INVESTIGATION OF THE LEVELS AND DIVERSITY OF HETEROTROPHIC BACTERIA IN DRINKING WATER BIOFILMS OF POTCHEFSTROOM, NORTH-WEST PROVINCE, RSA

by

Elsie Petronella Vos (B.Sc NWU-PUK)

Submitted in partial fulfilment of requirements for the degree of

MASTER OF ENVIRONMENTAL SCIENCE (M.ENV.SCI),

School of Environmental Science and Development: Microbiology
Faculty of Natural Science
North-West University: Potchefstroom Campus
Potchefstroom

Supervisor: Prof. C.C. Bezuidenhout

NOVEMBER 2007

DECLARATION

I declare that the dissertation for the degree of Master of Environmental Science (M.Env.Sc) at the North-West University: Potchefstroom Campus hereby submitted, has not been submitted by me for a degree at this or another University, that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

....

Elsie Petronella Vos **Date**

ACKNOWLEDGEMENTS

I would like to express my appreciation to the following people for their support during this study:

My supervisor, Prof. Carlos Bezuidenhout, for his guidance, motivation, positive and friendly attitude, advice, supervision and input during my research, and compilation of this dissertation.

My parents, for making me believe in myself and encouraging me. Thanks for your unending love. My dad thanks for your guidance, and for being there and standing strong for us especially the past year when things were very difficult during the illness of my mother. Thanks for your faith in me.

Darius, just for being there for me, thank you. You are my best friend.

My heavenly Father. Without Him all things in life are impossible. Thanks for the strength.

The National Research Foundation, South Africa, for financial support during this study

ABSTRACT

Heterotrophic bacteria in drinking water and implications thereof is a controversial subject. Though this group of organisms is generally considered not harmful, the occurrence and harmlessness of this group of bacteria in drinking water was recently reconsidered by several leading scientists. High levels of these bacteria in drinking water could be an indication of either inefficient purification, regrowth or that contamination from external sources occurred. The potential implications and effects of such bacteria in biofilms within distribution systems remain undetermined. The aim of the present study was firstly to determine the diversity and levels of antibiotic resistant bacteria in drinking water biofilms in Potchefstroom and secondly to determine whether these isolates are potential pathogens. There were two main objectives. The first objective was to determine the diversity, levels and characteristics of heterotrophic bacteria in biofilms from home water filtering systems. Here, 144 and 381 bacterial colonies were respectively isolated from 2 home water filtering devices and a biofilm device, using standard microbiological culturing and sub-culturing procedures. Using biochemical methods the following Gram-negative species were identified: *Enterobacter* spp, *Citrobacter* spp, *Aeromonas hydrophilia*, *Providentia* spp. No Gram-positive species were identified. Among the isolates from the home water filtering systems 88.2% were resistant to tetracycline, 6.6% to vancomycin (Gram-positive only), 56.0% to erythromycin, 69.0% to ampicillin, 34.0% to neomycin, 9.0% to chloramphenicol, and 4.1% to gentamycin. Isolates were also tested for haemolytic activity (potentially pathogenic features) and a considerable number showed α-or β-hemolysis. Scanning electron microscopy results indicated that relatively large particulate matter was present in the water and that typical multi-species biofilms formed within the water filtering devices. The second objective of the study was to determine the diversity, levels and characteristics of heterotrophic bacteria in biofilms

isolated from an *in situ* biofilm device. The 381 isolates obtained from the biofilm device included *Pseudomonas* spp, *Enterobacter* spp. and *Exiguobacterium* spp. as well as some unidentified Gram-positive species. Among these isolates, 34.4% were resistant to ampicillin, 5.6% to penicillin (Gram-positive only), 36.7% to tetracycline and 5.6% to vancomycin (Gram-postive only). Most of the isolates obtained over the 12 week period showed α- or β- hemolysis. The majority of isolates from the biofilm device were tolerant to high levels of copper (minimum inhibitory concentrations > 5mM). The results also demonstrated that some of the isolates were simultaneously tolerant to high concentrations of heavy metals and resistant to multiple antibiotics. It was not determined whether a significant correlation existed between these two parameters. The results presented in this study may not indicate risk to consumers of Potchefstroom water. However, the presence of some of the species in the drinking water biofilms is cause for concern and this aspect should be further investigated. Other aspects that require attention include the source of the bacteria, the population dynamics thereof in the water distribution system and the dynamics of genetic elements that could be responsible for the heavy metal tolerance and antibiotic resistance.

OPSOMMING

Die teenwoordigheid van heterotrofiese bakterieë in drink water en die implikasies daarvan, is 'n kontraversiële onderwerp. Hoewel hierdie groep bakterieë oor die algemeen nie as skadelik beskou word nie, het verskeie vooraanstaande wetenskaplikes onlangs die voorkoms en onskadelikheid van hierdie groep bakterieë in drink water in heroorweging geneem. Hoë vlakke heterotrofiese bakterieë in drink water mag 'n aanduiding wees van onvoldoende watersuiwering, hergroei of dat moontlike kontaminasie vanuit 'n eksterne bron plaasgevind het. Die potentiële implikasies en uitwerking van sulke bakterieë in biofilms binne die verspreiding sisteme bly steeds onbekend. Die doel van hierdie studie was eerstens om die diversiteit en vlakke van antibiotikum-weerstandbiedende bakterieë in drink water biofilms in Potchefstroom te bepaal, en tweedens om vas te stel of hierdie bakterieë potentiële patogene is. Daar was twee doelwitte. Die eerste doelwit was om die diversiteit, vlakke en eienskappe van heterotrofiese bakterieë in biofilms van huishoudelike water filtrering sisteme te bepaal. 'n Totaal van 144 en 381 bakteriële kolonies is respektiewelik uit twee huishoudelike water filtrerings sisteme en 'n biofilm apparaat geïsoleer deur standaard mikrobiologiese kwekings prosedures toe te pas. Deur gebruik te maak van biochemiese toetse is die volgende Gram-negatiewe spesies geidentifiseerd: *Enterobacter* spp, *Citrobacter* spp, *Aeromonas hydrophilia* en *Providentia* spp. Geen Gram-positiewe spesies is geïdentifiseer nie. Onder die isolate uit die huishoudelike water filtrering sisteme was 88.2% weerstandbiedend teen tetrasiklien, 6.6% teen vankomisien (slegs Gram-positiewe isolate), 56.0% teen eritromisien, 69.0% teen ampisillien, 34.0% teen neomisien, 9.0% teen chloramfenikol en 4.1% teen gentamisien. Isolate is ook getoets vir hemolitiese aktiwiteit (aanduidend van potentiële patogenisiteit) en 'n aansienlike getal α-and β-hemoliese vertoon. Resultate van die skanderings-elektron mikroskopie het aangedui dat relatiewe groot partikulêre materie in die drink water was en dat tipiese

meervoudige spesie biofilms binne die water filtrerings sisteme gevorm het. Die tweede doelwit van die studie was om die diversiteit, vlakke en eienskappe van heterotrofiese bakterieë in biofilms wat vanuit 'n *in situ* biofilm apparaat geïsoleer is, te bepaal. Die 381 isolate wat uit die biofilm apparaat verkry is, sluit in: *Pseudomonas* spp, *Enterobacter* spp en *Exiguobacteium* spp asook as ongeidentifiseerde Gram-positiewe spesies. Van hierdie isolate was 34.4% weerstandbiedend teen ampisillien, 5.6% teen penisillien (slegs Gram-positiewe isolate), 36.7% teen tetrasiklien en 5.6% teen vankomisien (slegs Gram-positiewe isolate). Meeste van die isolate wat oor die 12 weke periode verkry is, het α- en β-hemolitiese eienskappe getoon. Die meerderheid van die isolate uit die biofilm apparaat was tolerant teen hoë koper vlakke. (Die minimum inhiberende konsentrasies > 5mM). Die resultate het ook getoon dat sommige van die isolate gelyktydig tolerant was teen hoë konsentrasies swaar metale sowel as weerstandig teen meervoudige antibiotikums. Of die verwantskap tussen hierdie twee veranderlikes betekenisvol was, is nie bepaal nie. Die resultate van hierdie studie dui dalk nie op ñ risiko vir die gebruikers van Potchefstroom se water nie. Alhoewel, die teenwoordigheid van sommige van die spesies in die drink water biofilms, is rede tot kommer, en hierdie aspek behoort verder ondersoek te word. Ander apekte wat aandag verg sluit in die bron van hierdie bakterieë in drink water, die populasie dinamika daarvan in die water verspreiding sisteem, en die dinamika van genetiese elemente wat moontlik verantwoordelik kan wees vir swaar metaal toleransie en antibiotikum weerstandbiedendheid.

TABLE OF CONTENT

DECLARATION ii

ACKNOWLEDGEMENTS iii

ABSTRACT iv

OPSOMMING vi

TABLE OF CONTENT viii

LIST OF FIGURES xiii

LIST OF TABLES xvi

CHAPTER 1 1

INTRODUCTION 1

1.1 GENERAL INTRODUCTION AND PROBLEM STATEMENT 1

1.2 RESEARCH AIM AND OBJECTIVES 5

CHAPTER 2 6

LITERATURE REVIEW 6

2.1 INTRODUCTION 6

2.2. HETEROTROPHIC BACTERIA AND IMMUNOCOMPROMISED INDIVIDUALS 7

2.3 CHARACTERISTICS OF BIOFILMS IN WATER DISTRIBUTION SYSTEMS 11

2.4 ANTIMICROBIAL RESISTANCE of MICROBES IN BIOFILMS .. 15

2.5 EFFECTS OF CHLORINE ON REGROWTH OF MICROORGANISMS IN WATER DISTRIBUTION SYSTEMS 16

2.6 ANTIBIOTIC RESISTANCE AND HEAVY METAL TOLERANCE OF HETEROTROPHIC BACTERIA FROM THE NORTH-WEST PROVINCE 17

2.7 METHODS AND PRINCIPLES OF EXPERIMENTAL WORK 19

2.7.1 Generation and collection of biofilm sample 19
2.7.2 Isolation and cultivation of heterotrophic bacteria 20
2.7.3 Analytical profile index 20 E (API 20E) and triple sugar iron (TSI) agar 21
2.7.4 Determination of antimicrobial susceptibility 22
2.7.5 Hemolysis 22
2.7.6 Heavy metal tolerance determination based on the Minimum Inhibitory Concentration (MIC) 22
2.7.7 Scanning electron microscopy (SEM) 23
2.8 SUMMARY 24
CHAPTER 3 26
DIVERSITY AND CHARACTERISTICS OF HETEROTROPHIC BACTERIA FROM HOME WATER FILTERING SYSTEMS 26
3.1 INTRODUCTION 26
3.1.1 Drinking water purification 27
3.1.2 Microorganisms present in drinking water systems 29
3.1.3 Increased interest in the use of home water filter systems 29
3.1.4 Home water filters 30
a) Activated carbon filters 32
b) Reverse osmosis 33
3.1.5 Aim and Objectives 35
3.2 MATERIALS AND METHODS 36
3.2.1 Sampling: home water filtering system 36
3.2.2 Preliminary identification 36
3.2.3 TSI agar slants 37
3.2.4 Analytical Profile Index (API 20 E) 37

3.2.5 Motility test 38
3.2.6 Hemolysis on blood agar (5%) 38
3.2.7 Kirby Bauer disk diffusion method 38
3.2.8 Scanning electron microscopy (SEM) 40
3.3 RESULTS 40
3.3.1 Isolation and preliminary identification of isolates 40
3.3.2 Identification by TSI and API 20E 43
3.3.3 Antibiotic resistance patterns 45
3.3.4 Scanning electron microscopy (SEM) 49
3.3.5 Summary of results 58
3.4 DISCUSSION 59
3.4.1 Isolation of heterotrophic bacteria from activated carbon and reverse osmosis home water filtering units 59
3.4.2 Presence of Gram-negative bacilli 60
a) Enterobacter spp. 60
b) Citrobacter spp 61
c) Providencia alcalifaciens 62
d) Aeromonas hydrophilia 62
3.4.3 Unidentified Gram-positive Bacilli 63
3.4.4 Levels and diversity 63
3.4.5 Antibiotic resistance and potential pathogenic characteristics of isolates 65
3.5 SUMMARY AND CONCLUSION 66
CHAPTER 4 68
DIVERSITY AND CHARACTERISTICS OF HETEROTROPHIC BACTERIA IN A WATER DISTRIBUTION SYSTEM 68

4.1. INTRODUCTION 68

4.1.1 Available energy sources in the distribution system 69

4.1.2 Disinfection efficiency 69

4.1.3 Corrosion control 70

4.1.4 Construction materials 71

4.1.5 Operational characteristics 72

4.1.6 Microorganisms entering the distribution system 73

4.1.7 Cleaning and maintenance water mains 73

4.1.8 Studies of biofilms *in situ* in water distribution systems 74

4.1.9 Aim and objectives 74

4.2. MATERIALS AND METHODS 76

4.2.1. Biofilm sampling 76

4.2.2 Determination of physico-chemical parameters of drinking water 77

4.2.3 Preliminary identification and characterization of isolates 77

4.2.4 Molecular identification of isolates 77

(i) DNA extraction 77

(ii) PCR (Polymerase Chain Reaction) 78

(iii) Electrophoresis 78

4.2.5 Minimum inhibitory concentration (MIC) for Copper 79

4.2.6 Statistical analysis 79

4.3 RESULTS 80

4.3.1 Physico-chemical parameters of the drinking water of the J.S van der Merwe building 80

4.3.2 Isolation and preliminary identification of isolates 81

4.3.3 Identification by TSI and API 20E 82

4.3.4. Antibiotic resistance patterns 83

4.3.5 Minimum inhibitory concentrations (MIC) of isolates. 84
4.3.6 Scanning electron microscopy (SEM) 85
4.3.7 Summary of results 91
4.4 DISCUSSION 93
4.4.1 Physico-chemical characteristics of the drinking water 93
4.4.2 Levels and diversity heterotrophic bacteria from metal discs 94
4.4.3 Identification, antibiotic resistance and potential pathogenic characteristics of isolates 97
a) Pseudomonas spp 97
b) Exiguobacterium spp 98
c) Enterobacter spp 99
4.4.6 Minimum inhibitory concentrations (MIC) of copper for isolates ... 100
4.5 SUMMARY AND CONCLUSION 102
CHAPTER 5 104
FINAL CONCLUSION AND PROSPECTS 104
5.1 Diversity, levels and characteristics of HPC bacteria in biofilms from home water filtering systems 104
5.2 Diversity, levels and characteristics of HPC bacteria in biofilms isolated from an *in situ* biofilm device 105
PROSPECTS FOR FURTHER STUDY 106
REFERENCES 107
APPENDIX A 131

LIST OF FIGURES

Figure 2.1- Scanning electron micrograph of a three-day old bacterial biofilm forming on the feedwater surface of a cellulose acetate RO membrane used to treat municipal wastewater at Water Factory 21, Fountain Valley, California, USA. (www.trusseltech.com/images/RO_Membrane.jpg)..... 13

Figure 2.2- A diagrammatic explanation of the several phases followed after adhesion to the formation of a complete biofilm (1, initial adsorption to surface; 2, cell-cell growth population growth / reproduction; 3, production of an extra-cellular polysaccharide substances / irreversible adhesion; 4, trapped biofilm bacteria form a community that controls the structural complexity of the biofilm; 5, cells are dislodged and dispersed to new areas where they can adhere (biology.binghamton.edu/davies/research.htm)..... 15

Figure 3.1- A photo of the activated carbon filter (http://www.amazon.com/WaterPik-Instapure-faucet-mount filter/dp/B000LNO6BA) 32

Figure 3.2- A schematically representation of Reverse Osmosis (www.waterforlife.ca/images/Radiogram.jpg) 34

Figure 3.3- Percentage isolates from RO home water filtering system that were resistant, susceptible and intermediate resistant to all antibiotics tested. (Gentamycin=GM; Kanamycin= K; Ampicillin=AP; Neomycin=NE; Streptomycin=S; Chloramphenicol=C; Ciproflaxin= CIP; Tetracycline=T; Erythromycin=E; Vancomycin= VA) 46

Figure 3.4- Percentage isolates from activated carbon home water filtering system that were resistant, susceptible and intermediate resistant to all antibiotics tested. (Gentamycin=GM; Kanamycin= K; Ampicillin=AP; Neomycin=NE; Streptomycin=S; Chloramphenicol=C; Ciproflaxin= CIP; Tetracycline=T; Erythromycin=E; Vancomycin= VA)..... 47

Figure 3.5- Electron microscopy of the first phase of the home water filtering system (100X magnification)..... 50

Figure 3.6- Enlargement of the filtered substances and microorganisms on phase 1 of the home water filtering system (2000X magnification)..... 51

Figure 3.7- Enlargement of the material from the second phase of the home water filtering system (100X magnification)..... 52

Figure 3.8- Enlargement of the biological activity on the 2nd phase of the home water filtering system (5000X magnification)..... 53

Figure 3.9- Enlargement of the biological activity on the 2nd phase of the home water filtering system (20 000X magnification)..... 54

Figure 3.10- Enlargement of the material of the final phase of the home water filtering system (50X magnification)..... 55

Figure 3.11- Enlargement of the biological growth on the last phase of the home water filtering system (12 000X magnification)..... 56

Figure 3.12- Enlargement of the biological growth on the last phase of the home water filtering system (24 000X magnification)..... 57

Figure 4.1- Schematic representation of the design of the biofilm device. 76

Figure 4.2- Percentages of isolates from the red copper discs, after 6 week exposure to drinking water that was resistant, susceptible and intermediate resistant to all tested antibiotics..... 84

Figure 4.3- A SEM micrograph of a galvanized disc from the biofilm apparatus (12 000X magnification)..... 86

Figure 4.4- A SEM micrograph of a red copper disc from the biofilm device (12 000X magnification)..... 87

Figure 4.5- A SEM micrograph of a yellow copper disc from the biofilm device (12 000X magnification)..... 88

Figure 4.6- SEM micrograph of a red copper disc from the biofilm device after 6 week of exposure to drinking water (12 000X magnification). 89

Figure 4.7- A SEM micrograph of a red copper disc from the biofilm device after a 6 week period (20 000X magnification). 90

Figure 4.8- A SEM micrograph of a red copper disc after an 8 week period of exposure to drinking water (20 000X magnification). 91

LIST OF TABLES

Table 2.1- Various genera of bacteria associated with drinking water (compiled from LeChavallier *et al.*, 1980; Herson and Victoreen, 1980; Briganti and Wacker, 1995; Obi *et al.*, 2007). 9

Table 3.2- Antibiotics used in this study. The concentration [μg] as indicated on the discs and the inhibition zone classification is also provided using NCCLS (1999) data. 39

Table 3.3- Summarized primary characteristics of the HPC isolates from a biofilm that formed on a reverse osmosis home water filtering system. The levels of the various morphotypes are also provided. 42

Table 3.4- Summarized primary characteristics of the isolates from a biofilm that formed on a home activated carbon filter. The levels of the various morphotypes are also provided. 43

Table 3.5- Identification of the isolates from the reverse osmosis and activated carbon home water filtering systems. Haemolytic and antibiotic resistance for the various species/morphotypes data are also indicated. 44

Table 4.1- Physical characteristics of the Potchefstroom drinking water as measured in the JS van der Merwe building. 80

Table 4.2- Summarized colony morphology and characteristics of the isolates from a biofilm that formed in a biofilm apparatus. The levels of the various morphotypes are also provided. 81

Table 4.3- Identification of the isolates from the biofilm apparatus unit. Haemolysis, antibiotic resistance patterns and the MICs of the isolates are also indicated. 83

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION AND PROBLEM STATEMENT

Intake of good quality water is essential in our every day life. It is recommended that our drinking water intake should be at least 8 glasses per day (http://www.dwlz.com/WWinfo/water.html). Although recent debates (Valtin, 2007) argue against this rule, humans still require a considerable amount of good quality water per day to keep healthy.

The quality of the water is influenced by several factors including physical, chemical and microbiological ones. Chemical and physical factors of the water may directly influence the microbiology thereof and the importance of the latter quality have, for a long time been recognised (Geldreich, 1989). Already 1000's of years BC the treatment of water by sunlight and charcoal was considered to produce good quality drinking water. However, modern disinfection strategies date back to the early 1900's (US EPA, 2000). Only as recently as two to three decades ago has drinking water microbiology emerged, from an extended period of contentment with conventional disinfection processes, as an important factor that should be dealt with in a serious manner (Geldreich, 1989). This was because of knowledge regarding emergence of "new pathogenic and opportunistic pathogenic bacteria" and the potential association with water sources and biofilms in drinking water systems.

Heterotrophic plate count (HPC) bacteria are regarded as harmless bacteria that exist in all sources of water. These are bacterial species that require organic material for growth (WHO, 2003). They could be present in drinking water at relatively high densities. Normally 100 to 500 colony forming units (cfu) per millilitre of sample are allowable.

However, up to 10 000 cfu/ml of sample in 1.0% of samples are acceptable (Allen *et al.*, 1980; DWAF, 1996; WHO, 2002). The levels of HPC are used as measure of the effectiveness of disinfection process (Allen *et al.*, 2004) but could also be as a result of re-growth of bacteria that are not killed during the disinfection process. Such bacteria may enter the viable-but-non-culturable (VBNC) metabolic state (LeClerc, 2003). The harmlessness of HPC had been questioned because they have been associated with illnesses and infections in and could be a threat to human health (LeChevallier *et al.*, 1980; Rusin *et al.,* 1997; Edberg and Allen, 2004). Several older and relatively recent studies demonstrated potential health risks associated with this group of bacteria (LeChevallier *et al.*, 1980; Schwartz *et al.*, 2003; Kalmbach *et al.*, 1997; Muyima, & Ngcakani, 1998; Pavlov *et al.*, 2003). The 2002 World Health Organisation (WHO) report on HPC in drinking water concluded that not enough evidence is available to associate this group of bacteria with human health risk (WHO, 2002). However, the report recognised that the development and application of molecular techniques may provide additional public health information. An aspect that was not addressed in this report is the possible link between HPC in drinking water and the transfer of antibiotic resistance genes from the source water. Using molecular methods, Schwartz *et al.* (2003) showed that genes responsible for antimicrobial resistance in indicator bacteria found in source waters could be isolated from HPC bacteria in drinking water suggesting a horizontal transfer of these antibiotic resistance genes from the indicator bacteria. This type of gene transfer is common in nature (Toussaint and Merlin, 2002; Licht *et al.*, 2003).

Antibiotic resistance is a serious and increasing problem that is mainly caused by the misuse and overuse of antimicrobial agents and environmental pollutants (Nue, 1992; Mah and Memish, 2000). Thus antimicrobial substances such as antiseptics, detergents,

pesticides, heavy metals etc. may also select for antibiotic resistant bacteria (McArthur and Tuckfield, 2000; Badar *et al.*, 2001; Silver *et al.*, 2001). Elevated levels of these substances in the environment select for organisms that are able to tolerate them i.e. resistant organisms (Nies, 1999). Where source water is exposed to pollution by these substances the chances of finding high levels of antibiotic resistant bacteria are also increased (McArthur and Tuckfield, 2000).

What is of concern are the levels of antibiotic resistance and pathogenic potential of HPC bacteria isolated from drinking water as well as the transfer of potentially harmful genes from bacteria present in source water. This is an aspect that the WHO report on HPC of 2002 (WHO, 2002) did not consider. In a South African study, Pavlov *et al.* (2003) demonstrated that over 50.0 % of HPC isolated from drinking water had pathogenic features. Of these, over 50.0 % were also resistant to the beta-lactam antibiotics they were tested against.

Heterotrophic plate count bacteria may form biofilms in drinking water distribution systems that may be resistant to antibiotics that are generally used for infection management in humans (Schwartz *et al.* 2003). The closeness of the bacteria in these biofilm communities and their capability of exchanging DNA in forms of plasmids and transposons, notorious for carrying antibiotic resistance, virulence, pathogenicity, metal tolerance and other genes (Hogan and Kolter, 2002), can cause resistance to pass very rapidly. Bacteria that contain such genetic materials may also be more prone to survive unfavourable conditions, such as those created by disinfection, more readily (Mah and O'Toole, 2001).

It is thus important to gain sufficient knowledge about the HPC bacteria in drinking water and to determine the antibiotic resistance patterns, metal tolerance levels and pathogenicity potential of these isolates. The experts agree (WHO, 2002; 2003) that such surveillance studies are crucial to expanding our understanding of drinking water distribution system ecology.

The Mooi River catchment area of the North-West Province, which includes the towns of Potchefstroom and Carltonville is an area of economic prosperity and population growth, supporting gold mining and agricultural industries (NWP-SOTE, 2002) These cities provide "safe" treated drinking water to their inhabitants. However, the source water within this catchment area is exposed to pollution from these mining, agricultural and industrial activities (Erdmann, 1999; Venter, 2001). The municipality of Potchefstroom is concerned about the high levels of heavy metals in the source and possibly drinking water of the city. They took legal action against some mines that were suspected of polluting the water source of the town with heavy metals (Van Aardt, 2007).

Several researchers have demonstrated the relationship that exists between the occurrence of metal tolerant bacteria and antibiotic resistant bacteria in aquatic environments (Sabry *et al.*, 1997; Hernandez *et al.*, 1998; Miranda and Castillo, 1998; Nies, 1999; McArthur and Tuckfield, 2000; Badar *et al.*, 2001; Silver *et al.*, 2001). The levels of antibiotic resistant HPC (that are simultaneously metal tolerant and pathogenic) in source and drinking water for particularly biofilms within the distribution system Potchefstroom is undetermined.

1.2 RESEARCH AIM AND OBJECTIVES

The main aim of this study was to determine the diversity and levels of heterotrophic bacteria in drinking water biofilms in Potchefstroom and also determine whether these isolates are potential pathogens.

Objectives were:

1. To determine the diversity, levels and characteristics of HPC bacteria in biofilms from home water filtering systems.
2. To determine the diversity, levels and characteristics of HPC bacteria in biofilms isolated from an *in situ* biofilm device.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Heterotrophic bacterial species are autochthonous in terrestrial and aquatic environments (Allen *et al.*, 2004). They have the ability to utilize simple organic compounds for growth and may occur in relatively high densities in drinking water (LeChevallier *et al.*, 1980). This has raised special concerns about their potential health hazards and several studies dealt with this aspect (LeChevallier *et al.*, 1980; Falkinheim III *et al.*, 2001; Haung *et al.*, 2002; WHO, 2002; Pavlov *et al.*, 2003; Allen *et al.*, 2004).

Levels of heterotrophic plate count bacteria can be used to assess the general microbial quality of drinking water (Reasoner, 1990; WHO, 2003). High heterotrophic plate counts in treated water may indicate inadequate water treatment, post treatment contamination or bacterial re-growth in the distribution system (Reasoner, 1990; Allen *et al.*, 2004) but do not necessarily mean that the water poses a risk to human health (Edberg *et al.*, 1996; Allen *et al.*, 2004). However, a study by Pavlov *et al.* (2003) on potentially pathogenic features of heterotrophic plate count bacteria isolated from treated and untreated drinking water presented an alarming picture. Of 339 HPC isolated 188 (55.5%) showed α- or β hemolysis activities. The latter feature is associated with pathogenicity. Based on this and previous related studies Pavlov *et al.* (2003) suggested that a need existed for more detailed studies on the potential health risks of heterotrophic bacteria in treated drinking water supplies. These authors also suggested that there may be a need for re-evaluation of the current microbial water quality guidelines in South-Africa.

2.2. HETEROTROPHIC BACTERIA AND IMMUNOCOMPROMISED INDIVIDUALS

Some studies have indicated a relationship between drinking water, diarrhoea and HIV (Eisenberg *et al.*, 2002; Obi *et al.*, 2007). Results from these studies suggested that drinking water may act as a potential conduit for diarrhoeal disease agents. However, Eisenberg *et al.* (2002) also demonstrated that other factors may also contribute to diarrhoea in HIV patients. Obi *et al.* (2007) demonstrated the diversity levels of various heterotrophic bacteria in diarrhoeal HIV positive and HIV negative individuals. These authors were concerned about the prevalence of one of these bacterial species (*Aeromonas* spp.) that could be waterborne.

According to worldwide HIV and aids epidemic statistics, 4.1 million people became infected with the human immunodeficiency virus during 2005 (http://www.journaids.org/statistics.php#globalstats). Based on statistical data and projection from the World Health Organization (WHO, 2007), approximately 3 million annual HIV/AIDS deaths will occur by 2010. However, this may increase to approximately 4 million by 2015 and to close to 7 million by 2030. These statistics do not include other HIV/AIDS related deaths. Based on the South-African National HIV survey of 2005, it was estimated that 10.8% (approximately 4.6 million) South-Africans over the age of 2 years were living with HIV (Shisana *et al.*, 2005). The 2005 causes of death report by Statistics South Africa (Stats. S.A, 2005) indicated that the leading cause of death in this country was by infectious and parasitic diseases (23.8 %). Diseases of the blood and immune mechanisms contributed 3.3 % and infectious intestinal diseases contributed 4.8 % of all deaths. The latter diseases are also the fourth highest cause of death (4.6 %) in the North-West Province, South Africa. From these statistics it is clear that infectious diseases play a crucial role in mortality in South

Africa and the situation may be similar in the North-West Province. Although no direct evidence is available that link drinking water and HIV/AIDS mortality, studies on various aspects such as diarrhoea suggest a potential pathway (Eisenberg *et al.*, 2002; Obi *et al.*, 2007). Some of the heterotrophic bacterial species listed in Table 2.1 have been associated with diarrhoea in HIV positive and negative individuals in the Limpopo Province of South Africa (Obi *et al.*, 2007).

These statistics presented here paint a bleak picture. This is especially worrisome when one considers that a large proportion of rural communities in the North-West Province still rely on untreated surface- and groundwater as the only source of drinking water (NWP-SOTE, 2002). Among these rural and urban communities the immuno-compromised component is increasing, thus facing the risk of becoming infected with infectious opportunistic pathogens through drinking water. It is thus essential that drinking water free from opportunistic pathogens be available to the South African public, in general, but for these immuno-compromised individuals in particular. Effective water treatment techniques should ensure that these immuno-compromised individuals are protected from potentially infective agents.

Table 2.1 provides a list of bacteria that had been associated with drinking water systems (LeChevallier *et al.*, 1980; Herson and Victoreen, 1980; Briganti and Wacker, 1995; Obi *et al.*, 2007). Some of the species in this list are known opportunistic pathogens and they have the ability to form biofilms. The potential health impacts of selected species are discussed below.

Pseudomonas spp. is an environmental bacterium and some of the species, particularly *P. aeruginosa* are known opportunistic pathogens. The latter species has for

considerable time been associated with infections in immuno-compromised individuals (Prescott *et al.*, 2005). They are also well known for their invasion of burned areas on the skin and are responsible for urinary tract infections and high mortality levels in patients suffering with cystic fibrosis (Prescott *et al.*, 2005). In a study by Messi *et al.* (2005), *Pseudomonas* spp. was the most predominant isolate among antibiotic resistant heterotrophic bacteria from mineral water. They found that this organism was, among all the various species, the one species that was resistant to the largest number of antibiotics.

Table 2.1- Various genera of bacteria associated with drinking water (compiled from LeChevallier *et al.*, 1980; Herson and Victoreen, 1980; Briganti and Wacker, 1995; Obi *et al.*, 2007).

Acinetobacter spp.	*Enterobacter cloacae*
Actinomycetes spp.	*Escherichia coli*
Aeromonas spp.	*Klebsiella pneumonia*
Aeromonas hydrophila	*Mycobacterium* spp.
Alcaligenes spp.	*Pseudomonas* spp.
Arthrobacter spp.	*Pseudomona cepacia*
Bacillus spp.	*Pseudomona fluorescens*
Camphylobacter spp.	*Pseudomona maltophilia*
Citrobacter freundi	*Salmonella* spp.
Corynebacterium	*Serratia liquefaciens*
Enterobacter agglomerans	*Shigella* spp.

Aeromonas spp. also occurs naturally in water and is also an opportunistic pathogen. They have been isolated from drinking water worldwide and are capable of surviving and growing in drinking water systems (WHO, 2003). *Aeromonas* spp. are normally responsible for illnesses such as gastroenteritis and enteritis but are of low virulence. Although it requires high levels of infectious *Aeromonas* spp. agents to cause disease, the presence of small quantities of this bacterium in drinking water should not be overlooked (Allen *et al.*, 2004). Obi *et al.* (2007) argued that the association of *Aeromonas* spp. and diarrhoea in HIV patients may not be by chance and should be further investigated.

Enterobacter cloacaea and *Enterobacter agglomerans* have been associated with diseases in several studies which highlighted the significance of their presence in drinking water (Van Nierop *et al.*, 1998; De Man *et al.*, 2001). *Enterobacter* spp. are commonly known for the colonization of especially hospitalized patients. They are thus known as opportunistic pathogens and more likely cause disease in persons with compromised immune system than in healthy individuals.

Mycobacterium spp. are slow growing organisms occurring in the environment and are capable of causing disease in humans e.g. *Mycobacterium tuberculosis*. Various *Mycobacterium* spp. have been isolated from drinking water biofilms on a regular basis (Schwartz *et al.*, 1998). *Mycobacterium avium* can cause disease after ingestion of contaminated water or inhalation of water vapour containing the bacterium. Although it is possible for this bacterium to colonize the pharynx without causing any disease, HIV patients are very susceptible to disease caused by this bacterium (WHO, 2003).

Klebsiella spp. and *Citrobacter* spp. are also heterotrophic bacteria that have been associated with infections in immuno-compromised individuals (Fisman and Kaye, 2000; Underwood, 2004). A waterborne outbreak of *Klebsiella* spp. in a Durban hospital has lead to deaths amongst neonates (Pillay and Horner, 2005)

Thus in the context of, and in relation to the potential risk of un- or under-treated as well as treated drinking water consumption, four HIV disease stages were proposed (Engelhart *et al.*, 2001). In the first stage the HIV patient should just avoid drinking water from untreated sources. The individuals in the fourth stage (full blown AIDS) should only drink sterile fluids. It is thus important to deliver to all communities, water that is of an extremely good quality.

2.3 CHARACTERISTICS OF BIOFILMS IN WATER DISTRIBUTION SYSTEMS

Any available organic (natural organic matter; NOM) and inorganic nutrients in treated drinking water are utilized by bacteria that survived the water treatment process. This is referred to as regrowth/aftergrowth in the distribution system, which means that bacterial counts could start increasing in water some time after leaving the treatment plant (Momba and Binda, 2002). The removal of NOM in order to decrease the regrowth potential of bacteria in the water distribution system is thus a very important goal for all water treatment plants (Volk and LeChevallier, 1999; DWAF, 2002). Bacteria that regrow normally form a biofilm when they adhere/attach to the surface of the distribution pipes. The formation of three-dimensional structures within biofilms is a complex process (Figure 2.1). This process involves several molecular events that initiate mechanisms for adhesion, aggregation and community expansion (Figure 2.1; O'Toole *et al.*, 2000; Schembri *et al.*, 2002; 2003).

Biofilms can be expected to form any place where a surface comes into contact with water (Mah and O'Toole, 2001). When biofilms form in our water distribution system it can cause several negative aspects (Block, 1995). Cells in biofilms normally express characteristics different from planktonic cells. One of these characteristic is that these sessile cells (biofilm based cells) are more resistant (1000 fold) to antimicrobial agents than planktonic cells. This means that 10^3 times higher doses of antimicrobials are needed to kill biofilm organisms compared with planktonic ones (Mah and O'Toole, 2001; Schembri *et al.*, 2003).

Formation of extra-cellular polymeric substances (EPS) is another unique characteristic of biofilm communities. It can be associated with the formation of three dimensional structures and probably enhances the resistance against antibacterial agents (Schembri *et al.*, 2003). Such EPS also allow cells in a biofilm to form highly organized and structured communities. The EPS may account for 50% to 90% of the total organic carbon of biofilms (Flemming *et al.*, 2000). Furthermore, biofilms may contain single or multiple bacterial species (Allen *et al.*, 1980; Ridgeway and Olsen, 1981; Mah and O'Toole, 2001). A SEM micrograph is given in Figure 2.1 of a three day old biofilm from a wastewater treatment system. This micrograph represents a developing multiple species biofilm.

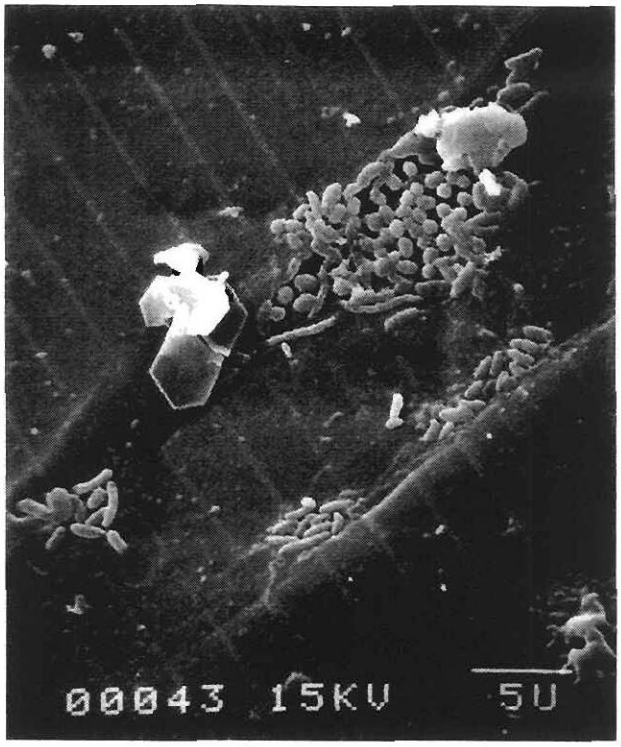


Figure 2.1- Scanning electron micrograph of a three-day old bacterial biofilm forming on the feedwater surface of a cellulose acetate RO membrane used to treat municipal wastewater at Water Factory 21, Fountain Valley, California, USA. (www.trusseltech.com/images/RO_Membrane.jpg)

The first step of bacterial colonization on a surface is adhesion or attachment (Figure 2.2). This is mostly due filamentous and primarily proteinaceous activities (Jones and Isaacson, 1983). Bacterial adhesions and the formation of biofilms are affected by different parameters such as the surface properties, charge, hydrophobicity and hydrodynamics (Jones and Isaacson, 1983; Klemm and Schembri, 2000).

After adhesion a more complex micro-colony structure is formed (Figure 2.2). Such proliferation of bacteria in the distribution system contributes to alterations in the physico-chemical and biological characteristics of water (Abouzaid *et al.*, 1996). A

series of molecular events involving multiple factors, which will be different among different bacterial species, are responsible for the development of surface attached bacterial micro-colonies to highly organized community structures (Figure 2.2). The solid surface may have a few properties that are important in the attachment process, for example, the extent of microbial colonization appears to increase as the surface roughness increases (Characklis *et al.*, 1990).

Cells in a biofilm have the ability to co-exist in a co-operative way, which is partly explained by the fact that bacteria may identify potential growth surface by sensing nearby bacteria. Cell-to-cell signalling mechanisms plays a very important role in such sensing and can also promote bacterial attachment to a solid surface (Espinosa-Urgel and Ramos, 2003; Harsley, 2003). Other characteristics that play a very important role in such a co-operative existence of cells include chemotaxis, motility, and co-regulation of metabolic interactions. These characteristics also contribute to the fact that large micro-colonies develops over a short period of time (Schembri *et al.*, 2003).

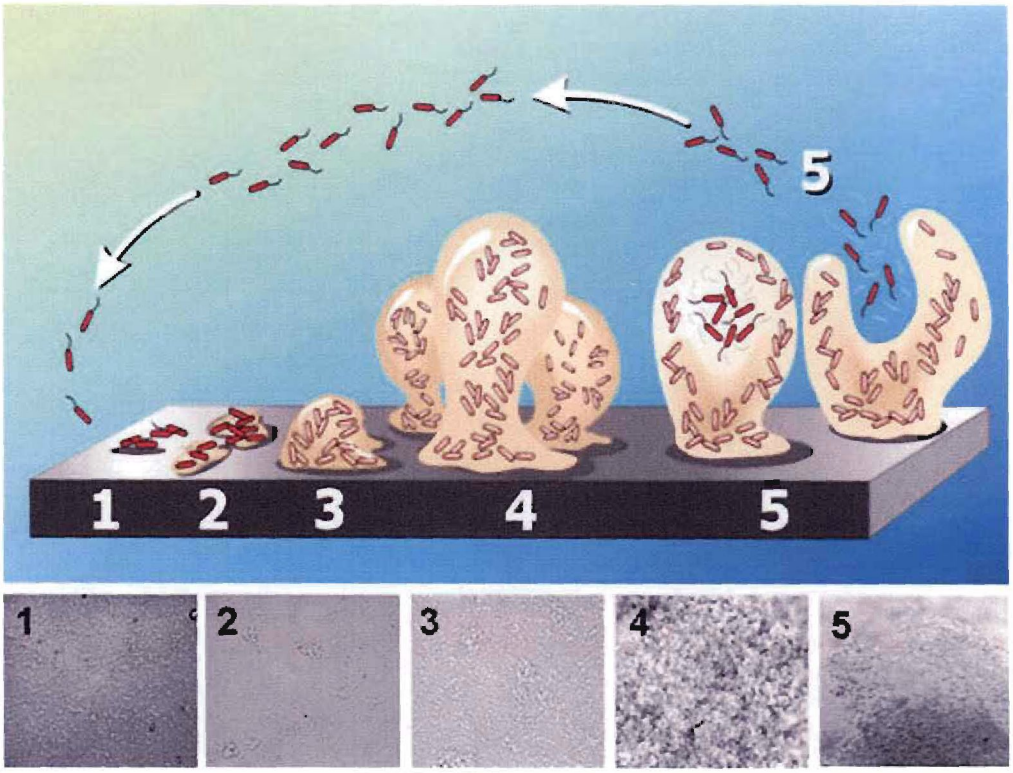


Figure 2.2- A diagrammatic explanation of the several phases followed after adhesion to the formation of a complete biofilm (1, initial adsorption to surface; 2, cell-cell growth population growth / reproduction; 3, production of an extra-cellular polysaccharide substances / irreversible adhesion; 4, trapped biofilm bacteria form a community that controls the structural complexity of the biofilm; 5, cells are dislodged and dispersed to new areas where they can adhere (biology.binghamton.edu/davies/research.htm).

2.4 ANTIMICROBIAL RESISTANCE OF MICROBES IN BIOFILMS

Best known mechanisms of antibiotic resistance, such as efflux pumps, target mutations and the production of modifying enzymes do not seem to be responsible for the protection of bacteria in a biofilm (Walsh, 2000). Mechanisms such as stress responses, penetration potential, and modification of the antimicrobial may be responsible for resistance to such antimicrobial substances (Walsh, 2000).

Osmotic stress responses are examples of responses that could contribute to resistance by changing the relative proportions of porins in a way that reduces cell envelope permeability to antimicrobials (De Beer *et al.*, 1994; Steward and Costerton, 2001). General stress responses initiated by growth within a biofilm have influences on the growth rate of cells within biofilms. These stress responses result in physiological changes that act to protect the bacterial cells from the various environmental stresses (Brown and Barker, 1999). This may partially explain the protection of such cells from the harmful effects of heat and cold shock, osmotic changes, changes in pH and the activity of many chemical agents (Hengge-Aronis, 1996).

Slow or incomplete penetration of the antimicrobial into the biofilm may also be a possibility of protection of the cells in the biofilm from the effects of antimicrobial agents (Mah and O'Toole, 2001). Penetration of antimicrobials can also be profoundly retarded if deactivation of the antimicrobial occurs in the biofilm matrix (Steward and Costerton, 2001).

2.5 EFFECTS OF CHLORINE ON REGROWTH OF MICROORGANISMS IN WATER DISTRIBUTION SYSTEMS

Chlorine is a highly reactive substance and an effective disinfectant. It is used against viruses and bacteria in water treatment and purification of drinking water (WHO, 2004). Free chlorine residuals declines in the water distributions system and the concentration usually drop from more than 1.0 mg/l to below 0.1mg/l after about a 10-h residence time. Factors such as pipe material, biofilm in the distribution system and distance from the treatment plant play an important role in chlorine reduction (Lu *et al.*, 1995; Vasconcelos *et al.*, 1997; Prevost *et al.*, 1998). Low concentrations of chlorine are not effective in biofilms and certain microorganisms can survive or multiply in the presence

of low concentrations of chlorine (LeChevallier *et al.*, 1980; 1988a, b; 1990a, b; Herson *et al.*, 1991). It has also been indicated that cells in a biofilm are as much as 3000 times more resistant to free chlorine than their planktonic counterparts (LeChevallier *et al.*, 1988a).

2.6 ANTIBIOTIC RESISTANCE AND HEAVY METAL TOLERANCE OF HETEROTROPHIC BACTERIA FROM THE NORTH-WEST PROVINCE

Agricultural and commercial farming practices in the North-West Province involve utilization of large amounts of antibiotics (Mulamattathil *et al.*, 2000) and pesticides. Increased levels of these substance in the environment may lead to increased antibiotic resistance of microorganisms in particular, water sources such as rivers and dams (Smith *et al.*, 2002). A study by Mulamattathil *et al.* (2000) has demonstrated the impact of antibiotic use in the poultry industry in Mafikeng/Mmabatho (North-West Province) on the level of antibiotic resistance of environmental bacteria. This study also showed that bacteria treated with chlorine at a chicken processing plant survived this treatment process and were detected in the effluent. On the other hand, antibiotic resistance studies of meat-, and dairy products in the North-West Province also showed high levels of multiple antibiotic resistance amongst bacteria isolated from such sources (Beirowski, 2002; Watermeyer, 2002; Ramatlhape, 2005; Kwenamore, 2007). The research of Mulamattathil (2000), Beirowski (2002), Ramatlhape (2005), de Wet (2006), Pantshwa (2006) and Kwenamore (2007) are examples of studies demonstrating high levels of antibiotic resistance in bacteria associated with the environment, food and water sources in the North-West Province.

The majority of elements found in the periodic table are heavy metals. A large number of these are essential micronutrients of living organisms and are important role players in essential enzymatic activities (Nies, 1999). Most heavy metals can be toxic at high enough concentrations. For this reason heavy metals are also used as antimicrobial agents and are part of pesticides (Nies, 1999).

Heavy metals such as uranium, mercury, arsenic, copper, chrome, vanadium, cadmium, lead and zinc showed elevated levels in sediment, water, and in tissues of aquatic animals in the Mooi River catchment area (Erdman 1999; Venter 2001) This phenomenon was attributed to pollution from mining, agricultural and other anthropogenic activities.

Mechanisms that are responsible for the tolerance of antimicrobial chemical substances, including heavy metals, are in many cases similar to those responsible for antibiotic resistance (Nies, 1999; Silver *et al.*, 2001). Molecular studies have demonstrated that these mechanisms could be non-specific (Hernandez *et al.*, 1998; Putman *et al.*, 2000) or could be of genetic origin and carried on exchangeable DNA fragments such as plasmids and transposons (Nies, 1999; Putman *et al.*, 2000; Silver *et al.*, 2001). Such genes can then be inducibly expressed by elevated levels of the substance that caused the selective pressure or by a different one such as an antibiotic (Hernandez *et al.*, 1998). Evidence also demonstrated that exchange of such material occurs between different species (horizontal genetic transfer) and stable inheritance between successive generations (vertical genetic transfer) (McCormick, 1996; Putman *et al.*, 2000; Smith *et al.*, 2002; Licht *et al.*, 2003).

The pollution and high concentrations of heavy metals in the environment have different effects and implications on the environment. These effects may be less beneficial as the presence of metal resistance may contribute to the increase in antibiotic resistance as well as higher HPC bacteria levels in treated drinking water.

Copper pipes are used to distribute drinking water. Resistance to this metal is thus of special concern. A study by Lin and Olsen (1995) on bacteria that were isolated from the water distribution system, showed that as high as 62.0% of all isolates were resistant to copper. High levels of copper resistant HPC bacteria in potable water is of concern to the health of water consumers, because they may also poses related drug resistance and potential pathogenicity characteristics.

2.7 METHODS AND PRINCIPLES OF EXPERIMENTAL WORK

2.7.1 Generation and collection of biofilm sample

The protocols used for determining HPC bacteria levels in biofilms from water distribution systems normally include the use of devices to generate the biofilm first. Kalmbach *et al.* (1997) used a Robbins device which consisted of stainless steel cylinders (180mm by 150mm), with ten threaded holes. During the sampling, biofilms were removed after different exposure times and immediately placed in sterile drinking water. Furthermore, they used a sterile plastic scraper to detach bacteria from the slide surfaces and then made serial dilutions of the bacterial suspensions to plate on R2A agar. These were incubated between 4-7 days at 37°C to enumerate the HPC.

Camper *et al.* (1986), in a study that investigated bacteria associated granular activated carbon particles in drinking water filters, removed the filters and cut it into small fragments. These were placed in beakers containing cold sterile, distilled water,

followed by vigorously shaking to dislodge organisms from the filter. The analysis approach was similar to the above mentioned one of Kalmbach *et al.* (1997).

2.7.2 Isolation and cultivation of heterotrophic bacteria

The growth rate, survival, and activities of heterotrophic bacteria in water are mainly dependant on the chemical, biological and physical interactions of the aquatic environment in which they are present (Vitanage *et al.*, 2004) The three most common alternative methods for determination of heterotrophic plate counts are pour plates, spread plates and membrane filtration methods (Reasoner, 1990). It has been suggested that the highest HPC are obtained by the streak plate method and long incubation periods on non-selective media with low substrate concentrations are recommended (Foot and Taylor, 1949; Jones 1970; Fiksdal *et al.*, 1982; Maki *et al.*, 1986).

Thus, not only the technique but also considerations of culture medium, temperature, and incubation time are important factors to consider when HPC tests are conducted. The use of nutrient agar is highly recommended as it is a non-selective medium and suitable for the isolation and cultivation of a high diversity of micro-organisms (Walsh *et al.*, 2003; WHO, 2003). However, low nutrient media are better preferred for enumeration of water based bacteria where the most commonly used heterotrophic medium is R2A. It was designed specifically as a low-nutrient, low ionic strength formulation to isolate bacteria that have a water based lifestyle. Low temperature incubation (20-28°C) and longer incubation time (5-7 days) favours the growth of water-based bacteria (Reasoner *et al.*, 1990).

Although HPC media such as nutrient agar, R2A agar and M-HPC agar are used for the growth and isolation of heterotrophic bacteria, many clinical important HPC bacteria

may not grow on such media (WHO, 2003). It may thus be important to use selective media if specific microorganisms are targeted. Examples of such selective media include mFC for the enumeration of faecal coliforms, Aeromonas selective media the detection of this species and King B medium for detection of *Pseudomonas* spp. (WHO, 2003).

2.7.3 Analytical profile index 20 E (API 20E) and triple sugar iron (TSI) agar

Triple sugar iron agar (TSI Agar) is used for the differentiation and preliminary identification of Gram-negative bacilli potentially from *Enterobacteriaceae*. The TSI agar slants contain three sugars (1.0% lactose and sucrose, and 0.1% glucose). The pH indicator phenol red is included to monitor carbohydrate fermentation and ferrous ammonium sulphate for detection of hydrogen sulphide production (Harley and Prescott, 2002). The slants are incubated for 18-48 hours at 37°C and then examined for sugar fermentation, gas (splitting of agar) and H_2S production (blackening of agar). Acid formed in the medium as indicated by a yellow colour change (Ewing, 1985; Harley and Prescott, 2002).

Analytical profile index (API) kits are biochemical test kits that can be used to identify bacteria. These are based on the biochemical fermentation reactions. The API 20E system is devised for the identification of *Enterobacteriaceae* and related bacteria. It is a plastic strip that consists of 20 compartments that contains a dehydrated substrate for the different biochemical classification tests. After incubation, the reactions are recorded and a seven digit profile is generated. The latter could then be used for identification purposes using the API 20E manual (bioMerieux, Inc. Hazelwood) or related software.

2.7.4 Determination of antimicrobial susceptibility

The Kirby- Bauer disc susceptibility technique is one of the most common and widely used techniques to determine the antibiotic susceptibility of an isolate. This technique is based on the principle that the antibiotic will diffuse into the Mueller-Hinton agar, where it will interact with the bacteria that were spread on the media (Bauer *et al.*, 1966). This method is favoured because a large number of isolates can be tested for susceptibility to several antibiotics. The incubation temperature is 37°C and results are normally available within a 24 hour incubation period.

2.7.5 Hemolysis

Hemolysis is the result of breakage of the red blood cell membranes due to activity of toxin produced by pathogens (Harley and Prescott, 2002). When these strains are grown on blood agar the growth patterns can be classified as either alpha (α) or beta (β) hemolysis. The latter is because of yellowish halo of complete clearing against a red background. Partial - or alpha hemolysis is when a turbid halo with a green cast around the colonies is formed (Harley and Prescott, 2002). The recommended media when performing hemolysis contains either sheep or horse blood. However, a recent study performed by Anand *et al.* (2000) demonstrated that sheep and horse blood can be replaced with pig and goat blood.

2.7.6 Heavy metal tolerance determination based on the Minimum Inhibitory Concentration (MIC)

Several methods exist to determine the heavy metal MIC of bacteria (Dressler *et al.*, 1991; Karbasizaed *et al.*, 2003). A most commonly recommended method is the use of broth dilution (Karbasizaed *et al.*, 2003). Experimental tubes are prepared by supplementing Mueller-Hinton medium with metal salts of different cationic

concentrations. One millimetre of the test organism suspension ($1x10^6$ CFU/ml) is then added to each tube which is then incubated for 18hours at 35°C after which visual turbidity is recorded and compared to controls. Dressler *et al.* (1991), on the other hand used a solid media approach. In this, solid tris-buffered mineral salts agar containing 0.4% sodium succinate and metal salts in different concentrations were inoculated with bacteria after incubation at 30°C for 24hours. Although both methods were successful, the microdilution method has the advantage of performing the experiment in small volumes (200µl) and triplicates experiments can easily be set up.

2.7.7 Scanning electron microscopy (SEM)

For a long time scanning electron microscopy (SEM) has played a central role in structural characterisation of material surfaces (http://www.azom.com/details.asp?ArticleID=1556). During this procedure the surface of the material is bombarded with a beam of electrons and detecting those that are emitted or backscattered. This allows microscopists to see down to resolutions of a few nanometres, giving intricate details of the structure of the material (http://www.azom.com/details.asp?ArticleID=1556). However, due to specific requirements certain types of materials have always proved difficult or impossible to image e.g. the coating can obscure the fine surface detail on some material. Also difficulties arise with imaging of wet and damp samples such as biological tissue/material. These problems can be overcome by using a environmental scanning electron microscope (ESEM) or in a conventional SEM that used sample preparation techniques based on ESEM (http://www.azom.com/details.asp?ArticleID=1556).

Advantages of the ESEM includes that non-conducting samples can be investigated without coating and that measurements can be made under controlled atmospheres (Kaegi and Holzer, 2003). Not withstanding these advantages, specimen damaging can

still occur, even under low vacuum and acceleration voltages associated with the ESEM (Kaegi and Holzer, 2003), whereas conventional SEM samples are vacuumed and need to be critically dried or cryogenically frozen.

Surman *et al.* (1996) investigated and compared the use of various types of advanced microscopy techniques for the study of biofilms and found that ESEM enabled one to study the surface topology of biofilms at high magnification. Recently, Lessa *et al.* (2007) demonstrated the usefulness of using SEM in studying dental biofilms *in vivo*.

2.8 SUMMARY

In this chapter an overview, from literature and recent studies, were presented to provide a theoretical base for this study. Some questions about heterotrophic plate count bacteria and the use of such data to assess the general microbial quality of drinking water was dealt with. It also dealt with potential opportunistic pathogens that may occur amongst the HPC bacteria. An attempt was also made to deal with this in the context of HIV and AIDS as well as mortality statistics. This was done to focus the attention on the need to provide drinking water of good quality to all communities.

The regrowth potential of HPC bacteria, after water purification and disinfection, and the potential of these bacteria to form biofilms in drinking water distribution systems, was addressed. A brief overview of biofilm formation and the protective properties of the biofilm on the microbial communities contained within it, were also provided. From relatively recent studies, a brief overview was provided about antibiotic use and heavy metal pollution in the North-West Province, South Africa. The common selective pressure that these pollutant types could provide for resistance to antimicrobial substances was discussed. Drinking water biofilms may thus contain HPC bacteria tolerant to disinfection substances and may become dislodged. Having pathogenic

potential and being resistant to antibiotics, these bacteria in drinking water could have detrimental effects on sections of the population.

To conclude this chapter, the following:

1. levels and characteristics of HPC bacteria from drinking water biofilms in the Potchefstroom is undetermined.
2. in the context of existing knowledge, baseline data for this should be obtained i.e. a need for the present study.
3. standard procedures and methods as highlighted in Section 2.7 are available to conduct such a study.

CHAPTER 3

DIVERSITY AND CHARACTERISTICS OF HETEROTROPHIC BACTERIA FROM HOME WATER FILTERING SYSTEMS

3.1 INTRODUCTION

Sufficient potable water must be provided to consumers (WHO, 2006). In developed and most developing economies, water is processed through multiple steps before it is dispatched to the water consumer (WHO, 2006). During purification, substances such as algae, bacteria, fungi, minerals, viruses and chemical pollutants should be removed (WHO, 2006). When the treatment does not succeed in providing good quality drinking water, communities may be exposed to risks of outbreaks of intestinal and other infectious diseases (WHO, 2006). Furthermore, the aesthetic quality of water to consumers is also a concern of the water supply agency (DWAF, 2002). Once purified, the water is supplied to consumers via a distribution system. The water that reaches consumers could have been impacted on by various factors within the distribution system that negatively affect the general quality of the final product. These factors include material and age of the distribution system, but also the quality of the source water and the efficiency of the treatment processes (DWAF, 2002; Momba *et al.*, 2002). For this reason affluent persons may prefer bottled or home filtered water for drinking and cooking purposes.

It is suggested that the market for bottled water is the fastest growing industry in South-Africa (Neall, 2000).The perception amongst consumers are that this water is safer than tap water (Ehlers *et al.*, 2000). The Department of Water Affairs and Forestry (DWAF), refer to the use of bottled water as alternative to tap water, expensive, and only useful in cases of a drinking water emergency. One litre of bottled water cost roughly ten rand.

On the other hand, it costs less than 1 cent to fill a one litre bottle from the tap (DWAF, 2005).

The city council of Potchefstroom assures that the provided drinking water is safe and of a very high quality (Van Aardt, 2007). However, the aesthetic quality is not always pleasing. Smell and taste is sometimes impaired and for this reason several companies selling water filtering systems are operational in the Potchefstroom area. Thus, in this part of the research a comparative study of the diversity and characteristics of microorganisms isolated from biofilms from two examples of home water filtering systems were conducted. A brief overview of water purification and home water filters is provided as a prelude to the methods used, results obtained and discussion thereof.

3.1.1 Drinking water purification

Waterworks may make use of different types of water purification procedures and the treatment strategy is a management decision (DWAF, 2002). The process in general starts with the intake of the source water, followed by pre-treatment, mixing, coagulation and/or flocculation, sedimentation, filtration, disinfection and finally distribution to the consumer (DWAF, 2002).

The first step basically involves the treatment of incoming water with a coagulant (aluminium sulphate, ferric chloride or lime) to remove colloidal particles. Then coagulation and/or flocculation follow. Coagulation is a process used to enhance the interaction of small particles to form larger particles. Flocculation is the more physical process of producing inter-particle contacts that lead to formation of large particles. After coagulation, the flocs are removed by sedimentation, followed by rapid sand filtration. Sedimentation is a solid liquid separation process, in which particles settle

under the influence of gravity. The sedimentation process is thus responsible for the removal of chemical precipitates, fine clay and organic particles such as dead organisms (DWAF, 2002; WH0, 2004). Filtration is the step before disinfection that further enhances the purity of the water by removing and controlling biological contamination and turbidity. Microbial pathogens can be removed from drinking water because of effective filtration methods that act as barriers for these microbes (www.aces.edu/pubs/docs/A/ANR-0790/WQ2.1.5). Different types of filtration materials are used including sand, activated carbon, fibrous, cartridge, etc (DWAF, 2002; WHO, 2004)

Disinfection is the final and most important step before the water is distributed to the water consumer. At this point most of the impurities have been removed by the initial steps. The main goal of the disinfection step is to destroy pathogenic and opportunistic pathogenic bacteria (DWAF, 2002; WHO 2004).

Various disinfection processes exist. Some includes pretreatment oxidation, primary disinfection and secondary disinfection (DWAF, 2002). The disinfectants used are chlorine gas, chloramines, ozone and UV radiation although some waterworks may not use ozone and UV radiation (DWAF, 2002; WHO, 2004). Chlorination is the most commonly used disinfectant, not only because of its very efficient performance but it is also relatively cost effective (DWAF, 2002). When water is chlorinated with chlorine gas, the reaction between chlorine gas and water forms HOCl and HCl. In turn, a hypochlorite ion (OCl^-) and hydrogen ion (H^+) form when HOCl dissolves. The chlorine which is responsible for the toxic and very reactive action against several components of the bacterial cell is, OCl^- and HOCl (WHO, 2004). A human health risk associated with chlorine treatment of drinking water is the formation of organohalogens such as

trihalomethanes and haloacetic acids that may be mutagenic (Gagnon *et al.*, 2005). Factors such as the concentrations of the disinfectant, contact time, temperature and pH, have a direct effect on the efficiency of the disinfection process (WHO, 2004).

3.1.2 Microorganisms present in drinking water systems

Bacteria that survive the water purification process, or have the ability to regrow, may occur in our drinking water (LeChevallier *et al.*, 1980; Rand *et al.*, 2007; Srinivasan and Harrington, 2007). This is a practical problem for drinking water supply agencies. Although their concentration may not be high, it is important to monitor their levels not only from a health perspective but also from an operational perspective (Rand *et al.*, 2007; Srinivasan and Harrington, 2007). Several studies have been done over the years to investigate the occurrence of heterotrophic bacteria in drinking water and the potential pathogenic properties of these bacteria (LeChevallier *et al.*, 1980; Reasoner, 1990; Allen *et al.*, 2004; Pavlov *et al.*, 2004; Messi *et al.*, 2005). Recent studies were also concerned with biofouling, microbial induced corrosion and types of disinfection by products that could be formed by various treatment scenarios (Rand *et al.*, 2007; Srinivasan and Harrington, 2007).

3.1.3 Increased interest in the use of home water filter systems

The use of home water filters has become more common over the years due to the great concern about drinking water quality and implications for human health (Gelt, 1996). Several studies have reported the presence of various hazardous compounds in treated drinking water (Kraybill, 1981; Kruithof, 1985; Peters *et al.*, 1990; De Marini *et al.*, 1995; Filipic *et al.*, 1995; Rehena *et al.*, 1996). These include both organic and inorganic contaminants which could be removed by simple home water filtering systems (Ishizake *et al.*, 1983). Thus contaminants most commonly removed by home

water filtering systems are microbes, radionuclide, organic and inorganic contaminants, disinfectants, and disinfection byproducts. Toxic metal ions like copper (Cu^{2+}), lead (Pb^{2+}) and zinc (Zn^{2+}) sometimes commonly found in drinking water can also be removed by such filters (Ahmedna *et al.*, 2004).

Another aspect that is of concern and may also have an influence on the quality of the water is the presence of chlorine and chlorine byproducts. These substances may be mutagenic as well as carcinogenic (Carraro *et al.*, 2000). Carraro *et al.* (2000) demonstrated that chemical treatment of water lead to mutagen production which could be efficiently reduced by filtration. This is only one of the many reasons why filtered water is preferred over the use of tap water by many water consumers.

A study was performed by Sheffer *et al.* (2004) in a hospital building that was colonized with *Legionella pneumophila*. In their study all samples were cultured for *Legionella* spp, HPC bacteria, and *Mycobacterium* spp. A total of 594 samples were collected and analyzed. They demonstrated that point-of use filters managed a greater that 99.0% reduction in HPC bacteria levels. This study also showed that point-of use filters units can be very practical and can also be used to prevent exposure of high risks patients to waterborne pathogens without modification or disinfection of the entire potable water system.

3.1.4 Home water filters

Filters, distillers and softeners are three main types of home water treatment systems that have been available for a long time (Ingersoll, 1981). The most commonly used water filtering systems are filters containing either activated carbon filters, fibre filters or using reverse osmosis (www.heartspring.net/water_filters_guide.html). Activated

carbon filters are most commonly used since it is relatively inexpensive (www.cyber-nook.com/water/Solutions.html#carbon). Reverse osmosis systems on the other hand, are the more expensive filtering systems (www.cyber-nook.com/water/Solutions.html#ro).

The variety of filter devices that are available is because of the different filter media used, types of chemicals that are removed, and their location in the home. Although these filters seem to be very practical in removing particles and contaminants, they are not 100% efficient in removing all known hazardous contaminants such as arsenic, barium, chromium, coliform bacteria etc.(www.cyber-nook.com/water/Solutions.html#carbon). Common filter brands that are currently available on the market are the Envirofilter, Omnifilter, BRITA, PuR and the Teledyne (Ahmedna *et al.*, 2004).

Figure 3.1- A photo of the activated carbon filter (http://www.amazon.com/WaterPik-Instapure-faucet-mount filter/dp/B000LNO6BA)

a) Activated carbon filters

Activated carbon filters (Figure 3.1) that are used in home water treatment may contain granular activated carbon (GAC) or powdered block carbon, which are contaminant removers. These types of filters normally remove volatile organic chemicals, chlorine, benzene, trihalomethanes compounds and heavy metals (Wallis *et al.*, 1974). Carbon particles with a positively charged surface area, draw the negatively charged contaminants (chemicals) towards it, and therefore explain why this reaction results in water of a higher quality (Franzblau *et al.*, 1984). Contact time between the water and the activated carbon is essential because an increased contact time results in increased removal of contaminants (www.cyber-nook.com/water/Solutions.html#carbon). The

main reason why carbon is used is because of its powerful absorbent quality. One disadvantage is that these filters should be replaced very frequently (www.cyber-nook.com/water/Solutions.html#carbon).

b) Reverse osmosis

One of the most efficient filtration techniques is reverse osmosis. This is because of its high range of contaminant removal. Reverse osmosis systems are composed of granular activated carbon (GAC) pre- filters, a reverse osmosis membrane, a storage tank, and a faucet that deliver purified water (www.cyber-nook.com/water/Solutions.html#ro). The use of pre-filters is essential to avoid any degradation of the membranes by chlorine and other contaminants (www.cyber-nook.com/water/Solutions.html#ro).

Membranes of the reverse osmosis units are semi permeable, and allow water to pass through it. The membrane physically rejects the passing through of any solutes that are larger than the pores of the semi-permeable membrane. Reverse osmosis membranes retains particles that are 0.0005 microns and larger. It will thus retain bacteria (0.2-1 micron) and viruses (0.02-0.4 microns). All contaminants then flow downstream (www.cyber-nook.com/water/Solutions.html#ro). A schematically representation of the basic concepts of the reverse osmosis filtration unit is given in Figure. 3.2. Thin film composite (TFC) and cellulose triacetate (CTA), are two types of reverse osmosis membranes that are available. The former film is more efficient in removing contaminants (Water Quality Research Council, 1991).

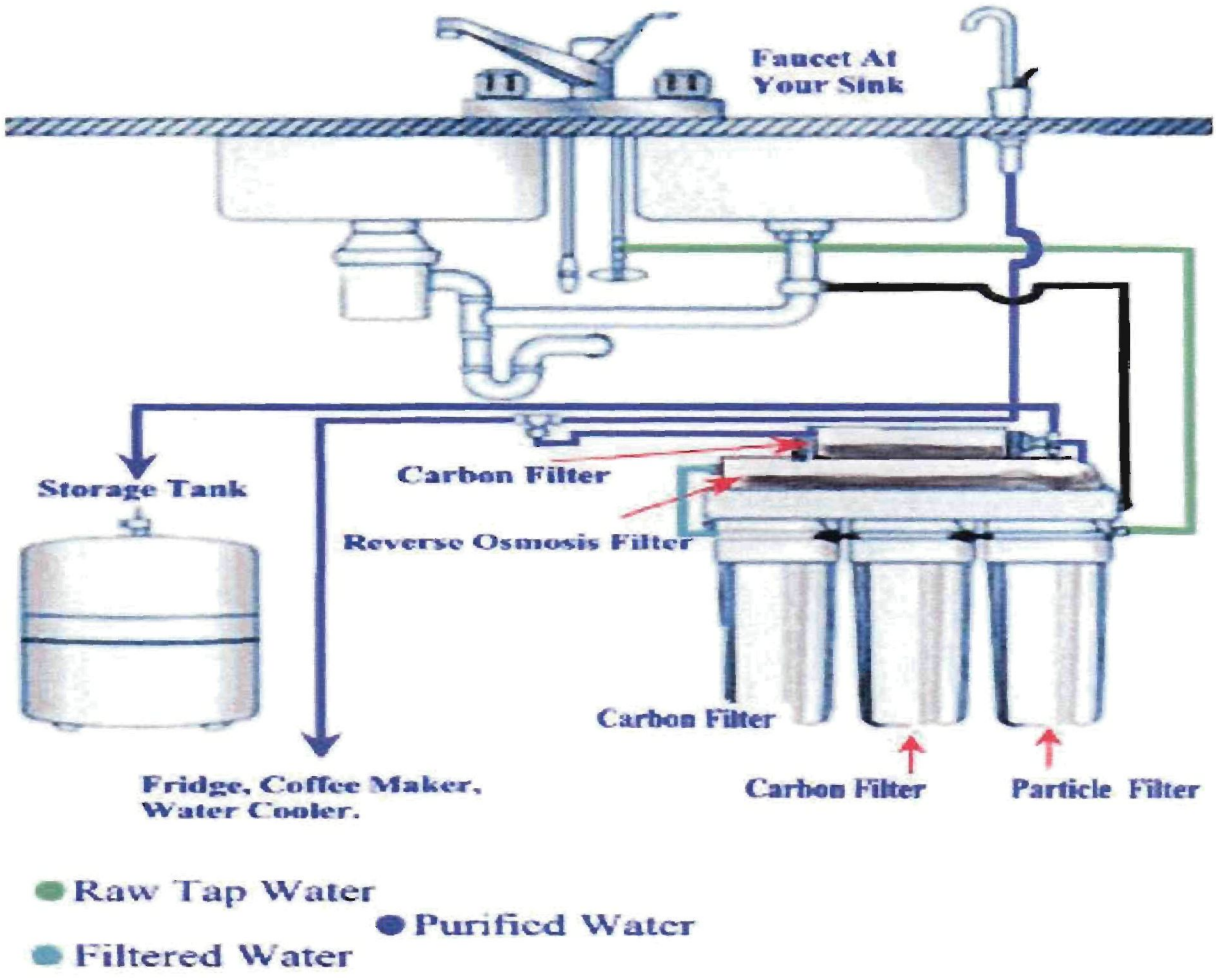


Figure 3.2- A schematically representation of reverse osmosis filtration unit (www.waterforlife.ca/images/Radiogram.jpg)

Neither of the two water filtering systems described requires electricity, but essential accessories for reverse osmosis systems make them initially relatively more expensive than activated carbon filters (www.cyber-nook.com/water/Solutions.html#ro). However, in the long term reverse osmosis systems become more cost effective. Disadvantages of reverse osmosis systems include the requirement for several filters (sediment and carbon pre- filtration to prevent membrane fouling) that needs to be replaced regularly (every 6 months). The filtration process can be very slow, and the reverse osmosis device, including all its parts, can take up considerable space if kitchen space is limited (www.cyber-nook.com/water/Solutions.html#ro).

3.1.5 Aim and Objectives

The aim of this part of the study was to investigate the diversity and characteristics of HPC bacteria isolated from home water filtering systems (one activated carbon and one reverse osmosis filter).

The objectives were to:

- isolate, identify and determine levels and diversity of HPC bacteria from activated carbon and reverse osmosis filters.
- determine the antibiotic susceptibility/resistance patterns of HPC isolates from both filters.
- determine pathogenicity potential of isolates from both filters.
- make use of scanning electron microscopy (SEM) to study the biofilm structure on the filters (phases) of the reverse osmosis system.

3.2 MATERIALS AND METHODS

3.2.1 Sampling: home water filtering system

Filters were collected from a reverse osmosis home water filtering system after a period of 6 months of operation. The experimental period was March 2005 to April 2006. It is recommended by the manufacturer that the cartridges should be removed and replaced every 6 months due to the decrease in efficiency of the filter after this period. After removed, these filters were placed in plastic containers and transported to the laboratory. Sterile swabs were used to transfer biofilm from different filters (phases) of the filtering systems directly onto various selective and non-selective media. Media used were nutrient agar, mEndo agar, mFc agar, and *Aeromonas* selective agar and were obtained from Merck (South Africa). Plates were incubated for 24 hours at 37°C, except for the mFc media which were incubated at 45°C.

3.2.2 Preliminary identification

Colony morphology characteristics (mainly different pigmentation characters and colony shapes) were used to divide these isolates in various morphological groups. All isolates were Gram-stained using the procedure as described in Harley and Prescott, (2002). In addition to Gram-negative staining samples were also capsule-stained for glycocalyx characteristics. The staining procedure was Anthony's capsule staining with crystal violet and 20.0% copper sulfate (Harley and Prescott, 2002) Endospore-staining was performed using the Schaeffer-Fulton or Wirtz-Conklin method (Harley and Prescott, 2002).

3.2.3 TSI agar slants

Triple sugar iron agar (TSI agar) was used for the differentiation and preliminary identification of Gram-negative bacilli potentially from the family *Enterobacteriaceae.* To inoculate, a sterile inoculation needle containing an isolated colony was streaked onto the slant surface in a zigzag pattern. The agar was then stabbed deeply with the needle. The slants were incubated for 18-48 hours at 37°C and then examined for sugar fermentation, gas (splitting of agar) and H_2S production (blackening of agar). Acid formed in the medium as indicated by a yellow colour change (Ewing, 1985; Harley and Prescott, 2002). The guidelines of Harley and Prescott (2002), were used for interpreting the observations.

3.2.4 Analytical Profile Index (API 20 E)

The API 20 E system (bioMerieux, Inc. Hazelwood) was the procedure followed in the identification of *Enterobacteriaceae* and other Gram-negative bacteria. Isolates were inoculated in 5ml saline solution (0.89% $^{w}/_{v}$ NaCl). After the bacterial-saline suspension was well mixed, a sterile pipette was used to transfer the suspension to the cupule and microtubes according to instructions of manufacturer. Tap water was dispensed into the incubation tray to ensure a humid atmosphere during the 24 hours incubation period. The ADC, LDC, ODC, H_2S and URE microtubes was slightly under filled which was followed by complete filling of the cupule section with mineral oil to create anaerobic conditions. A lid was then placed on the incubation tray and incubated at 37°C for 24 hours. Reactions were recorded after incubation. A seven digit profile was then generated and used for identification according to the manufacturers specifications.

3.2.5 Motility test

Motility tests were done on all isolates using procedures from Harley and Prescott, (2002). The isolates were aseptically stabbed in motility test media where motile bacteria were observed by migration from the inoculation line and non motile bacteria were observed by growth along the line of inoculation (Harley and Prescott, 2002).

3.2.6 Hemolysis on blood agar (5%)

To test the isolates for potentially pathogenic features, pure cultures were streaked aseptically on blood agar plates (5.0% sheep blood). Blood agar plates were obtained from NHLS, Johannesburg. The incubation period was for 24hours at 37°C. Different types of hemolysis (β-hemolysis-with a sharply defined zone and α-hemolysis with a greenish- brown zone) were recorded. Isolates were classified as being potentially pathogenic or non-pathogenic, based on whether hemolysis occurred or not (Harley and Prescott, 2002).

3.2.7 Kirby Bauer disk diffusion method

Mueller-Hinton is the most commonly used media in this technique. The bacterium is swabbed on the agar and the antibiotic discs are placed on top. Discs containing the antibiotics were stored at 4°C and only warmed to room temperature before use. Antibiotics to which the organisms are susceptible are identified by clear areas/circles around the discs, which indicate growth inhibition. The diameter of the zone of inhibition (in mm), is an indication of the degree of susceptibility of the organism to the particular antibiotic. The inhibition zone can be used to classify the bacterium as resistant or susceptible using NCCLS 1999, guidelines (Table 3.2).

The antibiotics in Table 3.2 were chosen because they are commonly used during treatment of human diseases. They have also been used in previous studies of antibiotic resistance in aquatic environments (Mulamattathil, 2000; Kwenamore, 2007). Results based on the inhibition zone indicate if the organism is sensitive/susceptible (S), intermediate resistant (I), or resistant (R), towards an antibiotic. The abbreviations of the antibiotics were used as indicated on the antibiotic discs. This is according to the manufacturers (MAST Diagnostics Group Ltd, UK).

Table 3.2- Antibiotics used in this study. The concentration [µg] as indicated on the discs and the inhibition zone classification is also provided using NCCLS (1999) data.

Class of antibiotic	**Antibiotic Used**	**Abbreviation**	**[µg]**	**R (mm)**	**I (mm)**	**S (mm)**
B-Lactams	Ampicillin	AP	10µg	≤16	-	≥17
	Penicillin	PG	10units	≤14	-	≥22
Chloramphenicols	Chloramphenicol	C	30µg	≤12	13-17	≥18
Quinolones	Ciprofloxacin	CIP	5µg	≤15	16-20	≥21
Macrolide	Erythromycin	E	15µg	≤13	14-22	≥23
Amino glycosides	Gentamycin	GM	10µg	≤12	13-14	≥15
	Kanamycin	K	30µg	≤13	14-17	≥18
	Neomycin	NE	30µg	≤13	13-16	≥17
	Streptomycin	S	300µg	≤6	7-9	≥10
Tetracycline	Tetracycline	T	30µg	≤14	15-18	≥19
Glycopeptides	Vancomycin	VA	30µg	≤14	15-16	≥17

3.2.8 Scanning electron microscopy (SEM)

SEM was used to study the biofilm occurring on the filters. The potential biofilm segment was initially fixed in 70.0% acetone for 15 minutes and then fixed in sequentially increasing ethanol concentrations; first with 70.0% ethanol for 10 minutes then in 90.0% ethanol for 10 minutes and twice in 100% ethanol for 10 minutes each (Tiedt, 2006)

It was also important, to prevent the biofilm from coming into contact with air. The material was then critical dried with liquid carbon dioxide gas and then placed on SEM stubs with double sided carbon band. This step was followed by carbon and metal vaporization, respectively (Tiedt, 2006).

After the vaporization was completed it was ready to be investigated under the electron microscope. The materials were first viewed at 50X and 100X enlargements. The biological activity of the RO filter system was viewed under a 100X, 1600X, 5000X and a 24 000X enlargement. No electron microscopy was done on the activated carbon filter home system.

3.3 RESULTS

3.3.1 Isolation and preliminary identification of isolates

The isolates from the reverse osmosis system obtained during the two sampling periods were divided into 10 types based on colony morphology. Only 3 different colony types were isolated from the activated carbon filter system. The levels and different primary characteristics of these isolates are summarized in Tables 3.3 (reverse osmosis home water filtering system) and 3.4 (activated carbon filtering systems).

A total of 94 original colonies were obtained from the reverse osmosis unit (Table 3.3). According to the results, 84.0% of the isolates were Gram-negative and 16.0 % were Gram-positive. None of the isolates were motile and 30.0% of them showed hemolysis (Table 3.5).

A representation of the characteristics of isolates from the second home water filter (carbon filter) is given in Table 3.4. A total of 50 original colonies were observed from the carbon filter home filtering unit. (The different levels of each isolate are indicated in Table 3.4). These results indicate that 60.0% of the colonies isolated from the carbon filter unit were Gram-negative and only 40.0% were Gram-positive. These isolates were all non-motile and all showed potential pathogenic characteristics.

Table 3.3- Summarized primary characteristics of the HPC isolates from a biofilm that formed on a reverse osmosis home water filtering system. The levels of the various morphotypes are also provided.

Isolate Code	Colony Morphology	Pigmentation	Gram Staining	Capsule Staining	Motility	Number of Isolates (%)
BDP203	Small, smooth, raised, round, shiny	Deep dark pink	-	-	-	37(39.4)
BB03	Medium, smooth, raised, round, shiny	Blue	-	-	-	2(2.1)
BLP03	Small, smooth, raised, round, shiny	Light pink	-	-	-	21(22.3)
BA03	Small, circular, raised, dull, smooth	Light green	-	-	-	6(6.4)
BG103	Medium, circular, smooth, raised, dull	Yellow	+	+(?)	-	6(6.4)
BG203	Medium, circular, smooth, raised, dull	Yellow	+	+(?)	-	4(4.3)
BG303	Medium, circular, smooth, flat, dull	Yellow	-	-	-	7(7.4)
BG403	Medium, circular, smooth, flat	Yellow	-	-	-	6(6.4)
BW03	Small, punctiform, smooth, raised	White	+	-	-	5(5.3)

Table 3.4- Summarized primary characteristics of the isolates from a biofilm that formed on a home activated carbon filter. The levels of the various morphotypes are also provided.

Isolate Code	Colony Morpology	Pigmentation	Gram Staining	Capsule Staining	Motility	Number of Isolates (%)
W09	Small, smooth, round, raised, shiny	White	+	-	-	5(10)
VW09	Medium, smooth, raised, round, dull	Dull white	-	-	-	20(40)
G09	Small, smooth, round, shiny	Bright Yellow	-	-	-	25(50)

3.3.2 Identification by TSI and API 20E

These techniques were used for the identification of enteric bacteria. It was done for all Gram-negative rods. Interpretation of the TSI results was based on four aspects including the colours of the slants and butts, gas formation which was observed by splitting of the agar and H_2S gas formation. See Appendix A for observations.

Seven different Gram-negative isolate types were further identified using TSI and API 20E methods as described in Sections 3.3.3 and 3.3.4. According to the results of the API 20E (Table 3.5), the following bacteria were isolated from the reverse osmosis filter: *Enterobacter agglomerans*, *Aeromonas hydrophilia*, *Citrobacter* spp., and *Enterobacter cloacae*. The API 20E results were to some extend supported by the TSI results. The seven Gram-negative isolate types thus only represented 4 different bacterial species. None of the Gram-positive isolates were identified.

Table 3.5- Identification of the isolates from the reverse osmosis and activated carbon home water filtering systems. Haemolytic and antibiotic resistance for the various species/morphotypes data are also indicated.

Identification (Reverse Osmosis Filter)

Isolate Code	API 20E	TSI	Hemolysis	Antibiotic Resistance Patterns
BDP203	*Enterobacter agglomerans*	*Enterobacter* spp.	-	K, NE, AP, T, E
BB03	*Aeromonas hydrophilia*	*Enterobacter* spp.	-	K, AP, T, E
BLP03	*Citrobacter* spp.	*Enterobacter/ Citrobacter*	-	K, AP, T, E
BA03	Not determined	Not determined	-	K, AP, T, E
BG103	Not determined	Not determined	β	GM, K, NE, C, AP, T, VA, E
BG203	Not determined	Not determined	α	K, AP, T, VA, E
BG303	*Enterobacter cloacae*	*Enterobacter/ Shigella*	-	C, AP
BG403	*Enterobacter cloacae*	*Enterobacter/ Shigella*	α & β	NE, AP, T
BW03	Not determined	Not determined	α & β	AP

Identification (Activated Carbon Filter)

G 09	*Enterobacter cloacae*	*Serratia* spp.	α & β	T
VW09	*Providencia alcalifaciens*	*Serratia* spp.	β	K, AP
W09	Not determined	Not determined	-	PG, T

The activated carbon filter had only 2 Gram-negative bacterial types. They were identified as *Enterobacter cloacae* and *Providencia alcalifaciens* by the API 20E system. In this case the TSI results did not support the API 20E results. *Enterobacter cloacae* was the one species isolated from both water filtering systems.

Among the isolates from the RO system, *Enterobacter agglomerans* levels were the highest (39.4%). Levels of *Enterobacter agglomerans* were between 1.7 and 18.3 times higher than levels of *Enterobacter cloacae* (22.3%), *Citrobacter* spp (7.45%) and *Aeromonas hydrophilia* (2.12%). Sixteen percent of the isolates were Gram-positive species.

No *Enterobacter agglomerans* was isolated from the activate carbon filter system. However, from this filter system *Prov. alcalifaciens* was the most predominantly isolated (50.0%) heterotrophic species. Five percent isolates from the carbon water filtering system were Gram-positive species and *Enterobacter cloacae* constituted 40.0% of the isolates.

3.3.3 Antibiotic resistance patterns

Ten different antibiotics were used to determine the susceptibility of all Gram-positive and Gram-negative isolates. Figure 3.3 shows the percentage of isolates from the RO system that was susceptible or resistant to the tested antibiotics.

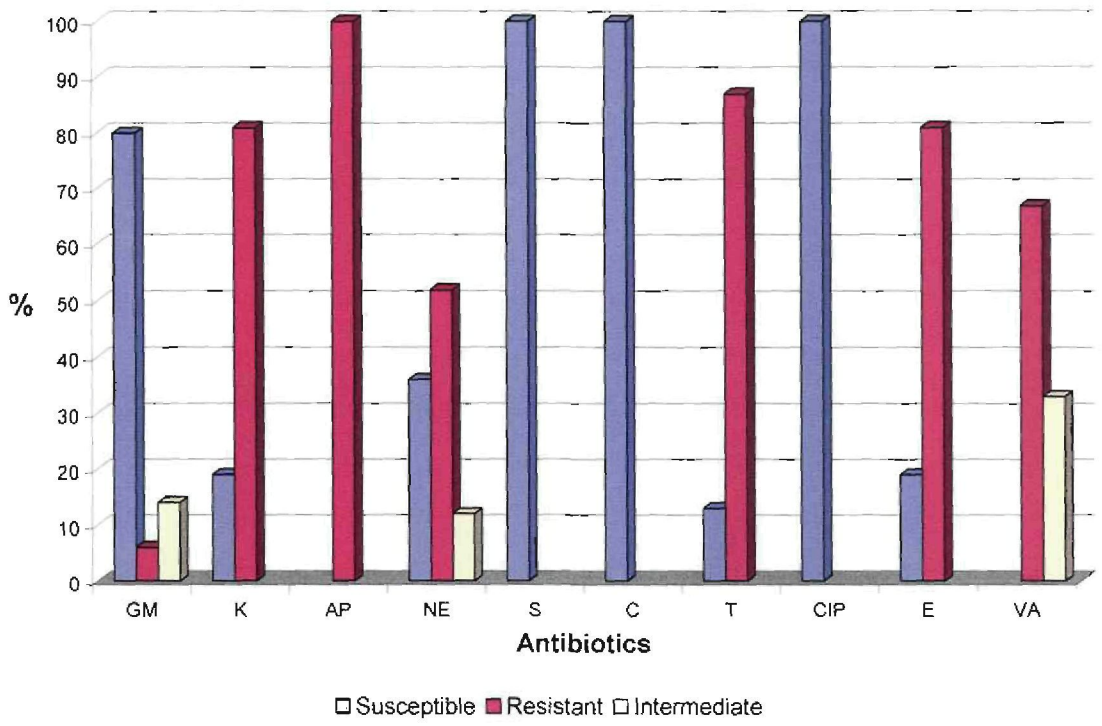


Figure 3.3- Percentage isolates from RO home water filtering system that were resistant, susceptible and intermediate resistant to all antibiotics tested. (Gentamycin=GM; Kanamycin=K; Ampicillin=AP; Neomycin=NE; Streptomycin=S; Chloramphenicol=C; Ciproflaxin=CIP; Tetracycline=T; Erythromycin=E; Vancomycin=VA)

According to results, it is evident that isolates (50 to 100%) were resistant to a large number of the antibiotics. These included kanamycin (K; 80.0%), ampicillin (AP; 100%), neomycin (NE; 50.0%), tetracycline (T; 85.0%), erythromycin (E; 80.0%) and vancomycin (VA; 65.0%). On the other hand, all of the isolates were susceptible for chloramphenicol (C), ciproflaxin (CIP) and streptomycin (S). Intermediate resistance was observed for only to 3 antibiotics [(gentamycin (GM), neomycin (NE) and vancomycin (VA)]. Only Gram-positive isolates were tested for susceptibility to vancomycin (Figure 3.3).

Figure 3.4 shows the percentage of isolates from the activated carbon system that was resistant, susceptible or intermediate resistant to the tested antibiotics.

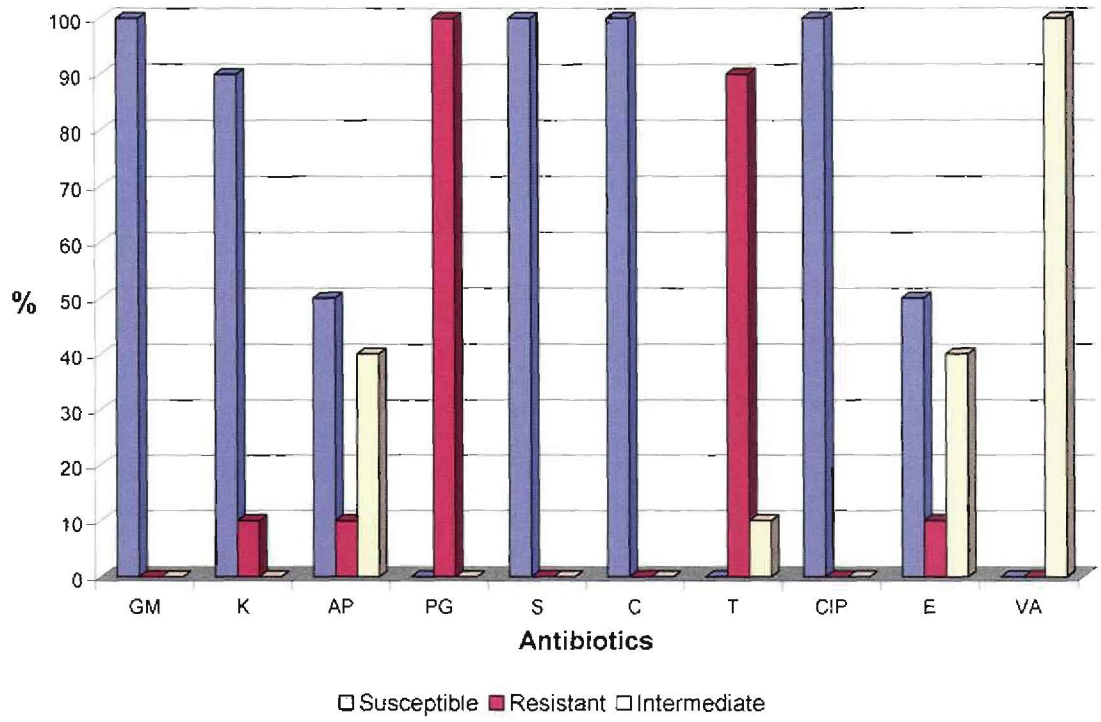


Figure 3.4- Percentage isolates from activated carbon home water filtering system that were resistant, susceptible and intermediate resistant to all antibiotics tested. (Gentamycin=GM; Kanamycin=K; Ampicillin=AP; Neomycin=NE; Streptomycin=S; Chloramphenicol=C; Ciproflaxin=CIP; Tetracycline=T; Erythromycin=E; Vancomycin=VA)

All isolates were susceptible to gentamycin (GM), streptomycin (S), chloramphenicol (C) and ciproflaxin (CIP). Forty to eighty percent of isolates was susceptible to kanamycin (K), ampicillin (AP) and erythromycin (E). All Gram-positive isolates were intermediate resistant to vancomycin (VA) and penicillin G (PG). Ninety percent of isolates was resistant to tetracycline (T) and 10.0% to ampicillin (AP), kanamycin (K)

and erythromycin. Forty percent of the isolates were intermediate resistant to ampicillin (AP) and erythromycin (E).

From the results presented in the preceding sections as well as in Appendix A, it is evident that 90.0% of isolates from the RO filter were resistant to multiple antibiotics. Forty percent from the isolates from the carbon filter were resistant to at least 2 antibiotics.

From Table 3.5 it is evident that isolates BB03, BLP03 and BA03 had the same antibiotic resistant profiles. The antibiotic resistant (AR) profiles of BDP103 and BDP203 were similar.

Six different AR profiles, designated capital Roman numerals, were observed among isolates from the reverse osmosis home water filter. These 6 profiles included **I** (K- NE- AP- T- E), **II** (K- AP- T- E), **III** (GM- K- NE- C- AP- T- VA- E), **IV** (K- AP- T- VA- E), **V** (C- AP), **VI** (NE- AP- T). The most predominant AR profiles amongst the isolates from the reverse osmosis filter were **I** (K-AP-T-E) and **II** (K-NE-AP-T-E) indicating that most isolates were resistant to kanamycin (K), ampicillin (AP), tetracycline (T) and erythromycin (E).

Two different AR profiles were observed among isolates from the activated carbon filter systems. These AR profiles included **VII** (K-AP) and **VIII** (PG- T). None of isolate types had similar AR profiles. The most predominant AR profile from the activated carbon filter system was K-AP.

In the overall study the maximum number of antibiotics to which isolates were resistant to was eight and the profile was GM-K-NE-C-AP-T-VA-E. This isolate was Gram-positive and unidentified. Among the Gram-negative bacteria, *Enterobacter agglomerans* was resistant to 5 antibiotics (K-NE-AP-T-E). A Gram-positive unidentified isolate was also resistant to the same number of antibiotics (K-AP-T-VA-E).

3.3.4 Scanning electron microscopy (SEM)

Figures 3.5 to 3.12 represent SEM micrographs depicting the biological and other activities observed on the surfaces of the filters occurring in the various filtering phases of the home reverse osmosis (RO) filter system.

Bladsy 50

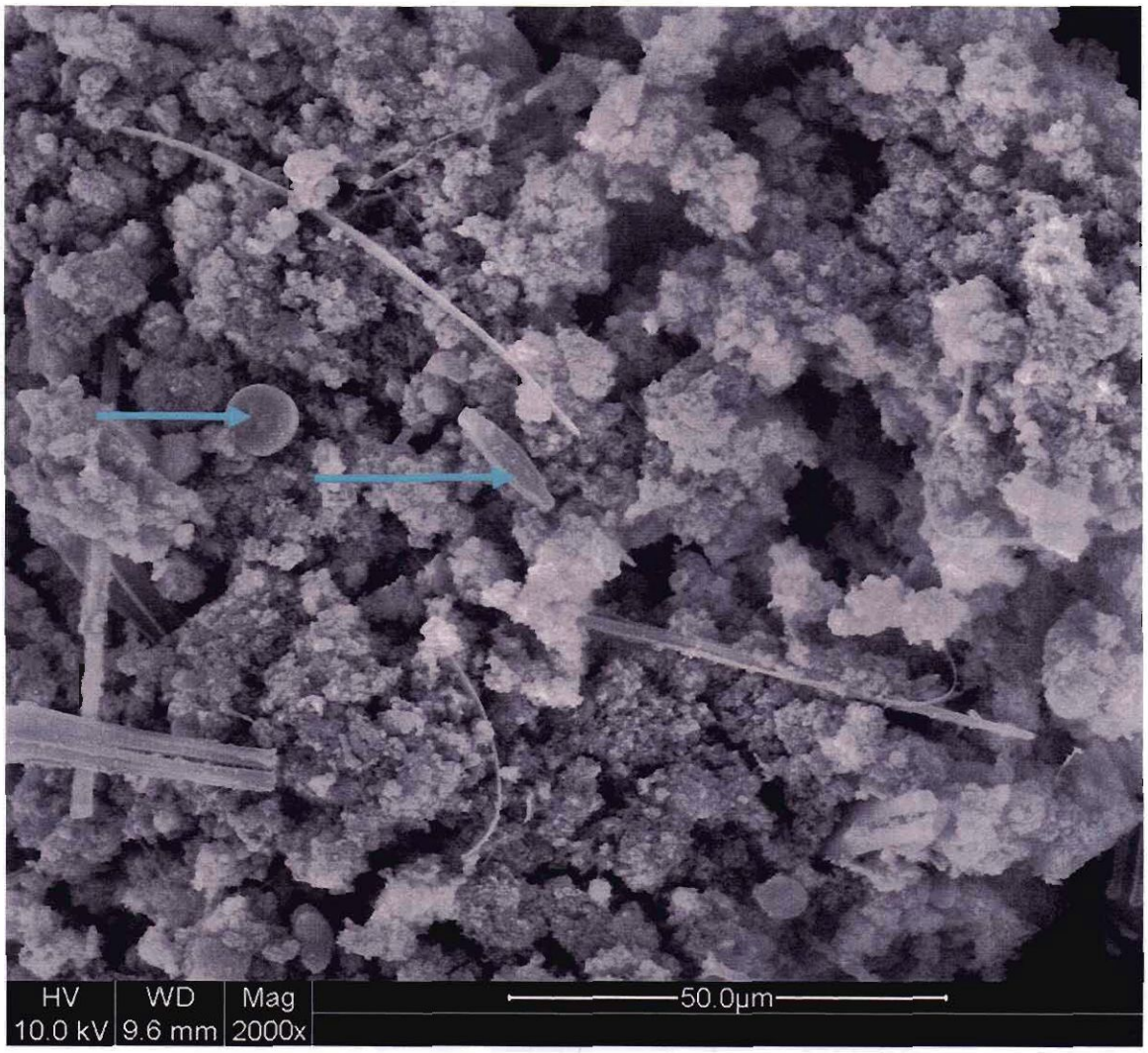


Figure 3.6- Enlargement of the filtered substances and microorganisms on phase 1 of the home water filtering system (2000X magnification).

In Figure 3.6 a higher magnification (2000X) of the filter is provided in order to show the diatoms (turquoise arrow) amongst the other deposits. At this magnification no biofilm could be detected (Figure 3.6). A possible reason is that the first phase is a 10 µm filter that mainly removes larger particles whereas the smaller objects can move through to phases 2 and 3.

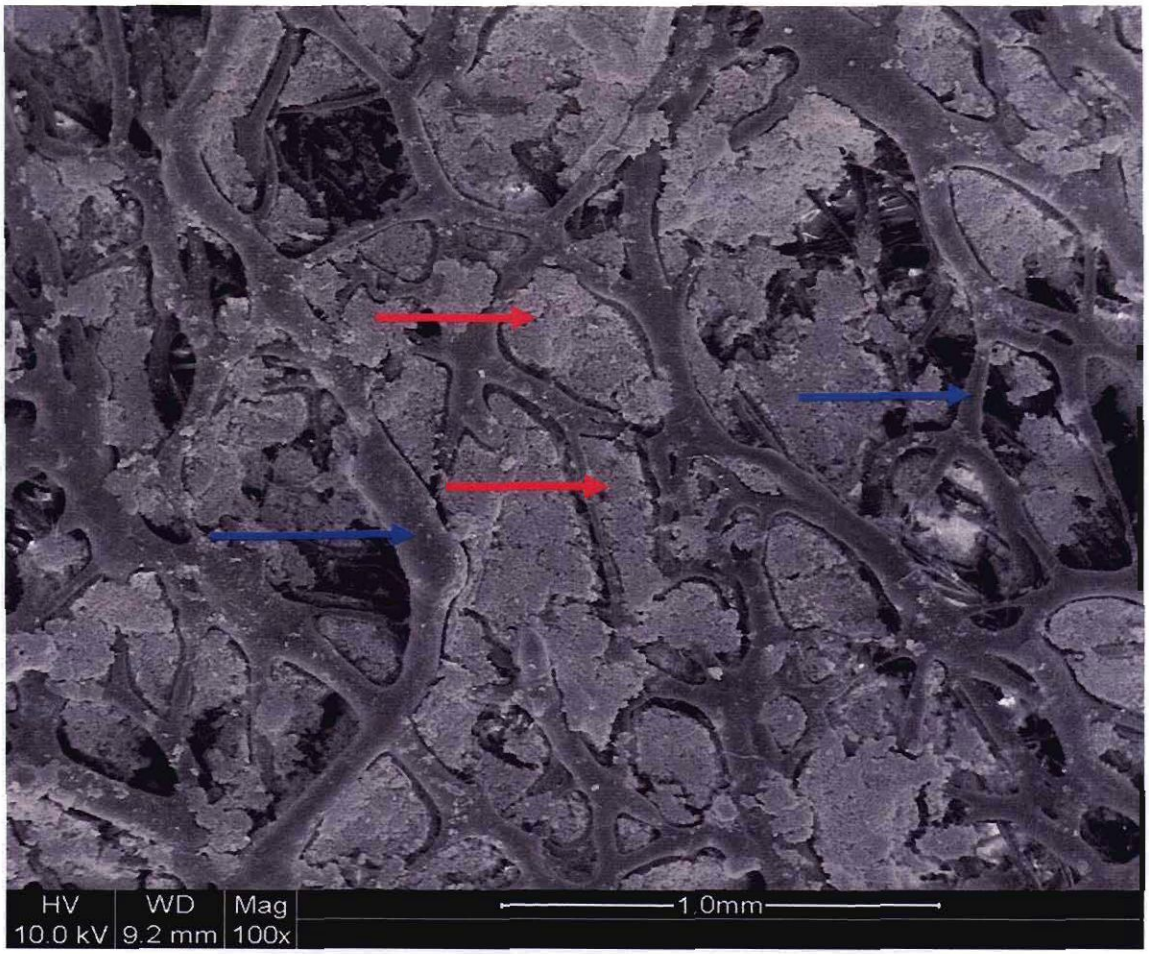


Figure 3.7- Enlargement of the material from the second phase of the home water filtering system (100X magnification).

Figure 3.7 shows the second phase (5 μm) of the filtering system. In this picture the darker lines indicates the material of the filter (blue arrow) whereas the area between the darker lines is an indication of the retentate (red arrows). Although the matrix of this phase was smaller than that of the first phase (Figure 3.5) the general appearance was similar. This second phase removes smaller particles from the filtrate of the first phase.

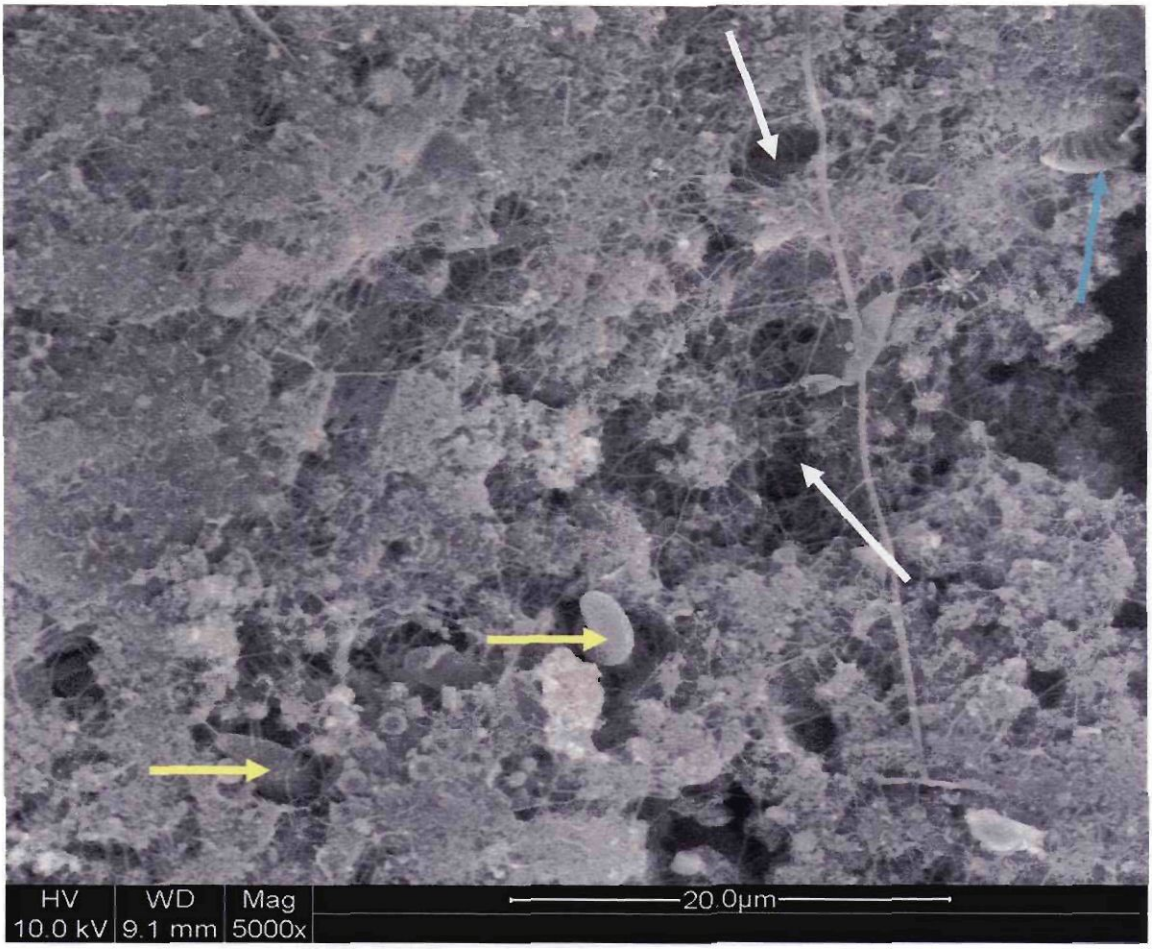


Figure 3.8- Enlargement of the biological activity on the 2nd phase of the home water filtering system (5000X magnification).

In Figure 3.8 (5000X magnification) some diatoms (turquoise arrow) and some curved shaped structures (yellow arrows; potential bacteria?) were observed. It is not clear if there was any biofilm formed at this stage. However, the abundant filamentous materials forming a matrix could be evidence of extra-cellular polysaccharide (EPS; white arrows). In the following micrograph (Figure 3.9) the bacterial structures are more clearly demonstrated.

Figure 3.9- Enlargement of the biological activity on the 2nd phase of the home water filtering system (20 000X magnification).

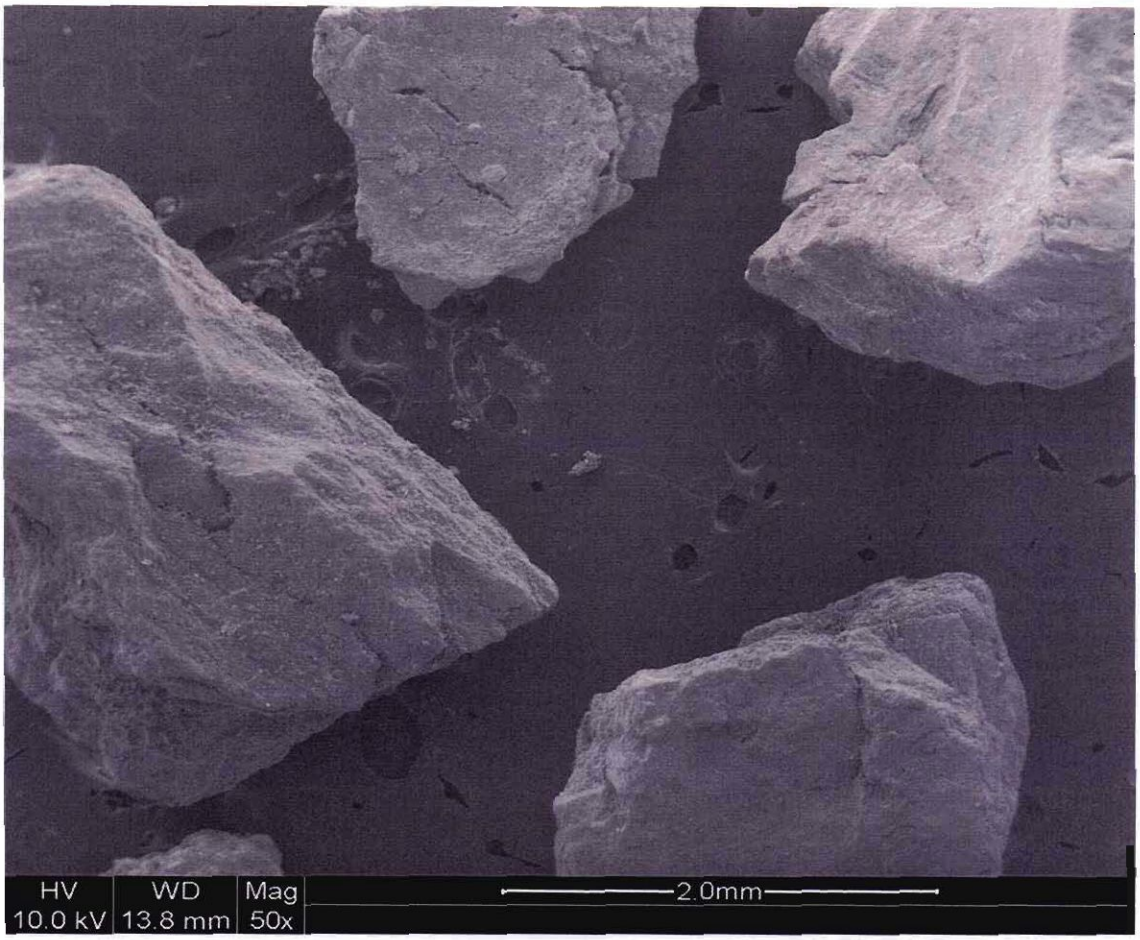


Figure 3.10- Enlargement of the material of the final phase of the home water filtering system (50X magnification).

The final filtration step before the membrane reverse osmosis is an activated carbon filtration step. This filter consists of activated carbon particles and it is responsible for the adsorption of organic compounds. In the SEM micrographs it was clearly seen that the material contains a very rough surface area (Figure 3.10) and that biofilm (Figure 3.11 to 12) was formed in spaces and cracks where there are no direct or slower water flow.

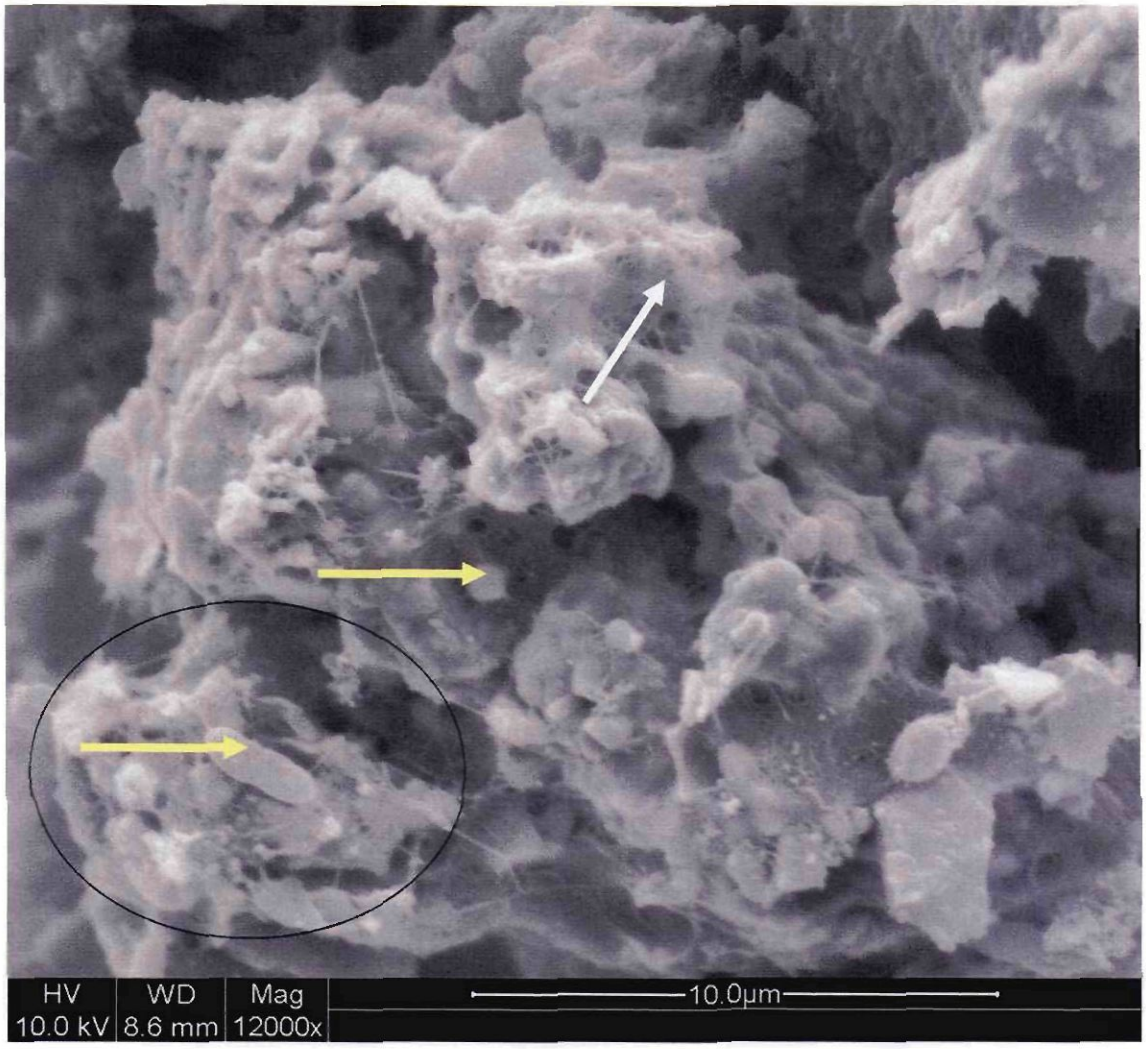


Figure 3.11- Enlargement of the biological growth on the last phase of the home water filtering system (12 000X magnification).

In Figures 3.11 and 3.12 better presentations of a developing biofilm are depicted. Biofilms represent complex colony structures (Figure 3.11; black circle) which are the result of bacterial cells attaching to a surface and forming a matrix. If one refers back to Figure 2.1 and compares the information with this micrograph in Figure 3.11 then a classical mixed species biofilm is represented in the latter, corresponding to stages 4 and 5 of the illustration in Figure 2.1.

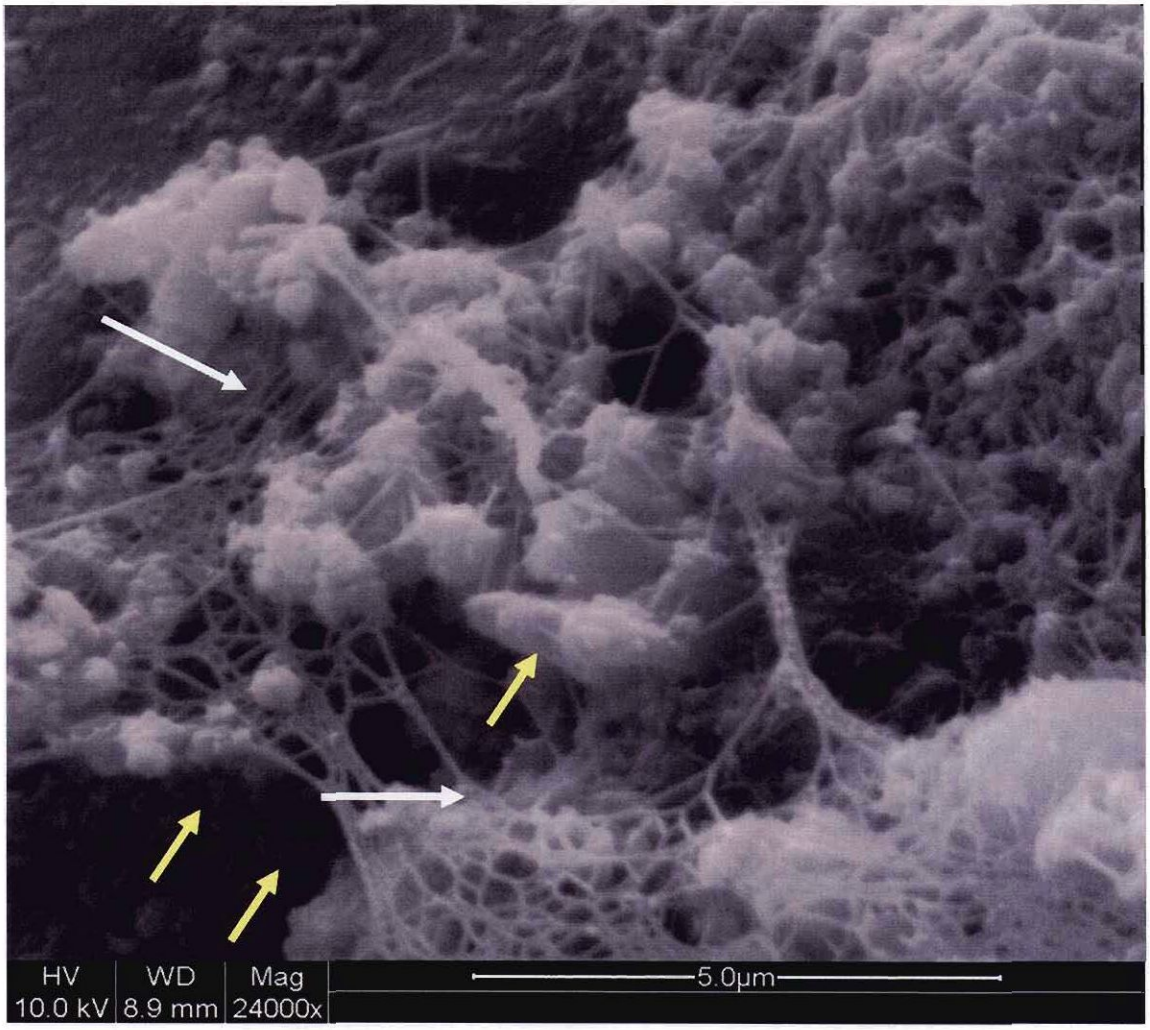


Figure 3.12- Enlargement of the biological growth on the last phase of the home water filtering system (24 000X magnification).

In this case the cells attached to the surface area of the activated carbon particles. Another important characteristic of biofilms is the EPS (extra-cellular polysaccharide) which can also observed in Figures 3.11 and 3.12. The EPS forms an adhesive matrix and is mainly responsible for the protection of the cells in such a biofilm (LeChevallier *et al.*, 1988b). Extra-cellular polysaccharides can also be responsible for communication among the cells through biochemical signals. This layer provides protection of the cells against antimicrobial substance which may include disinfectants such as chlorine

(Steward and Costerton, 2001). Classical EPS formation is evident in Figures 3.11 and 3.12.

3.3.5 Summary of results

In this study 12 heterotrophic morphological types were isolated. Nine of these were Gram-negative and identified by the API 20E system. They were identified as *Enterobacter agglomerans*, *Aeromonas hydrophilia*, *Citrobacter* spp. *Enterobacter cloacae* and *Providencia alcalifaciens*. The Gram-positive isolates were not identified. Eight of the morphological types showed pathogenic characteristics but only two were Gram-negative. These were *Enterobacter cloacae* and *Providencia alcalifaciens*. *Enterobacter cloacae* were detected in both home water filter systems. None of the isolates were motile but a large percentage of them were resistant to several antibiotics. Nine different antibiotic resistance profiles were observed among isolates from the reverse osmosis home water filter and the activated carbon home water filter. Two were from the activated carbon filter and seven from the RO filter.

At high magnification the SEM micrographs clearly indicated biological activity in the form of active biofilm. Evidence was provided that the biofilms occurred at various stages of development and that classical features of mature bioflims were present. Further more, at low magnification, the presence of large particles and diatoms were observed.

3.4 DISCUSSION

3.4.1 Isolation of heterotrophic bacteria from activated carbon and reverse osmosis home water filtering units

Both filters were sampled for bacteria and biofilm after two 6 month periods because it is the most efficient period of operation before the filters need to be replaced. The isolates on the RO home water filtering system were more diverse than the isolates on the activated carbon home water filtering system.

The plate culturing results were supported by the SEM results that demonstrated biological activities in the home water filtering systems. The highest level of biological activity was observed on phase three of the RO filtering unit, the activated carbon filter. This can be explained by the fact that in such granular filtration, the water passes through a filter consisting of a packed bed of granular materials. The microbes or microbe-associated particles are removed as they deposit on the filter medium (WHO, 2004). This last phase involves two steps and two main principles by which the contaminants are removed from water. Firstly the organic compounds are removed by adsorption and secondly residual disinfections e.g. chlorine, are removed by catalytic reduction.

Camper *et al.*, (1986) performed a study on associated bacteria on granular activated carbon particles in drinking water. Their scanning electron microscopy demonstrated micro-colonies of bacteria on the particle surfaces. These bacterial colonies were mostly observed in the cracks on the carbon surfaces. The finding of Camper *et al.*, (1986) was similar to the findings in the present study.

The presence of biofilms that formed in the water distribution system can have both a negative and positive effect. On the positive side bacteria occurring in the biofilm may remove natural organic matter (NOM) in the water where as the negative aspect is that biofilms can act as a reservoir of opportunistic pathogens (Szewzyk *et al.*, 2000).

3.4.2 Presence of Gram-negative bacilli

Gram-negative isolates included *Enterobacter agglomerans*, *Citrobacter* spp., *Aeromonas hydrophilia*, *Enterobacter cloacae* and *Providencia alcalifaciens*. These species have been isolated from drinking and source water samples in previous studies (LeChevallier *et al.*, 1983; Camper *et al.*, 1986; Nevondo and Cloete, 1999). Nevondo and Cloete (1999) performed a study on 5 different water sources in South-Africa and isolated organisms including *Aeromonas hydrophilia*, *Pseudomonas* spp., *Alcaligens* spp., *Klebsiella* spp., *Citrobacter* spp., *Enterobacter cloacae*, *Enterobacter agglomerans* and *E. coli*. *Aeromonas* spp was the most predominantly species found. In the present study *Aeromonas hydrophilia* was the less predominant isolated species.

a) Enterobacter spp.

Enterobacter agglomerans are commonly found in human and animal feaces, on plants and in water (Graham and Hodgkiss, 1967). The *E. agglomerans* isolates from his study showed no potential pathogenic characteristics although these organisms are well known for causing human infections. From as early as 1972, reports (Bottone and Schniereson, 1972) have been published about human infections caused by *Enterobacter agglomerans*. These species have been associated with arthritis infections including knee arthritis and septic mono-arthritis (Flatauer and Khan, 1978; Olenginski *et al.*, 1991; De Champs *et al.*, 2000). In a more recent study on infected infants deaths, Van Rostenberghe *et al.* (2006) found that in 7 out of 8 cases the cause of death was

infection by *Enterobacter* spp. from nutrition solutions. The level of *E. agglomerans* isolated in this study was considerably high which indicate a health risk to water consumers.

Furthermore, in the present study *Enterbacter clocaea* was isolated from both water filtering systems and these isolates demonstrated potential pathogenic features. This was the third most commonly isolated heterotrophic species. *Enterobacter clocaea* and *Enterobacter aerogenes* are clinically the most common species of the genus *Enterobacter* (Davin-Regli *et al.*, 1997). This bacterium had been associated with an outbreak of necrotizing enterocolitis in the neonatal intensive care unit of a provincial hospital in Gauteng, South Africa. The results of the investigation on this outbreak were reported by Van Nierop *et al.* (1998). In their study they demonstrated that isolates from *Enterobacter cloacae* from blood samples of patients were genotypically the same as *Enterobacter cloacae* isolates from 6 environmental samples. The detection of *Enterobacter cloacae* in the present study is a case of concern especially for immunocompromised water consumers. Although these bacteria were detected in the biofilms, it could pose a hazard if it becomes dislodged (especially if they show potential pathogenic characteristics) and land in the bulk water for immediate consumption.

b) Citrobacter spp.

Citrobacter species are commonly found in the environment and also in the human intestinal tract (Fisman and Kaye, 2000; Underwood, 2004). The most commonly isolated species is normally *C. freundii* (HPA, 2001). The isolates from this study did not show any potential pathogenic characteristics. *Citrobacter* spp. has been associated with diarrhoea and secondary infections in debilitated persons, infections of the urinary

tract, and infant meningitis. They also occasionally cause severe bloodstream infections (bacteraemia). In this study *Citrobacter* spp. was the second most commonly isolated bacteria species and this further increases health risk of water consumers, especially those with an impaired low immune system.

c) Providencia alcalifaciens

Prov. alcalifaciens was isolated only from the activated carbon filter. This species normally occurs in water, wastewater and groundwater (John and Soucek, 1996). Isolates of this bacterium in the present study had potential pathogenic characteristics. Several studies about the clinical effects of this bacterium have been reported since earlier years (Ridge and Thomas, 1955; Omland, 1960; Bhat *et al.*, 1971; Washington *et al.*, 1973). From these studies it is evident that this bacterium is associated with infections of the intestinal and urinary tract. It was also linked to sporadic cases of gastroenteritis, diarrhea and traveler's diarrhoea (Janda *et al.* 1998). Detection of these organisms in drinking water biofilm of Potchefstroom is a cause of concern.

d) Aeromonas hydrophilia

Aeromonas spp. is commonly associated with environmental waters (WHO, 2003). Detection of low levels of these organisms in the present study may not be a concern since only high levels of infectious agents are required for infection. However, *Aeromonas* spp. is known as common enteric pathogens (Duncan, 1998). Acute or chronic gastrointestinal illness, septicaemia in immunocompromised individuals and water associated wound infections are well known results from infections caused by *Aeromonas* spp. (Semel and Trenholme, 1990). Vally *et al.* (2004) reported on an outbreak of *Aeromonas hydrophilia* wound infections associated with mud football. The study was initiated after a total of 26 people were presented to the emergency

department of the local hospital in the rural town of Collie in south Western Australia. Many were infected with scratches and pustules spread over their bodies. They proposed that though the exposure of these patients to contaminated mud is likely to have been the source of infection, exposure of skin lesions to contaminated river water may also have played a role in this outbreak. In a more recent study it has been revealed that *Aeromonas* spp were frequently associated in cases of diarrhoea and household drinking water in HIV patients in rural communities in Limpopo Province (Obi *et al.*, 2007).

3.4.3 Unidentified Gram-positive Bacilli

In total 10.0% and 16.0% Gram-positive isolates were respectively isolated from the activated carbon and reverse osmosis filters. All Gram-positive isolates were *Bacillus* spp. which is generally known to produce spores that are resistant to unfavourable conditions such as environments with low levels of usable organic matter (WHO, 2006). The spores associated with bacilli also enhance resistance to disinfection processes. *Bacillus* spp. is classified into two subgroups *B. polymyxa, B. Subtilis* (including *B. cereus* and *B. licheniformis*), *B. brevis* and *B. anthracis.* According to the WHO, (2006), these bacteria (*Bacillus* spp.) are readily detected in most drinking water supplies. However, water has not been identified as a source of pathogenic forms of this species.

3.4.4 Levels and diversity

The levels and diversity of the isolates in the two filter systems that were studied here, varied considerably. This was expected since these were completely different systems in so far as construction, dimensions and operation were concerned. The systems were also

installed at considerable distances from the water purification plant. It is still worth commenting on the levels and diversity of bacteria isolated from these two systems.

Among the isolates from the RO system, *Enterobacter agglomerans* levels were the highest (39.4%), followed by *Enterobacter cloacae* (22.3%), *Citrobacter* spp. (7.45%). and *Aeromonas hydrophilia* (2.12%). On the other hand, from the activate carbon filter system *Prov. alcalifaciens* was the most predominantly isolated (50.0%) followed by *Enterobacter cloacae* which constituted 40.0% of the isolates. In a study by LeChevallier *et al.* (1983) *Enterobacter agglomerans* was the most common isolated facultative anaerobic coliforms and constituted 51.6% of the isolates from drinking, chlorinated surface and untreated drinking water. In this study *Enterobacter cloacae*, and *Citrobacter* spp. were also among the facultative anaerobic bacteria isolated. *Aeromonas hydrophilia* were among the aerobic bacteria isolated.

In the present study the levels of *Enterobacter agglomerans* were 12 times higher than *Enterobacter cloacae*. A study by Camper *et al.* (1986) showed that *Enterobacter cloacae* was isolated in higher levels than *Enterobacter agglomerans* from particles in finished drinking water. According to the Camper study *Enterobacter agglomerans*, *Enterobacter cloacae*, *Aeromonas hydrophilia* and *Alcaligenes* spp. are bacteria that are capable of surviving disinfection under conditions of 2mg of free residual chlorine per liter even 30 minutes after contact time. Thus if these species are present in drinking water biofilms there is a greater opportunity for them to survive. According to Momba *et al.* (2003) it has been established that for each planktonic bacterium detected, there might be roughly 1000 organisms present in a biofilm. This could explain the absence of these species in the planktonic phase of the water but the presence in the biofilms.

3.4.5 Antibiotic resistance and potential pathogenic characteristics of isolates

The 2004 WHO Expert Committee on HPC bacteria in drinking water (WHO, 2004), indicated that insufficient data are available to demonstrate that heterotrophic bacteria present in drinking water may pose health risks. The results from this study are providing support for the opposite. In sub-Saharan countries such as South Africa where the HIV rate is considerably high, it could become problematic if water is supplied to consumers, containing HPC bacteria that may be opportunistic pathogens and particularly if these organisms are also resistant to several antibiotics. Based on haemolysis data, results from this study indicated that thirty three percent of isolates displayed potential pathogenic characteristics. Furthermore, a considerable number of the isolates were also resistant to multiple antibiotics. A combination of MAR and potential pathogenic characteristics may have dramatic consequences for the consumers, when conditions are conducive.

Enterobacter spp. from this study, isolated from both water filters, were all resistant to more than one antibiotic. In their study, Chollet *et al.*, (2002) also found that *Enterobacter cloacae* and *Enterobacter agglomerans* are generally resistant to more than one antibiotic. In the case of the present study *Enterobacter agglomerans* isolates were resistant to five of the ten tested antibiotics. Other opportunistic pathogens such as *Aeromonas hydrophilia*, *Citrobacter* spp. and *Providencia alcalifaciens* were also resistant to more than one antibiotic. Isolates of the former two species were resistant to 4 antibiotics. Of the Gram-positive isolates that were generally resistant to more than one antibiotic, 75.0 % had potential pathogenic features. Some were resistant to between 5 and 8 of the 10 tested antibiotics. The antibiotics included β-lactam and vancomycin as well as several from the various antibiotic groups including macrolides, aminoglycosides, tetracyclines and chloramphenicols.

In previous studies genera that were isolated from mineral water and rivers that showed resistance to a large number of antibiotics were *Pseudomonas*, *Alcaligenes*, *Acinetobacter*, *Citrobacter*, *Enterobacter* and *Serratia* (Ash *et al.*, 2002; Messi *et al.*, 2005). In a study by Mudryk (2002), it was also found that most of the bacterial species were resistant to between 4 and 6 antibiotics. The findings of these studies are thus similar to findings of the present study, in which isolates were resistant to several antibiotics. Isolates from the present study, however, occurred in water biofilms, where they existed in such close proximity (Figures 3.11 and 3.12). This may have provided the opportunity for transfer genetic elements responsible for antibiotic resistance amongst species (Nies, 1999).

Antibiotic resistant organisms that also have pathogenic features have been considered a problem for a long time (Cassel, 1997; Allen *et al.*, 2004). Some of the species in this study, isolated from the biofilms of water filter systems, are well known opportunistic pathogens and were resistant to several of the antibiotics commonly used in treatment of human infections. This observation may be cause for concern if such species would also occur in the biofilm of the general drinking water distribution system, particularly if such species become dislodged and are dispersed through the distribution system.

3.5 SUMMARY AND CONCLUSION

The aim of this part of the study was to investigate the diversity and characteristics of HPC bacteria isolated from home water filtering systems. These systems were point of use systems and inserted in the drinking water distribution system of Potchefstroom.

A total of 144 HPC were isolated from 2 different home water filtering systems using microbiological plating methods. Reasons for the variability in levels and diversity of

isolates form the two systems were discussed. Implications of the presence of some of the isolated species, particularly potential opportunistic pathogenic bacteria were also discussed, using relevant literature. The study indicated that HPC bacteria present in our drinking water may be retained by home water filtering systems and from biofilms. Although their origin remains unknown, the characteristic of such bacteria indicated potential risk to water consumers in general but the immuno-suppressed and immuno-compromised in particular. This is because of pathogenic features of these isolates that are coupled to multiple antibiotic resistance. Antibiotics to which isolates were resistant to included ones generally used for treatment of human infections such as ampicillin, penicillin G, chloramphenicol, erythromycin, gentamycin, kanamycin, neomycin, tetracycline and vancomycin.

Scanning electron microscopy studies showed typical biofilm structures on the various components of the RO filter units. The proximity of the various bacterial species in such biofilms was demonstrated. The implications and role of such biofilms in dissemination of mobile genetic elements, particularly those involved in transfer of pathogenic and antibiotic resistance features, were considered.

CHAPTER 4

DIVERSITY AND CHARACTERISTICS OF HETEROTROPHIC BACTERIA IN A WATER DISTRIBUTION SYSTEM

4.1. INTRODUCTION

The distribution of good quality potable water is very important and has social and economic implications (Momba and Makala, 2004). A distribution system could stretch over a few hundred kilometers, depending on the size of the city or town. Damage or vandalism to the distribution pipes, could contribute to (direct or indirect) bacterial contamination of the potable water (Geldreich and LeChevallier, 1999; Vitanage *et al.*, 2004). The maintenance of the distribution system is thus important to prevent any contamination of the water in the distribution system (Vitanage *et al.*, 2004; Vreeburg and Boxall, 2007).

For various reasons the quality of treated water may change during distribution (Momba and Makala, 2004). It is thus important to understand which factors influence bacterial regrowth in distribution systems and to monitor and manage these. When the water leaves the purification plant, it is normally free from pathogenic bacteria (Vitanage *et al.*, 2004). However, the responsibility of the water supply agency does not stop here. These agencies still have responsibility to prevent any possible contamination which could lead to degradation of the water quality as well as potential public health risks (Vitanage *et al.*, 2004; Vreeburg and Boxall, 2007).

Several studies were performed over the years to investigate the occurrence and effects of biofilms in water distribution systems (LeChevallier, 1990a, b; Momba *et al.*, 1998, 1999, 2002; Schwartz *et al.*, 2003; Momba and Makala, 2004; Codony *et al.*, 2005;

Morton *et al.*, 2005). In preceding chapters an overview of the implications of such biofilms in drinking water systems were provided.

4.1.1 Available energy sources in the distribution system

The survival of heterotrophic bacteria that form biofilms in the distribution system causes several water quality problems including off-tastes, odors and colored water problems (LeChevallier *et al.* 1996). Assimilable organic carbon (AOC) levels in the water distribution system may contribute to increased levels of heterotrophic bacteria proliferation (LeChevallier *et al.* 1996). Many bacterial species may survive in disinfected drinking water by creating environments that protect themselves from the disinfectants such as chlorine (Morton *et al.*, 2005). These species also rely on available energy sources other than AOC. In the study by Morton *et al.* (2005), the implications of nutrient released from iron metal sources were investigated. These authors suggested that corroding steel may serve as a source of fixed carbon, nitrogen and phosphorus for microorganisms. All these elements are essential for bacterial regrowth. An observation made by Morton *et al.* (2005) was that the levels of phosphorus released from the corroding iron were corresponding to the levels of phosphorus needed to sustain sufficient development of bacterial biofilm in a distribution system.

4.1.2 Disinfection efficiency

The disinfectant type and level have an effect on the level of biological activity in a drinking water distribution system (LeChevallier *et al.*, 1996). An investigation on the disinfection efficiency of free chlorine and monochlorine in a model distribution system was performed by LeChevallier *et al.* (1990a). In this study it was demonstrated that the composition of pipe material has a major influence on disinfection efficiency and that the accumulation of corrosion products on iron pipes interfered with free chlorine

disinfection. Gagnon *et al.* (2005) also demonstrated the efficiency if disinfectant in relation to the type of pipe material used. The two studies mentioned here and others such as those by Momba and Makala (2004) suggested that although a number of factors may contribute to bacterial growth in distribution systems that higher levels of HPC bacteria could also be attributed to the impact of pipe material on the disinfection efficiency.

4.1.3 Corrosion control

Another aspect that is of great concern during water distribution is corrosion (Zhang *et al.*, 2007). Corrosion can be either internal or external. Internal corrosion slows the water flow and therefore provides a more protective surface area that could favour the formation and growth of biofilms (WHO, 2004). When internal and/or external corrosion occur it often causes leaks and bursts which could contribute to some level of contamination of treated water (LeChevallier *et al.*, 1993; WHO, 2004; Zhang *et al.*, 2007). It is therefore important that the corrosion potential of the water with respect to distribution system materials should be controlled and monitored (Zhang *et al.*, 2007). The most common biofilm organisms that are associated with such problems are *Actinomycetes, Streptomyces, Nocardia* and *Arhrobacter* (Geldreich, 1990).

Dislodging of corrosion material could also lead to discolouration of the drinking water and would lead to serious complaints by water consumers (Vreeburg and Boxall, 2007). Furthermore, corrosion scales also impact negatively on the efficiency of disinfection (Zhang *et al.*, 2007).

4.1.4 Construction materials

Eleven types of pipe materials are generally used in distribution systems in South-Africa (Momba and Makala, 2004). These include: PVC (25.0%), asbestos cement (21.0%), asbestos (19.0%), unplasticised polyvinylchloride (16.0%), steels (8.0%), cement (4.0%), bitumen coated (3.0%), high density polyethylene (2.0%), copper (1.0%), galvanized mild steel (1.0%), mortor lined steel (1.0%) and cast iron (1.0%) (Momba and Makala, 2004). The survival and multiplication of heterotrophic bacteria on selected pipe materials was investigated by Momba and Makala, (2004), and it was found that bacterial colonization was observed on all tested pipes. The plastic based pipe materials indicated a higher mean of HPC bacteria than the cement based pipe materials and it was also suggested by these investigators that cement and asbestos cement pipes are a better choice for the distribution of chlorine-monochloramine treated water.

Copper is well known for its antimicrobial effect (Nies, 1999). The combination of this and the potential corrosion resistance made copper probably one of the best main piping materials.(http://www.allbusiness.com/construction/construction-buildings-residential/4021027-1.html). Several recent studies were concerned with the interface between microbiological and chemical processes on the surface of copper pipes and the influence of water characteristics on these (Lehtola *et al.*, 2004; Li *et al.*, 2004; Li *et al.*, 2007; Reyes *et al.*, 2007; Zhang *et al.*, 2007). A study by Li *et al.* (2004) demonstrated the impact of organic material on the corrosion potential of copper. They demonstrated that in soft water, organic substance that simulated extra-cellular polymeric substances (EPS), affected corrosion significantly. Lehtola *et al.* (2004) compared the chemistry and microbiology of copper and plastic pipes. They found that biofilm formation was slower on copper pipes compared to plastic ones during the first 200 days. However,

after this period no differences were observed in terms of biofilm and bacterial levels. In this study (Lehtola *et al.*, 2004) it was also demonstrated that pipe material influenced the bacterial community (particularly Gram-negative) structure. More recently Li *et al.* (2007) demonstrated that copper and its corrosion products impact on the formation and distribution of haloacetic acids. Reyes *et al.* (2007) investigated the influence of microbial biofilms on microbiological induced corrosion in copper pipes of rural Chilean houses. Their study showed that certain waters types (pH below 6.5) accelerated corrosion.

4.1.5 Operational characteristics

Operational conditions within the distribution system may also have an influence on the level of biological activity (LeChevallier *et al.*, 1996). Parameters such as the transit time, system condition, hydraulic conditions and initial physical, chemical and microbial characteristics of the treated water influence the level of microbiological activity in a distribution system. Chemical, physical and biological characteristics include parameters such as pH, oxygen levels, temperature, and levels of available nutrient (Vitanage *et al.*, 2004). Water temperature is a very important factor that contributes to the occurrence of microbial activity in drinking water. An increase in the water temperature can directly be associated with an increase in biological activity. In drinking water distribution systems, microbiological activity can be observed above 15°C (LeChevallier *et al.*, 1996).

Bacterial proliferation can be reduced by minimizing the particles leaving the treatment plant, the amount of particulate, colloidal and dissolved iron, manganese and aluminium compounds and the factors that cause increased use of the residual disinfectant (WHO, 2004). In the preceding chapter the micrographs (Figures 3.5 to 3.8) demonstrated that

the levels of particulate matter in Potchefstroom drinking water distribution are considerably high.

4.1.6 Microorganisms entering the distribution system

The presence of microorganisms in the distribution can be explained in several ways. Firstly, Sources of contamination are directly associated with the construction or repair of water mains. During main breaks and repairs or when new mains are installed, microorganisms will unavoidably enter the distribution system (Kirmeyer *et al.*, 2001). Secondly, there is survival and regrowth of bacteria after the treatment process. This aftergrowth could result in biofilm formation in the distribution mains and eventually result in the degradation of the bacteriological quality of potable water (Nagy and Olsen, 1985; Kirmeyer *et al.*, 2001). To ensure water of high quality and free from any possible health related risks, it is thus an important part of water distribution management to monitor bacterial or any other form contamination. The former aspect should not be neglected.

4.1.7 Cleaning and maintenance water mains

The inspection and cleaning of the water distribution pipes should be frequent. However, economical considerations and inconsistent results from generally monitoring processes may indicate the need for cleaning regimes to be instituted (Vreeburg and Boxall, 2007).

It is important that any visible defects are reported to authorities to avoid any possible contamination. Once a biofilm is formed in the distribution system is very difficult to remove and numerous consequences of biofilms in the distribution may be initiated (Vreeburg and Boxall, 2007). In a study by Clark *et al.* (1998), contamination of the

water distribution system rather than poorly treated water, was responsible for twenty four percent of waterborne diseases. Vitanage *et al.* (2004) as well as Vreeburg and Boxall (2007) deals with the implications of maintenance of the distribution systems on the quality of drinking water.

Repairing or replacement of a water distribution system can have a dramatic economical effect. In 1997 it was estimated by the U.S Environmental Protection Agency (EPA), that such rehabilitation could cost around $138 billion (US EPA, 1997).

4.1.8 Studies of biofilms *in situ* in water distribution systems

Specially designed devices and adaptation of water pipes are frequently used to study biofilms *in situ* (LeChevallier *et al.*, 1990a; Schwartz *et al.*, 2003; Emtiazi *et al.* 2004; Martiny *et al.*, 2005). These devices are specially designed for easy access and investigation of the distribution system. They are also useful for monitoring impacts of fluctuating operating conditions. In this way several aspects of concern such monitoring levels and diversity of bacteria in biofilms, corrosion potential as well as corrosion events can be monitored and studied. Such devices have also been employed to investigate the disinfection efficiency of free chlorine and monochloramine (LeChevallier *et al.*, 1990a, b) as well as distribution of disinfectant byproducts (Li *et al.*, 2007). Schwartz *et al.* (2003) and Emtiazi *et al.* (2004) used configurations of Robbins devices to study effects of water quality parameters on bacterial growth and biofilms formation in the distribution system.

4.1.9 Aim and objectives

The aim of this study was to investigate the characteristics of HPC bacteria isolated from red copper discs from a biofilm device that were installed directly into the main

distribution system of an academic building (J.S van der Merwe building) at the North-West University, Potchefstroom Campus.

The objectives were to:

- monitor the physico-chemical characteristics of the drinking water in the J.S van der Merwe building, NWU.
- isolate and identify HPC bacteria from the red copper discs that were exposed to bulk drinking water.
- determine the levels and diversity of these HPC isolated from the copper discs.
- use scanning electron microscopy (SEM) to study the biofilm structure on the copper disc.
- determine the antibiotic resistance pattern and pathogenicity potential of isolates.
- determine copper minimum inhibitory concentration (MIC) of selected isolates.

4.2. MATERIALS AND METHODS

4.2.1. Biofilm sampling

This study was conducted from July to October 2006. A biofilm device (Figure 4.1) was designed, constructed and inserted into the main water supply of the J.S Van der Merwe building at the North-West University, Potchefstroom. The construction of the biofilm device is detailed in Figure 4.1. The 6 metal discs (15 X 20 mm) were placed horizontal in the device. Water flow direction was controlled by three main valves (A, B and C). During the period of operation valve A was closed and B and C opened. This allowed for an actual water flow scenario. Sampling was at 4, 6 and 8 weeks of operation.

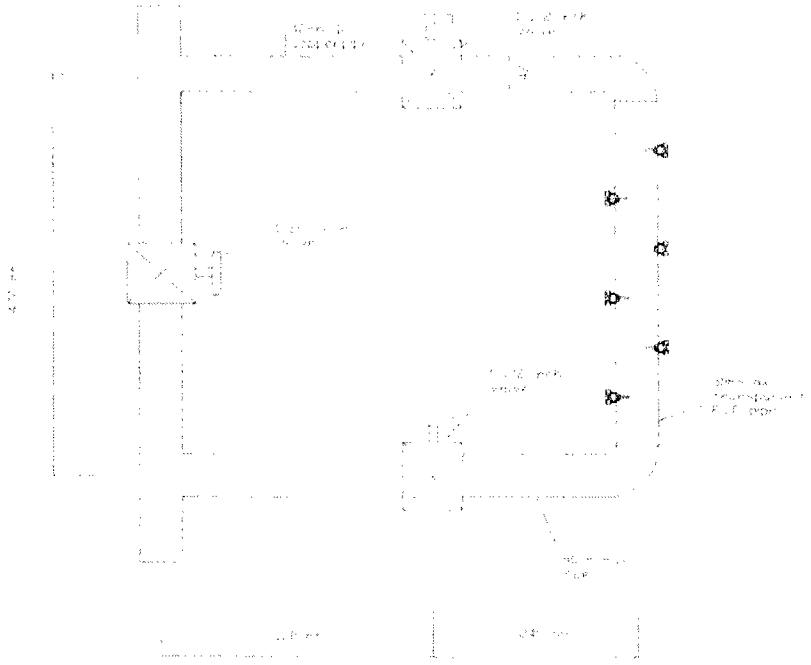


Figure 4.1- Schematic representation of the design of the biofilm device.

The first set of six metal discs that were inserted consisted of 2 galvanized, 2 yellow copper and 2 red copper ones. They were exposed to the drinking water for 4 weeks. In the second experiment only red copper discs (6) were used. Three of these removed

after 6 weeks. The rest of the red copper discs were exposed to drinking water for further 2 weeks.

4.2.2 Determination of physico-chemical parameters of drinking water

The pH, temperature, salinity and TDS were measured twice a week over an 8 week period. A calibrated combined conductivity and dissolved oxygen sensor (ConOx) supplied by WTW Measurement Systems (Germany) was used to measure these parameters.

4.2.3 Preliminary identification and characterization of isolates

Sterile swabs were used to transfer the surface material from the copper discs to nutrient agar. The plates were incubated at 37°C (high humidity) for 7 to 11 days. For preliminary identification and characterization, the following tests were performed; Gram staining, TSI agar, API 20E, motility, hemolysis, antibiotic resistance and scanning electron microscopy. See Sections 3.2.2 to 3.3.8 for the details of these methods.

4.2.4 Molecular identification of isolates

(i) DNA extraction

For the extraction of DNA, the peq GOLD Bacterial DNA kit (PEQLAB Biotechnology, Germany) was used according to the protocol of the manufacturer. This method combines physical, chemical, enzyme and silica gel column procedures. For the determining the quality and quantification both UV and electrophoresis (See Section 4.2.4 (iii)) methods were used. For the UV procedures, 1:100 dilution were prepared and absorbance measured at 260 and 280 nm. The former was used to calculated the DNA concentration using the standard $1A_{260} = 50$ µg/ml (Zhou *et al.*, 1996). To confirm

successful DNA extraction, the extracts were subjected to electrophoresis on a 1.0 % ($^{w}/_{v}$) agarose gel and stained with ethidium bromide.

(ii) PCR (Polymerase Chain Reaction)

Individual 16S ribosomal DNA fragments were amplified using the universal eubacterial primer combination GM5F (5'-CCT ACG GGA GGC AGC AG-3') and 907R (5'-CCG TCA ATT CCT TTG AGT TT-3') (Muyzer *et al.*, 1993). The primers were synthesised by Inqaba Biotech (South Africa). Each 25µl reaction contained 1X PCR mastermix (Fermentas,US, supplied by Inqaba Biotec, South Africa; 1 unit of *Taq* polymerase, dNTPs and 1.5 M of Mg_2Cl) appropriate volume of PCR quality water and 50 pmol primer mix. Additionally 50 ng of BSA was added to the 25µl PCR mixture. One hundred nanogram of template DNA was used in each reaction. PCR was performed with the I-Cycler from Bio-Rad (UK). PCR conditions for each of the 35 cycles consisted of an annealing temperature of 55°C for 30 seconds, primer extension temperature at 72°C for 1 minute and denaturing at 95°C for 30 seconds. An additional initial denaturation step of 95°C for 5 minutes and a final extension of 72°C for 5 minutes was included.

(iii) Electrophoresis

Electrophoresis was conducted in a wide mini-sub cell GT electrophoresis system (Bio-Rad, UK) for 45 minutes at 80 V, using 1 x TAE (40mM Tris, 1mM EDTA and 20mM glacial acetic acid, pH 8.0) as electrophoresis buffer. A Gene Genius Bio Imaging System (Syngene Synoptics, UK) was used to capture the image using GeneSnap (version 6.00.22) software. Each gel contained a 100 base pair DNA molecular weight standard (Fermentas, supplied by Inqaba biotech, South Africa). The image was

analysed using GeneTools (version 3.00.22) software (Syngene, Synoptics, UK) to determine the size of the bands in each lane.

4.2.5 Minimum inhibitory concentration (MIC) for Copper

Representatives of all isolate types were selected and used to determine the MIC of these isolates to copper. Fresh overnight liquid cultures (10 µl) were inoculated into 100 µl of nutrient broth containing appropriate concentrations of copper. The copper concentrations ranged from 0.01 mM to 5 mM. Microdilution experiments (total volume 150 µl) were performed in 96 well plates. Incubation was at 30°C and A_{600} readings were taken a time 0 hours, 24 hours, 48 hours and 106 hours. A Power Woxex multichannel spectrophotometer (Bio-Tek, SA) was used to measure the absorbance. See Appendix A for detailed experimental set-up. Appropriate blanks and controls were also used. Experiments were done in triplicate. The averages were then calculated. Averages were then used to draw the graphs. See Appendix A for the raw data

4.2.6 Statistical analysis

Where appropriate average and standard deviations were determined using Microsoft Excel. In other appropriate cases STATISTICA 7.0 (StatSoft, US) was used to analyze the results.

4.3 RESULTS

In the preliminary investigation that was over a 4 week period (July 2006) the levels of bacteria on the red copper discs were higher than on the galvanised steel and yellow copper discs (also Figures 4.4 to 4.6). Since copper pipes are generally used in plumbing in houses and buildings and also the observation that higher levels of HPC bacteria were detected on the red copper discs in this study it was decided to continue with the next part of the experiment using only copper discs.

4.3.1 Physico-chemical parameters of the drinking water of the J.S van der Merwe building

In this part of the study the water pH, temperature, salinity, conductivity and TDS were measured twice weekly, over an eight week period, and the averages of the results summarized in Table 4.1. The pH of tap water was constantly above 8. It ranged between 8.06 and 8.31 during the 8 week period. The lowest temperature during the study period was 18°C (week 2) where as the highest was 24°C. The TDS value of the water over an 8 week period was between 641mg/l and 764 mg/l. (See Section 4.4 for a more detailed discussion).

Table 4.1- Physical characteristics of the Potchefstroom drinking water as measured in the JS van der Merwe building.

	Average pH	Average Temperature (°C)	Average TDS (mg/l)
Week 1	8.08	18.75	682
Week 2	8.06	18.00	688
Week 3	8.07	20.80	681
Week 4	8.07	21.50	685
Week 5	8.31	19.05	690
Week 6	8.21	20.70	690
Week 7	8.10	22.95	764
Week 8	8.19	24.05	641

4.3.2 Isolation and preliminary identification of isolates

The isolates from the biofilm apparatus were roughly divided into different morphotypes based on colony morphology and pigmentation. This was similar to the procedure used in Chapter 3. Characteristics of the isolates from the biofilm apparatus are summarized in Table 4.2. The first two morphotypes (LG104 and OR104) listed in Table 4.2 are Gram-positive and were from the first four weeks (preliminary part) of the study. These were isolated from the red copper discs.

Table 4.2- Summarized colony morphology and characteristics of the isolates from a biofilm that formed in a biofilm apparatus. The levels of the various morphotypes are also provided.

Isolate name	Colony Morphology	Pigmentation	Gram Staining	Capsule Staining	Motility	Number of isolates (%)
LG104	Medium, smooth, Irregular and dull.	Light Yellow	+	-	-	4(33.3)
OR104	Medium, smooth, round and dull, raised	A very light orange to yellow	+	-	-	8(66.7)
VWO6	Medium, round, dull, smooth	white	+	-		116 (44.5)
HG06	Small, round, smooth,	Bright Yellow	_	-	-	123 (47.5)
WO6	Small, round, smooth	None	-	-	-	5(1.9)
OR06	Medium, smooth, round, dull raised	Orange	+	-	-	15(5.8)
HG 08	Small, round, smooth	Bright Yellow	-	-	-	110 (100)

A total of 381 original colonies were observed over the entire operational and sampling period and representatives isolated and purified by sub-culturing. The different levels of each isolate are also indicated in Table 4.2. According to the results (Table 4.1), 62.47%

of isolates were Gram-negative and 37.53% were Gram-positive. During the first 6 weeks 5 morphotypes were isolated. Orange pigmented isolates were isolated during both sample periods. However, after an 8 week period only one morphotype that were bright yellow pigmented, was observed. This morphotype was also observed during the 6 week samples. Although this study was conducted over a very short period the plate count results of HPC bacteria demonstrate potential pioneer population dynamics.

4.3.3 Identification by TSI and API 20E

Triple sugar iron test and API 20E is normally used for the identification of enteric bacteria. Based on colony morphology and coloration, 3 Gram-negative bacterial types were isolated. One of the Gram-negative bacilli, the bright yellow morphotype, was identified by both TSI and API 20E systems as *Enterobacter* species. The detailed results are shown in Appendix A. The orange isolates (OR104 and OR06) and the white isolates (W06) were identified by sequence data of a16S rRNA gene fragment. The orange ones were identified as *Exiguobacterium* spp. and the white ones as *Pseudomonas* spp. The details of the molecular identification are provided in Appendix A.

Table 4.3- Identification of the isolates from the biofilm apparatus unit. Haemolysis, and antibiotic resistance patterns as well as the MICs of the isolates are also indicated.

Isolate	Identification				
	API 20E	TSI	Haemolysis	Antibiotic Patterns	MIC(mM)
LG104	Not determined	Not determined	β	-	> 5
OR104	Not determined	Not determined	β	T	?
VWO6	Not determined	Not determined	-	PG-AP-T	>5
HG 06	*Enterobacter cloacae*	*Shigella or Serratia*	-	-	>5
W O6	Questionable	Questionable	-	T	<3
OR 06	Not determined	Not determined	α & β	PG-AP-T-VA	>5
HG 08	*Enterobacter cloacae*	*Shigella or Serratia*	-	-	<0.75

None of the Gram-negative isolates showed haemolytic features. On the other hand, some of the Gram-positive representative isolates showed haemolysis (potential pathogenic features; Table 4.3).

4.3.4. Antibiotic resistance patterns

Among the isolates from the preliminary study (four week old biofilm) only the OR104 isolates were resistant to tetracycline (Appendix A). The rest of the isolates from this part of the study were susceptible to all the antibiotics used.

The isolates from the second part of the study, the discs that were exposed for 6 weeks, were resistant to certain antibiotics. The antibiotic susceptibility data of the isolates from the 6 week biofilm are presented in Figure 4.2. Thirty three percent of these isolates were resistant to tetracycline and 50.6% were resistant to ampicillin. All Gram-positive isolates were resistant to penicillin G and 11.45% to vancomycin. The HG 08 isolates were from the week 8 sampling period and were not resistant to any of the tested antibiotics.

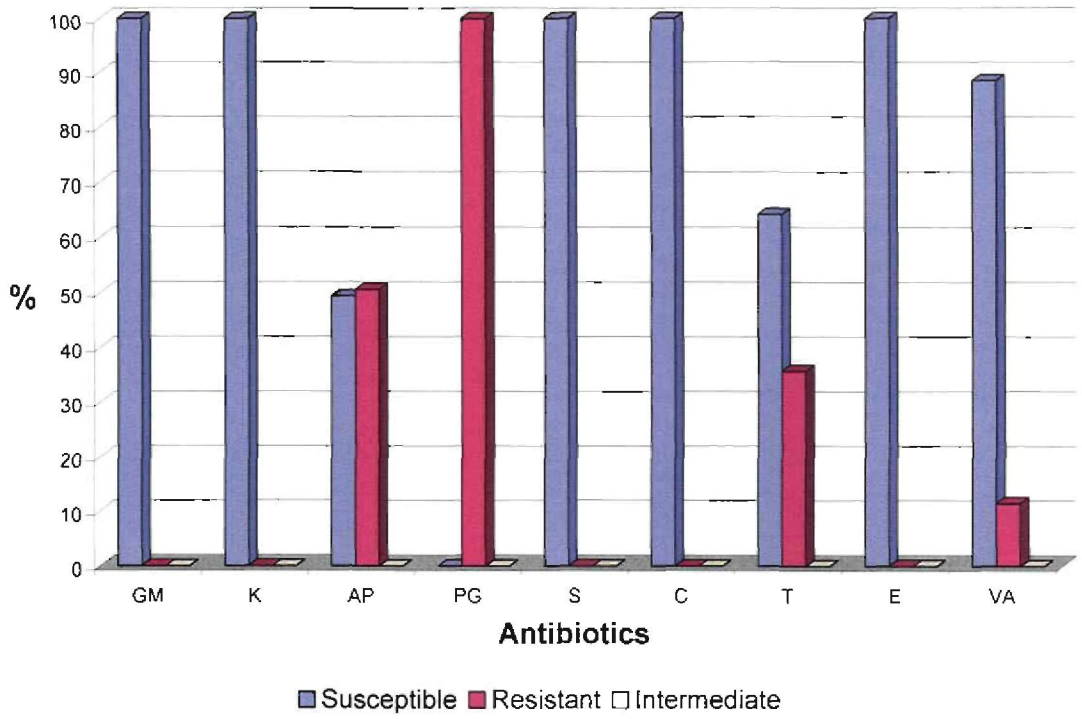


Figure 4.2- Percentages of isolates from the red copper discs, after 6 week exposure to drinking water that was resistant, susceptible and intermediate resistant to all tested antibiotics.

Table 4.2 shows the phenotypic antibiotic resistance profiles of the isolates from the biofilm device. Two different patterns **IX** (PG-AP-T), and **X** (PG-AP-T-VA) were observed among the Gram-positive isolates. The *Exiguobacterium* spp displayed profile **X** (PG-AP-T-VA) and the isolates identified as *Pseudomonas* spp. isolates were resistant to only tetracycline.

4.3.5 Minimum inhibitory concentrations (MIC) of isolates.

The detailed MIC results for the representative isolates are shown in Appendix A. According to the results, four of the isolates (LG04, VW06, HG06 and OR06) had a copper MIC above 5mM. The lowest observed MIC was smaller than 0.75mM and that

was for the bright yellow isolates (HG08) isolated during the 8 week sampling (Table 4.2).

4.3.6 Scanning electron microscopy (SEM)

Figures 4.3 to 4.6 represent the SEM micrographs of the preliminary experiment. The 3 different metal discs were exposed to Potchefstroom drinking water. Some biological activity on the galvanized steel discs is indicated in Figure 4.3. Crystals (potentially corrosion products) were also observed on these discs. Bacteria were present on the surface and are indicated by the yellow arrows. Extra-cellular polymeric substances (EPS) are indicated by white arrows. These observations could be proof of biological activity (corrosion) on these discs when they were exposed to the drinking water for 4 weeks.

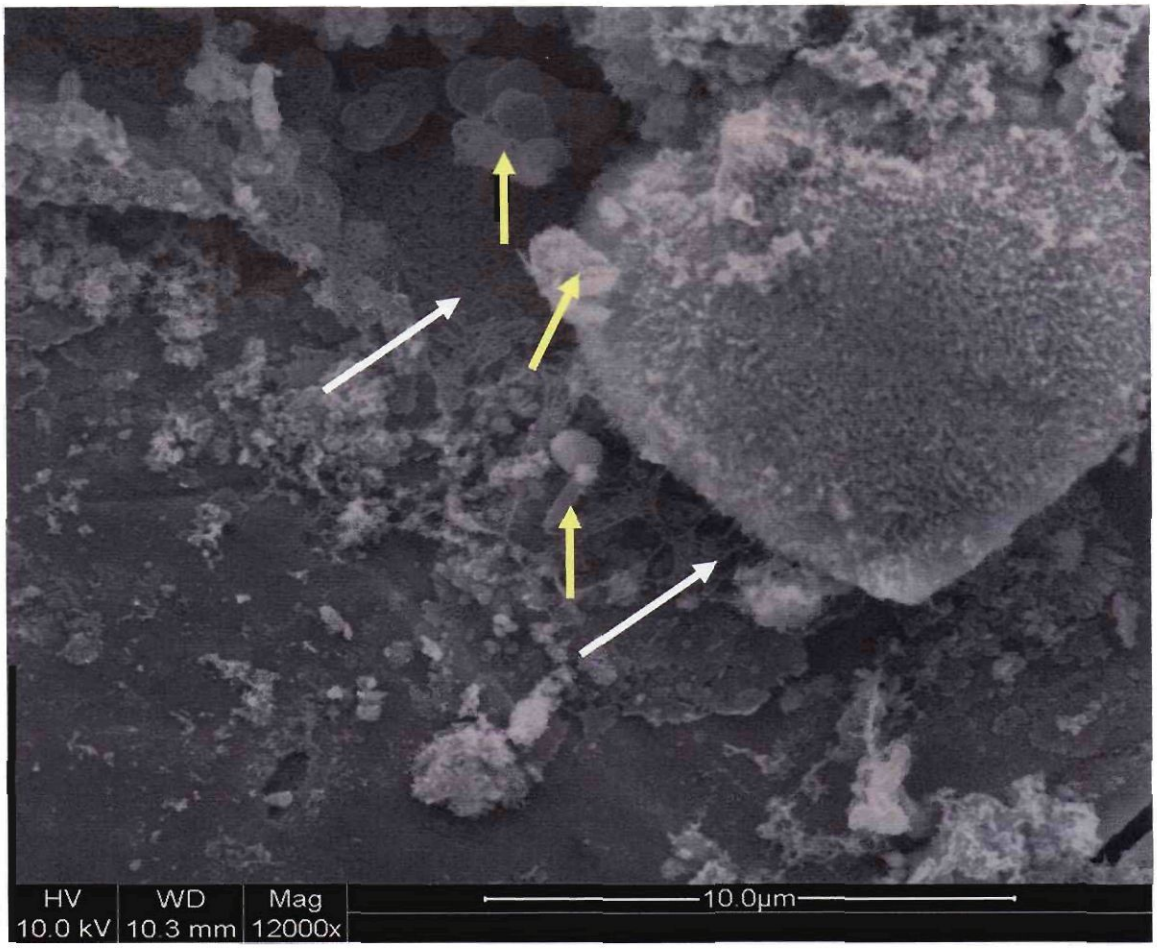


Figure 4.3- A SEM micrograph of a galvanized disc from the biofilm apparatus (12 000X magnification).

Figure 4.4 is a micrograph of the surface of a red copper disc of a 4 week biofilm. From the micrograph it is evident that bacilli started to form a uniform layer. The surface of disc appears to be in the process of being corroded.

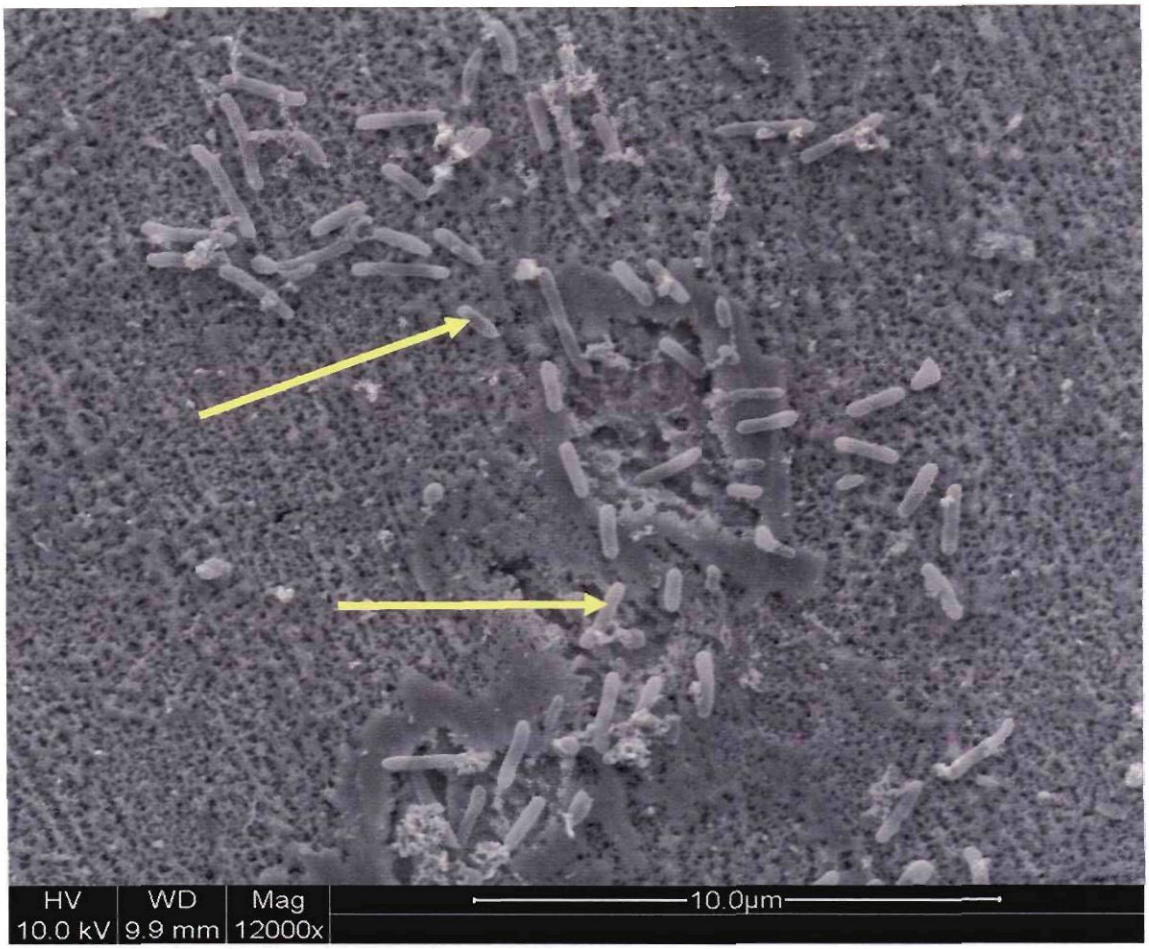


Figure 4.4- A SEM micrograph of a red copper disc from the biofilm device (12 000X magnification).

Biological activity on the yellow copper discs was less obvious than the other discs (Figure 4.5). Large crevices in the surface (green arrows) of these discs were also observed. There very few bacilli-like structures.

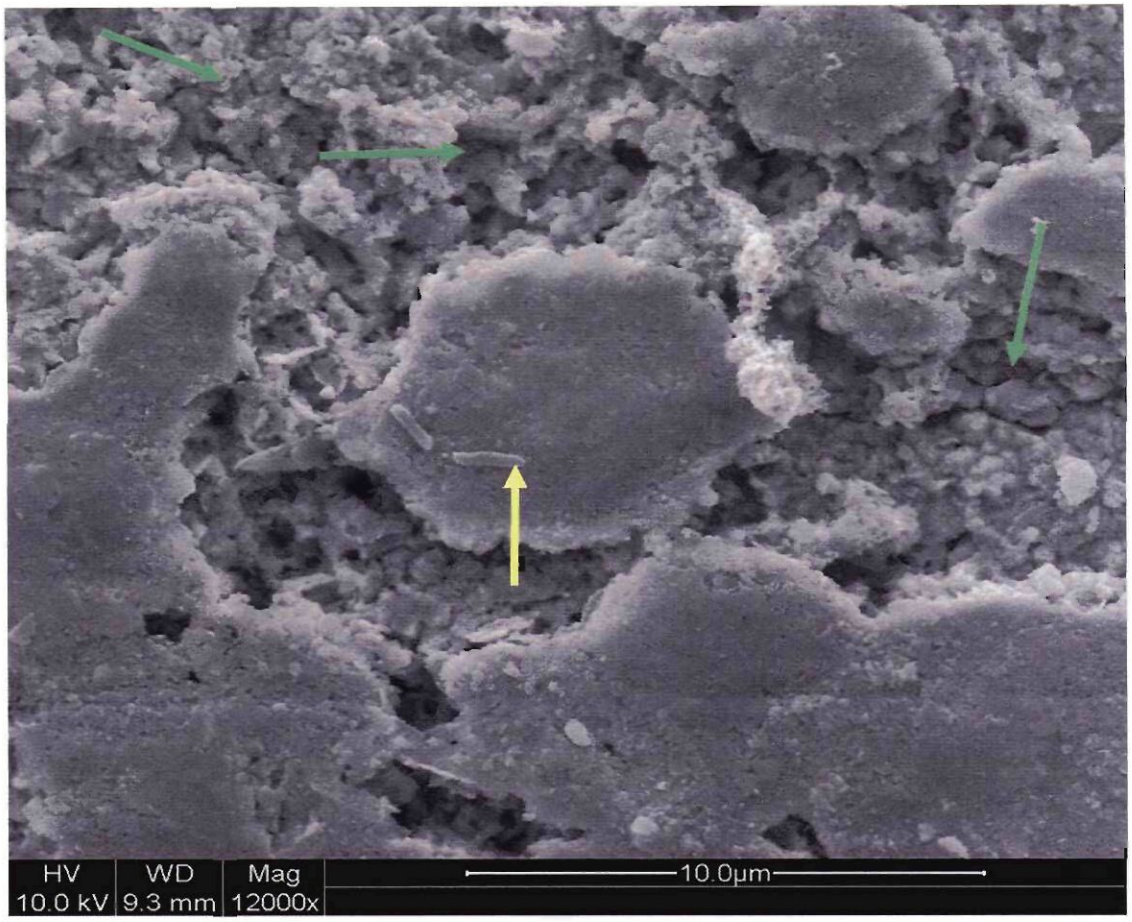


Figure 4.5- A SEM micrograph of a yellow copper disc from the biofilm device (12 000X magnification).

After the initial 4 week preliminary experiment, in which metal discs were exposed to drinking water, an experiment over 8 weeks was conducted. This was from mid August to mid October 2006. The SEM results are depicted in Figures 4.6 to 4.9.

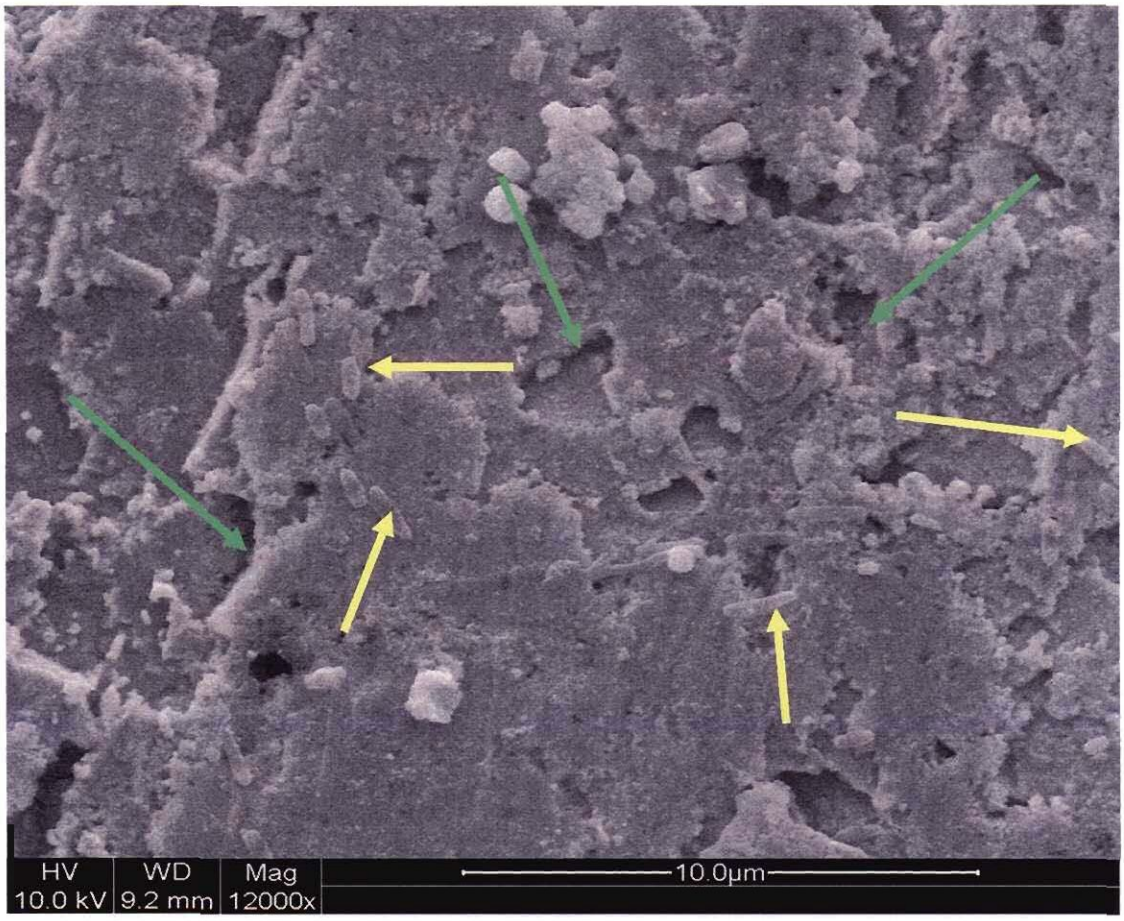


Figure 4.6- SEM micrograph of a red copper disc from the biofilm device after 6 week of exposure to drinking water (12 000X magnification).

In Figure 4.6 the corrosion of the red copper discs is visible when one compares this micrograph to the one in Figure 4.4. The surface area of this disc is very rough and uneven. It would normally be expected that the rough surface area and crevices (Figure 4.6; green arrows) would be the ideal environment for microorganisms to colonize because they are not exposed to the strong direct water flow. Few bacilli were present on the surface (Figure 4.6; yellow arrows).

The image in Figure 4.7 also indicates the biological activity on a red copper disc after a period of 6 weeks of exposure to drinking water. Magnification in this case was 20 000X. Once again the bacterial growth was confined to single bacteria attached to the

disc surface (Figure 4.7; yellow arrows). The bacterial attachment that was observed was mainly on the rough areas of the discs. Signs of corrosion was clearly visible (Figure 4.7; green allows).

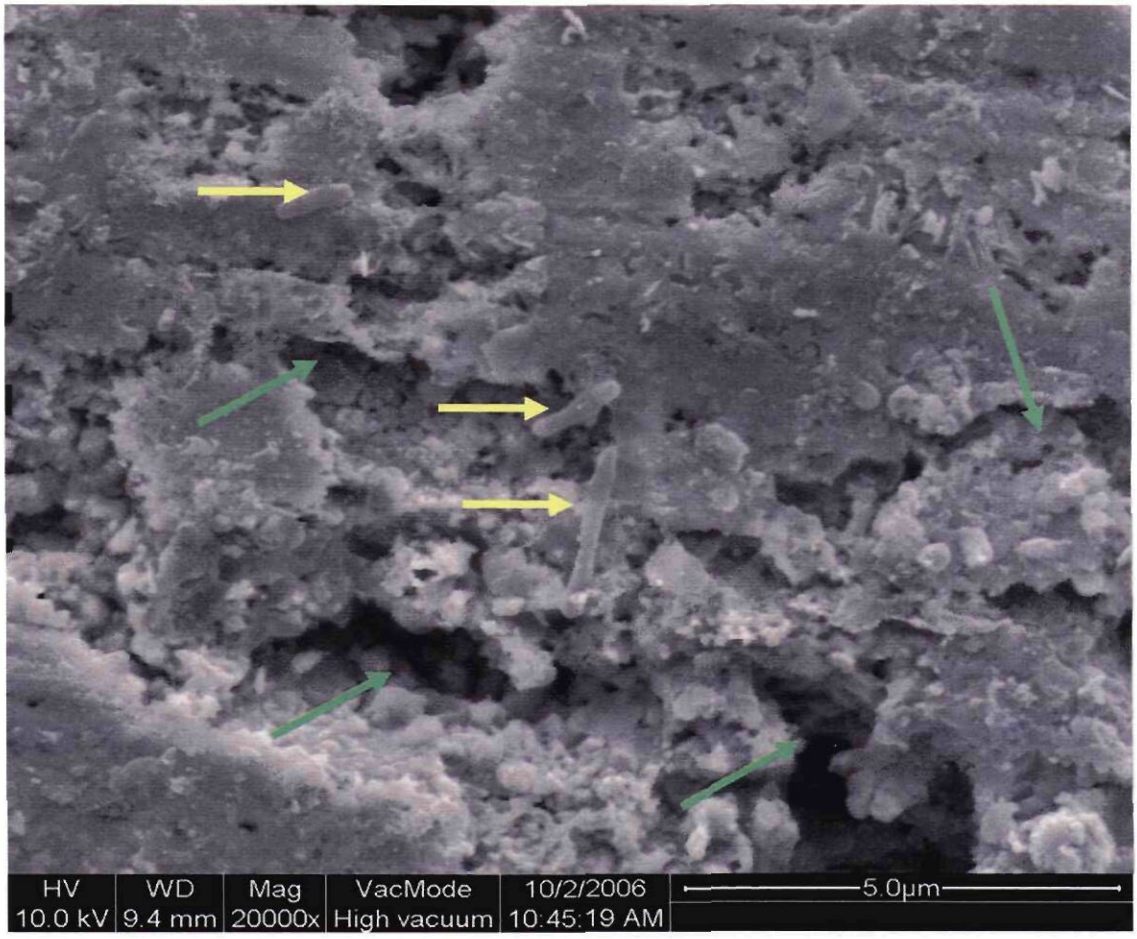


Figure 4.7- A SEM micrograph of a red copper disc from the biofilm device after a 6 week period (20 000X magnification).

After an 8 week period of exposure to drinking water the discs (Figure 4.8) were clearly more corroded when one compared them to those of 6 weeks of exposure to drinking water (Figures 4.5 and 4.6).

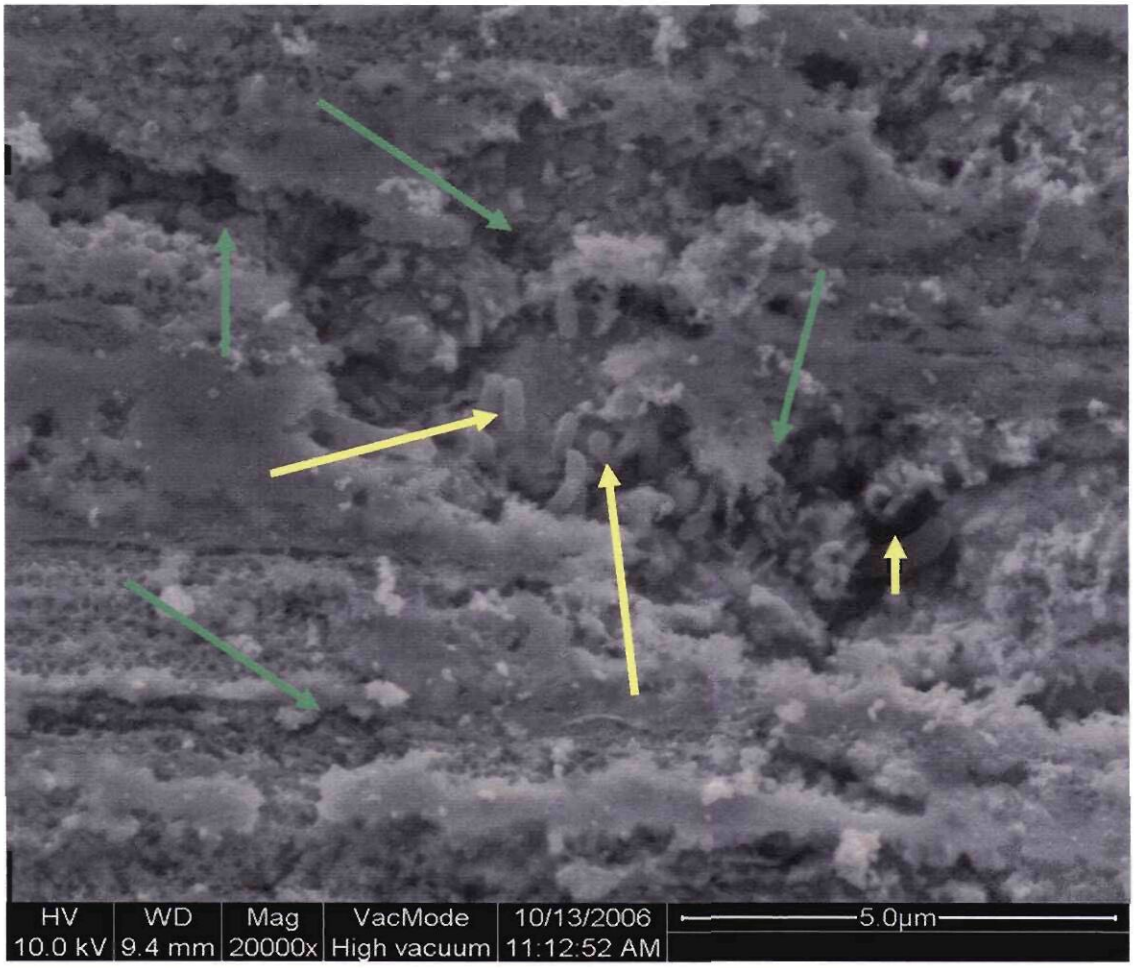


Figure 4.8- A SEM micrograph of a red copper disc after an 8 week period of exposure to drinking water (20 000X magnification).

Once again bacterial attachment was in crevices and on rough areas (Figure 4.8; yellow arrows). Large crevices and crystals (potential corrosion material) were again observed (green arrows). After 8 weeks no bacteria were observed at a 12 000X magnification but only at a 20 000 X magnification. On these discs the bacterial growth were confined to the crevices (Figure 4.8).

4.3.7 Summary of results

In this part of the study some physico-chemical characteristics of the drinking water were determined and indicated that the pH was above 8, temperature above 15°C and

total dissolved solids below 800 mg/l. The various metals discs that were exposed to this water were differently affected when these were simultaneously placed in the bulk drinking water. The surfaces of galvanised steel discs showed more corrosion activity than the red copper discs. Heterotrophic bacteria were only isolated from the red copper discs during the preliminary and the subsequent experiments. Some of these were identified, characterised for antibiotic and metal tolerance. The HPC were classified into 5 morphotypes based on colony morphology. Among these, three genera (*Enterobacter* spp., *Exiguobacterium* spp. and *Pseudomonas* spp.) were identified using biochemical and molecular methods. The *Exiguobacterium* spp. isolated from the red copper disc after 6 weeks of exposure to drinking water was resistant to 4 antibiotics and were tolerant to high (>5 mM) concentrations of copper ($CuSO_4$). This species also showed potential pathogenic features.

At high magnification the SEM micrographs clearly indicated biological activity in the form of corrosion. Single layers of bacilli were also observed. Only on the galvanised steel disc were typical EPS structures observed.

4.4 DISCUSSION

4.4.1 Physico-chemical characteristics of the drinking water

The physico-chemical analysis (pH, temperature and TDS) of the bulk drinking water indicated that the water was of a relatively good quality according the South African (DWAF, 2002; SANS 241, 2005) and international standards (WHO, 2006). Potchefstroom drinking water is from dolomitic origin and for this reason the pH is above 8 and TDS is relatively high (van Aardt, 2007). Salts like calcium, magnesium, potassium, chlorides and sulphate are normally responsible for influencing the TDS values. High TDS (>1 000 mg/l) is responsible for scaling and may result in pitting corrosion of metal distribution pipes (WHO, 2006). Scaling on the discs used in the present study can be observed in Figures 4.5–4.8. Although the high TDS value in the present study may be responsible for the excessive scaling observed in latter figures, it should not be considered a significant health impact for water consumers. Other implications in regards to high TDS values may be of an aesthetic nature (odour, or bad taste, colour or formation of a precipitate when frozen).

Water temperature and pH plays a very important role in the growth and colonization of bacteria in the distribution system (WHO, 2006). During the experimental period the measured temperatures were optimal for bacterial growth >15°C (LeChevallier *et al*, 1996). High water temperatures may contribute to corrosion problems which could in turn be responsible for increased biofilm formation, when kept in mind that corrosion may provide a more protective surface area in favour of biofilm formation (Zhang *et al.*, 2007). Corrosion as an effect of high water temperatures may also influence the quality of drinking water, due to some level of contamination (Zhang *et al.*, 2007). Although the highest measured temperatures (22.95°C and 24.05°C), were determined during the last two weeks of the study, this was also the period where the lowest diversity of

bacteria was observed. On the other hand, the number of isolates was relatively high during this period. The high temperature in the present study may have contributed to the observed corrosion/scaling of the discs.

The pH of the water on the other hand, has no direct impact on consumers although it is an important parameter when considering the efficiency of the disinfection process (WHO, 2006). A pH of around 8 is recommended by WHO, (2006), to ensure an effective disinfection process with chlorine. The pH values in the present study ranged between 8.06 and 8.21, which are thus in favour of an effective disinfection process. A low pH on the other hand is likely to be corrosive and is not recommended in terms of minimizing the corrosion of water mains and pipes in household water systems (WHO, 2006)

4.4.2 Levels and diversity heterotrophic bacteria from metal discs

Metal discs that were exposed to bulk drinking water of Potchefstroom were sampled and investigated for biofilms after 4 weeks. From the results it was evident that only on galvanised steel discs evidence of biofilm formation was observed. Since most of the drinking water distribution pipes in houses and building in Potchefstroom are red copper pipes it was decided to use only this material the subsequent experiment. During this subsequent study no biofilms were observed but only single layers of bacilli. This was also more clearly on the red copper compared to yellow copper and galvanized discs.

Hallam *et al.* (2001) suggested that developing time of biofilms grown under laboratory conditions and biofilms in the distribution system may vary significantly. A two week period may be sufficient for certain biofilms to develop under laboratory conditions,

which on the other hand may not be the case for biofilms in the distribution system (LeChevallier *et al.*, 1990b; Hallam *et al.*, 2001). In the present study only the initial phases of biofilm formation was observed as indicated by SEM micrographs. This observation suggests that 8 weeks were insufficient for biofilm development on the red copper discs. When Lehtola *et al.* (2004) compared biofilm formation on copper pipes to those on plastic (polyethelene) pies they found that bacterial number and biofilm formation increase slower on the copper pipes over a 200 day period (28.5 weeks). However, after this period the biofilm and number of bacteria isolated from both pipe materials were similar.

In the present study the swabbing technique was used to collect cells from the metal plates. It has been suggested that this technique may not be accurate enough to investigate the actual levels of HPC in water distribution systems (Hallam, *et al.*, 2001). This may explain the low numbers and diversity of bacteria isolated in the present study. This technique was used though, in order to investigate and determine the representative levels of bacteria that survive in nutrient poor environments.

The levels and diversity of bacteria in the water distribution system can depend on several factors such as the water temperature, pH, available nutrients level (LeChevallier *et al.*, 1990b) as well as pipe material and residual disinfectant levels (Geldreich *et al.*, 1977). In the present study, the levels and diversity over the sampling period varied. *Exiguobacterium* spp. (66.6%), *Pseudomonas* spp. (1.9%) and *Enterobacter* spp. (5.8%) were isolated and identified. Unidentified species included LG04 (33.3%), VW06 (44.5%) and HG06 (47.5%). Only one bacterial type (HG08) was isolated after the 8 week incubation period. The HG morphotype was the most predominant one observed over the experimental period.

Block *et al.* (1995), in their study on the biodiversity in drinking water found that bacterial species isolated from drinking water was as those found in natural sources. Studies have also indicated that from 10^1 to 10^7 cfu/cm^2 of drinking water biofilm could be enumerated (LeChevallier *et al.*, 1983; Lehtola *et al.*, 2004). It was also suggested by them that organisms occur in high densities and levels in the distribution system. In the present study diversity levels and densities were low.

The difference in bacterial densities between water distribution systems can depend very much on the type of disinfectant that is generally used. Free chlorine has a fast reaction rate where as chloramines, on the other hand, has a slower reaction rate. It is therefore expected that density of bacteria from the biofilm will be higher in systems that are disinfected with free chlorine in comparison to a lower biofilm density in systems that are disinfected with chloramines (De Beer *et al.*, 1994; Chen and Steward 1996). In Potchefstroom free chlorine is used as a disinfectant. The sampling point was ±2 km from the purification plant and it was expected for the residual chlorine to be relatively high. This parameter, however, was not measured.

During studies where the levels and diversity of heterotrophic bacteria are investigated, it is always important to bear in mind that under certain conditions bacteria may enter into a physiological state where they are viable but not culturable; (VBNC). In this state the metabolism is lowered and is typical when certain metabolic stresses such as starvation, low temperatures etc. are experienced (LeClerc, 2003; WHO, 2006). This can result the underestimating of total number of bacteria observed on plate counts in comparison to the more accurate number of bacteria that are truly present in the water distribution system (LeClerc, 2003).

Another factor that may influence the levels and diversity of HPC bacteria in the water distribution system is the pipe material (Momba *et al.*, 2004; Lehtola *et al.*, 2004). Activities and composition of biofilm populations are strongly influenced by pipe surfaces where iron pipe surfaces compared to smoother PVC piping contribute to a higher level of diversity of microbial population (Lehtola *et al.*, 2004; Reyes *et al.*, 2007). The rougher surface area may play a role in this (LeChevallier *et al.*, 1993).

Because corrosion slows water flow and serves as a protective surface for microbes, it can also contribute to an enhanced proliferation of biofilms in the water distribution system (O'Connor and Banerji, 1984; Lehtola *et al.*, 2004). In the present study high levels of corrosion (pitting corrosion) had been observed in the SEM micrographs of the metal discs. Corrosion of the pipe materials can be explained by the galvanic effect of different pipe materials on each other but also the effect of organic material such as extra-cellular polymeric substances (Li *et al.*, 2004). Thus bacterial activities may also contribute to corrosion. Bacilli were observed on all the metals that were tested and plating methods confirmed these observations.

4.4.3 Identification, antibiotic resistance and potential pathogenic characteristics of isolates

a) Pseudomonas spp

The growth and presence of bacteria such as *Pseudomonas* spp. are expected in water distribution systems because of their ability to grow better than other bacteria in environments with a low biodegradable organic matter (BDOC) level (Ribas *et al.*, 2000; WHO 2004, 2006). *Pseudomonas* spp. together with *Aeromonas* spp. are two types of rapidly growing heterotrophic bacteria found in potable water, that are capable of causing infections if they are present in infectious doses (LeChevallier, 1982; WHO

2004; 2006). A study was performed by Ribas *et al.,* (2000) in which they demonstrated that *Pseudomonas* may be a better indicator of potential regrowth in water distribution systems than *Aeromonas*. The *Pseudomonas* genera have been associated with opportunistic wound infections (Kudinha *et al.*, 2000; WHO 2006). In a more recent study it was demonstrated by Schaber *et al.* (2007) that *Pseudomonas aeruginosa* are capable of forming a biofilm within 8hours of infection, which also demonstrate that biofilms may contribute to bacterial colonization in acute infections.

The presence of *Pseudomonas* in drinking water may be of concern. The findings of Rusin *et al.* (1997) indicated that exposure to high levels of *Pseudomonas*, may contribute to increased risk of infection. *Pseudomonas* was one of the most virulent isolated HPC bacteria from selected drinking water supplies in South-Africa (Pavlov *et al.,* 2004). They demonstrated that this specie produce extra cellular enzymes involved in bacterial pathogenesis such as proteienase, gelatinase, DNase lipase and fibrinolysin.

The morphotype isolated in this study that were identified as *Pseudomonas* spp. did not display haemolysis features (potential pathogenic) and was only resistant to one antibiotic. It also constituted only 1.9 % of the total number of bacteria isolated during the entire period. Even though the species was tolerant to high levels of copper (MIC 2.5<3 mM) it did not constitute any health risk.

b) Exiguobacterium spp

Exiguobacterium spp. has been isolated from places such as the Yellow Sea, near Mokpo City in Korea (In-Gi *et al.*, 2005) and has recently also been isolated and identified by molecular approaches from goat's milk during a lactation year (Callon *et al.*, 2007). In-Gi *et al.* (2005), proposed 2 novel species in the genus *Exiguobacterium*

including *Exiguobacterium aestuarri* spp. and *Exiguobacterium marinum* spp. They proposed that cells from *E. aestuarri* are Gram variable and facultative anaerobic but that they grow better under aerobic conditions. Their colonies were circular, glossy and raised after incubation of 2 days at 30°C under aerobic conditions. The optimal pH for growth of these cells was pH 6.5 - 8.5. In the present study morphological characteristics were similar except that colonies were dull. Pigmentation of the cells was shades of orange. This was also similar to findings in the study performed by In-Gi *et al.* (2005). On the other hand, the time and temperature of incubation in the present study were slightly different. In this study it took an incubation period of 6-7 days at 37°C before any colonies were observed on solid media.

Exiguobacterium spp. is not known as an opportunistic pathogen but showed potential pathogenic features in the present study. These isolates showed α and β hemolysis and were resistant to 5 of the nine antibiotics tested. This species was also tolerant to high levels of copper (>5 mM).

c) Enterobacter spp

The most common species among the isolates from the red copper discs were identified as *Enterobacter cloacae*. All these isolates were susceptible to all the antibiotics they were tested against. The potential implications of the presence of this species in drinking water were discussed in Chapter 3. Although the *Enterobacter* spp. isolated from copper discs in this part of the study did not pose human health risks, they were the most frequently isolated species.

4.4.6 Minimum inhibitory concentrations (MIC) of copper for isolates

The majority of the isolate types in the present study that were isolated from red copper discs could tolerate high concentrations of copper (MIC >5Mm). This was anticipated since the biofilms were developed on copper discs. However, the *Enterobacter* spp. isolated from week 6 (HG06) and those from week 8 (HG08) gave conflicting MIC results. All the latter isolates were relatively sensitive to copper (MIC<0.75). This conflict could not be explained and no further investigation was conducted.

The presence and growth of bacteria on the copper discs, as indicated by electron microscopy and cultivation methods, may be the result of copper resistance among these bacteria. Nies (1999) indicated that free copper levels within bacterial colonies must be limited and that energy-dependent efflux systems are required for the removal of these. Such efflux systems may also be expressed by plasmids (Nies, 1999). Copper accumulation methods are also used by some species to tolerate high levels of copper in the environment (Dell'Amico *et al.*, 2006). Since this was a preliminary study, it was not within the scope to investigate whether plasmids were present or which method was responsible for the metal tolerance observed. This should be investigated in follow-up studies.

Recent studies (Turpeinen *et al.*, 2004; Dell'Amico *et al.*, 2006) indicated that several Gram-negative and Gram-positive species were associated with heavy metal contaminated (copper included) soils. These included the species identified in this study. Previous studies (Kunito *et al.*, 1999; Dell'Amico *et al.*, 2006) have indicated that all aspects of microbial presence in heavy metal contaminated environments could be affected by the presence and levels of heavy metals. These include bacterial numbers, biomass, activity and diversity. Higher levels of copper tolerance (10 times

higher, depending experimental conditions) were also observed in bacterial communities in soil compared to pure cultures (Kunito *et al.*, 1999; Dell'Amico *et al.*, 2006).

Potchefstroom's raw water sources (dams in the Mooi River system) are surrounded by mines, agriculture as well as other industrial activities (Van Aardt, 2007). It is estimated that daily 139 megaliter of ground water from these mines are pumped into the Wonderfontein, a tributary of the Mooi River,. High levels of certain heavy metals, including copper (5.4 mg/l) are present in the effluent from the mines (Van Aardt and Hough, 2007). The pollution potential has raised special concerns to the municipality of Potchefstroom (Van Aardt 2007). It is known that heavy metals are normally completely precipitated in hardwater and is mobilised when the pH drops below 5. The water of the Mooi River System is from dolomitic origin and classified as very hard (total alkalinity as $CaCO_3$ = 252 mg/l). However, the studies by van Aardt and others (reviewed in Van Aardt and Hough, 2007) indicated that the levels of metals such as lead and cadmium were found to be very high in the sediment of the Klerkskraal and Boskop dams in the Mooi River Sytem but in low concentrations in the bulk water. On the other hand, the study by Van Aardt and Hough, (2007) showed that the copper levels were high in the bulk water and this was ascribed to copper not precipitating completely, even in very hard water. These studies thus demonstrated that copper levels in the drinking water sources of Potchefstroom are elevated. It would thus not be uncommon for heterotrophic bacteria that are tolerant to copper to colonise copper surfaces in the drinking water distribution system of the town in a short period as indicated by the present study.

In a previous study corrosion problems had also been associated with the occurrence of copper tolerant bacteria in a water distribution system (Lin and Olsen, 1995). Here it was demonstrated that, of bacteria isolated from a water distribution system experiencing problems with copper corrosion, 62.0% were copper resistant. The extent of copper tolerance among various heterotrophic bacteria from the Potchefstroom distribution system is not understood. The approach and methods used in this study could provide an opportunity to investigate the occurrence and impact of copper tolerant bacteria in the Potchefstroom and other systems.

4.5 SUMMARY AND CONCLUSION

The aim of this study was to investigate the characteristics of HPC bacteria isolated from red copper discs from a biofilm device that were installed directly into the main distribution system of an academic building (J.S van der Merwe building) at the NWU-Potchefstroom. Results presented and discussed showed physico-chemical characteristics of the water in the Potchefstroom distribution system. Using SEM bacterial growth was observed on metal, particularly red copper, discs that were placed in the bulk water systems. Heterotrophic bacteria were isolated from these discs and some of these were tolerant to high concentrations of copper as well as certain antibiotics. Scanning electron micrographs also indicated corrosion effects on these discs.

The main conclusion from this part of the study is that the HPC bacteria from the Potchefstroom bulk water may not be as harmless as generally thought. Although the origin of the bacteria was not determined, these were detected in what appeared to be the initial stages of a biofilm. Further research needs to be conducted on the levels and diversity of the bacteria that occur on these discs and their source should be traced.

Isolation procedures to capture the viable but not culturable bacteria (e.g. enrichment) should also be considered. Future studies could also consider determining the biofilm formation as well as copper corrosion potential of the isolated bacteria.

It should also be considered to investigate the levels and diversity of facultative anaerobic and strict anaerobic bacteria in biofilms as well. The latter could shed light on the corrosion potential of bacteria within the biofilm of inner surfaces of the distribution system.

Studies to determine the effects of metals on isolated bacteria could also be conducted. This could include research on the presence of plasmids and/or other genetic material that could be involved in tolerance to heavy metals and antibiotics. Several studies suggested or demonstrated that it may not be far fetched to anticipate co-selection of metal tolerance and antibiotic resistance in bacteria (Dhakephalkar and Chopade, 1994; McArthur and Tuckfield, 2000; Spain and Alm, 2003). Some of the mechanisms that organisms display to these substances are overlapping and if the selection pressure to one or the other is maintained then the section of the population of tolerant HPC bacteria will be maintained. This could have implications for human health.

Growth studies linked to transmission electron microscopy and real-time reverse transcription PCR investigations to determine whether the metal is accumulated or whether potentially efflux pumps are involved could also be considered. Information about the various aspects recommended would be useful in model to determine whether the municipality of this town (Potchefstroom) should consider upgrading the water purification plant or not.

CHAPTER 5

FINAL CONCLUSION AND PROSPECTS

Biofilms are ubiquitous in drinking water distribution systems and evidence is provided that bacterial and viral pathogens as well as oocysts of pathogenic waterborne protozoans may survive within them (Angles *et al.*, 2007). Detachment of such biofilm may thus place consumers of such drinking water at risk of contracting waterborne diarrhoeal disease. Furthermore, exocellular polymeric substance may cause a rapid decline in disinfectant levels (Manuel *et al.*, 2007). Such substances may also affect corrosion rates within the distribution system (Li *et al.*, 2004).

The main aim of this study was to determine the diversity and levels of antibiotic resistant bacteria in drinking water biofilms in Potchefstroom and also determine whether these isolates are potential pathogens. Two main well motivated objectives were set for this study. The results of these were presented as two separate studies in Chapters 3 and 4. Trends and conclusions are thus briefly discussed in the following sections:

5.1 Diversity, levels and characteristics of HPC bacteria in biofilms from home water filtering systems

HPC bacteria were isolated from both activated carbon as well as RO filter systems. A total of 144 HPC bacteria were isolated. The implications of the presence of some of the isolated species, particularly potential opportunistic pathogenic bacteria were also discussed, using relevant literature. Although their origin remains unknown, the characteristic of such bacteria indicated a potential health risk to water consumers in general but the immuno-suppressed and immuno-compromised in particular. This is because of pathogenic features of these isolates that are coupled with multiple antibiotic

resistance. Antibiotics to which isolates were resistant to included ones generally used for treatment of human infections such as ampicillin, penicillin G, chloramphenicol, erythromycin, gentamycin, kanamycin, neomycin, tetracycline and vancomycin.

Scanning electron microscopy studies showed typical biofilm structures on the various components of the RO filter unit. The proximity of the various bacterial species in such biofilms was demonstrated. The implications and role of such biofilms in dissemination of mobile genetic elements, particularly those involved in transfer of pathogenic and antibiotic resistance features, were considered.

5.2 Diversity, levels and characteristics of HPC bacteria in biofilms isolated from an *in situ* biofilm device

Results presented and discussed showed physico-chemical characteristics of the water in the Potchefstroom distribution system is of a fair quality. Scanning electron microscopy data showed bacterial growth on metal discs that were exposed to bulk drinking water for periods ranging from 4 to 8 weeks. Some of the HPC bacteria that were isolated from these discs were tolerant to high concentrations of copper (MIC > 5 mM) as well as certain antibiotics. Scanning electron micrographs also indicated corrosion effects on these discs.

Not much similarity was observed between the species isolated from water filters and those isolated from the copper discs. The different experimental materials may have had an influence on the diversity of the isolates.

The main conclusion from the study is that the HPC bacteria from the Potchefstroom bulk water as well as biofilms may not be as harmless as generally thought. Although

the health risk potential of many of these isolates was not a cause for concern, the bacteria could be involved with microbially induced corrosion.

PROSPECTS FOR FURTHER STUDY

Future studies could also consider determining the biofilm formation as well as copper corrosion potential of the isolated bacteria. Sampling strategies should be improved enhance the detection levels of the viable bacteria. It should also be considered to investigate the levels and diversity of facultative anaerobic and strict anaerobic bacteria. The latter could shed light on the corrosion potential of bacteria within the biofilm of inner surfaces of distribution surfaces.

Studies to determine the effects of metals on isolated bacteria could also be conducted. This could include search for the presence of plasmids and/or other genetic material that could be involved in tolerance to heavy metals and antibiotics.

A number of precautions can be made by the water consumers in order to avoid any health risks associated with the presence of these micro-organisms. People suffering from HIV should be aware of their disease status to avoid using any water that could be harmful to their health. It may be suggested that point-of-use water filtration systems, such as those in this study could be used to purify the water further, if possible.

A more detailed follow up study including investigation of different compartments of RO filtration units should be conducted over different time periods in order to determine the evolution of the characteristics of all isolates from this drinking water distribution system. It is also recommended that the source of contamination or regrowth should be determined.

REFERENCES

A guide to water filters and water treatment. http//heartspring.net/water-filters-guide.html. (Accessed on 21May 2005)

Abouzaid, H., El Mghari Tabib, M., Outair, A., Laraki, L., and Benabdallah, S. 1996. Development of water quality in a distribution network: A case study from the Bouznika Centre, Morocco. *Water Supply.* **14**(3-4): 464-471

Activated carbon filters. http//www.cyber-nook/water/Solutions.html#carbon. (Accessed on 13 October 2007)

Ahmedna, M., Marshall, W.E., Husseiny, A A., Rao, R.M., and Goktepe, I. 2004. The use of nutshell carbons in drinking water filters for removal of trace metals. *Water Research.* **38**(4): 1062-1068

Allen, M.J., Edberg, S.C., and Reasonar, D.J. 2004. Heterotrophic plate count bacteria-what is their significance in drinking water. *International Journal of Food Microbiology.* **92**(3): 265-274

Allen, M.J., Taylor, R.H. and Geldrich, E.E. 1980. The occurrence of microorganisms in water main encrustations. *Journal of American Water Works Association.* **72**: 614-625

Anand, C., Gordon, R., Shaw, H., Fonseca, K. and Olsen, M. 2000. Pig and goat blood as substitutes for sheepblood in blood- supplemented agar media. *Journal of Clinical Microbiology.* **38**(2): 591-594

Angles, M.L., Chandy, J.P., Cox, P.T., Fisher, I.H. and Warnecke, M.R. 2007. Implications of biofilm-associated waterborne Cryptosporidium oocysts for the water industry. *Trends in Parasitology.* **23**(8): 352-356

Anonymous. http://www.waterforlife.ca/images/Radiogram.jpg. (Accessed on 23 August 2007)

Ash, R.J., Mauck, B. and Morgan, M. 2002. Antibiotic resistance of gram negative bacteria in rivers in the United States. *Emerging Infectious diseases*. **8**(7):713-716

Badar, U., Abbas, R., and Ahmed, N. 2001. Characterization of copper and chromate resistant bacteria isolated from Karachi tanneries effluents. *In*: Ahmed, N., Qureshi, F.M., and Khan, O.Y. (editors.). Industrial and environmental biotechnology. Horizon Scientific Press, Norfolk. 45-53

Bauer, A.W., Kirby, W.M., Sherris, J.C. and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*. **45**(4): 493-496

Beirowski, V. 2002. The incidence of antibiotic resistant bacteria in milk and dairy products. M.Sc dissertation, Potchefstroom University for CHE, South Africa.

Bhat, P., Myer, R.M., and Feldman, R.A. 1971. Providence group of organisms in the aetiologyof juvenile diarrhoea. *Indian Journal of Medical Research*. **59**:1010-1018

Block, J.C., Sibille, I., Gatel, D., Reasonar, D.J., Lykins, B., and Clark, R.M. 1995. Biodiversity in drinking water distribution systems: A brief review. The microbiological quality of water. From IWISA and FBA Specialized Conference, London, UK. December

Bottone, E., and Schniereson, S.S. 1972. An emerging human pathogen. *American Journal of Clinical Pathology*. **57:** 400-405

Briganti, L.A., and Wacker, S.C. 1995. Fatty acid profiling and the identification of environmental bacteria for drinking water utilities. AWWA Research Foundation and American Water Works Association, Denver. 89

Brown, M.R. and Barker, J. 1999. Unexplored reservoirs of pathogenic bacteria: protozoa and biofilms. *Trends in Microbiology*. 7: 46-50

Callon, C., Duthoit, F., Delbes, C., Ferrand, M., Le Frileux, Y., De Cremouz, R., and Montel, M-C. 2007. Stability of microbial communities in goat milk during a lactation year: Molecular approaches. *Systematic and Applied Microbiology.* Doi:10.1016/j.syapm.2007.05.004

Camper, A.K., LeChevallier, M.W., Broadaway, S.C., and McFeters, G.A. 1986. Bacteria associated with granular activated carbon particles in drinking water. *Applied Environmental Microbiology.* **52**(3): 434-438

Carraro, E., Bugliosi, E.H., Meucci, L., Baiocchi, C., and Gilli, G. 2000, Biological drinking water treatment process, with special reference to mutagenicity. *Water Research.* **34**(11): 3042-3054

Cassel, G.H. 1997. Emergent antibiotic resistance: health risks and economic impact. *FEMS Immunology and Medical Microbiology*: **18**(4):271-274

Characklis, W.G., McFeters, G.A., and Marshall, K.C. 1990. Physiological ecology in biofilm systems. *In:* Characklis, W.G., and Marshall, K.C. (editors). Biofilms. John Wiley and Sons, New York. 341-394

Chen, X., and Steward, P.S. 1996. Chlorine penetration into artificial biofilm is limited by a reaction diffusion interaction. *Environmental Science and Technology.* **30**: 2087-2083.

Chollet, R., Bollet, C., Chevalier, J., Mallea, M., Pages, J-M., and Davin-Regli, A. 2002. mar-Operon involved in multidrug resistance of *Enterobacter aerogenes. American Society for Microbiology.* **46**(4): 1093-1097

Clark, R.M., Tafuri, A.N., Yezzi, J.J., Haught, R.C., and Meckes, M.C. 1998. Urban drinking water distribution systems: A U.S perspective. Proceedings of the Conference entitled Regional Water System Management: Water Conservation, Water Supply and Sytem Integration, held in Valencia, Spain

Codony, F., Morato, J., and Mas, J. 2005. Role of discontinuous chlorination on microbial production by drinking water biofilms. *Water Research.* **39**(9): 1896-1906

Davin-Regli A., Bosi, C., Charrel, R, Ageron, E., Papazian, L., Grimont, P.A.D., Cremieux, A., and Bollet, C. 1997. A nosocomial outbreak due to *Enterobacter cloacae* strains with the *E. hormaechei* genotype in patients treated with fluoroquinolones. *Journal of Clinical Microbiology* **35**(4): 1008-1010

De Beer, D., Stoodley, P., Roe, F., and Lewandowski, Z. 1994. Effects of biofilm structure on oxygen distribution and mass transport. *Biotechnology and Bioengineering.* **43**: 1131-1138

De Champs, C., Le Seeaux, S., and Dubost, J.J. 2000. Isolation of *Pantoea agglomerans* after plant thorn and wood silver injuries. *Journal of Clinical Microbiology.* **38**: 460-461

De Man, P., Van der Veeke, E., Leemreijze, M., van Leeuwen, W., Vos, G., van der Anker, J., Verbrugh, H., and van Belkum, A. 2001. *Enterobacter* species in a pediatric hospital: Horizontal transfer or selection in individual patients. *The Journal of Infectious Diseases.* **184**: 211-214

De Marini, D.M., Abu-Shakara, A., Felton, C.F., Patterson, K.S., and Shelton, M.L. 1995. Mutation spectra in Salomonella of chlorinated, chloraminated, or ozonated drinking waterextracts: comparison to MX. *Environmental Molecular Mutagenesis.* **26**(4): 270-285.

De Wet, J. 2006. The incidence of antibiotic resistant bacteria in industrial and residential air. M.Env.Sc. dissertation, North-West University, Potchefstroom Campus, South Africa.

DeBeer, D., Srinivasan, R., and Steward, P.S. 1994. Direct measurement of chlorine penetration into biofilms during disinfection. *Applied and Environmental Microbiology.* **60**(12): 4339-4344

Dell' Amico, E., Mazzocchi, M., Cavalca, L., Allievi, L., and Andreoni, V. 2006. Assessment of bacterial community structure in a long-term copper-polluted ex-vineyard soil. *Microbiological Research*, doi:10.1016/j.micres.2006.09.03

Department of Water Affairs and Forestry (DWAF). 1996. South African Water Quality Guidelines for Domestic Use. (2nd Edition)

Department of Water Affairs and Forestry (DWAF). 2002. Nation water resource quality status report: Inorganic chemical water quality of surface water resources in S.A.-The big picture.

Department of Water Affairs and Forestry (DWAF). 2005. Drinking water quality in South Africa. A consumer's guide. http://www.dwaf.gov.za/iwqs/report.html. (Accessed on 16 September 2006)

Dhakephalkar, P.K., and Copade, B.A. 1994. High levels of multiple metal resistance and its correlation to antibiotic resistance in environmental isolates of Acinetobacter. *Biometals.* 7(1): 67-74

Dressler, C., Kues, U., Nies, D.H., and Barbel, F. 1991. Determinants encoding resistance to several heavy metals in newly isolated copper resistant bacteria. *Applied and Environmental Microbiology.* **57**(11): 3079-3085

Drinking water standards. What happens at a water treatment plant. http://www.aces.edu/pubs/docs/A/ANR-0790/WQ2.1.5). (Accessed on 3 June 2006)

Duncan, H.E., and Edberg, S.C. 1995. Host-microbe interaction in the gastrointestinal tract. *Critical Reviews in Microbiology.* **21**: 85-100

Duncan, I.B.R. 1998. Waterborne Klebsiella and human disease. *Toxicity Assessment* **3**(5): 581-598

Drinking water treatment methods. http//www.cyber-nook.com/water/Silutions.html#ro. (Accessed on 13 October 2007)

Edberg, S.C., Gallo, P., and Kontnick, C. 1996. Analysis of the virulence characteristics of bacteria isolated from bottled water cooler, and tap water. *Microbiol Ecology in Health and Disease.* **9**: 67-77

Edberg, S.C., and Allen, M.J. 2004. Virulence and risk from drinking water of heterotrophic plate count bacteria in human population groups. *International Journal of Food Microbiology*. **92**. 255-263.

Ehlers, M.M., Van Zyl, W.B., Manz, W., and Schleifer, K.H. 2000. Microbiological safety of drinking water. *Annual Reviews in Microbiology*. **54**: 81-127

Eisenberg, J.N.S., Wade, T.J., Hubbard, A., Abrams, D.I., Leiser, R.J., Charles, S.,Vu, M., Saha, S., Wright, C.C., Levy, D.A., Jensen, P., and Colford jr, J.M. 2002. Associations between water-treatment methods and diarrhoea in HIV-positive individuals. *Epidemiology and Infection*. **129**: 315-323

Emtiazi, F., Schwartz, T., Marten, S.M., Sidenstein, P.K., and Obst, U. 2004. Investigation of natural biofilms formed during the production of drinking water from surface water embankment filtration. *Water Research*. **38**(5): 1197-1206

Engelhart, S., Glasmacher, A., Kaufmann, F. and Exner, M. 2001. Protecting vulnerable groups in the home: the interface institutions and the domestic setting. *Journal of Infection*. **43**: 57-59

Environmental scanning electron microscopy –ESEM http://www.azom.com/details.asp?ArticleID=1556. (Accessed on 10 July 2006)

Erdman, R. 1999. Die voorkoms van kadnium, koper, lood en sink in die weefsel van di varswater vis, *Labeo capensis* en bodemsedimente van uit die damme in die Mooirivier. M.Sc verhandeling, Potchefstroomse Universiteit vir CHO, Potchefstroom, Suid Afrika.

Espinosa-Urgel, M., and Ramos, J-L. 2003. Genetics of biofilm formation. *In*: Biofilms in medicine, industry and environmental biotechnology. Lens, P., Moran, A.P., Mahony, T., Stoodley, P., and O'Flaherty, V. (editors). IWA Publishers, Londen, UK.47-59

Ewing, W.H.1985. Edwards and Ewing's identification of *Enterobacteriaceae,* 4th Edition. Elseriver Science Publishing Co., Inc., New York, N.Y.

Falkinheim III, J.O., Norton, C.D., and LeChevallier, M.W. 2001. Factors influencing numbers of *Mycobacterium avium, Mycobacterium intracellulare,* and other *Mycobacteria* in drinking water distribution systems. *Applied Environmental Microbiology*. **67**:1225-1231

Fiksdal, U.V., Vik, E.A., Mills, A., and Staley, J.T. 1982. Non-standard methods for enumerating bacteria in drinking water. *Journal of American Water Works Association.* **74**: 313-318

Filipic, M., Cantor, K.P., Glavic, D., and Planina, P. 1995. Toxic and genotoxic activity of water samples from the river Ljublijanica. *Bulletin of Environmental Contamination Contamination and Toxicology.* **55:** 237-244

Fisman, D. N., and Kaye, K. M. 2000. Once daily dosing of aminoglycoside antibiotics. *Infectious Disease* **14**: 475-487

Flatauer, F.E., and Khan, M.A. 1978. Septic arthritis caused by *Enterobacter agglomerans*. *Archive of Internal Medicine*. **138**: 788

Flemming, H-C, Wingender, J., Griegbe, C., and Mayer C. 2000. Physico-chemical properties of biofilms. *In*: Evans., L.V. (editor). Biofilms: recent advances in their study and control. Amsterdam: Harwood Academic Publishers. 19-34

Foot, C.H., and Taylor, C.B. 1949. The influence of the consumption of the medium on the growth of bacteria from water. *Process Water Quality and Technology Conference*. Vol: 11-13

Franzblau, S.G., Hinnebusch, B.J., Kelley, L.M., and Sinclair, N.A. 1984. Effect of noncoliforms on coliform detection in potable groundwater: Improve recovery with an anaerobic membrane filter technique. *Applied Environmental Microbiology*. **48**: 142-148

Gagnon, G.A., Rand, J.L., O'Leary, K.C., Rygel, A.C., Chauret, C., and Andrews, R.C. 2005. Disinfectant efficacy of chlorite and chlorine dioxide in drinking water biofilms. *Water Research*. **39**(9): 1809-1817

Geldreich, E.E. 1989. Drinking water microbiology-new directions towards water quality enhancement. *International Journal of Food Microiology*. **9**: 295-312

Geldreich, E.E. 1990. Microbial quality control in distribution systems. Water quality and treatment, 4th Edition. *In:* Pontius, F.W. (editor). American Water Works Association, McGraw-Hill, Inc., New York. 1113-1158

Geldreich, E.E., and LeChevallier, M.W. 1999. Microbial water quality in distribution systems. *In:* Letterman, R.D. (editor). Water Quality and Treatment, 5th Edition. Mcgraw-Hill. New York. 1-49

Geldreich, E.E., Nash, H.D., and Spino, D. 1977. Characterizing bacterial populations in treated water supplies: a progress report. *Process Water Quality and Technology Conference.* Kansas City, MO. 3

Gelt, J. 1996. Consumers increasingly use bottled water, home water treatment systems to avoid direct tap water. *Arroyo*. **9**(1): 1-9

Glasmacher, A., Engelhart, S., and Exner, M. 2003. Infections from HPC organisms in drinking water amongst the immunocompromised. *In*: Bartman, J., Cotruvo, J., Exner, M., Fricker, C., and Glasmacher, A. (editors). WHO - Heterotrophic plate counts and drinking water safety, IWA Publishing, London. 137-144

Graham, D. C., and Hodgkiss, W. 1967. Identity of Gram negative, yellow pigmented, fermentative bacteria isolated from plants and animals. *Journal of Applied Bacteriology*. **30**:175-189

Hallam, N.B., West, J.R., Forster, C.F and Simms, J. 2001. The potential for biofilm growth in water distribution systems. *Water Research*. **35**(17): 4063-4071

Harley, J.P., and Prescott, L.M. 2002. Laboratory exercises in microbiology. (5th Edition). Boston: McGraw-Hill, US.

Harsley, R.M. 2003. Bacterial Motility on a surface: Many ways to a common goal. *Annual Review of Microbiology*. **57**: 294-273

Haung, S.S., Labus, B.J., Samuel, M.C., Wan, D.T., and Reingold, A.L. 2002. Antibiotic resistance patterns of bacterial isolates from blood in San Francisco Country, California, 1996-1999. *Emerging Infectious Diseases.* **8**: 195-201

Health Protection Agency. 2001. http://www.hpa.org.uk/infections/topics_az/citrobacter/Default.htm. (Accessed on 12May, 2007)

Hengge-Aronis, R. 1996. Regulation of gene expression during entry into stationary phase. *In*: Neidhardt, F C., Curtiss III, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E. (editors). *Escherichia coli* and *Salmonella*: cellular and molecular biology. 2nd Edition. *American Society for Microbiology*. Washington, D.C, US.1497–1512

Hernandez, A., Mellado, R.P., and Martinez, J.L. 1998. Metal Accumulation and Vanadium-Induced Multidrug Resistance by Environmental Isolates of *Escherichia hermannii* and *Enterobacter cloacae*. *Applied and Environmental Microbiology.* **64**: 4317-4320

Herson, D.S., and Victoreen, H.T. 1980. Hindrance of coliform recovery by turbidity and non-coliforms. *Environmental Protection Agency Report. EPA-600/2-80-097.* 34

Herson, D.S., Marshall, D.R., Baker, K.H., and Victoreen, H.T. 1991. Association of microorganisms with surfaces in distribution systems. *Journal of American Water Works Association.* **83**: 103-106

Hogan, D.A., and Kolter, R. 2002. Pseudomonas-Candida interactions: an ecological role for virulence factors. *Science*. **296:** 2229-2232

Ingersoll, J. H. 1981."Water Filters - Should You Install One?" *Popular Mechanics*, **156**: 73-81

In-Gi, K., Mi-Hwa, L., Seo-Toun, J., Jae-Jun, S., Tae-Kwang, O. and Jung-Hoon, Y. 2005. *Exiguobacterium aestuarii* sp.nov. and *Exiguobacterium marinum* sp.nov., isolated from a tidal flat of the Yellow Sea in Korea. *International Journal of Systematic and Evolutionary Microbiology*. **55**: 885-889

Ishizake, C., Marti, I., and Ruiz, M. 1983. Effect of surface characteristics of activated carbon on the adsorption of chloroform from aqueous solution. *In:* McGuire, M.J., and Suffet, I.H. (editors.). Treatment of water by granular activated carbon. Advances in Chemistry Series. *American Chemical Society*, Washington, D.C. US. 95-106

Janda, M.J., Abbot, S.L., Woodward, D., and Khashe, S. 1998. Invasion of HEp-2 and other eukaryotic cell lines by providenciae: further evidence supporting the role of *Providencia alcalifaciens* in bacterial gastroenteritis. *Currrent Microbiology*. **38**:159-165

John, C., and Soucek, A. Gram-negativne fakultativne anaerobni tycky. 1996. *In*: Bednar, M., Frankova, V., and Schindler, J. (editors). Lekarska mikrobiologie. Vol 1.Prague: Marvil; 261-283

Jones, J.G. 1970. Studies on freshwater bacteria: Effect of medium composition and method on estimates of bacterial population. *Journal of Applied Bacteriology*. **33**: 679-686

Jones, G.W., and Isaacson, R.E. 1983. Proteinaceous bacterial adhesins and their receptors. *Critical Reviews in Microbiology*, **10**(3): 229-60

Kaegi, R., and Holzer, L. 2003. Transfer of a single particle for combined ESEM and TEM analysis. *Atmospheric Environment*. **37**: 1-7

Kalmbach, S., Manz, W., and Szewzyk, U. 1997. Dynamics of biofilm formation in drinking water: Phylogenetic affiliation and metabolic potential of single cells assessed by formazan reduction and in situ hybridization. *FEMS Microbiology*. **22**: 265-279

Karbasizaed, V., Badami, N., and Emtiazi, G. 2003. Antimicrobial, heavy metal resistance and plasmid profile of coliforms isolated from nosocomial infections in a hospital in Isfahan, Iran. *African Journal of Biotechnology*. **2**(10): 379-383

Kirmeyer, G.J., Friedman, M., Martel, K., Howie, D., Lechevallier, M., Abbaszadegan, Karim, M., Funk, J., and Harbour, J. 2001. Pathogen intrusion into the distribution system. Research foundation and the American Water Works Association, Denver. AWWA. 2-15

Klemm, P., and Schembri, M.A. 2000. Bacterial adhesins: structure and function. *International Journal of Medical Microbiology.* **290**: 27-35

Kraybill, H.F. 1981. Carcinogenesis of synthetic organic chemicals in drinking water. *Journal of American Water Works Association.* **73**: 370-372

Kruithof, J.C. 1985. Influence of water treatment process on the formation of organic halogens and mutagenic activity by post chlorination. *Water Chlorination.* **5**: 113-134

Kudinha, T., Tswana, S.A., and Simango C. 2000. Virulence properties of *Aeromonas* strains from humans, animals and water. *The South African Journal of Epidemiology and Infection.* **15**: 94-97

Kunito, T., Senoo, K., Saeki, K. Oyaizu, H., and Matsumoto, S. 1999. Usefulness of the sensitivity-resistance index to estimate the toxicity of copper on bacteria in copper-contaminated soils. *Ecotoxicology and Environmental Safety*, **44**: 182-189.

Kwenamore, K.M. 2007. Investigation of levels of antibiotic resistant *Enterococcus* spp. and coliform bacteria from ground water sources within Ditsobotla and Molopo districts of the North-West Province, South Africa. M.Sc in Agriculture, North-West University, Mafikeng Campus, South Africa.

LeChevallier, M., Seidler, R., and Evans, T. 1980. Enumeration and characterization of standard plate count bacteria in chlorinated and raw water supplies. *Applied and Environmental Microbiology.* **40**: 922-930

LeChevallier, M.W., Evans, T.M., Seidler, R.J., Daily, O.P., Merrel, B.R., Rollins, D.M., and Joseph, S.W. 1982. *Aeromonas sobria* in chlorinated drinking water supplies. *Microbial Ecology.* **8**:325-333

LeChevallier, M.W., Cameron, S.C., and McFeters, G.A. 1983. Comparison of verification procedures for the membrane filter total coliform technique. *Applied Environmental Microbiology.* **45**(3):1126-1128.

LeChevallier, M.W., Cawthon, C.D., and Lee, R.G. 1988a. Factors promoting survival of bacteria in chlorinated water supplies. *Applied and Environmental Microbiology.* **54**: 649-654

LeChevallier, M.W., Cawthon, C.D., and Lee, R.G. 1988b. Inactivation of biofilm bacteria. *Applied and Environmental Microbiology.* **54**: 2492-2499

LeChevallier, M.W. 1989. Treatment to meet the microbiological MCL in the face of a coliform regrowth problem. Proceedings. AWWA Water Quality Technology. Conference. Philadelphia, PA. American Water Works Association, Denver, CO.

LeChevallier, M.W., Lowry, C.D., and Lee, R.G. 1990a. Disinfecting biofilms in model distribution system. *Journal of American Water Works Association.* **82**: 87-99

LeChevallier, M.W., Schultz, W.H., and Lee, R.G. 1990b. Bacterial nutrients in drinking water. *Applied and Environmental Microbiology.* **53**(3): 857-862

LeChevallier, M.W., Lowry, C.D., Lee, R.G., and Gibbon, D.L. 1993. Examining the relationship between iron corrosion and the disinfection of biofilm bacteria. *Journal of the American Water Works Association.* **85**(7): 111-123

LeChevallier, M.W., Welch, N.J., and Smith, D.B. 1996. Full scale studies of factors related to coliform regrowth in drinking water. *Applied and Environmental Microbiology.* **62**(7): 2201-2211

LeClerc, H. 2003.Relationship between common water bacteria and patogens in drinking water. *In*: Bartman, J., Cotruvo, J., Fricker, C., and. Glasmacher, A. (editors). (WHO). Heterotrophic Plate Counts and drinking water safety. IWA Publishing, London, UK. 80-118

Lehtola, M.J., Miettinen, I.T., Keinanen, M.M., Kekki, T.K., Laine, O., Hirvonen, A., Vartiainen, T. and Martikainen, P.J. 2004. Microbiology, chemistry, and biofilm development in a pilot drinking water distribution system with copper and plastic pipes. *Water Research.* **38:** 3769-3779

Lessa, F., Enoki, C., Ito, I., Faria, G., Matsumoto, M., and Nelson-Filho, P. 2007. *In vivo* evaluation of the bacterial contamination and disinfection of acrylic base plates of removable orthodontic appliances. *American Journal of Orthodontics and Dental Orthopedics.* **131**(6):705.

Li, B., Liu, H., Gu, J., and Qu, J. 2007. The formation and distribution of haloacetic acids in copper pipe during chlorination. *Journal of Hazardous. Materials.* doi:10.1016/j.jhazmat.2007.06.090

Li, S., Ni, L., Sun, C., and Wang, L. 2004. Influence of organic matter on orthophosphate corrosion inhibition for copper pipe in soft water. *Corrosion Science.* **46**: 137-145

Licht, T.R., Struve, C., Christensen, B.B., Poulsen, R.L., Molin, S., and Krogfelt, K.A. 2003. Evidence of increased spread and establishment of plasmid RP4 in the intestine under sub-inhibitory tetracycline concentrations. *FEMS Microbiology Ecology* **1491:** 1-7

Lin, C., and Olson, B.H. 1995. Occurrence of cop-like resistance genes among bacteria isolated from a water distribution system. *Canadian Journal of Micobiology.* **41**: 642-646

Lu, C., Biswas, P., and Clark, R.M. 1995. Simultaneous transport of substrates, disinfectants and microorganism in water pipes. *Water Research.* **29**(3): 881-894

Mah, M.W., and Memish, Z.A. 2000. Antibiotic resistance. An impeding crisis. Suadi Medical Journal. **21**(12): 1125-1129

Mah, T-F. C., and O'Toole, G.A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology.* **9**(1): 5-11

Maki, J.S., LaCroix, S.J., Hopkins, B.S., and Staley, J.T. 1986. Recovery and diversity of heterotrophic bacteria from chlorinated drinking waters. *Journal of Applied and Environmental Microbiology*. **51**: 1047-1055

Manuel, C.M., Nunes, O.C., and Melo, L.F. 2007. Dynamics of drinking water biofilm in flow/non-flow conditions. *Water Research.* **41**: 551-562

Martiny, A.C., Albrechtsen, H-J., Arvin, E., and Molin, S. 2005. Identification of bacteria in biofilm and bulk water samples from a nonclorinated model drinking water distribution system: Detection of a large nitrogenous population associated with *nitrospira* spp. *Applied and Environmental Microbiology*. **71**(12): 8611-8617

McArthur, J.V., and Tuckfield, R.C. 2000. Spatial Patterns in Antibiotic Resistance among Stream Bacteria: Effects of Industrial Pollution. *Applied and Environmental Microbiology.* **66**(9): 3722-3726

McCormick, J.B. 1996. Epidemiology of emerging/remerging antimicrobial-resistant bacterial pathogens, *Current Opinion in Microbiology* **1**:125-129

Messi, P., Guerrieri, E., and Bondi, M. 2005. Antibiotic Resistance of antibacterial activity in heterotrophic bacteria of mineral water origin. *Science of the Total Environment*. **346**(1): 213-219

Microbiology, bacterial phyiology, biofilm research. http://www.biology.binghamton.edu/davies/research.htm. (Accessed on 15 November 2006)

Miranda, C.D., and Castillo, G. 1998. Resistance to antibiotic and heavy metals of motile aeromonads from Chilean freshwater. *The Science of the Total Environment.* 224 (1-3): 167-676

Momba, M.N.B., Cloete, T.E., Venter, S.N., and Kfir,R. 1999. Examination of the behaviour of *E.coli* in biofilms established in laboratory scale units receiving chlorinated and chloraminated water. *Water Research.* **33**(13): 2937-2940

Momba, M.N.B., and Binda, M.A. 2002. Combining chlorination and chloramination processes for the inhibition of biofilm formation in drinking water surface water system models. *Journal of Applied Microbiology.* **91**: 1-8

Momba, M.N.B., and Makala, N. 2004. Comparing the effect of various pipe materials on biofilm formation in chlorinated a combined chlorine-chloraminated water system. *WaterSA.* **30**(2): 175-178

Momba, M.N.B., Cloete, T.E., Venter, S.N., and Kfir, R. 1998. Evaluation of the impact of disinfection process on the formation of biofilms in potable surface water distribution systems. *Water Science and Technology.* **38**(8-9): 283-289

Momba, M.N.B., Makala, N., Tyafa, Z., Brauckaert, B.M., Bucley, C.A., and Thompson, P.A. 2002. Improving the efficiency and sustainability of disinfection at a rural water treatment plant. *WaterSA.* **30**(5):69-74

Morton, S.C., Zhang, Y., and Edwards, M.A. 2005. Implications of nutrient release from iron metal for microbial regrowth in water distribution systems. *Water Research.* **39**(13): 2883-2892

Mulamattathil, S.G. 2000. Antibiotic resistance patterns of faecal coliforms in the Molopo river at Mafikeng. M.Sc dissertaion, Potchefstroom University for CHE, Potchefstroom, SA.

Mulamattathil, S.G., Esterhuysen, H.A., and Pretorius, P.J. 2000. Antibiotic resistant Gram-negative bacteria in a virtually closed water reticulation system, *Journal of Applied Microbiology* **88**: 930 – 937

Muyima, N.Y.O., and Ngcakani, F. 1998. Indicator bacteria and regrowth potential of the drinking water in Alice, Eastern Cape. *Water SA*. **24**(1): 29-34

Muyzer, G., De Waal, E.C., and Utterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S rRNA. *Applied Environmental Microbiology.* **59**: 695-700

Nagy, L.A., and Olsen, B.H. 1985. Occurrence and significance of bacteria, fungi, and yeasts associated with distribution pipe surfaces. *Water Supply* **11**(3-4): 365-376

NCCLS. 1999. Performance standards for antimicrobial susceptibility testing:Ninth information supplement. **19**(1): 38-84

Neall, B. 2000. New Bottling line for Valvita. *South-African Food Review*. **27**: 23-24

Nevondo, T.S., and Cloete, T.E. 1999. Bacterial and chemical quality of water supply in the Dertig village settlement. *WaterSA.* **5**(2): 215-220

Nies, D.H. 1999. Microbial heavy metal resistance. *Journal of Applied Microbiology and Biotechnology.* **51**: 730-750

North West Province- State of the Environment Report. (NWP-SOTE). 2002. www.nwpg.gov.za/soer/FullReport/toc.html. (Accessed on 2 September 2007)

Nue, H.C. 1992. The crisis in antibiotic resistance. *Science*. **257**:1064-1073

O'Connor, J.T., and Banerji, S.K. 1984. Biologically mediated corrosion and water quality deterioration in distrtibution systems. U.S.Environmental Protection Agency Report, EPA-600/52-84-056. Cincinnati, US. 442

O'Toole, G.A., Kaplan, H.B., and Kolter, R. 2000. Biofilm formation as microbial development. *Annual Review of Microbiology.* **54**: 49-79

Obi, C.L., Ramalivhana, J., Momba, M.N.B., and Igumbor J. 2007. Scope and frequency of enteric bacterial pathogens isolated from HIV/AIDS patients and their household drinking water in Limpopo Province. *WaterSA*. **33**(4): 539-548

Olenginski, T.P., Bush, D.C., and Harrington, T.M. 1991. Plant thorn synovitis: an uncommon cause of monoarthritis. *Seminars in Arthritis and Rheumatism.* **21**: 40-46

Omland, T. 1960. Nosocomial urinary tract infections caused by *Proteus rettgeri. Acta Pathology Microbiology Scandinavian.* **42**: 221-230

Panthswa, M.J. 2006. Investigation of faecal pollution and occurrence of antibiotic resistant bacteria as a function of a changed environment. M.Env.Sc. dissertation, North-West University, Potchefstroom Campus, South Africa.

Pavlov, D., de Wet, C.M.E., Grabow, W.O.K., and Ehlers, M.M. 2003. Determination of cytotoxicity and invasiveness of heterotrophic plate count bacteria isolated from drinking water. *Water Supply.* **2**(3):155

Pavlov, D., de Wet, C.M.E., Grabow, W.O.K., and Ehlers, M.M. 2004. Potentially pathogenic features of heterotrophic plate count bacteria isolated from treated and untreated drinking water. *International Journal of Food Microbiology.* **92**(3): 275-287

Peters, R.J.B., De Leer, E.W.B., and De Galan, L. 1990. Dihaloacetonitriles in Dutch drinking water. *Water Research.* **24**: 797-800

Pillay, T., and Horner, B. 2005. *Klebsiella* death 2 years ago 'recovered up'. http://www.dispatch.co.za/2005/07/25/South Africa/ cover.html. (Accessed 25 September 2007)

Prescott, L.M., Harley, J.P. and D.A Klein. 2005 Laboratory exercises in Microbiology: 5th Edition. Boston: McGraw-Hill, US.

Prevost, M., Rompre, A., Coalier, J., Servais, P., Laurent, P., Clement, B., and Servais, P. 1998. Suspended bacterial biomass and activity in full scale drinking water distribution systems: impact of water treatment. *Water Research.* **32**(5): 1393-1406

Putman, M., Van Veen, H.W., Konings, W.N. 2000. Molecular properties of bacterial multidrug transporters. *Microbiology and Molecular Biology Reviews.* **64**(4): 672-693

Ramatlhape, L.L. 2005. The assessment of multiple antibiotic resistant enterococci in communal and commercial cattle faecal samples and their water sources in Mafikeng, North-West Province, RSA. M.Sc in Agriculture, North-West University, Mafikeng Campus, South Africa.

Rand, J.L., Hofmann, R., Alam, M.Z.B., Chauret, C., Cantwell, R., Andrews, R.C., and Gagnon, G.A. 2007. A field study evaluation for mitigating biofouling with chlorine dioxide or chlorine integrated with UV disinfection. *Water Research.* **41**: 1939-1948

Reasoner, D.R. 1990. Monitoring heterotrophic bacteria in potable water. *In:* Mcfeters, G.A. (editor). Drinking water microbiology- progress and recent developments, Springer –Verlag, New York. 452-477

Rehena, Z., Malik, A., and Ahmad, M. 1996. Genotoxicity of the Ganges water at Narora (UP), India. *Mutation Research.* **367**: 187-193

Reyes, A., Letelier, M.V., De la Iglesia, R., Gonzalez, B., and Lagos, G. 2007. Microbiology induced corrosion of copper pipes in low pH. *International Biodeterioration & Biodegradation*, doi:10.1016/j.ibiod.2007.06.001

Ribas, F., Perramon, J., Terradillos, A., Frias, J., and Lucena, F. 2000. The Pseudomonas group as an indicator of potential regrowth in water distribution systems. *Journal of Applied Microbiology.* **88**(4): 704

Ridge, L.E.L., and Thomas, M.E.M. 1955. Infection with the Providence type of paracolon bacillus in a residential nursery. *Journal of Pathology Bacteriology.* **69**: 335-336

Ridgeway, H.F., and Olsen, B.H. 1981. Scanning electron microscope evidence for bacterial colonization of a drinking water distribution system. *Applied and Environmental Microbiology.* **41**:274-287

Rusin, P.A., Rose, J.B., Haas, C.N., and Gerba, C.P. 1997. Risk assessment of opportunistic bacterial pathogens in drinking water. *Reviews of Environmental Contamination Toxicology.* **152**: 57-83

Sabry, S.A., Ghozian, H.A., and Abou-Zeid, D-M. 1997. Metal tolerance and antibiotic resistance patterns of a bacterial population isolated from seawater. *Journal of Applied Bacteriology.* **82:** 245-252.

Schaber, J.A., Triffo, W.J., Suh, S.J., Oliver, J.W., Hastert, M.C., Griswold, J.A., Auer, M., Hamwood, A.N., and Rumbaugh, K.P. 2007. *Pseudomonas aeruginosa* forms biofilms in acute infection independent of cell to cell signaling. *Infection and Immunity.* **75**(8): 3715-3721

Schembri, M.A., Givskof, M., and Klemm, P. 2002. An attractive surface: Gram negative bacterial biofilms. *Science Signal, Transduction Knowledge Environment* **132**: 6.

Schembri, M.A., Kjaergaard, K., and Klemm, P. 2003. Global gene expression in *Escherichia coli* biofilms. *Molecular Microbiology.* **48**: 253

Schwartz, T., Hoffmann, S., and Obst, U. 2003. Formation of natural biofilms during chlorine dioxide and UV disinfection in a public drinking water distribution system. *Journal of Applied Microbiology.* **95**(3): 591-601

Schwartz, T., Kalmbach, S., Hoffmann, S., Szewzyk, U., and Obst, U. 1998. PCR-based detection of mycobacteria in biofilms from a drinking water distribution system. *Journal of Microbiological Methods* **34:** 113–123

Semel, J.D., and Trenholme, G. 1990. *Aeromonas hydrophilia* water associated traumatic wound infections: A review. *Journal Trauma.* **30**: 324-327

Sheffer, P., Stout, T., Muder, R., and. Wagener M. 2004. Efficacy of New Point-of- Use Water Filters To Prevent Exposure to Legionella and Waterborne Bacteria. *Journal of American Infection Control.* **32**(3): 87

Shisana, O., Rehle, T., Simbayi, L., Parker, W., Zuma, K., Bhana, A., Connoly, C., Jooste, S., and, Pillay, V. 2005. South African national HIV prevalence, HIV incidence, Behaviour and Communiation Survey, HSRC Press. 156

Silver, S., Phung, L.T., Lo, J-F., and Gupta, A. 2001. Toxic metal resistances: Molecular biology and potential for bioremediation (33 - 41), *In:* Ahmed N, Qureshi F.M. and Khan O.Y. (editors) Industrial and Environmental Biotechnologies. Horizon Press, England. 33-41

Smith, D.L., Harris, A.D., Johnson, J.A., Silbergeld, E.K., and Morris, .J.G. 2002. Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria *Proceedings of the National Acadamy Sciences USA*. **99** (9): 6434–6439

South African National Standard. SANS 241:2005.6th.Edition. http://www.stansa.co.za

Spain, A., and Alm, E. 2003. Implications of microbial heavy metal tolerance in the Environment. *Reviews in Undergraduate Research.* **2**:1-6

Srinivasan, S., and Harrington, G.W. 2007. Biostability analysis for drinking water systems. *Water Research.* **41**: 2127-2138

Stats. SA (2005). Mortality and causes of death in South Africa, 2005: Findings from death notification. http://www.hst.org.za/indicators/StatsSA/P03093_2005.pdf (Accessed 25 September 2007)

Steward, P.S., and Costerton, J.W. 2001. Antibiotic resistance of bacteria in biofilms. *Lancet.* **358**: 135-138.

Surman, S.B., Walker, J.J., Goddard, D.T., Morton, L.H.G., Keevil, C.W., Weaver, W., Skinner, A., Hanson, K., Coldwell, D., and Kurtz, J. 1996. Comparison of microscope

techniques for the examination of biofilms. *Journal of Microbiological Methods*. **25**(1): 57-70

Szewzyk, U., Szewzyk, R., Manz, W., and Scheifer, K.H. 2000. Microbiological safety of drinking water. *Annual Review of Microbiology*. **54**: 81-127

The piping debate: copper vs CPVC. (http://www.allbusiness.com/construction/constructionbuildingsresidential/4021027-1.html. (Accessed on 10 January, 2008)

Tiedt, L. 2006. Electron Microscopy Unit. North-West University, Potchefstroom Campus, South Africa.

Toussaint, A., and Merlin, C. 2002. Mobile elements as a combination of functional modules. *Plasmid*. **47**(1):26-35

Turpeinen, R., Kairesalo, T., and Häggblom, M.M. 2004. Microbial community structure and activity in arsenic-, chromium- and copper-contaminated soils. *FEMS Microbial Ecology*, **47**(1): 39-50

Understanding a selecting an appropriate desalting process train for a specific application. http://www.trusseltech.com/images/RO_Membanes.jpg. (Accessed on 18 September 2007)

Underwood, S. 2004. *Citrobacter koseri* and *Citrobacter amalonaticus* isolates carry highly detegent –lactamase genes despite having high levels of biochemical similarity and 16S rRNA sequence homology. *Antimicrobial Chemotherapy*. **53**: 1076-1080

U.S. EPA 1997. Drinking water infrastructure needs survey- First report to congress. EPA/812/R-97/001. Office of Water, Washington, DC

U.S. EPA 2000. EPA Studies on sensitive subpopulations and drinking water contaminants. US Environmental protection Agency, Washington, DC.

Vally, H., Whittle, A., Cameron, S., Dowse, G.K., and Watson T. 2004. Outbreak of *Aeromonas hydrophilia* wound infections associated with mud football. *Clinical Infectious Diseases.* **38**: 1084-1089

Valtin, H. 2007. Drink at least eight glasses of water a day. Really? Is there scientific evidence for "8x8"? *American Journal of Physiology: Regulatory, Integrative and Comparitive Physiology.* **283**(5):993-1004

Van Aardt, W. 2007. Potch se drinkwater is skoon. *In*: Beeld. http://news24.com/Beeld/Opinie/Briewe.html. (Accessed 28 September 2007)

Van Aardt, W., and Hough, M. 2007. The effect of short-term Cu exposure on the oxygen consumption and Cu accumulation of mudfish (*Lebeo capensis*) and the largemouth bass (*Micropteris salmoides*) in hard water. *WaterSA*, **33**(5): 701-708

Van Nierop, W.H., Duse, A.G., Stewart, R.G., Bilgeri, Y.R., and. Koornhof, H. J. 1998. Molecular epidemiology of an outbreak of *Enterobacter cloacae* in the neonatal intensive care unit of a provincial hospital in Gauteng, South Africa. *Journal of Clinical Microbiology.* **36**(10): 3085-3087

Van Rostenbergh, H., Noraida, R., Wan Pauzi, W.I., Habsah, H., Zeehaida, M., Rosliza, A.R., Fatimah, I., Nik Sharimah, N.Y., and Maimunah, H. 2006. The clinical picture of neonatal infection with *Pantoea* Species. *Japenese Journal of Infectious Diseases.* **59**: 120-121

Vasconcelos, J.J., Rossman, L.A., Grayman, W.M., Boulos, P.F., and Clark, R.M. 1997 Kinetics of chlorine decay. *Journal of American Water Works Association.* **89**(7):54-65

Venter, L.C.R. 2001. Die effek van lood en waterkwaliteit op die biologie van die vleikurper, *Tilapia sparmanii.* M.Sc verhandeling, Potchefstroomse Universiteit vir CHO, Potchefstroom, South Africa.

Vitanage, D., Pamminger, F., Vourtsanis, T., and. Ainsworth R. 2004. Maintenance and survey of distribution systems. *In*: Bartman, J., Cotruvo, J., Exner, M., Fricker, C., and

Glasmacher, A. (editors). WHO, Safe piped water: Managing microbial water quality in piped distribution systems, IWA Publishing, London.

Volk, C.J., and LeChevallier, M.W. 1999. Impacts of the reduction of nutrient levels on bacterial water quality in distribution systems. *Applied Environmental Microbiology.* **65**(11): 4957-4966

Vreeburg, I.J.H.G., and Boxall, J.B. 2007. Discolouration in potable water distribution systems. A review. *Water Research.* **41**: 519-529

Wallis, C., Stagg, C.H., and Melnick, J.L. 1974. The hazards of incorporating charcoal filters into domestic water systems. *Water Research.* **8**: 111-113

Walsh, C. 2000. Molecular mechanisms that confer antibacterial drug resistance, *Nature.* **406**: 775-781

Walsh, S.E., Maillard, Y., Russel, A.D., Catrenich, C.E., Charbinneau, D.L. and Bartolo, R.G. 2003. Development of bacterial resistance to several biocides and effects on antibiotic susceptibility. *Journal of Hospital Infection.* **55**: 98-107

Washington, J.A., Senjem, D.H., Haldorson, A., Schutt, A.H., and Martin, W.J. 1973. Nosocomially acquired bacteriuria due to *Proteus rettgeri* and *Prov.stuartii. American Journal of Clinical Pathology.* **60**: 836-838

Water and 20 tips for getting your 8 glasses daily. http://www.dwlz.com/WWinfo/water.html). (Accessed on 16 September 2007)

WaterPik Instapure F2C faucet mount water filter. http://www.amazon.com/Waterpi-Instapure-faucet-mount-filter/dp/B000LN06BA). (Accessed on 18 May 2005)

Water Quality Research Council. 1991. Water Review: Residential reverse osmosis. **9**:2

Watermeyer, J. 2002. The incidence of antibiotic resistance bacteria in red meat. M.Sc dissertation, Potchefstroom University for CHE, South Africa.

World Health Organisation (WHO). 2002. Heterotrophic plate count measurement in drinking water safety management. Report on expert meeting. Geneva.

World Health Organization (WHO). 2003. Heterotrophic plate counts and drinking water safety: Edited by Bartran,J., Cotruvo, J.,Exner,M., Fricker,C.,and Glasmacher,A. Published by IWA Publishing, London, UK.

World Health Organization (WHO). 2004. Water treatment and Pathogen Control: Process Efficiency in Achieving Safe Drinking Water. *In:* LeChevallier, M.W., and Kwok-Keung, A. (editors). IWA Publishing, London, UK.

World Health Organization (WHO). 2006. Guidelines for dinking water quality [electronic resource], incorporating first addendum. Vol.1, Recommendations. 3rd Edition. ISBN 9241546964

World Health Organization (WHO). 2007. Ten statistical highlights in global public health http://www.who.int/whosis/whostat2007_10highlights.pdf. (Accessed 25 September 2007)

Zhang, Z, Stout, J.E., Yu, V.L., and Vidic, R. 2007. Effect of pipe corrosion scales on chlorine dioxide consumption in dinking water distribution systems. *Water Research.* doi: 10.1016/j.watres.2007.07.054

Zhou, J., Bruns, M.A., and Tiedje, J.M. 1996. DNA Recovery from soils of diverse composition. *Applied Environmental Microbiology.* **62**(2): 316-322

APPENDIX A

Part 1

Antibiotic Resistance Data

Home Water Filtering Systems

Table A1- Isolates from the reverse osmosis home water filtering system –March {(3 Months), (r = resistant; s= susceptible; i = intermediate)}

Tested Antibiotics	GM	K	Ne	S	C	AP	T	CIP	VA	E
BDP203	s	r	r	s	i	r	r	s	-	r
BDP203	s	r	r	s	i	r	r	s	-	r
BDP203	s	r	r	s	i	r	r	s	-	r
BB103	s	r	s	s	s	r	r	s	-	r
BB203	s	r	s	s	s	r	r	s	-	r
BB303	s	r	s	s	s	r	r	s	-	r
BLP103	s	r	s	s	s	r	r	s	-	r
BLP203	s	r	s	s	s	r	r	s	-	r
BLP303	s	r	s	s	s	r	r	s	-	r
BA103	s	r	s	s	i	r	r	s	-	r
BA203	s	r	s	s	i	r	r	s	-	r
BA303	s	r	s	s	i	r	r	s	-	r
BG1103	r	r	r	s	r	r	r	s	r	r
BG1203	r	r	r	s	r	r	r	s	r	r
BG1303	r	r	r	s	r	r	r	s	r	r
BG2103	s	r	i	s	i	r	r	s	r	r
BG2203	s	r	i	s	i	r	r	s	r	r
BG2303	s	r	i	s	i	r	r	s	r	r
BG3103	i	s	s	s	r	r	s	s	-	s
BG3203	i	s	s	s	r	r	s	s	-	s
BG3303	i	s	s	s	r	r	s	s	-	s
BG4103	i	s	r	s	i	r	r	s	-	s
BG4203	i	s	r	s	i	r	r	s	-	s
BG4303	i	s	r	s	i	r	r	s	-	s
BW103	s	s	s	s	s	r	s	s	i	s
BW203	s	s	s	s	s	r	s	s	i	s
BW303	s	s	s	s	s	r	s	s	i	s

Table A2- Isolates from the carbon home water filtering system –Augustus (6 Months)

Tested Antibiotics	GM	K	PG	S	C	AP	T	CIP	VA	E
VW091	s	r	-	s	s	r	i	s	-	r
VW092	s	r	-	s	s	r	i	s	-	r
VW093	s	r	-	s	s	r	i	s	-	r
VW094	s	r	-	s	s	r	i	s	-	r
VW095	s	r	-	s	s	r	i	s	-	r
G091	s	s	s	s	s	s	r	s	-	s
G092	s	s	s	s	s	s	r	s	-	s
G093	s	s	s	s	s	s	r	s	-	s
G094	s	s	s	s	s	s	r	s	-	s
G005	s	s	s	s	s	s	r	s	-	s
W091	s	s	r	s	s	i	r	s	i	i
W092	s	s	r	s	s	i	r	s	i	i
W093	s	s	r	s	s	i	r	s	i	i
W094	s	s	r	s	s	i	r	s	i	i
W095	s	s	r	s	s	i	r	s	i	i

Antibiotic Resistance Data
Biofilm Device

Table A3- Isolates from the 4 week sample period.

Tested Antibiotics	GM	K	PG	S	C	AP	T	CIP	VA	E
OR104	s	s	S	s	s	s	s	s	s	s
OR204	s	s	s	s	s	s	s	s	s	s
OR304	s	s	s	s	s	s	s	s	s	s
OR404	s	s	s	s	s	s	s	s	s	s
OR504	s	s	s	s	s	s	s	s	s	s
LG104	s	s	s	s	s	s	r	s	s	s
LG204	s	s	s	s	s	s	r	s	s	s
LG304	s	s	s	s	s	s	r	s	s	s
LG404	s	s	s	s	s	s	r	s	s	s
LG504	s	s	s	s	s	s	r	s	s	s

Table A4- Isolates from the 6 week sample period.

Tested Antibiotics	GM	K	PG	S	C	AP	T	VA	E
VW106	s	s	r	s	s	r	r	s	s
VW206	s	s	r	s	s	r	r	s	s
VW306	s	s	r	s	s	r	r	s	s
VW406	s	s	r	s	s	r	r	s	s
VW506	s	s	r	s	s	r	r	s	s
HG106	s	s	-	s	s	s	s	-	s
HG206	s	s	-	s	s	s	s	-	s
HG306	s	s	-	s	s	s	s	-	s
HG406	s	s	-	s	s	s	s	-	s
HG506	s	s	-	s	s	s	s	-	s
W106	s	s	-	s	s	s	r	-	s
W206	s	s	-	s	s	s	r	-	s
W306	s	s	-	s	s	s	r	-	s
W406	s	s	-	s	s	s	r	-	s
W506	s	s	-	s	s	s	r	-	s
OR106	s	s	r	s	s	r	r	r	s
OR206	s	s	r	s	s	r	r	r	s
OR306	s	s	r	s	s	r	r	r	s
OR406	s	s	r	s	s	r	r	r	s
OR506	s	s	r	s	s	r	r	r	s

Table A5- Isolates from the 8 week sample period.

Tested Antibiotics	GM	K	PG	S	C	AP	T	VA	E
HG1	S	S	-	S	S	S	S	-	S
HG2	S	S	-	S	S	S	S	-	S
HG3	S	S	-	S	S	S	S	-	S
HG4	S	S	-	S	S	S	S	-	S
HG5	S	S	-	S	S	S	S	-	S

Part 2

Physical characteristics of Potchefstroom drinking water

Table A6- Raw data (pH, temperature, conductivity, TDS and salinity) values.

Week	pH		Average	Temperature (°C)		Average	Conductivity (µs/cm)		Average	TDS		Average	Salinity [nLF]	
1	8.07	8.09	8.08	19.2	18.3	18.75	677	687	682	677	687	682	0.3	0.3
2	8.07	8.04	8.06	18.2	17.8	18	687	689	688	687	689	688	0.3	0.3
3	8.05	8.08	8.06	20.4	21.2	20.8	679	683	681	679	683	681	0.3	0.3
4	8.08	8.05	8.06	22.4	20.6	21.5	682	688	685	682	688	685	0.3	0.3
5	8.22	8.4	8.31	18.3	19.8	19.05	693	687	690	693	687	690	0.3	0.3
6	8.11	8.3	8.26	21.4	20	20.7	693	687	690	693	687	690	0.3	0.3
7	8.1	8.1	8.1	23.8	22.1	22.95	786	742	764	786	742	764	0.3	0.3
8	8.231	8.156	8.19	24.5	23.6	24.05	627	654	640.5	627	654	640.5	0.3	0.3

Part 3

Heavy Metal Tolerance Data

Table A7- Format indicating the various volumes of metal suspension, media and bacteria loaded into the 96 well plates to yield the different copper concentrations.

	[Copper]	V Metal(µl)	V Water(µl)	V Media(µl)	V Bact(µl)	V Final(µl)
1	0.75	0.11	39.89	100	10	150
2	1	0.15	39.85	100	10	150
3	1.5	0.225	39.775	100	10	150
4	2	0.3	39.7	100	10	150
5	2.5	0.375	39.625	100	10	150
6	3	0.45	39.55	100	10	150
7	3.5	0.525	39.475	100	10	150
8	4	0.6	39.4	100	10	150
9	4.5	0.675	39.325	100	10	150
10	5	0.75	39.25	100	10	150
11	Blank		40	110	0	150
12	Control		40	100	10	150

Table A8- Average OD_{600nm} reading of each isolate at each copper concentration.

[Copper]	0.75	1	1.5	2	2.5	3	3.5	4	4.5	5
OR 04	0.131	0.130	0.134	0.127	0.117	0.136	0.162	0.145	0.177	0.229
W 06	0.147	0.172	0.121	0.139	0.127	0.113	0.089	0.095	0.091	0.08
VW06	0.168	0.142	0.149	0.183	0.196	0.210	0.182	0.196	0.185	0.274
OR 06	0.096	0.08	0.081	0.096	0.162	0.134	0.127	0.112	0.132	0.201
HG 06	0.159	0.125	0.124	0.262	0.194	0.130	0.197	0.146	0.106	0.136
HG08	0.063	0.057	0.046	0.048	0.051	0.053	0.061	0.061	0.065	0.070
LG 04	0.151	0.194	0.205	0.176	0.174	0.134	0.147	0.130	0.151	0.169

Table A9- Raw heavy metal tolerance data (Optical density readings at different copper concentrations)

Isolates from week 4 sampling											
[Copper]	**0.75**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**C**
LG 0	0.119	0.115	0.1155	0.1185	0.118	0.086	0.1195	0.0735	0.067	0.107	0.234
LG 24	0.076	0.1	0.067	0.0807	0.078	0.085	0.078	0.073	0.109	0.095	0.359667
LG 48	0.14	0.18	0.225	0.2065	0.161	0.142	0.158	0.15	0.17	0.156	0.747667
LG 106	0.2673	0.3796	0.412	0.298	0.337	0.224	0.234	0.224	0.257	0.317	0.969667
OR 0	0.079	0.076	0.079	0.07	0.067	0.067	0.078	0.081	0.083	0.084	0.22
OR 24	0.095	0.097	0.099	0.093	0.075	0.087	0.073	0.065	0.074	0.091	0.380667
OR 48	0.154	0.151	0.154	0.136	0.11	0.113	0.221	0.112	0.181	0.213	0.456667
OR 106	0.196	0.199	0.204	0.209	0.216	0.278	0.274	0.321	0.37	0.526	0.717667
Isolates from week 6 sampling											
W 0	0.086	0.086	0.044	0.084	0.052	0.044	0.073	0.061	0.074	0.035	0.64
W 24	0.103	0.146	0.141	0.124	0.095	0.065	0.052	0.058	0.052	0.052	0.657
W48	0.181	0.248	0.122	0.18	0.189	0.196	0.125	0.166	0.144	0.152	0.859667
W 106	0.217	0.206	0.175	0.168	0.17	0.145	0.106	0.095	0.093	0.081	0.778333
VW 0	0.097	0.084	0.085	0.11	0.112	0.135	0.131	0.151	0.119	0.111	0.3325
VW 24	0.126	0.125	0.122	0.174	0.182	0.183	0.139	0.16	0.165	0.173	0.326
VW 48	0.204	0.173	0.187	0.229	0.237	0.232	0.204	0.216	0.209	0.323	0.353667
VW 106	0.246	0.187	0.203	0.217	0.254	0.289	0.253	0.257	0.247	0.487	1.011333
OR 0	0.099	0.09	0.092	0.106	0.111	0.149	0.148	0.15	0.162	0.175	0.317
OR 24	0.064	0.057	0.058	0.073	0.159	0.112	0.11	0.093	0.115	0.097	0.355
OR 48	0.093	0.078	0.081	0.097	0.158	0.13	0.123	0.101	0.122	0.211	0.735667

OR 106	0.127	0.095	0.092	0.108	0.219	0.143	0.127	0.103	0.13	0.32	0.808667
HG 0	0.086	0.079	0.085	0.102	0.139	0.082	0.099	0.094	0.093	0.06	0.2498
HG 24	0.099	0.074	0.081	0.198	0.179	0.086	0.172	0.095	0.062	0.08	0.298
HG 48	0.17	0.136	0.143	0.346	0.228	0.142	0.248	0.154	0.119	0.186	0.452667
HG 106	0.281	0.21	0.186	0.4	0.231	0.209	0.269	0.24	0.15	0.219	0.454667
Isolates from week 8 sampling											
HG 0	0.006	0.0163	0.0193	0.031	0.045	0.128	0.061	0.046	0.046	0.056	0.307667
HG 24	0.095	0.095	0.064	0.062	0.063	0.074	0.066	0.077	0.075	0.078	0.319
HG 48	0.075	0.058	0.049	0.049	0.047	0.047	0.043	0.057	0.066	0.053	0.363667
HG 106	0.075	0.057	0.051	0.05	0.047	0.05	0.073	0.062	0.071	0.092	0.386067
HG 0	0.006	0.0163	0.0193	0.031	0.045	0.128	0.061	0.046	0.046	0.056	0.307667

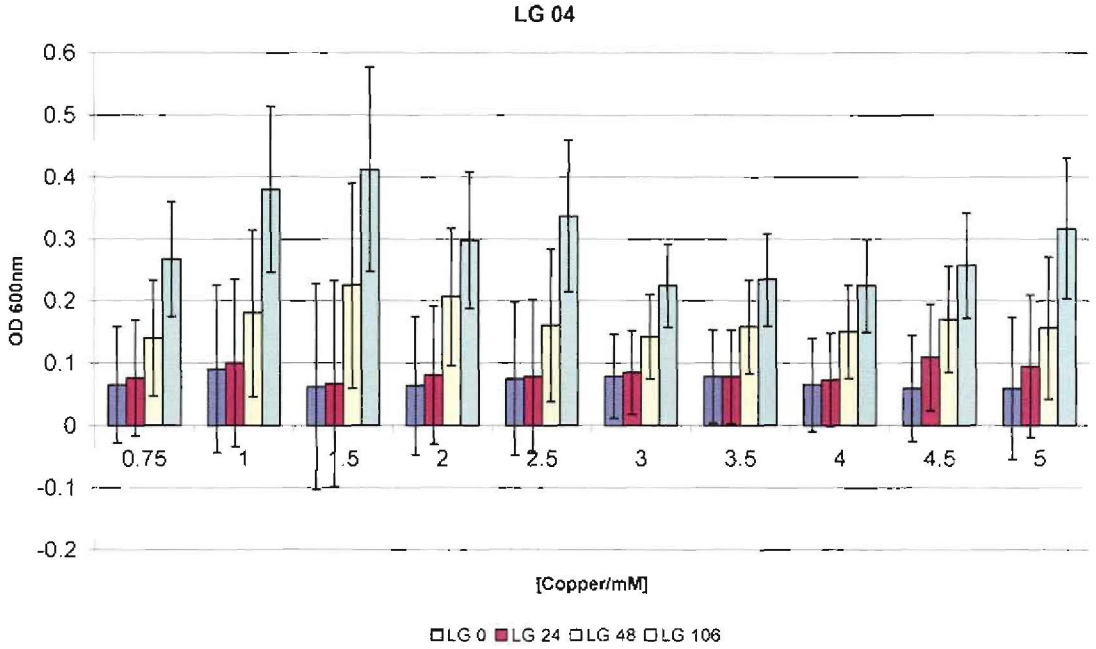


Figure A1- OD readings of LG 04 isolates after incubation periods of 0, 24, 48 and 106 h at different copper concentrations at 680nm

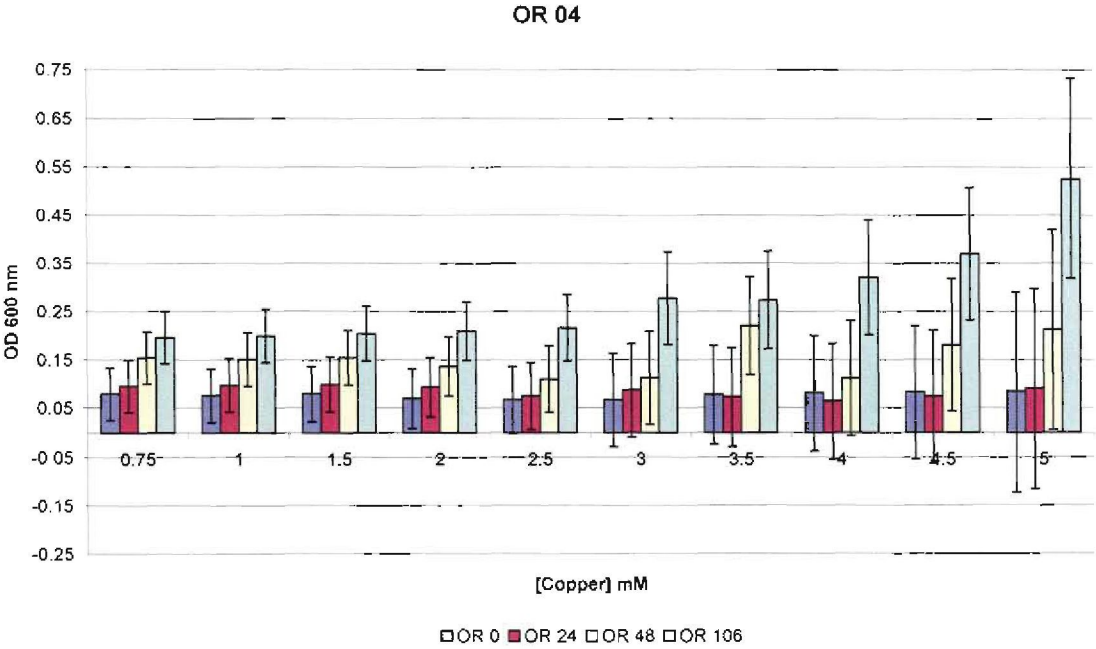


Figure A2- OD readings of OR 04 isolates after incubation periods of 0, 24, 48 and 106 h at different copper concentrations at 680nm

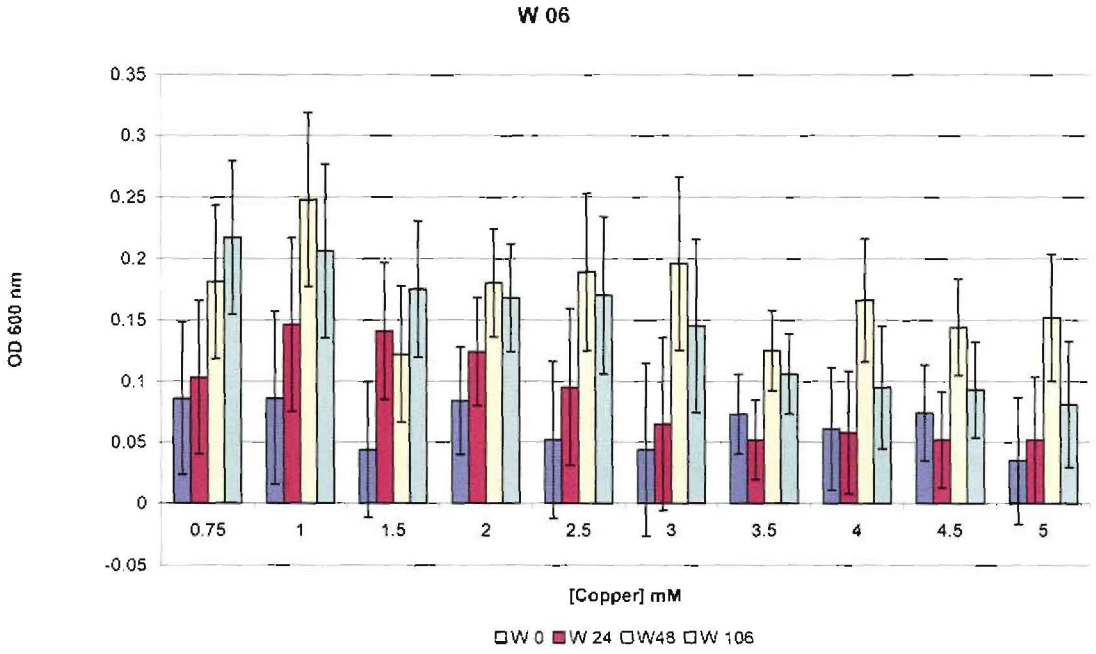


Figure A3- OD readings of W 06 isolates after incubation periods of 0, 24, 48 and 106 h at different copper concentrations at 680nm

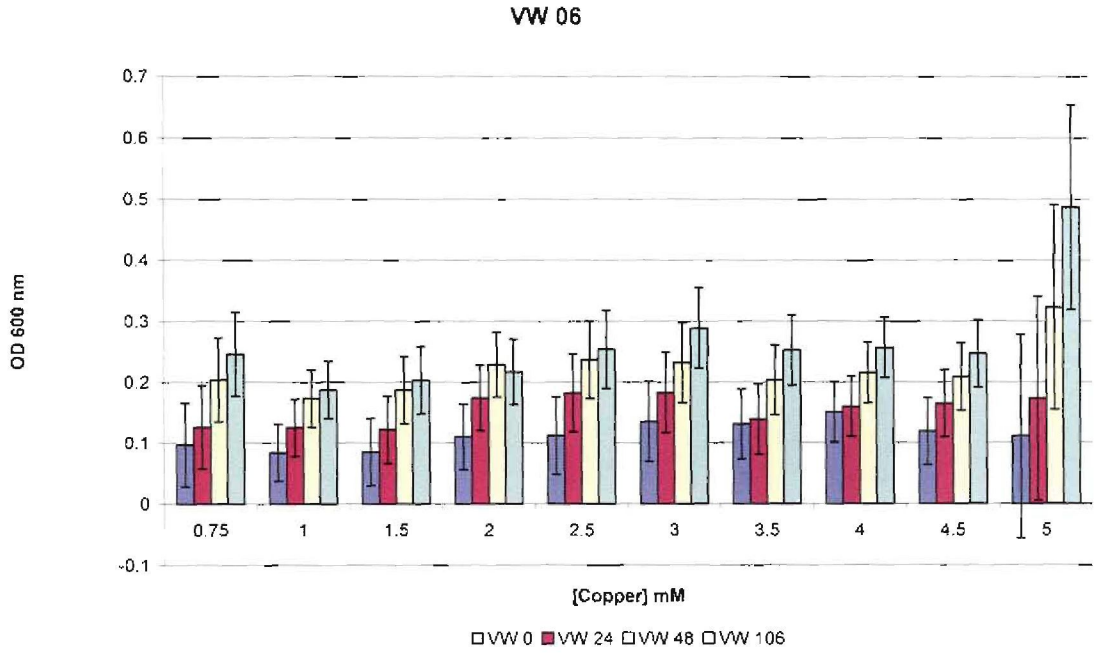


Figure A4- OD readings of VW 06 isolates after incubation periods of 0, 24, 48 and 106 h at different copper concentrations at 680nm

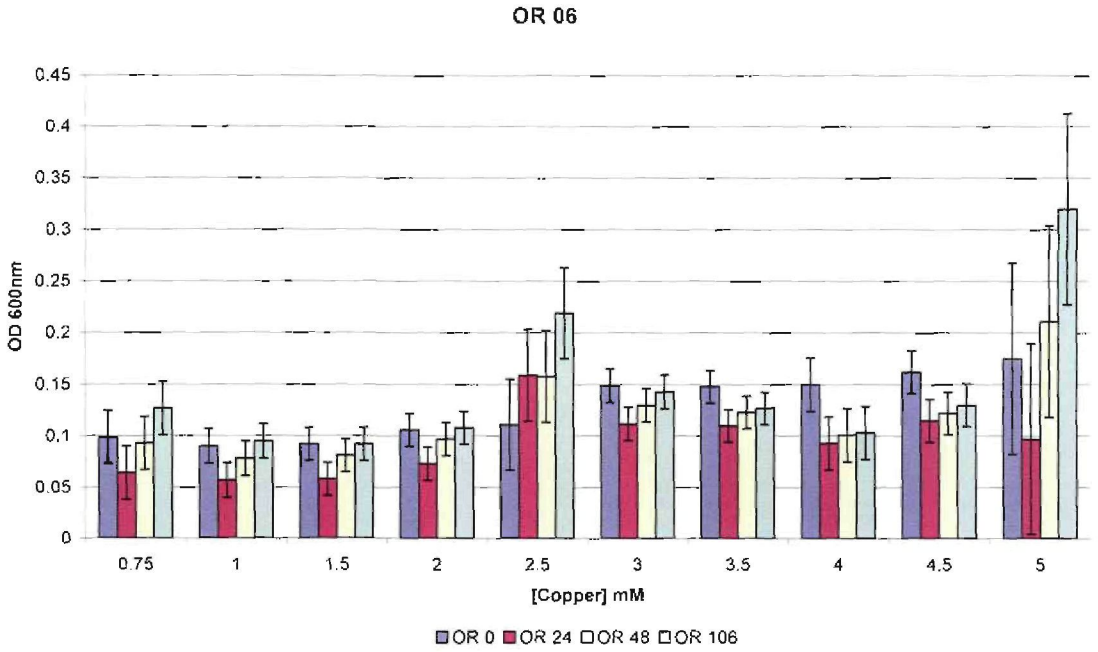


Figure A5- OD readings of OR 06 isolates after incubation periods of 0, 24, 48 and 106 h at different copper concentrations at 680nm

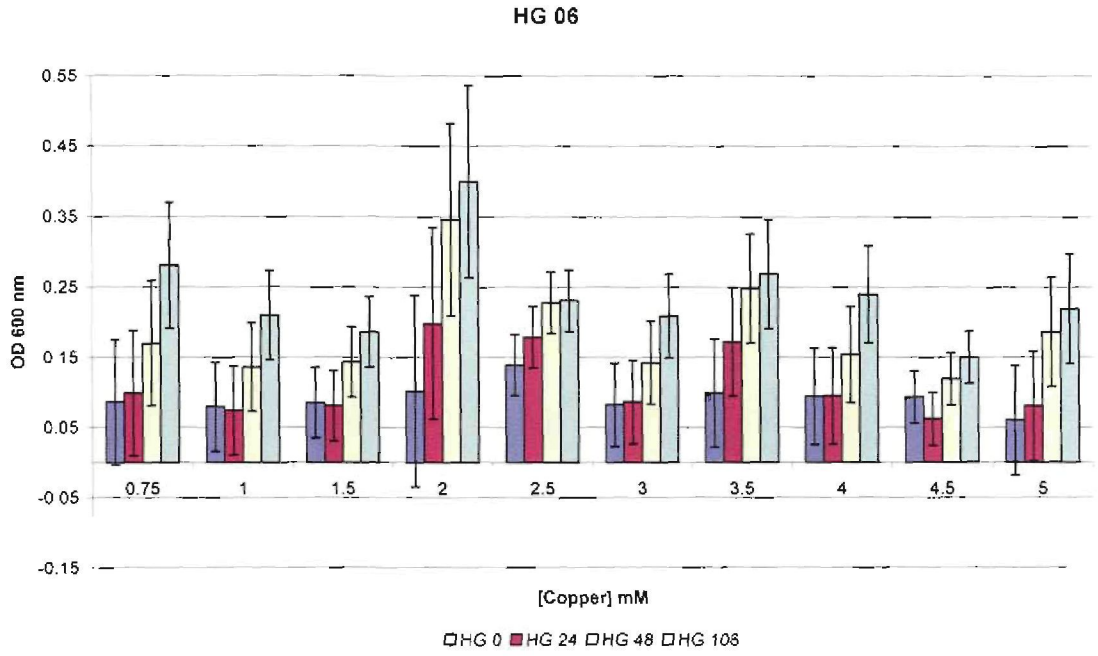


Figure A6 - OD readings of OR 06 isolates after incubation periods of 0, 24, 48 and 106 h at different copper concentrations at 680nm

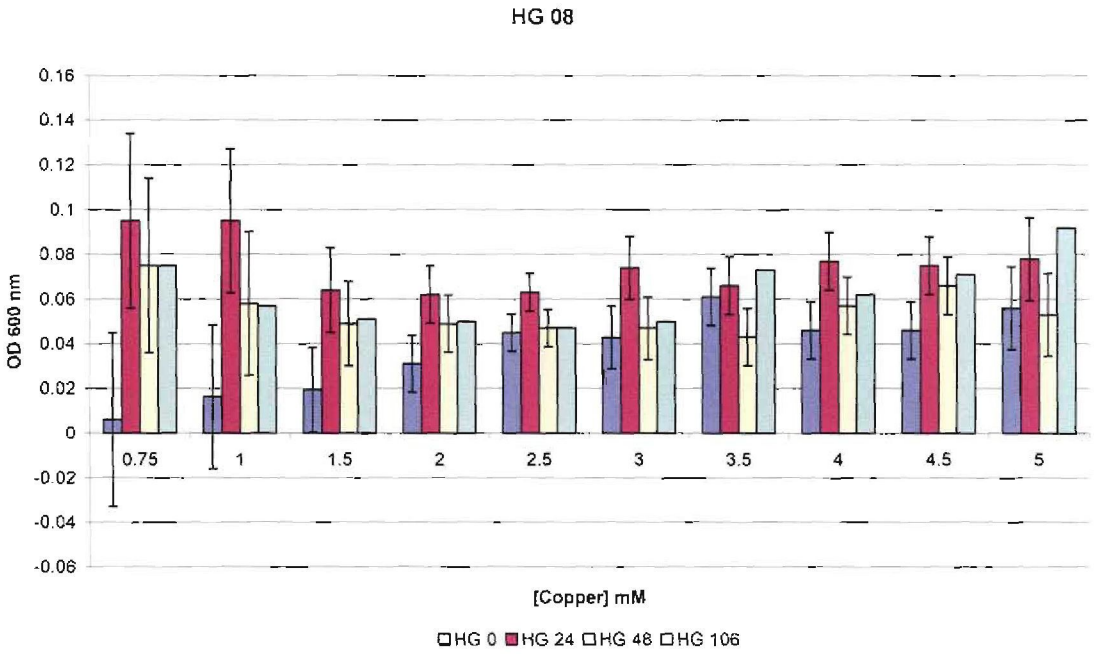


Figure A7- OD readings of OR 06 isolates after incubation periods of 0, 24, 48 and 106 h at different copper concentrations at 680nm

Part 4

Bacterial Identifications

TSI Results

Table A10 -TSI Agar Results (RO Filter)

Isolate Name	**TSI Slants**				**Possible Identification.**		
	Butt	Slant	H_2S	Gas			
BBDP103	A	A	N	Y	Enterobacter		Klebsiella
BBDP203	A	A	N	Y	Enterobacter		Klebsiella
BBBO3	A	A	N	Y	Enterobacter		Klebsiella
BLP03	A	A	N	Y	Enterobacter	Citrobacter	Klebsiella
BA03	A	A	N	N			
BBG03	A	K	N	N	Shigella	Enterobacter	
BBG04	A	K	N	N	Shigella	Enterobacter	

Table A11- TSI Agar Results (Carbon Filter)

Isolate Name	**TSI Slants**				**Possible Identification.**
	Butt	**Slant**	**H_2S**	**Gas**	
VW09	A	A	N	N	*Serratia*
G09	A	A	N	N	*Serratia*

API 20 E Results (Filters)

Table A12- Results of the API 20 E system (RO Filter)

	BDP103	BDP203	BB103	BLP103	BG303	BG403
ONPG	+	+	+	+	-	-
ADH	-	-	+	-	+	+
LDC	-	-	+	-	-	-
ODC	-	-	-	+	+	+
CIT	+	+	+	+	+	+
H_2S	-	-	-	-	-	-
URE	-	-	-	-	-	-
TDA	+	+	-	-	-	-
IND	-	-	-	-	-	-
VP	+	+	-	-	-	-
GEL	-	-	+	-	+	+
GLU	+	+	+	+	+	+
MAN	+	+	+	+	-	-
INO	-	-	-	-	+	+
SOR	-	-	-	+	+	+
RHA	+	+	-	+	+	+
SAC	-	-	-	-	+	+
MEL	-	-	-	-	+	+
AMY	-	-	+	+	+	+
ARA	-	-	-	+	+	+
OXI	+	+	-	-	-	-
Name	*Enterobacter. agglomerans*	*Enterobacter. agglomerans*	*Aeromonas hydrophilia*	*Citrobacter* spp.	*Enterobacter cloacae*	*Enterobacter cloacae*

API 20 E Results (Device)

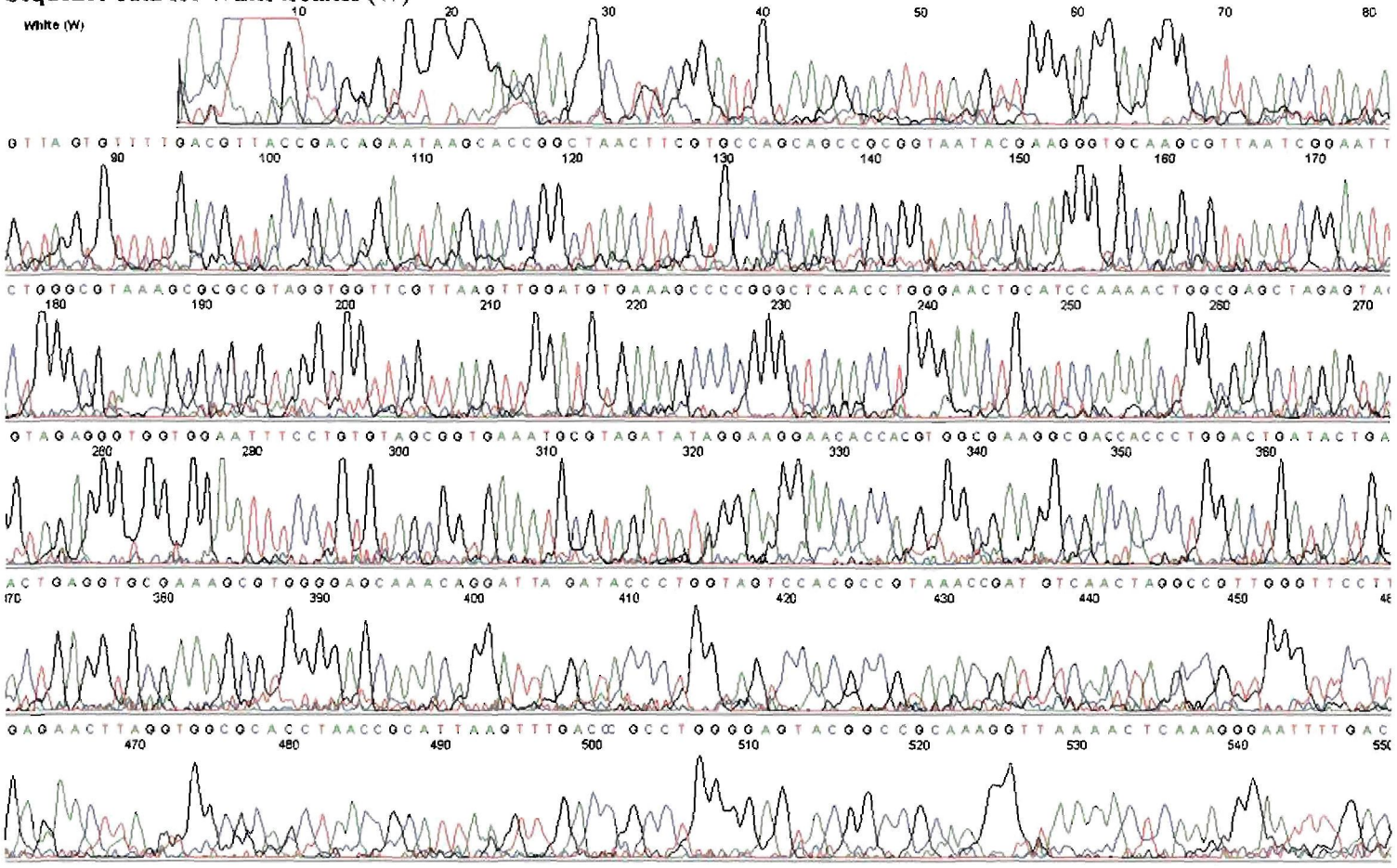
Table A14- Results of the API 20 E system (Gram negative isolates from biofilm device)

	HG 06	HG 08
ONPG	+	+
ADH	+	+
LDC	-	-
ODC	+	+
CIT	+	+
H_2S	-	-
URE	-	-
TDA	-	-
IND	-	-
VP	+	+
GEL	+	+
GLU	+	+
MAN	+	+
INO	-	-
SOR	+	+
RHA	+	+
SAC	+	+
MEL	+	+
AMY	+	+
ARA	+	+
OXI	-	-
Name	*Enterobacter cloacae*	*Enterobacter cloacae*

Table A15- TSI results for isolates from the biofilm device

Isolate Name	TSI Slants				Possible Identification.
	Butt	**Slant**	**H_2S**	**Gas**	
LG104					
OR104					
VWO6			-	-	
HG06	A	K	-	-	*Shigella or Serratia*
WO6	A	K	+	+/-	*Pseudomonas aeruginosa*
OR06					
HG08	A	K	-	-	*Shigella or Serratia*

Sequence data for White isolates (W)



Sequence data for Orange isolates (OR)