution (DWAF 1996). Controversy exists in the literature over the importance of HPC bacteria as a microbial water quality indicator of human health significance (Payment *et al.* 1991; Ford 1999; Quiroz 1999; Thompson 1999; Stelma *et al.* 2004; Donskey 2006). Although certain HPC bacterial species are opportunistic pathogens, clinical evidence of these species causing gastrointestinal tract infections is lacking (Edberg *et al.* 1997; Edberg & Allen 2004; Allen *et al.* 2004). However, if HPC bacterial levels are high, this could then mean that an organic nutrient source is available. The COD levels at these boreholes were low (2.3 to 3.5 mg/l) but it indicates that there were supplies of organic nutrients available to maintain HPC bacteria in the borehole water.

Beside the levels of HPC bacteria, diversity of species is also important. This is particularly so for the Sub-Saharan African region where malnutrition and high levels of HIV-AIDS affect large sections of the population. In this case, opportunistic pathogenic species that may occur are of great concern (Ford 1999; Quiroz 1999; Paulse *et al.* 2009).

The most abundant genus identified was *Pseudomonas*. Other genera included *Flavobacterium*, *Comamonas*, *Chryseobacterium*, *Bacillus*, and *Leifsonia*. Individual representatives from the genera *Aeromonas*, *Fermicutes*,

*Delftia*, *Massilia*, *Siphonobacter*, *Dechlorosoma*, *Brevundimonas*, *Lysinibacillus* and *Chitinophaga* were also isolated and identified. Opportunistic pathogens that were enumerated as HPC bacteria in previous studies include *Pseudomonas aeruginosa*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Acinetobacter*, *Flavobacterium*, *Bacillus cerues* and *Mycobacterium* (Payment *et al.* 1991; Ford 1999; Quiroz 1999; Thompson 1999; Stelma *et al.* 2004; Donskey 2006). The opportunistic pathogens *Flavobacterium*, *Bacillus cerues* and *Aeromonas* were identified in the present study. Antibiotic resistance of the HPC bacteria varied, but similarities in resistance to some of the antibiotics were observed. Resistance to cephalothin and amoxicillin was observed amongst most of the isolates and also to erythromycin and streptomycin to a lesser degree.

FLA are unicellular eukaryotes that are predators of bacteria (Moliner *et al.* 2010) feeding through phagocytosis. The bacterial cells are engulfed and digested by enzymes (Greub & Raoult 2004). For bacteria to be able to survive in the phagolytic environment of the amoeba, they have developed certain mechanisms of resistance. Specific mechanisms include the resistance of microbicidal effectors in the phagocytes of the amoeba, the ability to replicate in the intracellular environment or the secretion of toxins that kill the amoebae before phagocytosis can be completed (Greub & Raoult 2004; Thomas *et al.* 2006; Cosson & Soldati 2008). The mechanism of phagocytosis used by FLA is similar to that of human macrophages (Greub & Raoult 2004; Thomas *et al.* 2006; Cosson & Soldati 2008). ARB may therefore possess an increased ability to resist phagocytosis by cells in the human immune system. Studies also demonstrated that resistance to FLA may contribute to antibiotic resistance (Walchonik *et al.* 1999; Greub & Raoult 2004; Thomas *et al.* 2006). Horizontal gene transfer has been identified as a process that is favoured in the intracellular environment of amoebae (Pagnier *et al.* 2008; Moliner *et al.* 2010). Genes are selected and incorporated into the DNA of ARB that would help ARB to survive the macrophage lifestyle (Moliner *et al.* 2010). All of these factors may contribute to the pathogenicity of ARB to humans.

In this study *Pseudomonas* sp. was the most abundant ARB species identified. Other species identified included *Alcaligenes* sp. (2 isolates), *Ochrobactrum* (3 isolates) and *Achromobacter* (2 isolates). *Pseudomonas* spp., *Alcaligenes*

*faecalis* and *Ochrobactrum* spp. are known ARB species and these may be opportunistic pathogens. Amongst the ARB, the opportunistic pathogen *Pseudomonas aeruginosa* was isolated from two boreholes. Results from this study demonstrated that ARBs were resistant to more antibiotic groups than the HPC bacteria that were isolated from the bulk water compartments. All of the ARBs were resistant to aminoglycosides (streptomycin), chloramphenicol (chloramphenicol) and  $\beta$ -Lactam (cephalothin) groups. However, all the HPC bacteria from the same borehole were only resistant to the  $\beta$ -lactams (cephalothin and amoxicillin).

Studies by Walchoniak *et al.* (1999) and Thomas *et al.* (2006) proposed that the internal environment of amoebae may contribute to increased antibiotic resistance of ARB. This is achieved either by horizontal gene transfer between ARBs, the amoeba host or by transformation by taking up free DNA of digested non-resistant bacteria (Moliner *et al.* 2010). Greub & Raoult (2004) also proposed that amoebae may have a decreased rate of uptake of antibiotics, thus allowing ARB to adapt to antibiotics at low concentrations. The authors (Greub & Raoult 2004) also stated that amoebae may be able to inactivate antibiotics in their intracellular environment. This metabolic feature may then be acquired by ARB through horizontal gene transfer. All of these may explain why ARB isolated from amoebae in this study were resistant to more antibiotics than the HPC bacteria from bulk water of the same borehole. All of these mechanisms may also increase the pathogenicity of ARB, and therefore ARB should be considered as emerging human pathogens (Pagnier *et al.* 2008).

Many of the ARB isolated in this study were previously reported (Barker & Brown 1994; Pagnier *et al.* 2008). However, no reports could be found for the presence of ARB in borehole water in the North West province of South Africa. Furthermore, a number of the ARB species identified in this study could also not be accounted for in literature. This is cause for concern since the amoeba-resistant genotype may be associated with pathogenicity and virulence. At the time of sampling the specific boreholes of this study were not used as domestic drinking sources. However, the results demonstrate that borehole water in the Mooi River system may be contaminated with bacterial species that may have human health implications.

Faecal coliforms were enumerated from three (Trim-park, School, Cemetery) of the boreholes. The selected isolates that were further identified by mPCR were positive for *E. coli* marker genes. It is not uncommon to isolate faecal coliforms from a selection of boreholes in the North West province of South Africa. Mpenyana-Monyatsi & Momba (2012) and Ferreira (2011) also demonstrated that many of the boreholes that they sampled were positive for faecal coliforms. Furthermore, the results of Ferreira also indicated the presence of faecal streptococci in boreholes that were sampled. The presence of both faecal coliforms (potentially all *E. coli*) and faecal streptococci in the boreholes supports the notion that these boreholes were faecally polluted. This may be indicative that faecally transmitted pathogens may also be present in the same water source. The risk then arises that these pathogens may become ARBs. Furthermore, opportunistic pathogens may become more virulent and this may cause increased adverse health effects to groundwater consumers.

## CONCLUSIONS

Results from this study indicate that groundwater from the Mooi River catchment is vulnerable to physico-chemical as well as microbiological pollution. Based on microbial parameters, none of the boreholes was suitable for drinking water purposes. Three of the five boreholes were positive for faecal indicator bacteria and high numbers of HPC bacteria were detected in all. All of the HPC bacteria were resistant to varying numbers of antibiotics and so were the potential *E. coli* isolates. *Pseudomonas plecoglossicida* was identified as a HPC bacterium present in the bulk water as well as an ARB from the same borehole. This may indicate the possibility that bacteria from bulk water may become ARB. Interaction of bacteria with amoebae will result in the development of resistance to these protists. This may lead to the development of pathogenicity of non-pathogenic bacteria and increased pathogenicity of opportunistic pathogens. These results raise concern when faecal pollution is identified in water sources, as known human pathogens may also interact and develop resistance to FLA and consequently become more virulent. The interaction of the free-living amoeba and HPC bacteria should not be

underestimated and more research is required in this important field. General findings of this limited study demonstrate once again that groundwater supply should be carefully monitored for potential health risk parameters.

It is thus recommended that groundwater for human consumption is monitored and when necessary treated in the same manner as drinking water produced from surface water sources. Early detection of possible contamination can lead to faster implementation of corrective measures, preventing a disease outbreak. Amoebae can increase the pathogenicity of bacteria (non-pathogens as well as pathogens). Certain amoebae are also opportunistic human pathogens. Amoebae should therefore be considered for the standard battery of microbial tests for water quality. Clinical studies should also be performed on ARB to assess the pathogenicity of such bacteria and to measure the effectiveness of antimicrobial treatment. Such data would be important in cases of outbreaks due to such ARB.

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