The microbiological assessment of a biofiltration system in KwaZulu-Natal (South Africa) treating borehole water containing Mn (II) and Fe (II)

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Declaration 1 – Plagiarism

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Details of publications that form part and/or include research presented in this thesis:

Publication 1 (Chapter 3):

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

Additional data to this chapter is provided in the form of an appendix to chapter 3.

_____ _____

Lorika Selomi Beukes Date

Ms Lorika Beukes performed the experimental work and drafted the manuscript of the above publication.

_____ _____

Prof Stefan Schmidt Date

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This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has therefore been unavoidable.

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Abstract

In the following study, the potential role that microorganisms play in the removal of Mn (II) and Fe (II) was assessed using biofilter sand and water samples collected from a biofiltration system (operated by Umgeni Water in KwaZulu-Natal, Nottingham Road, at the Nottingham combined school, South Africa) treating borehole water containing manganese and iron. Initially the presence of Mn (II) and Fe (II) oxidizing bacteria was demonstrated in the biofiltration system. Thereafter, the contribution of individual microorganisms to the overall removal of manganese and iron was assessed in the laboratory by determining the difference in metal oxidation in the presence and absence of active bacteria at neutral pH, simulating conditions in the biofilter. Controls were run to verify the elimination via physiochemical reactions occurring within the biofiltration system. Finally a diversity snapshot of the bacteria present within the biofilter matrix was established via analysis of a clone library. Viable bacterial counts for the biofiltration system were established using MSVP (minimal salts vitamins pyruvate) medium - plus added manganese sulfate or iron sulfate targeting Mn (II) and Fe (II) oxidizing bacteria - and R2A for heterotrophic bacteria.

In the first experimental chapter, batch tests using MSVP were employed to determine manganese oxidation, by measuring the pH and ORP (oxidation reduction potential) in experimental flasks and controls over time. There was a clear drop in pH and a concomitant increase in ORP when an isolated manganese oxidizing strain (designated LB1) was grown in MSVP plus added manganese sulfate, indicating manganese oxidation. Based on physiological characteristics established by the VITEK-2 system as well as by 16S rRNA gene sequence analysis and MALDI-TOF (Matrix assisted laser desorption ionization-time of flight mass spectrometry) mass spectrometry of cell extracts, the isolate was identified as a member of the genus *Acinetobacter*. EDX (energy dispersive X-ray analysis) analysis of crystals formed in batch culture tests, containing MSVP plus either added manganese or iron sulfate, confirmed the ability of the isolate to oxidize both Mn (II) and Fe (II). The leucoberbelin blue colorimetric assay and batch tests using MSVP both demonstrated that in the presence of the isolated strain, *Acinetobacter* sp. LB1, the rate of Mn (II) oxidation at neutral pH was enhanced as compared to abiotic controls.

In the second experimental chapter the difference in Fe (II) oxidation between biological and abiological systems at neutral pH was determined using batch tests run with *Acinetobacter* sp. LB1 and Fe (II) in saline. In addition, the rate of Fe (II) oxidation was also determined at acidic pH and at alkaline pH in experimental and control flasks. To determine Fe (II) removal under conditions simulating those in the biofiltration system, batch tests were set up using borehole water freshly collected from the biofiltration system. In order to verify the contribution of native microorganisms in the borehole water to Fe (II) oxidation, these flasks were spiked with bacterial strains isolated from the biofiltration system - *Acinetobacter* sp. LB1 and *Burkholderia* sp. strain LB2 - and two known iron oxidizing strains *Leptothrix mobilis* (DSM 10617) and *Sphaerotilus natans* (DSM 565) were used to determine the contribution of reference iron oxidizers to Fe (II) oxidation. A separate set of the same flasks with the addition of filter sand was used to qualitatively demonstrate iron oxidation as it would occur within the biofiltration system. The ferrozine assay was employed to quantify the amount of Fe (II) in batch tests employing saline medium and in batch tests employing borehole water. EDX analysis was employed to confirm the presence of Fe (II) in oxidation products in the batch test flask with filter sand spiked with *Acinetobacter* sp. LB1.

In the presence of *Acinetobacte*r sp. LB1 at neutral pH in saline medium, the rate of Fe (II) oxidation was very similar to that in the abiological controls thus demonstrating that the presence of metabolically active microorganisms does not per se enhance the oxidation of Fe (II) like in the case of Mn (II) at neutral pH. Surprisingly, in the heat inactivated control, apparently the highest amount of Fe (II) was oxidized. As expected, at acidic pH very little oxidation of Fe (II) took place and at alkaline pH almost all Fe (II) in the flasks was removed and small amounts oxidized as determined by the amount of Fe (III) produced. Batch tests using borehole water proved that native microorganisms within the biofiltration system were more efficient in the oxidative removal of Fe (II) from the system, in comparison to the reference iron oxidizing strains. In the final experimental chapter, the presence of biofilms with actively metabolizing cells was examined on a pooled sample of biofilter matrix from the manganese and iron filter using CLSM (confocal laser scanning microscopy) image analysis. DNA was extracted from the biofilm material associated with biofilter matrix to establish a diversity snapshot of the bacteria present within the biofilter matrix.

ARDRA (amplified “rDNA” restriction analysis) analysis of the clone library revealed the presence of 15 unique OTU’s (operational taxonomic unit) based upon restriction patterns of amplified 16S rRNA genes of a total of 100 randomly selected clones. The majority of the clones were closely related to the genera *Nitrospira* and *Lactococcus*. Overall, 42% of the clones were assigned to the phylum *Proteobacteria*, 13% to the phylum *Actinobacteria*, 24% to the phylum *Firmicutes* and 21% to the phylum *Nitrospirae*. Overall, the results demonstrate that bacteria present within an established biofiltration system at neutral pH can contribute to the oxidative removal of Mn (II) and, apparently only to a smaller degree, to that of Fe (II) present in borehole water and that species within the proteobacterial genus *Acinetobacter* are potentially involved in the geochemical cycling of these two metals.

Keywords: Biofiltration, iron and manganese oxidation, *Acinetobacter* sp. LB1, batch tests, 16S rRNA, MALDI-TOF MS analysis, Mn (II) and Fe (II) colorimetric assays, EDX analysis, biofilm formation, CLSM image analysis, 16S rRNA clone library

Abbreviations: MSVP (minimal salts vitamins pyruvate), ORP (oxidation reduction potential), EDX (energy dispersive X-ray analysis), MALDI-TOF MS (Matrix assisted laser desorption ionization-time of flight mass spectrometry), rRNA (ribosomal RNA), ARDRA (amplified “rDNA” restriction analysis), CLSM (confocal laser scanning microscopy), OTU (operational taxonomic unit)

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Chapter 1

General Introduction

In order to ensure the safety and quality of potable water, it is important to maintain the naturally occurring biochemical reactions taking place within aquatic systems (Salomons and Förstner, 1984). Metals such as manganese and iron are classified as elements that form positive ions when in solution and their oxides typically form hydroxides in water (Tsezos and Volesky, 1982). Iron is a key component in many proteins that are necessary for microbial respiration and metabolism and both manganese and iron are essential trace elements in biological/biotechnological systems (Lovley, 2000). These two metals are prevalent in water bodies and their removal is typically mediated by a combination of microbial and abiotic oxidation reactions (Jakob, 1970). Manganese is naturally occurring, present in almost all environments and comprises approximately 0.1% of the Earth's crust (IPCS, 2004). It occurs in 11 oxidation states of which oxidation states 2 (most stable and predominate in nature), 4 and 7 are the most important (Gerber *et al.*, 2002).

In aquatic environments manganese exists in two major forms: Mn (II) and Mn (IV) and the conversion between these two forms takes place through oxidation and reduction reactions which are either abiotically or microbially mediated (IPCS, 2004). Manganese (Mn (II)) is more prevalent at low pH and redox potential (IPCS, 2004). Public concern about environmental pollution as well as new applications of manganese compounds such as potassium permanganate and cyclopentadienyl manganese tricarbonyl (CMT), have directed attention to the toxic properties of manganese compounds and the possible involvement of these compounds in causing malformations (Gerber *et al.*, 2002; De Meo *et al*., 1991). Biological manganese oxidation is carried out by microorganisms which are also responsible for the biological oxidation of Fe (II) except for the stalked members of the genus *Gallionella* which are strict iron oxidizers (Katsoyiannis and Zouboulis, 2004). *Leptothrix*, *Crenothrix*, *Hyphomicrobium*, *Siderocapsa* and *Metallogenium* (Katsoyiannis and Zouboulis, 2004) are some of the well-known manganese oxidizing genera.

Iron is a key metal in environmental microbe-metal interactions due to its abundance in the earth's crust and its ability to readily convert between the Fe (II) and Fe (III) states (Lovley, 2000). Lithotrophic Fe (II) oxidizing microorganisms (FOM) use Fe (II) as an electron donor to provide reducing equivalents for the assimilation of carbon into biomass (Weber *et al.*, 2006; Emerson *et al*., 2010) and Fe (III) is used as a terminal electron acceptor under anaerobic conditions by lithotrophic and heterotrophic Fe (III)-reducing microorganisms (FRM) (Weber *et al.*, 2006). Nitrate (NO_3^-) can also be used as a terminal electron acceptor for iron oxidation under anaerobic conditions, although the kinetics of Fe (II) oxidation by nitrate are much slower than for nitrite (NO_2^-) (Picardal, 2012). The iron bacteria are defined as a group of bacteria which utilize the oxidation of ferrous and/or manganous ions as an essential component in their metabolism and these bacteria typically get their apparent brown/rust-red colour from the production of ferric ions and/or manganic salts, either within the cell or attached on the outside (Cullimore and Mc Cann, 1977).

On the basis of acceptability aspects, the WHO (2011) recommends concentrations for drinking water not exceeding 0.3 mg/L for Fe (II) and 0.1 mg/L for Mn (II). Concentrations of Fe (II) exceeding the recommended level in water systems result in a brown/rust-red discoloration in the water and pipes and reduction in water flow rates, which is typically caused by coatings of iron bacteria inside the pipes (Cullimore and Mc Cann, 1977). High levels of manganese - ingested via drinking water - have recently been seen to negatively impact the health of school children (Bouchard *et al*., 2011). Both manganese and iron impart a metallic, bitter, astringent or medical taste to water and they both contribute to corrosion in water distribution systems (Cullimore and Mc Cann, 1977). As a result the quality of the water is reduced and therefore the need to explore sustainable biotechnological processes for water purification. To establish biotechnological processes for the aerobic treatment of manganese or iron contaminated water, it is important to assess the potential of microorganisms for the oxidative elimination of these two metals. Bacteria play important roles in the activation, modification and detoxification of both manganese and iron in aerated water through changing the valence states of these metals and subsequently converting them into insoluble compounds that are easily removed from water systems (Urrutia *et al.*, 1992). The manganese and iron oxidizing bacteria react with metal cations with an intimate association to their surfaces. This occurs via the interaction of negative charges of

anionic functional groups on the surface of bacteria leading to permanently or temporarily chelated metal cations (Salomons and Förstner, 1984). Whether a metal is going to be permanently or temporarily fixed by functional groups present at the bacterial surface, is governed by parameters such as the cell surface constant K_m - which is a measure of the affinity for a metal of interest - and by the pH and ORP of the system.

To date limited information on biological manganese oxidation exists as the process is considered more complex than biological iron oxidation. Water treatment systems frequently employ chemical reagents to remove manganese and iron from groundwater, but the results of such treatment are not regarded as sustainable (Burger *et al.*, 2008). This is due to an increase in the cost of operation as a result of costly chemicals for treatment and secondary impacts that arise from the formation of residuals and by-products (Gallard and von Gunten, 2002). Biological water treatment systems have proven to be more effective than chemical treatment for the removal of iron and manganese from borehole water (Burger *et al.*, 2008; Trevors, 1989). Biological treatment requires less attention during operation and results in a reduced amount of sludge due to no residuals or by-product formation which arise from the addition of chemicals to the water. Chemical treatment of water demands a substantial amount of time and labour, producing large masses of sludge which increases operation costs (Burger *et al.*, 2008). The above reasons validate the use of biological manganese and iron oxidation as a viable and more sustainable alternative as compared to the use of chemical reagents for the treatment of borehole water.

This study aimed to demonstrate the presence of Mn (II) and Fe (II) oxidizing bacteria in a biofiltration system (Nottingham road, KwaZulu Natal, South Africa) (see thesis supplementary figures S1 and S2) treating borehole water containing Mn (II) and Fe (II) at concentrations of 0.35 mg/L and 2-8 mg/L respectively. In addition, the contribution of these microorganisms to the overall removal of manganese and iron was assessed via qualitative and quantitative methods, taking into account the oxidative elimination via physiochemical reactions typically occurring within biofiltration systems. Finally a clone library was established from metabolically active biofilm material to assess the diversity of bacteria present within the biofilter matrix.

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Chapter 2: Literature Review

A. Manganese Oxidation

1. Microbial oxidation of manganese

Microorganisms involved in manganese oxidation include bacteria, fungi and algae. Bacteria and fungi are considered the main groups of manganese oxidizers whilst algae are usually found to enhance the oxidation of manganese in combination with bacteria (Stuet *et al*., 1996). Manganese oxidation can occur via enzymatic or non-enzymatic processes. The non-enzymatic manganese oxidation process involves the direct chemical oxidation of Mn (II) or the oxidation by metabolic end products (Linhardt, 1997). The enzymatic process is performed by three groups of microorganisms: (i) those that oxidize dissolved Mn (II), (ii) those that oxidize Mn (II) that is prebound to solids and (iii) organisms that oxidize dissolved Mn (II) by the metabolite H_2O_2 via catalase. The abiotic/non-enzymatic oxidation of Mn (II) to MnO_2 is slow and occurs by the chemical reaction of dissolved Mn (II) and oxygen in natural waters (Linhardt, 1997). The enzymatic/biological manganese oxidation of higher manganese oxides and hydroxides takes place faster than abiotic oxidation, with a wide variety of ubiquitous microorganisms capable of converting Mn (II) to solid Mn (III/IV) oxides (Ghiorse, 1984). The diversity of these microorganisms is based on the different mechanisms they use to biochemically induce the transformation of Mn (II) (Ehrlich, 1990). The common microbial manganese oxides are Birnessite (MnO_2), Manganite (MnOOH) and Hausmannite (Mn_3O_4) (Ehrlich and Newman, 2009; Schweisfurth and Gattow, 1966).

1a. Microbial Mn (II) oxidation

Heterotrophic microorganisms such as *Bacillus* sp., *Pseudomonas putida* and *Pedomicrobium* sp. were previously described as using a multicopper oxidase (MCO) in the oxidation of Mn (II) to Mn (IV) (Brouwers *et al*., 2000; Spiro *et al*., 2008). The genes that encode putative Mn (II)-oxidizing enzymes like *mofA* in *Leptothrix discophora*, *cumA* in *Pseudomonas putida* GB-1, and *moxA* in *Pedomicrobium* sp. strain ACM 3067, are thought to produce multicopper oxidases, this

was based on the presence of conserved, predicted amino acid motifs (El Gheriany *et al.*, 2009). Homogenates from the endospores of a *Bacillus* sp. contained distinct proteins that produced a solid brown precipitate when exposed to Mn (II) when non denaturing polyacrylamide gel electrophoresis was used (Francis *et al.*, 2002). Upon disruption of the MCO gene this *Bacillus* sp. lost the ability to oxidize manganese (Spiro *et al.*, 2008) which indicates that the MCO gene is responsible for manganese oxidation in its endospores (da Silva and Williams, 1991). Multicopper oxidases are described as one electron transferring enzymes were single electrons are transferred from the substrate to O_2 through intervening copper ions (da Silva and Williams, 1991).

For experimental purposes, a variety of poisons were tested by Rosson and Nealson (1982) to check whether they inhibit manganese oxidation. Azide can inhibit the formation of Mn (III) whilst the addition of Cu (II) enhances the formation thereof (Ehrlich and Newman, 2009; Spiro *et al.*, 2008). Whilst it was found that some poisons do not potentially interfere with manganese oxidation it was suggested that the added poisons may interfere in unknown ways with manganese adsorption due to the difference in ion-exchange capacities of manganates (Murray, 1974). In a separate study conducted by Ghiorse and Hirsch (1979), it was established that heat treatment did not completely inhibit manganese oxidation which indicated that the suspected MCO protein responsible for manganese oxidation is somewhat heat stable. Other compounds that were found to inhibit manganese oxidation were SDS, NaCl, cyanide and $HgCl_2$ (Boogerd and de Vrind, 1987; Ehrlich and Newman, 2009). The oxidation of Mn (II) to Mn (IV) is catalyzed via the following reaction (1).

$$
\begin{aligned}
2Mn^{2+} + 8H_2O &\longrightarrow 2Mn(OH)_4 + 4e^- + 8H^+ \\
2Mn(OH)_4 &\longrightarrow 2MnO_2 + 4H_2O \\
4e^- + 4H^+ + O_2 &\longrightarrow 2H_2O \\
\hline
2Mn^{2+} + 2H_2O + O_2 &\longrightarrow 2MnO_2 + 4H^+
\end{aligned}
\qquad (1)
$$

The end product of bacterial Mn (II) oxidation, Mn (IV), is a powerful oxidant and cannot be stabilized by carboxylate groups, which are weak donor ligands from protein side chains (Spiro *et al.*, 2008). Most Mn (IV) complexes are polynuclear and are stabilized by oxo bridges (Pizarro *et al.*, 2004). The Mn (IV) complexes further polymerize to form solid-phase MnO_2 complexes, in the absence of capping ligands (Pizarro *et al.*, 2004). If a bacterial MCO accommodates multiple Mn (III) ions in its holding sites, a polynuclear Mn (IV) complex may form as a nucleation site for MnO_2 nanoparticle formation (Brouwers *et al*., 2000; Spiro *et al.*, 2008). The first step in the formation could be further oxidation of Mn (III), either via the MCO or possibly by direct reaction with O_2 to form a polynuclear oxo-bridged Mn (IV) complex (Brouwers *et al*., 2000; Spiro *et al.*, 2008). Numerous Mn (III) ions can also disproportionate to a polynuclear Mn (IV) complex and Mn (II) ions, which are then reoxidized at the MCO substrate site, ensuring a continuous supply of Mn (III) ions (Spiro *et al.*, 2008). The polynuclear Mn (IV) complex formed at the nucleation site in each of these cases, would ultimately be released to grow into MnO_2 nanoparticles (Spiro *et al.*, 2008).

1b. Mn (II) oxidizing bacteria

Mn (II) oxidation is carried out by a variety of microorganisms. Microorganisms found in sites that are high in concentrations of manganese and iron form distinct metallic casts and can be recognized by their distinct morphotypes in natural samples (Emerson and Revsbech, 1994). The most common manganese oxidizing microorganism found in these sites is *Leptothrix ochracea* (Emerson and Revsbech, 1994). Other bacteria that oxidize manganese are *Hyphomicrobium manganoxidans*, *Pseudomonas putida* and *Leptothrix cholodinii*, which attack dissolved manganese enzymatically. *Arthrobacter* spp. and *Oceanospirillium* sp. are known to oxidize manganese prebound to manganese oxides while *Pseudomonas manganoxidans* and *Bacillus* sp. strain SG-1 oxidize manganese enzymatically (Ehrlich and Newman, 2009). In addition, the ability of an *Acinetobacter* species to oxidize Mn (II) was recently reported (Beukes and Schmidt, 2012). The *Sphaerotilus-Leptothrix* group of microorganisms is the most common group of manganese oxidizing microorganisms and the species within these genera share a number of common characteristics like the formation of a sheath, the requirement of vitamin B_{12} for growth and the formation of poly-β-hydroxybutyrate (PHB) as reserve material (vanVeen *et*

al., 1978). The bacteria of the *Sphaerotilus-Leptothrix* group of microorganisms typically occur in low numbers in slightly polluted or non-polluted waters (vanVeen *et al.*, 1978). *S. natans* possesses a pronounced response to organic nutrients, producing high yields of cell material. This is in contrast to *Leptothrix* sp. which hardly responds to added nutrients (vanVeen *et al.*, 1978). *S. natans* has much larger cells than most of the *Leptothrix* spp. and false branching only occurs in *S. natans* and in *L. lopholea* (Takeda *et al*., 2012; vanVeen *et al.*, 1978).

The presence of a sheath has nutritional and ecological consequences for members of the *Sphaerotilus-Leptothrix* group of bacteria. Their growth in slow running waters low in nutrients requires a sheath enabling the bacteria to attach themselves to solid surfaces during nutrient depletion. The sheath also protects the bacteria against phages and bacterial or eukaryotic predators (vanVeen *et al.*, 1978). Cell propagation of sheath-forming bacteria is not necessarily dependant on the presence of the sheath, as can be concluded from the ability of the sheath-less mutants of this group of microorganisms to grow and divide (Takeda *et al*., 2012; Mulder and vanVeen, 1963). *Leptothrix cholodnii*, which was previously classified as *Leptothrix discophora* (Spring *et al*., 1996) is the most common species that is able to form a sheath and maintain it under laboratory conditions (Emerson and Ghiorse, 1992; Takeda *et al*., 2010, 2012).

1c. Mn (II) oxidizing fungi and algae

Fungi and algae (to a lesser extent) are also important groups of manganese oxidizing microorganisms. Like bacteria, these microorganisms also play an important role in the production of biogenic Mn oxides. Fungi that oxidize manganese are ubiquitous in nature, and have been isolated from freshwater systems, Mn nodules (Cahyani *et al*., 2009), soil environments (Santelli *et al*., 2010) including building materials (de la Torre and Gomez-Alarcon, 1994). They are able to thrive in these environments because they possess multiple mechanisms to tolerate environmental stresses such as nutrient fluctuations, desiccation or high levels of metals (Santelli *et al*., 2010). They have thus been implicated in the remediation of a wide range of pollutants and it was previously found that fungi contributed to the remediation of Mn-contaminated mine drainage and were also found growing in a Mn-attenuating bioreactor for treatment of mine waters (Mariner *et al*., 2008). Some examples of fungi involved in manganese

oxidation include *Plectosphaerella cucumerina* and *Stilbella aciculosa*, both isolated from mine waters, which both belong to the phylum *Ascomycota* (Mariner *et al*., 2008). These two fungi are soil inhabitants (de Hoog *et al*., 2000) that have never previously been known to oxidize manganese and were not phylogenetically related to known manganese oxidizing species.

Similarly, fungi isolated from freshwater and marine systems have been identified as ascomycetes via analysis of the 18S rRNA genes (Tebo *et al*., 2005). The main proteins involved in bacterial manganese oxidation are also involved in fungal manganese oxidation. An extracellular protein involved in manganese oxide formation found in *L. discophora* SS-1 was also found in an ascomycete; strain KR21-2, previously investigated by Tani *et al*. (2003). The manganese oxidation inhibitor for bacteria, azide, was also found to inhibit manganese oxidation in the ascomycete strain KR21-2 (Tebo *et al*., 2005). This suggests that the same protein - MCO-type enzyme - responsible for manganese oxidation in bacteria is also responsible for manganese oxidation in fungi. Fungi also use heme-containing Mn peroxidases and laccases, used in the degradation of lignin, for manganese oxidation (Tebo *et al*., 2005). The peroxide-oxidized enzyme and most laccase enzymes catalyse the oxidation of Mn (II) to Mn (III) (Schlosser and Höfer, 2002).

In terms of metal toxicity, fungi are considered more tolerant than bacteria to high concentrations of metals in the environment (Chander *et al*., 2001). Fungi and bacteria that oxidize manganese are heterotrophs that do not gain energy from the oxidation of manganese but rather benefit from the presence of Mn oxide minerals, through the uptake of dissolved Mn (II) or via scavenging of reactive oxygen species (Tebo *et al*., 1997). Besides their ability to oxidize Mn (II), fungi can also mediate the reduction of this metal via abiotic reactions and/or with enzymatic reduction (Gomah *et al*., 1980; Ghiorse and Ehrlich, 1976). In culture media the oxidation of manganese by fungi can be detected in a similar manner to that of bacteria, by using a triphenyl-compound (Leucoberbelin blue) which is oxidized by Mn IV and insensitive to Mn (II) and other metals in trace quantities (Altmann, 1972; Krumbein and Altmann, 1973).

2. Bacteriogenic manganese oxides

2a. Properties of bacteriogenic manganese oxides

Bacteriogenic/biogenic manganese oxides (manganese dioxide and manganese tetroxide) produced as a result of Mn (II) oxidation by bacteria are used in the oxidative removal of toxic metals from contaminated soils and wastewaters and have been studied extensively (Bargar *et al.*, 2009). These manganese oxides have highly reactive surfaces and thus contribute to the remediation of water contaminated with toxic metals, by scavenging metals such as Pb, Ni, Co and Zn (Peña *et al*., 2010; Takahashi *et al*., 2007). The reactivities of biogenic manganese oxides in comparison to abiotically produced manganese oxides is greater due to the smaller size, increased surface area, disorder and/or the sheet symmetry distortion of biogenic manganese oxides (Hochella *et al*., 2008). Manganese oxides are poorly soluble in water and are used in the manufacturing of catalysts, colorants, metal sorbents and batteries (IPCS, 2004) and they can also be used as a terminal electron acceptor by other bacteria in respiration (Spiro *et al.*, 2008). Due to its high surface area and oxidizing potential, MnO_2 is capable of efficiently degrading biologically recalcitrant organic molecules such as benzene and naphthalene to lower-molecular-mass compounds, indicating the potential of these compounds in bioremediation of xenobiotic organic compounds (Forrez *et al*., 2010; Spiro *et al.*, 2008). Whilst MnO_2 is the stable form of manganese it can however be reduced to Mn (II) in the presence of exogenous ligands or UV rays, which helps to regulate the bioavailability of Mn (II) (Spiro *et al.*, 2008).

2b. Structural composition of bacteriogenic manganese oxides

The manganese dioxides produced by microorganisms consist of stacked hexagonal sheets of octahedral MnO_6 and are categorized as having either a layer or tunnel structure (Jurgensen *et al*., 2004; Santelli *et al*., 2011; Spiro *et al.*, 2008). The layer-type oxides - phyllomanganates - which are most abundant in soils, nodules and rock varnishes, are poorly crystalline and highly reactive with metal cations (Fig. 1). This is the most dominant bacteriogenic manganese oxide formed at circumneutral pH which is also structurally similar to hexagonal birnessite (Bargar *et al*., 2005; Jurgensen *et al*., 2004). Tunnel structure oxides (Tectomanganates) are comprised of chains with

edge-sharing octahedral manganese complexes, linked through corner-sharing, forming square or rectangular cross-sections e.g., todorokite (Jurgensen *et al*., 2004; Santelli *et al*., 2011). Depending on the physical properties and mineral particle size of the manganese oxides, they are able to absorb a wide variety of cations onto their surfaces (Hochella *et al*., 2008). Cation vacancies and random stacking arrangements create structural defects on the layer-type oxides, which provide binding sites for exogenous metal ions, thus controlling the bioavailability of exogenous metal ions in the natural environment (Spiro *et al.*, 2008).

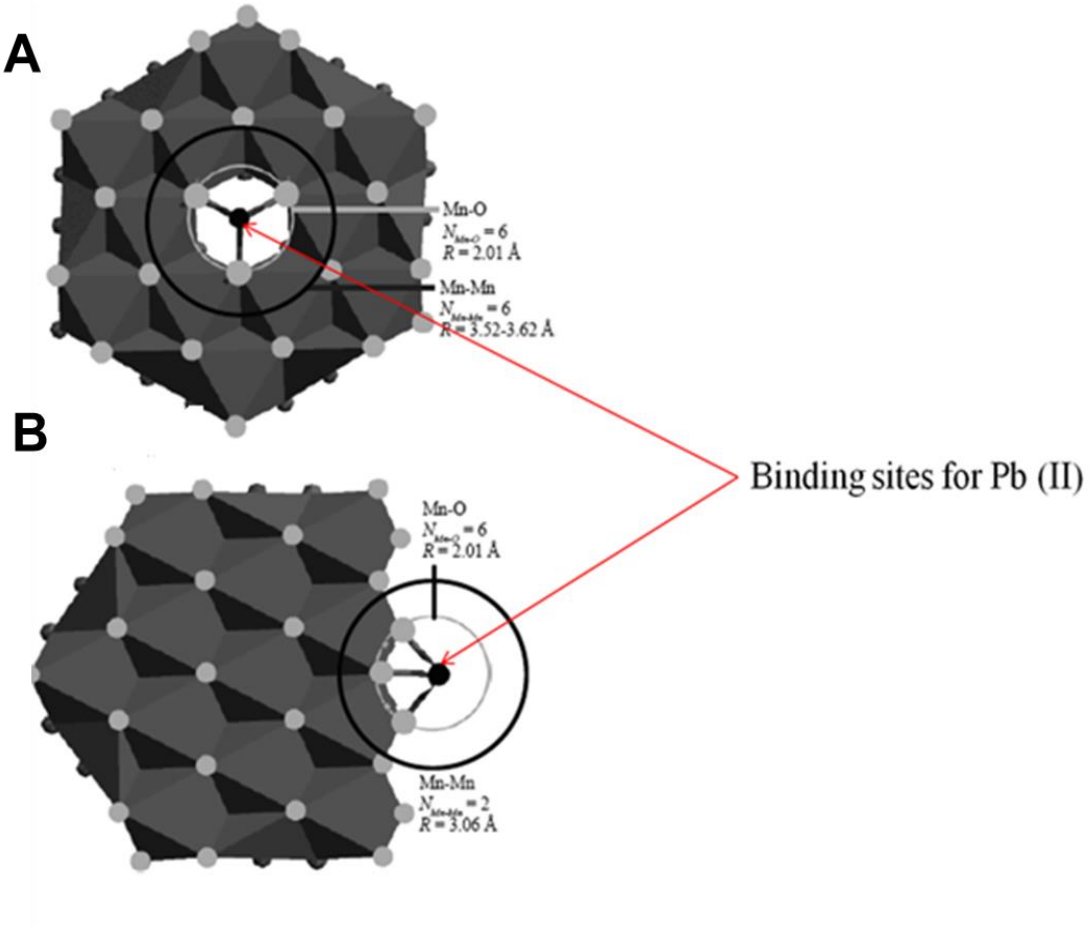

Figure 1 (Adapted from Spiro *et al.*, 2008): Binding of Pb (II) ions onto two crystal structures of bacterial manganese oxides. (A) triple-corner-sharing and (B) double-edge-sharing inner sphere surface complexes formed respectively above cation vacancy sites and at sheet edges.

In earlier studies conducted by Greene and Madgwick (1991), solid phases of manganese oxides were identified as Mn_3O_4 (Hausmannite), Buserite (a hydrated layer-type Mn (IV) oxide), Manganite (γ-MnOOH, a tunnel-type Mn (III) oxide) and Nsutite (γ-MnO_2, a tunnel-type Mn (IV) oxide). These are well-known products of biological and abiotic oxidation of Mn (II). In recent studies, the origin (via biological or abiotic oxidation) of the different minerals has been disproven except for Buserite. Buserite, most probably stemmed from the autocatalytic oxidation of adsorbed Mn (II) present on the surface of freshly precipitated Mn (IV) oxides (Bargar *et al.*, 2005). EXAFS (extended X-ray absorption fine structure) spectroscopy indicates that each Mn ion in Buserite is di-μ-oxo bridged to six Mn neighbours with a 2.82-2.90 Å Mn-Mn distance (Figure 2B) (Spiro *et al.*, 2008). In addition, 3.5-3.8 Å Mn-Mn distances were found in Buserite, which suggests the presence of Mn (III) positioned above or below the Mn (IV) vacancy sites (Figure 2A) (Gaillot *et al.*, 2003). The negative charge from Mn (IV) vacant sites can be overcome by the incorporation of hydrated metal cations, which results in basal plane spacings of 7-10 Å, depending on the degree of hydration (Post, 1999).

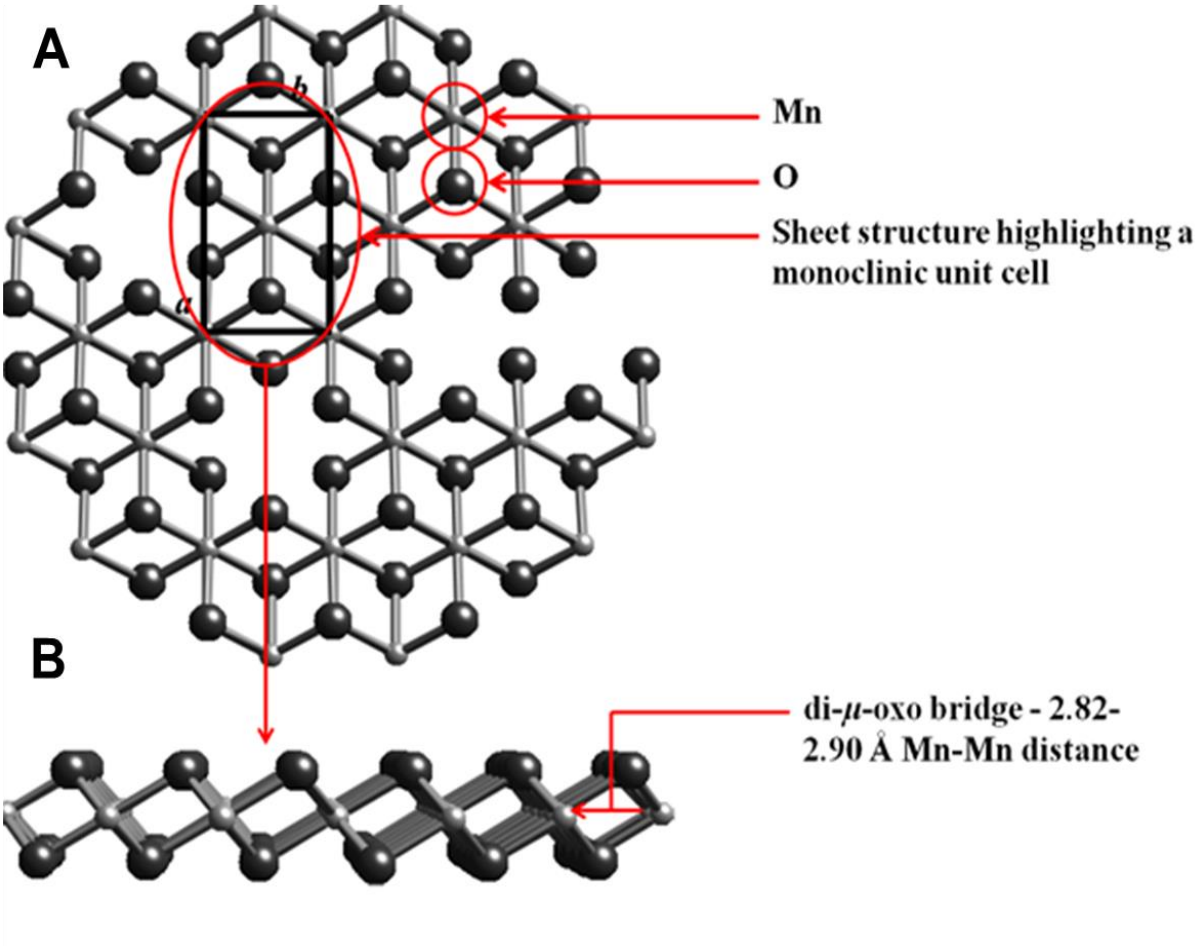

Figure 2 (Adapted from Spiro *et al.*, 2008): Crystal structure of the bacteriogenic Mn oxide - Buserite. (A) Sheet structure of Buserite, with hexagonal symmetry, highlighting a monoclinic unit cell (a-b). (B) Sheet structure (a-b), illustrating di-μ-oxo bridging between neighboring Mn atoms. Manganese atoms illustrated as large black circles and oxygen atoms as the grey smaller circles.

3. General properties of manganese compounds

3a. General toxicity associated with manganese compounds

Symptoms of manganese toxicity in plants consist of necrotic lesions, marginal chlorosis, and distorted development of the leaves (IPCS, 2004). In humans, manganese seems to be the one of the least toxic minerals from a nutritional point of view. There is no known toxicity arising from

"normal" manganese uptake via food or from taking reasonable amounts in supplements (Gerber *et al.*, 2002). Increased levels of manganese in the environment are no threat to man as long as they are not inhaled or ingested with contaminated drinking water (Gerber *et al.*, 2002). However, acute toxic effects and consequences have been seen after the administration of potassium permanganate (Henderson and Watt, 1951). The inhalation of manganese fumes can cause "metal fume" fever which is characterized by acute pneumonitis, tracheobronchitis and pulmonary oedema (Nemery, 1990). Chronic toxicity to the central nervous system (CNS) can also take place when manganese is inhaled, which is much more important than the acute toxicity (Huang *et al.*, 1989). In general, Mn toxicity results from exposure to high levels of manganese from various industrial settings like welding (Park *et al*., 2007), metal smelting, Mn mining (Myers *et al*. 2003a, b) and/or battery manufacturing (Bader *et al*., 1999). Cyclopentadienyl manganese tricarbonyl (CMT) which is used in petrol as a substitute for lead, produces convulsions and pulmonary oedema in Sprague-Dawley rats (Gerber *et al.*, 2002). The LD50s for an oral and intraperitoneal administration are 22 mg and 14 mg per kg body weight respectively (Penney *et al.*, 1985). The toxic effects of methylcyclopentadienyl manganese tricarbonyl (MMT) are similar as those of CMT and appear mainly in the lungs, liver and kidney (Hinderer, 1979).

In a previous report it was also found that an increase in the use of MMT (methylcyclopentadienyl) in gasoline had caused elevated levels of Mn in the environment and subsequently in the blood of children that resided in the surrounding environment (Batterman *et al.*, 2011; Röllin *et al.*, 2005). Chronic manganism affects carbohydrate metabolism, and patients with chronic manganism often have hypoglycaemia following a high glucose load (Gerber *et al.*, 2002). Whilst an essential element for nutrition in human beings, manganese has been reported to have toxic side effects (neurobehavioral development problems) on children after the intake of water containing high levels of Mn (II) that exceed the US EPA recommended level (Woolf *et al.*, 2002; Bouchard *et al.*, 2007). The recommended intake levels of manganese for children (9-13 yrs. old) are between 1.9 and 1.6 mg per day for males and females respectively (Batterman *et al.*, 2011). A very recent study conducted by Batterman *et al.* (2011) revealed that 8.1% of children tested in the study site in Durban (KwaZulu-Natal, South Africa), had blood manganese concentrations above the normal range (15 μg/L) reaching a maximum concentration

of 25 μg/L (Batterman *et al.*, 2011). The average manganese in the children's blood was around 10.1±3.4 μg/L (Batterman *et al.*, 2011).

3b. Mutagenic properties of manganese compounds

The presence of toxic manganese compounds causes a decrease in the fidelity of DNA replication by modifying the activity of DNA polymerase, however it does not seem to interfere with the repair of chemically induced DNA damage (van de Sande *et al.*, 1982). Mn (II) compounds and to a lesser extent Mn (VII) compounds, at micromolar concentrations can induce the cellular SOS repair when the normal progression fork is impeded (Olivier and Marzin, 1987). Divalent manganese compounds may also mediate in vitro mispairing of ethylating agents or aliphatic epoxides (Bhanot and Solomon, 1994). Manganese sulfate induces mutations in T4 phage DNA when developing in *Escherichia coli* and enhances UV induced mutagenesis (Rossman and Molina, 1986). Potassium permanganate can cause damage to the integrity of the DNA chain (De Meo *et al.*, 1991) but is less effective than $MnSO_4$ in the above mentioned aspects of mutagenicity (Gerber *et al.*, 2002). Although a proven mutagen, previous studies of cancer development after manganese exposure indicated that manganese is not a cancer risk in man (Gerber *et al.*, 2002).

4. Sources of manganese in the environment

Manganese is naturally occurring in numerous environments and the highest exposure of manganese is from industrial activities (such as ferroalloy production, iron and steel foundries, power plants, and coke ovens), combustion of fossil fuels, and reentrainment of manganese-containing soils (Lioy, 1983). Manganese occurs as a component of more than 100 minerals, such as sulfides, oxides, carbonates, silicates, phosphates and borates (NAS, 1973). The common manganese minerals include Pyrolusite (manganese dioxide), Rhodochrosite (manganese carbonate [$MnCO_3$]), Rhodonite (manganese silicate) and Hausmannite (manganese tetroxide) (NAS, 1973; Ehrlich, 1990).

5. Biochemical aspects of manganese

Metals of biological importance - required by microorganisms for structural or metabolic functions - are usually tolerated in higher quantities whilst the opposite is true for those without biological function (Haferburg and Kothe, 2007). Homeostasis is essential for controlling metal uptake by bacterial cells and bacteria have developed a modified regulatory system that controls metal uptake and excretion (Haferburg and Kothe, 2007). Manganese is a component of the eukaryotic mitochondrial enzymes pyruvate carboxylase, certain superoxide dismutases, glutamine synthetase, alkaline phosphatase and arginase and is responsible for the activation of a wide variety of enzymes (Gerber *et al.*, 2002). Manganese-containing superoxide dismutase intervenes in antioxidant activity and tumour defences (Gerber *et al.*, 2002) and is essential for normal bone structure and the formation of mucopolysaccharides (Keen and Leach, 1988). Whilst these are essential metal ions in microorganisms, manganese and iron have the potential of being toxic to the cells which means that homeostatic regulation of their concentrations is necessary (Jakubovics and Jenkinson, 2001).

The ionic radius of manganese is similar to that of magnesium, calcium and iron in aqueous solution, which allows the interchange of manganese and other cations in the metal binding sites of proteins (Jakubovics and Jenkinson, 2001). Manganese is essential for certain metabolic reactions like oxygenic photosynthesis in cyanobacteria (Christianson, 1997) and glycolysis in several Gram positive endospore forming bacteria and also plays an important role in the detoxification of reactive oxygen species such as peroxides and oxygen ions which are byproducts of metabolism (Jakubovics and Jenkinson, 2001; Chander *et al.*, 1998). In earlier research it was found that manganese promoted endospore formation and spore germination in *Bacillus* spp., contributing to the overall development cycle in this bacterial genus (Charney *et al.*, 1951; Gould, 1969). Oxidative stress is caused by the inability of the bacterium to efficiently resist or repair damage caused by reactive oxygen species like peroxides and oxygen ions and this is a major challenge facing bacteria growing in oxygenated environments (Jakubovics and Jenkinson, 2001). Manganese plays an important role in bacterial homeostasis by reducing oxidative stress through interaction with reactive oxygen species without generating harmful free

radicals within bacterial cells (Cornelis *et al*., 2011; Cheton and Archibald, 1988; Stadtman *et al.*, 1990).

6. Impact of manganese oxidizing bacteria on the corrosion of stainless steel

Manganese oxidizing bacteria play a vital role in the corrosion of stainless steel, which occurs as a result of the deposition of manganese oxides and hydroxides onto the surface of this metal through the activity of these microorganisms (Linhardt, 2004). The term used to describe the phenomenon of stainless steel corrosion is called ennoblement. This describes the shift from the free corrosion potential of stainless steel (i.e. abiotic) toward the anodic direction which is coincident with biofilm formation on metal surfaces (Linhardt, 2004). Corrosion by microorganisms consists of a biotic and an abiotic step. The biotic step takes place in the biofilm were biomineralization of MnO_2 takes place and the deposits placed directly on the surface of the metal (Linhardt, 2004). The abiotic step is the corrosion process, which is based on the electrochemical properties of the higher manganese oxides (Linhardt, 2004). The microorganisms modify the medium of the corrosion system by creating strong concentration gradients generating unexpected chemicals at the metals surface (Linhardt, 1997).

The two corrosion mechanisms relevant to metal depositing microorganisms are the accumulation of chlorides under deposits containing Fe (III) and Mn (IV), which are aggressive to stainless steel (Kobrin, 1976; Tatnall, 1981; Pope *et al.*, 1984) and the effect of the redox couples, Fe (II)/Fe (III) and Mn (II)/Mn (IV), which may influence the Eoc (open circuit potential), leading to pitting of the metal (Pope *et al.*, 1984; Duquette, 1986). The first mechanism appears to be unrealistic as high chloride concentrations are often a consequence of a corrosion reaction rather than the cause of the corrosion and are not found in deposits free of corrosion products (Linhardt, 1997). The second mechanism is considered more relevant as it is based on the redox properties of the deposits and has been previously proposed as a mechanism for corrosion by Linhardt (1994, 1996). The properties of MnO_2 that make it an effective cathodic material is its higher than practical oxygen potentials, it is not diffusion limited if reduced, it may act as an oxygen-electrode (Matsuki and Kamada, 1986) and the electronic conductivity allows a layer of MnO_2 to be reduced from the outer surface and not only at the interfacial layer (Linhardt, 1997).

B. Iron Oxidation

1. Bacterial oxidation of iron

In terms of biological reactivity, the most important oxidation states of iron are Fe (II) and Fe (III), the oxidized form and an abundant supply of Fe (II) must be available for bacteria to gain sufficient energy from the oxidation of Fe (II) (Cornelis *et al*., 2011; Liang *et al.*, 1993). However, at neutral pH and under fully aerated conditions, rapid chemical oxidation of Fe (II) takes place and this poses a problem for the bacteria. When Fe (II) enters a fully aerobic zone, its half-life is only in the range of minutes (Liang *et al.*, 1993). Microorganisms are able to overcome the inherent Fe (II) instability under fully aerated conditions by either thriving at very low pH, which is what *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* (both well-known for bioleaching of metals from sulfidic ores (Gehrke *et al*., 1998; Sand *et al*., 1992)) do, or in the case of *Gallionella ferruginea*, by growing at very low O_2 concentrations at circumneutral pH where the half-life of Fe (II) may be much longer (Liang *et al.*, 1993). A second problem faced by these microorganisms is that the product of the oxidation is an insoluble ferric hydroxide (Lovley, 2000; Ehrlich and Newman, 2009). To avoid a build-up of toxic levels of this oxidation product within the cells, iron oxidation must occur at the exterior of the cell surface and this requires that cells possess a chemical mechanism for transporting electrons to the cytoplasmic membrane where a chemiosmotic potential is established (Lovley, 2000; Ehrlich and Newman, 2009). At a pH range between 6 and 7, the Fe (II) will often be in the form of $FeCO_3$ and the Fe (III) in the form of an insoluble ferric hydroxide (2), thus the product of oxidation is continuously removed from solution (Widdel *et al.*, 1993). This process is somewhat important as it drives the oxidation of Fe (II) to Fe (III).

$$4\ FeCO_3 + O_2 + 6\ H_2O \longrightarrow 4\ Fe(OH)_3(s) + 4\ CO_2 \qquad (2)$$

1a. Bacterial Fe (II) oxidation mechanisms

Aerobic Fe (II) oxidation - Aerobic Fe (II) oxidation is the oxidation of Fe (II) by molecular oxygen to form a simple insoluble Fe (III) hydroxide (3). The aerobic oxidation of Fe (II) was

previously neglected due to the rapid rate of abiotic Fe (II) oxidation coupled to oxygen reduction (Davison and Seed, 1983). Environments that encourage aerobic Fe (II) oxidation are groundwater iron seeps, stream sediments, wetland surface sediments, irrigation ditches, cave walls, subsurface boreholes, municipal and industrial water distribution systems, deep ocean basalt and hydrothermal vents (Emerson and Weiss, 2004). Microaerophilic Fe (II) oxidizing microorganisms in the above environments compete successfully with the kinetics of abiotic Fe (II) oxidation through the conservation of energy from the oxidation of Fe (II) via the conversion of inorganic carbon into biomass (Emerson and Moyer, 1997; Weber *et al.*, 2006). The Fe (III)/Fe (II) couple has a reduction potential which is sufficient to provide reducing power between bacterial photosystems or alternative terminal electron acceptors which are involved in respiratory processes to sustain microbial growth (Weber *et al.*, 2006).

$$4\,Fe^{2+} + O_2 + 10\,H_2O \longrightarrow 4\,Fe(OH)_3\,(s) + 8\,H^+ \qquad (3)$$

Anaerobic, photoautotrophic Fe (II) oxidation – The first demonstration of microbially mediated Fe (II) oxidation in anoxic environments was the phototrophic anaerobic Fe (II) oxidation process (Widdel *et al.*, 1993). The microorganisms (FOM) involved in this process oxidize Fe (II) via the utilization of light energy to fix CO_2 into biomass (Weber *et al.*, 2006). The bacteria in this group include *Chlorobium ferrooxidans*, *Rhodomicrobium vannielii*, *Thiodictyon* spp., *Rhodopseudomonas palustris* and *Rhodovulum* spp. (Weber *et al.*, 2006). With the exception of *Rhodomicrobium vannielii,* these bacteria can completely oxidize Fe (II) to Fe (III) (Weber *et al.*, 2006). The incomplete oxidation of Fe (II) by *R. vannielii* was due to the encrustation of the cell wall of this bacterium with biogenic Fe (III) oxides which inhibited further metabolic activity (Widdel *et al.*, 1993). In order to prevent the cells from being encrusted with biogenic Fe (III) oxides, the production of low-molecular weight compounds that can solubilize these oxides has been suggested (Ehrenreich and Widdel, 1994). The phototrophic oxidation of Fe (II) results in the formation of poorly crystalline Fe (III) oxides but in the presence of metabolically active iron oxidizing microorganisms, they are transformed into the more crystalline Fe (III) oxide minerals such as lepidocrocite and goethite (Kappler and Newman, 2004). Phototrophic Fe (II) oxidation processes are typically limited by the maximum penetration of light at wavelengths between 275 and 700 nm to a depth of 200 µm in soil and

sediments (Ciania *et al.*, 2005). Phototrophic FOM are only of minor importance in the global iron biogeochemical cycling because they are unable to promote Fe (II) mineral dissolution and are limited by the solubility of the mineral (Kappler and Newman, 2004).

Anaerobic, nitrate-dependent Fe (II) oxidation – At neutral pH, the light-independent microbially mediated oxidation of iron coupled to nitrate reduction occurs and is thus not limited to anaerobic environments exposed to sunlight (Weber *et al.*, 2006). These reactions occur in various fresh water and saline environmental systems that support - in the presence of abundant nitrate - the nitrate Fe (II) oxidizing microbial communities which potentially contribute to the iron redox cycle (Chaudhuri *et al.*, 2001; Weber *et al.*, 2006). Light-independent reactions such as nitrate-dependent Fe (II) oxidation, have the potential to contribute to anaerobic Fe (II) oxidation on a global scale, provided that adequate concentrations of a suitable electron acceptor are readily available (Weber *et al.*, 2006). This is due to the ubiquity and diversity of anaerobic FOM. FOM, to date, have been demonstrated to exploit the favourable thermodynamics between Fe (II) (OH_3)/Fe (III) and nitrate reduction redox pairs (NO_3^-/½N_2, NO_3^-/NO_2^- and NO_3^-/NH_4^+) (Straub *et al.*, 1996) and also between Fe (II) (OH_3)/Fe (III) and perchlorate (ClO_4^-/Cl^-) and chlorate (ClO_3^-/Cl^-) (Bruce *et al.*, 1999). Fe (II) oxidation coupled to nitrate reduction yields enough energy to support carbon fixation and microbial growth (Weber *et al.*, 2006) (4). Nitrite (NO_2^-) and nitrogen gas (N_2) were thought to be the sole products of nitrate reduction until the recent demonstration of nitrate-dependent Fe (II) oxidation by *Geobacter metallireducens* (Straub *et al.*, 1996).

$$10\ FeCO_3 + 2\ NO_3^- + 24\ H_2O \longrightarrow 10\ Fe(OH)_3\ (s) + N_2 + 10\ HCO_3^- + 8H^+ \qquad (4)$$

1b. Representative neutrophilic, aerobic Fe (II) oxidizing bacteria

Iron bacteria are commonly referred to as metal depositing bacteria due to their ability to deposit iron hydroxides extracellularly. This phenomenon has caused them to become notorious in drinking water systems due to biofouling and corrosion (Little and Wagner, 1997; Tatnall, 1981).

***Sphaerotilus/Leptothrix* group** - This group of bacteria is found in rivers below sources of organic pollution, also in water distribution pipes and they are capable of both iron and manganese oxidation (Avery, 1970). They form brown fluffy filamentous layers that are visible with the naked eye. A microscopic inspection of this material reveals a tangled matrix of tubular sheaths encrusted with iron (vanVeen *et al.*, 1978; Emerson *et al.*, 2010). The *Sphaerotilus/Leptothrix* group prefers eutrophic environments and is often implicated in causing bulking problems in activated sludge and is well recognized for its capacity to deposit iron oxides on their sheaths. The sheaths are straight tubes that appear quite robust and refractile by phase-contrast microscopy and as the sheaths age, they continue to accumulate Fe (III) oxides. One consistent observation of *L. ochracea* morphotypes is that it is rare to see filaments of cells inside the sheaths (vanVeen *et al.*, 1978; Emerson *et al.*, 2010). It has been speculated that as this organism oxidizes iron, it deposits the ferric hydroxides on the sheath and in this way prevents itself from becoming encrusted in the Fe oxide precipitate (vanVeen *et al.*, 1978; Emerson *et al.*, 2010). Previous studies have suggested that *L. discophora*, which is a manganese oxidizer, possesses an Fe-oxidizing protein that is involved in the oxidation of Fe (II) to Fe (III) (Corstjens *et al.*, 1991).

***Gallionella* spp. and *Metallogenium* spp. –** *Gallionella* species are found in aquatic environments always associated with iron (Ridgway *et al.*, 1981). These organisms are auto-/mixotrophs, recognized by the helical stalk that they form and the cells typically consist of kidney-shaped mycoplasmodial-like cell bodies that lack the usual peptidoglycan component providing rigidity to the bacterial cell wall (Ridgway *et al.*, 1981; Herschel, 1999). This suggests an evolutionary kinship to the mycoplasmas and to *Metallogenium* spp., a related wall-less polymorphic iron oxidizing bacterium.

Metallogenium spp. consist of a single elongated stalk constructed from many helically wound mineralized fibrils which extend outwards from the convex side of the cell body (Ridgway *et al.*, 1981; Herschel, 1999). These organisms can grow in a pH range of 3.5-4.1 and are therefore somewhat intermediate between true acidophiles and neutrophiles (Ehrlich and Newman, 2009). The cells are bean-shaped and grow at the termini of the stalks; thus one cell is capable of producing large amounts of stalk material. The stalk is composed mainly of Fe (III) hydroxides

and continues to accumulate Fe (III) after the producing cells have left (Heldal and Tumyr, 1983). The stalk seems to form the substratum upon which further Fe (II) oxidation occurs and represents a survival structure, which the organism produces as a means of protection until more favourable growth conditions arise (Heldal and Tumyr, 1983). *Gallionella* spp. and certain other iron oxidizing bacterial genera derive their energy through a strictly chemolithotrophic process (Ridgway *et al.*, 1981). This process is known as the enzyme-mediated oxidation of ferrous ions with the concomitant fixation of carbon dioxide (Ridgway *et al.*, 1981). The result thereof is the precipitation of ferric salts in the hydroxide form, which colours cells brown or reddish-brown.

Other proteobacteria – A novel stalk-forming iron-oxidizing bacterium, strain R-1, was recently isolated from a freshwater iron seep (Krepski *et al*., 2012). This microorganism showed strong morphological similarities to *G. ferruginea* - like the production of stalks - but a low 16S rRNA gene sequence similarity of 93.55% to this strain (Krepski *et al*., 2012). This bacterium grew only on Fe (II) substrates at a maximum temperature of 35°C and in a pH range of 5.6 – 7.0 and was most closely related to uncultured bacteria from iron-rich groundwater springs in the betaproteobacterial group (Krepski *et al*., 2012).

1c. Growth requirements for iron oxidizing bacteria

Iron - Iron performs a key role in controlling the growth of microorganisms. In previous studies it was found that the large sized iron oxidizing bacteria that belong to the genera *Leptothrix* and *Gallionella* grew in static water conditions i.e. in ponds at circumneutral pH with 1.6 to 12 mg/L Fe (II), with growth inhibition taking place at ≥ l4 mg/L iron (Hasselbarth and Ludemann, 1972). In flowing water, encrustations of iron bacteria can be found if the iron concentration exceeds 0.2-0.5 mg/L due to the continuous flow of nutrients (Cullimore and Mc Cann, 1977; Hedrich *et al*., 2011).

Manganese - Most iron bacteria prefer to grow in media containing higher concentrations of iron than in media that containing equivalent molar concentrations of manganous ions (Cullimore and Mc Cann, 1977). However, manganese plays an important role in bacterial homeostasis by reducing oxidative stress in microorganisms (Cornelis *et al*., 2011).

pH - Heterotrophic iron bacteria generally grow well over a pH range of 5.4 to 7.2 (Hasselbarth and Ludemann, 1972). High alkaline conditions lead to the rapid oxidation of ferrous and manganous ions by normal physiochemical processes, making them less available as a potential energy source (Cullimore and Mc Cann, 1977). However, the acidophilic iron oxidizing bacteria such as *Acidithiobacillus* or their archael, thermophilic counterparts such as *Ferroplasma* spp., grow at a very low pH thus overcoming Fe (II) instability under fully aerated conditions (Hedrich *et al*., 2011).

Temperature and oxygen - Iron oxidizing bacteria are either aerobic or, given the instability of Fe (II) at neutral pH values, microaerophilic with massive growth of iron bacteria reported in wells containing less than 5 mg/L oxygen (Ehrlich and Newman, 2009; Hasselbarth and Ludemann, 1972). The common iron bacteria from the *Sphaerotilus-Leptothrix* group of bacteria, prefer temperatures ranging between 15 and 30°C (Hasselbarth and Ludemann, 1972). However, the anaerobic thermophilic oxidation of Fe (II) coupled to nitrate reduction at neutral pH has been reported for the archaeal species *Ferroglobus placidus* (Hafenbradl *et al*., 1996).

Carbon - Organic carbon in growth media required by heterotrophic iron bacteria such as *Leptothrix discophora* used in lab based studies, is typically provided in a variety of forms like citrate, acetate, glucose and peptone (Hasselbarth and Ludemann, 1972). The most common source of carbon in growth media is citrate, which is provided in the form of ferric ammonium citrate (Cullimore and Mc Cann, 1977; Hedrich *et al*., 2011). The total available organic carbon present in the water is a function of the flow rate of the water as well as concentration of carbon in the water. Therefore it is difficult to predict a threshold concentration of carbon below which iron oxidizing bacterial growth would be restricted or totally inhibited in flowing water (Cullimore and Mc Cann, 1977).

2. Bacteriogenic iron oxides

In the presence of molecular oxygen at circumneutral pH, the oxidation of Fe (II) is very rapid, resulting in the formation of poorly crystalline ferric oxyhydroxide, following Fe (III) hydrolysis. The reduction of these hydroxides is coupled to the degradation and/or sequestration of

contaminants within biological and engineered systems (Hansel and Lentini, 2011). The bacteriogenic iron oxides consists of a mixture of poorly crystalline hydrous ferric oxide, bacterial cells, organic material and are considered important sinks for inorganic contaminants and nutrients within soils, sediments and waters (Ferris *et al*., 1999; Hansel and Lentini, 2011). These oxides have been considered for environmental in situ remediation of contaminants due to their highly reactive surface properties and the wide range of functional groups provided by the organic fraction of these oxides (Ferris *et al*., 1999; Kennedy *et al*., 2011). Their high surface areas and density of reactive surface sites, allow the Fe (III) hydroxides to absorb numerous organics such as pesticides, nutrients and metals such as Pb, As and U (Cornell and Schwertmann, 2003). The common iron hydroxides are ferrihydrite, goethite, lepidocrocite and hematite. Ferrihydrite is considered the most bioavailable hydroxide from microbial Fe (III) formation (Lovley and Phillips, 1986). Fe (III) hydroxides consist of an array of Fe (III) and O^{2-} or OH^- ions and differ in how their basic structural units - Fe $(O/OH)_6$ or FeO_6 - are arranged in space (Hansel and Lentini, 2011).

Ferrihydrite ($Fe_5HO_8 \cdot 4H_2O$): This is a poorly crystalline and the least thermodynamically stable hydroxide form with the highest surface area of the four most common iron hydroxides. The exact formula for ferrihydrite is not yet established. Ferrihydrite is a group of oxyhydroxide minerals that lack long-range order and have varying degrees of crystallinity. It has a hexagonal crystal system and its dominant morphology is in the shape of spheres (Cornell and Schwertmann, 2003; Hansel and Lentini, 2011).

Goethite (α-FeOOH): The structure of this Fe (III) hydroxide is comprised of octahedral double chains linked through corners. It has an orthorhombic crystal system and exists in an acircular form (Cornell and Schwertmann, 2003; Hansel and Lentini, 2011).

Lepidocrocite (γ-FeOOH): This structure is composed of octahedral double chains in corrugated layers which are cross-linked through edges. It also has an orthorhombic crystal system and exists in the form of laths (Cornell and Schwertmann, 2003; Hansel and Lentini, 2011).

Hematite (α - Fe_2O_3): This is the most thermodynamically stable and well-structured crystalline Fe (III) hydroxide form. The structure of Hematite consists of octahedra linked through edge- and corner-sharing as well as face-sharing along the *c* axis. Like ferrihydrite, it has a hexagonal crystal system and exists in the form of plates (Cornell and Schwertmann, 2003; Hansel and Lentini, 2011).

The oxidized Fe (III) species can exist in a number of primary and secondary minerals including Fe-containing phyllosilicates and hydroxides. Above pH 4 and at low solubility, Fe (III) hydroxides are the predominant form of Fe (III) in most soils and sediments (Cornell and Schwertmann, 2003). The stability of the Fe (III) hydroxides is a function of the crystal structure and particle size which also determines the solubility of the phase. Solubility is a function of ionic strength, temperature, particle size and crystal defects, to name a few. The solubility of the common pure Fe (III) hydroxides progresses in the order of two-line ferrihydrite > six-line ferrihydrite > lepidocrocite > goethite and > hematite at circumneutral pH (Baes and Mesmer, 1976; Cornell and Schwertmann, 2003). Fe (III) hydroxides always contain co-precipitated ions in the environment and the trace metals that usually substitute into Fe (III) hydroxides are Cd (II), Co (III), Cr (III), Cu (II), Mn (III), Ni (II), V (III) and Zn (II) (Trolard *et al*., 1995; Hansel and Lentini, 2011). Most of these metals substitute to levels below 10% within Fe (III) hydroxides whilst manganese occupies a significant fraction of these Fe (III) sites (Hansel and Lentini, 2011).

3. General properties and biochemical aspects of iron

Iron plays a crucial role in energy production and is important for normal health and metabolism (Beard and Dawson, 1997). One of the major biotechnological applications for iron oxidizing bacteria is to solubilize metals from mineral ores making them accessible for chemical extraction. This process is called biomining or bioleaching and has been widely used for copper production and to a lesser extent for nickel, cobalt, uranium, zinc and gold (Rawlings and Johnson, 2007). Iron is also an essential cofactor for cytochromes and other iron-sulfur unit containing enzymes such as oxygenases and forms an essential structural component of catalase and peroxidase enzymes which prevent oxidative stress (Beard and Dawson, 1997). Whilst an

essential nutrient, iron, in excess accumulation can lead to the production of reactive oxygen species (ROS), such as peroxides and oxygen ions (Beard and Dawson, 1997). The excess accumulation of iron can result in or contribute to the development of diseases such as hemochromatosis, thalassemia and/or chronic liver disease (Beaton and Adams 2007; Kohgo *et al*., 2008). The deficiency of iron is however more common than iron overload and affects a large group of people worldwide, resulting in anaemic disease (Jarrah *et al*., 2007).

Bacterial iron homeostasis and responses to oxidative stress are interlinked and must be controlled for the effective functioning of the bacterial cell. Iron is an essential nutrient for the growth of microorganisms but is also a dangerous metal as it generates reactive oxygen species such as superoxide ions (O_2^-), hydrogen peroxide (H_2O_2) and the destructive hydroxyl radical ($OH^{\cdot}$), via the Fenton reaction (5). Bacteria must therefore control the uptake and storage of iron so as to avoid the build-up of reactive oxygen species that cause oxidative stress (Cornelis *et al*., 2011).

$$\begin{aligned} Fe^{3+} + O_2^- &\longrightarrow Fe^{2+} + O_2 \\ Fe^{2+} + H_2O_2 &\longrightarrow Fe^{3+} + OH^- + OH^{\cdot} \\ \hline O_2^- + H_2O_2 &\longrightarrow OH^{\cdot} + OH^- + O_2 \end{aligned} \qquad (5)$$

Reactive oxygen species cause damage to Fe-S clusters and can lead to protein carbonylation, Cys/Met-residue oxidation, membrane lipid peroxidation and cause DNA damage in bacterial cells (Imlay and Linn, 1988; Winterbourn, 1995). One of the ways that ROS are produced is by aerobes through incomplete reduction of O_2 during respiration processes (Cornelis *et al*., 2011; Imlay, 2002). Bacteria have thus developed mechanisms to resist oxidative stress through the production of enzymes such as superoxide dismutases (catalyze the dismutation of O_2), catalases (inactivate H_2O_2 and catalyze the reduction of alkyl hydroperoxides), alkylhydroperoxidases, superoxide reductases and peroxidases (inactivate H_2O_2 and catalyze the reduction of alkyl hydroperoxides) that degrade ROS (Hassett *et al*., 1995; Ueda *et al*., 2003). Iron is a major contributor to oxidative stress and is also required by some ROS-degrading enzymes, thus linking iron homeostasis and oxidative stress and highlighting the need for the two processes to

be carefully controlled in order to avoid the accumulation of potentially dangerous free iron in the cell (Cornelis *et al*., 2011; Faulkner and Helmann, 2011). One of the ways bacteria can protect themselves against ROS, is by sequestering iron and storing it in proteins like ferritins and bacterioferritins (Andrews, 1998). These proteins provide a source of iron when this metal becomes scarce and also protect the cell from ROS through the sequestration thereof (Andrews, 1998).

A variety of regulators can be used to control iron regulation in bacteria, of particular interest is the Fur protein, which regulates the uptake of iron. This is a conserved protein in many Gram positive and Gram negative bacteria and operates primarily as a repressor of iron uptake genes involved in siderophore biosynthesis or in transport across the membrane. Repression is achieved when Fur is associated with its co-repressor, Fe (II) (Bsat *et al*., 1998; Lee and Helmann, 2007). The Fur-Fe (II) complex controls expression by binding to the consensus sequence (iron or Fur box) and blocks access of the RNA polymerase to the promoter thus inhibiting transcription of the downstream genes. Thus iron uptake components are down regulated when iron levels become too high (Cornelis *et al*., 2009; Lee and Helmann, 2007). Whilst Fur typically behaves as a repressor, it has also been shown to positively regulate the expression of genes that encode iron proteins like bacterioferritins and the iron co-factored superoxide dismutase (Delany *et al*., 2004; Grifantini *et al*., 2004).

4. Impact of iron oxidizing bacteria in water systems and on the corrosion of carbon steel

Iron bacteria have caused problems in water distribution systems for many years, causing ‘red’ undrinkable water covered in slime (Cullimore and Mc Cann, 1977). The major problems in wells include plugging of screens; coating of pipe systems, impellers and motors, thereby reducing flow rates and the potability of water (Hasselbarth and Ludemann, 1972). Acidophilic iron oxidizing bacteria such as *Acidithiobacillus ferrooxidans*, including other sulfide-oxidizing bacteria, generate a very acidic iron oxidation product due to the simultaneous release of sulfuric acid, in the presence of oxygen (Cullimore and Mc Cann, 1977). Iron oxidizing bacteria consume oxygen creating microaerobic/anaerobic niches which are colonized by sulfate-reducing bacteria which are actively involved in corroding metal surfaces (Hamilton, 2003). The microorganisms

involved in the corrosion of steel, discolour the water through the release of iron from within the corrosion scales or as particulate matter when suspended and also contribute to the odour that accompanies the discoloured water. Manganese and iron oxidizing microorganisms that are usually implicated for problems in drinking water systems belong to genera such as *Pedomicrobium*, *Hyphomicrobium*, *Crenothrix*, *Leptothrix* and *Clonothrix* (Cerrato *et al*., 2010).

In a previous experiment conducted by Rao *et al*. (2000), it was found that filamentous sheathed sulfate reducing bacteria were encrusted with corrosion products of carbon steel coupons. Through this finding it was inferred that iron bacteria and sulfate reducing bacteria were responsible for the corrosion of carbon steel. A similar study by Starosvetsky *et al*. (2001) revealed that the addition of iron bacteria to a solution of sodium chloride in the presence of carbon steel induced corrosion and surface passivation. The key factor in the corrosion is the ability of iron bacteria to metabolize ferrous ions to ferric ions, followed by the formation of low density hydrated iron oxides in the tubercles of the metal (Emerson and Moyer, 1997). The sheaths of *Leptothrix* spp. form a membrane on the metal surface that is impermeable to oxygen, thus creating an anaerobic micro-environment that favours the growth of sulfate reducing bacteria.

An increase in pH in the solution in the vicinity of the metal is due to the cathodic reduction of oxygen and thus the metal will form metal cations at anodic sites (Borenstein, 1994; Mettel, 1998). In turn, the difference in potential between the iron surface outside and under the metal tubercle increases, thus accelerating the corrosion through the dissolution of iron oxides (Borenstein, 1994). The bacterial growth is thus pronounced leading to the accumulation and sedimentation of large amounts of ferric hydroxide (Borenstein, 1994; Rao *et al*., 2000). In the presence of iron oxidizing bacteria, corrosion occurs via the crevice corrosion mechanism, which partitions the metal into small anodic sites and large surrounding cathodic areas (Borenstein, 1994). The iron bacteria responsible for carbon steel corrosion are also capable of stainless steel corrosion and the corrosion of other passivated metals that are usually prone to crevice corrosion (Borenstein, 1994; Little and Wagner, 1997).

C. Microbe-Metal Interactions

1. Microbial uptake of metals

Due to the small size and consequently high surface area to volume ratio of bacteria, their surfaces provide a large area for contact with the surrounding environment (Haferburg and Kothe, 2007). Since bacteria probably lack highly specific metal uptake systems (Haferburg and Kothe, 2007), the negative net charge of the cell envelope of bacteria assists them in accumulating metal cations from the environment (Collins and Stotzky, 1992). Two important metal uptake systems that exist in bacteria are siderophore- and heme-mediated iron uptake (Cornelis *et al*., 2011). Siderophores are strong extracellular Fe (III) chelators that assist in the transport of Fe (III) into the bacterial cell, where it is reduced and Fe (II) is then released leaving behind the iron chelator which is left intact allowing recycling (Cornelis *et al*., 2011). Heme is an important source of iron for bacteria and is not found unbound due to its potential toxicity and hydrophobicity (Wyckoff *et al*., 2005). Bacteria obtain heme from the hosts that they colonize and it is first extracted from hemoproteins such as haemoglobin or hemopexin. Once inside the cytoplasm of the bacteria, the heme is broken down into biliverdin and CO via heme oxygenase or it can be de-ferrated, releasing Fe (II) and leaving the tetrapyrrole ring intact (Letoffe *et al*., 2009; Wandersman and Delepelaire, 2004). The metal absorption to the cell envelope is influenced by the cell envelope components such as phosphoryl groups of lipopolysaccharides, carboxylic groups of teichoic and teichuronic acids, or capsule forming extracellular polymers such as sheaths (Haferburg and Kothe, 2007). Metal accumulation can take place via two processes, passive attachment onto the bacterial cell or via the active uptake into the bacterial cell. The passive uptake of metals is normally the dominant mode of metal accumulation due to nutrient scarcity in many natural environments such as soils (Haferburg and Kothe, 2007). The active uptake process is usually slower, requires energy and is dependent on metal-specific transport systems (Gadd, 1988).

2. Microbial mechanisms providing metal resistance

Metal resistance seems to be more prevalent in environmental systems than in pure cultures (Sprocati *et al.*, 2006). An important resistance mechanism of bacteria to metals is the use of efflux transporters, characterized by a high substrate affinity. The transporters keep the metal concentration in the cytosol low via the excretion of over concentrated or toxic metals (Nies, 2003; Haferburg and Kothe, 2007). Another survival strategy is the release of metal binding compounds such as siderophores into the external environment of the bacterial cell where metals are then chelated and blocked from entering the cell (Haferburg and Kothe, 2007). This is an important mechanism as membrane transport systems of the cell cannot differentiate between potentially toxic and non-toxic metals (Haferburg and Kothe, 2007). A metal resistance mechanism for bacteria found in soil habitats is a combination of biosolubilization and bioprecipitation (Haferburg and Kothe, 2007), which typically involves the excretion of organic compounds that solubilize metals such as oxalates (Gadd, 1999). Some bacteria develop internal inclusion bodies e.g., polyphosphate granules, which bind the metal cations in the cytosol if they enter the bacterial cell and cannot be excreted via the efflux transporters (Gonzalez and Jensen, 1998). Another metal resistance mechanism, is the sorption of metals by the cell membrane in combination with the cell wall, which also facilitates bioreduction (Haferburg and Kothe, 2007). The four main metal resistance mechanisms employed by microbes are summarized in figure 3.

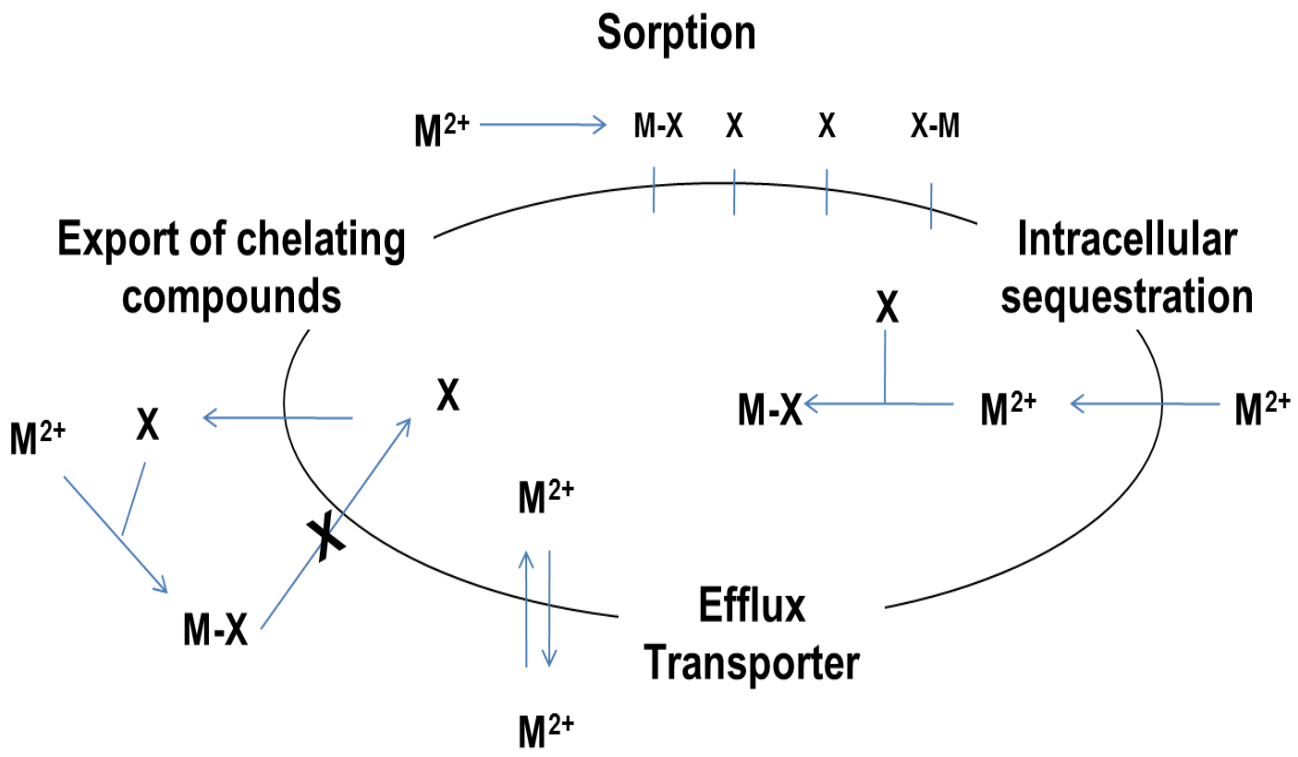

Figure 3 (Adapted from Haferburg and Kothe, 2007): Overview of the four microbial metal resistance mechanisms. (X) - Cell constituents interacting with metal cations, (M) - Metal cation.

3. Metal toxicity for microbes

The ionic form of a metal is its most active form. Properties used to predict the toxicity of a metal ion are related to the solubility, stability and electrochemical characteristics of the metal (Venugopal and Luckey, 1978). In biological systems, the toxicity of a metal ion is associated with the difference in binding of these ions to biological structures such as tissues, cells, organelles etc., the stability of the metal ligand bonds and the form of the metal ion in the target biological structure (Venugopal and Luckey, 1978). The biological activity of a dissolved metal is correlated to its free ion concentration and the electrochemical properties consist of the oxidation potential, ionization potential, electropositivity, electronegativity, electron affinity and the oxidation state of the metal (Walker *et al*., 2003). The oxidation potential of a metal couple is the tendency of a metal ion to undergo oxidation from a lower oxidation state to a higher

oxidation state (Walker *et al*., 2003). Whilst the ionization potential is the difference in energy between the ground state and the state of ionization and it gives an indication of the electron affinity or electronegativity of the metal ion (Walker *et al*., 2003). Electropositivity is defined as the ability of a metal ion to lose electrons whilst electronegativity is defined as the ability of a metal ion to gain electrons or similarly the power of an atom to attract electrons to itself from a ligand (Rossotti, 1960). Electron affinity of a metal is the energy released when atom and ion are in their lowest energy states.

Yatsimirskii (1994) described the oxidation state of a metal ion as the charge of the metal ion in a purely ionic model for the complex. Overall, the toxicity of a metal is a combination of the physical and chemical properties of the metal and the interaction between metals and their biological targets (Luckey and Venugopal, 1977). Fe (II) and Mn (II) are trace elements and generally have a low toxicity. The minimum inhibitory concentration (MIC) of Mn (II) in *E. coli* was determined as 20 mM (Mergeay *et al*., 1985). The toxic potential of manganese and most other metals is determined by their ability to form complex compounds (Nies, 1999). Fe (II) is rapidly oxidized under aerobic conditions to Fe (III), which has a very low solubility and under most circumstances it is generally not toxic to aerobic bacteria (Nies, 1999).

D. Environmental Biotechnology and Purification of Metal Contaminated Waters

Factors that render drinking water unsafe for human consumption are related to the disinfection stage, biofilm growth, nitrification, microbially mediated corrosion and the persistence of pathogens in drinking water distribution systems (Berry *et al.*, 2006; Kormas *et al.*, 2010). Not only do these factors impact on the health of those who consume the water but it also affects the aesthetic appeal of drinking water and undermines the confidence of consumers which in turn reflects badly on the efficiency of water utility companies (Kormas *et al.*, 2010). Purification of contaminated water using biofiltration systems is one of the processes viewed as a sustainable environmental biotechnology as it employs microbial communities that remove contaminants such as metals from water and provide society with clean drinking water. Environmental biotechnology in purification of groundwater involves oxidation and reduction reactions which

are beneficial to both microbe and man (Rittmann, 2010). The aim of this technology is to optimize systems performance wise by characterizing microbial communities in terms of: (1) the types of microorganisms present, (2) the metabolic functions these microorganisms carry out and (3) their interaction with each other and their surrounding environments (Rittmann, 2010).

Both biological and chemical methods are employed to remove manganese and iron from groundwater (Mamchenko *et al.*, 2009). During the biological treatment raw water is passed through a filter which contains both Mn- and Fe-oxidizing bacteria (Burger *et al.*, 2008). This water is passed through the filter with compressed air to ensure that heterotrophic organisms responsible for oxidation have enough oxygen to function normally (Burger *et al.*, 2008). The physical-chemical removal processes involves raising the oxidation-reduction potential (ORP) of the water so that the iron and manganese present will be converted into their insoluble oxidized forms (Gage *et al.*, 2001). An increase in the volumes of underground water used for consumption requires the development of new efficient but at the same time quicker methods for removal of manganese, iron, hydrogen sulfur and other impurities (Mamchenko *et al.*, 2009). Purification of water containing iron and manganese involves filtration through a granular filtering medium which is typically coated with a film of manganese oxides.

The presence of manganese oxides increase adhesion forces thereby improving the removal of impurities from the water through the interaction of van der Waals forces and ionic interactions (Mamchenko *et al.*, 2009). The filtering media includes Birm, Greensand, Filox, and Pyrolox (Mamchenko *et al.*, 2009). Iron must be removed first through one aeration-filtration step, because it is rapidly oxidized in the presence of oxygen and the product of Fe (II) oxidation serves as a catalyst for manganese oxidation (Gage *et al.*, 2001). Thereafter, the pH is raised by stripping carbon dioxide (CO_2) and manganese is removed with another aeration-filtration step (Gage *et al.*, 2001). Products of manganese and iron oxidation are removed from the filter via back washing and some of the remaining products settle on the filter matrix serving as an additional catalyst for further oxidation to take place (Mamchenko *et al.*, 2009). The purification of water contaminated with iron is not complicated but the presence of manganese in the water complicates the process as manganese is more stable than iron under aerobic conditions (Mamchenko *et al.*, 2009). Albeit the contribution of bacteria to the removal of such metals is

important, it is not clear for individual biofiltration systems to which degree the microbial activity contributes to the removal of Fe (II) and Mn (II) in these systems.

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Chapter 3

Isolation and characterization of a manganese-oxidizing bacterium from a biofiltration system for the treatment of borehole water in KwaZulu-Natal (South Africa)

The majority of this chapter dealing with the manganese oxidizing isolate Acinetobacter sp. strain LB1, has recently been published under the title: Isolation and characterization of a manganese oxidizing bacterium from a biofiltration system for the treatment of borehole water in KwaZulu-Natal (South Africa) in Engineering and Life Sciences (2012, 12:544–552) and the published manuscript is reproduced on the following pages. However, additional data not included in the published manuscript are presented in an appendix following this chapter.

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Research Article

Isolation and characterization of a manganese-oxidizing bacterium from a biofiltration system for the treatment of borehole water in KwaZulu-Natal (South Africa)

Using minimal salts medium containing vitamins and pyruvate (MSVP) plus added manganese sulfate, a manganese (II) oxidizing bacterial strain was isolated from water samples of a biofiltration system treating borehole water in KwaZulu-Natal, South Africa. The nonmotile Gram-negative and oxidase-negative isolate was subsequently characterized microbiologically. Based on its morphological and physiological characteristics and on the analysis of its 16S rRNA gene sequence, the isolate was assigned to the genus *Acinetobacter*. Growth of the isolated strain in MSVP with added manganese sulfate gave rise to a drop in pH and a concomitant increase in oxidation–reduction potential, which was absent in controls, thus indicating manganese oxidation. The ability of *Acinetobacter* sp. strain LB1 to oxidize Mn (II) was further verified using the leucoberbelin blue dye assay as well as by energy dispersive X-ray analysis of crystals formed in the medium. In addition, a biofilm assay indicated that this isolate can attach to solid surfaces such as a biofilter matrix, thus confirming its potential for biotechnological applications in the area of water purification.

Keywords: *Acinetobacter* sp. strain LB1 / Biofiltration / Leucoberbelin Blue / Manganese oxidation

Supporting information available online

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1 Introduction

Manganese represents the 12th most abundant element in the Earth's crust [1]. It can exist in 11 oxidation states ranging from –3 to +7 with two major forms in aquatic environments: Mn (II) and Mn (IV) [2]. Changes between the two major oxidation forms occur via oxidation and reduction reactions that may be abiotic or microbially mediated [2]. Manganese is in addition an essential cofactor for bacteria as this metal is involved in transcriptional regulation, developmental and metabolic processes, and at the same time protects bacterial cells against oxidative stress [3]. For South Africa, the Department of Water Affairs and Forestry [4] recommends a concentration for Mn (II) in potable water of below 0.05 mg/L. The presence of manganese (II) in drinking water has sparked a growing interest in Mn (II) removal techniques to treat groundwater, as the consumption of water high in manganese can exhibit toxic effects upon humans [5]. Bouchard et al. [6] reported only recently that the intake of manganese from ingested water can negatively impact on the IQ scores in school-age children and a recent study from Durban (South Africa) demonstrated elevated blood levels of manganese in about 8% of school children analyzed [7].

The first symptoms of the so called manganism are anorexia, weakness, and apathy while Parkinson symptoms such as tremors and muscle rigidity may appear in the later stages [5]. The oxidation of manganese (II) is generally very slow at neutral pH and would therefore require a very long time in the absence of catalysts or photochemical enhancements [8]. Unlike Fe (II), Mn (II) is quite stable under fully aerobic conditions at pH 7,

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Abbreviations: **EDX**, energy dispersive X-ray; **EPS**, extracellular polymeric substances; **ESEM**, environmental scanning electron microscopy; **MSVP**, minimal salts vitamins pyruvate; **ORP**, oxidation reduction potential; **TEM**, transmission electron microscopy

and only at pH $\geq$ 8 does chemical oxidation of manganese (II) begin to become appreciable [9]. Manganese often occurs in the same types of oxic–anoxic interfacial environments where iron is found; although it is generally 5 to 10 times less abundant, reflecting the relative total abundance of these two metals [10]. These properties have made it much easier to unequivocally demonstrate that manganese (II) oxidation can be biologically mediated [11].

Manganese removal systems typically rely on physiochemical reactions using manganese greensands (glauconite with manganese oxides of various Mn valence states), designed specifically to remove Mn (II), Fe (II), hydrogen sulfide, and arsenic [12] by employing intense aeration enabling chemical oxidation. Biofiltration systems were first developed to treat water rich in iron as this metal is not only more common but also because iron is more readily oxidized. Although these processes provide a feasible treatment option, they can be prone to operational problems. These are prolonged ripening periods for new filter media or turbidity problems when treating iron-rich groundwater, which are difficult to prevent even at low dissolved oxygen concentrations (i.e. <3 mg/L) thus producing effluents not always meeting water quality objectives [13].

Heterotrophic bacteria are well known to catalyze the oxidation of Mn (II) by direct and indirect processes [14]. An important side effect of the bacterial oxidation of manganese is its potential to trigger the so called microbially influenced corrosion of stainless steel via the deposition of manganese oxides and hydroxides onto the metal surface, a process that typically involves the formation of biofilms on the stainless steel surface [15]. As the Mn (IV) oxides generated via the bacterial oxidation of Mn (II) behave as strong oxidants, they might enable a cathodic reaction potentially driving anodic metal dissolution given direct contact with the metallic substrate [16].

Direct catalysis involves the production of polysaccharides or enzymes [17]. Extracellular polysaccharides may be found as a capsule attached to the bacteria or may be released to the environment as slime [18]. Such polysaccharides are important to the bacterium as they typically enable adhesion to surfaces (i.e. biofilm formation) and provide protection against desiccation [18]. Indirect catalysis of Mn (II) oxidation occurs when organisms modify the pH and redox conditions of the medium or release metabolic end products that chemically oxidize Mn (II) [17]. Thus, oxidation reduction potential (ORP) and pH measurements of microbial cultures provide a useful means for the confirmation of manganese oxidation in biological systems [14].

In addition to pH and ORP measurements, the dye leucoberbelin blue (N,N′-dimethylamino-p,p′-triphenylmethane-o′-sulfonic acid) can be used to reliably demonstrate the oxidation of Mn (II) to Mn (IV) in microbial culture media [19]. Due to the oxidation of leucoberbelin blue by Mn (III)/(IV) species to a blue reaction product with an absorption maximum at 618 nm, the oxidation of Mn (II) can be detected in microbial culture media in a pH range between 3.5 and 10 [19]. The primary objective of this study was to demonstrate the presence of manganese-oxidizing bacteria in a biofiltration system employed to remove iron and manganese from groundwater and to demonstrate the ability of isolates to perform the oxidation of manganese.

2 Materials and methods

2.1 Quantification of aerobic oligotrophic and manganese-oxidizing bacteria in biofilter water samples

R2A [20], a medium originally developed to enumerate oligotrophic bacteria, and minimal salts vitamins pyruvate (MSVP) agar [21] targeting heterotrophic bacteria, both with added manganese sulfate, were used to quantify manganese-oxidizing bacteria present in biofilter water samples. The MSVP medium was generated by adding filter sterilized (0.2 μm) stock solutions of sodium pyruvate (final concentration of 0.1 g/L in MSVP), $MnSO_4$ (manganese sulfate; final concentration of 1 mM in MSVP), and a vitamin solution (final concentration of 25 μL/L in MSVP) [21, 22] to the minimal salts after sterilization. In addition, the final medium contained 1 mg/L of Fe (II). Water samples used were collected at about 120 cm above the surface of the filter matrix in the biofiltration system treating borehole water (located in Nottingham Road, KwaZulu-Natal, South Africa). Decimal dilutions of samples were prepared using R2A and MSVP plus added manganese sulfate as diluents and 100 μL of appropriate decimal dilutions (typically in a range from 10^{-2} to 10^{-6}) were spread plated in duplicate using both R2A and MSVP agar. Plates were incubated at 25°C for 3 weeks in the dark to provide adequate time for the growth of slow-growing bacteria.

2.2 Microbiological characterization

Ten randomly selected colonies, originally obtained from solid MSVP medium with added manganese sulfate spread plated with biofilter water samples, were inoculated into 30 mL MSVP medium plus manganese sulfate in 100 mL Erlenmeyer flasks. An isolate showing the formation of a brown precipitate in the Erlenmeyer flask after 8 days incubation was selected for further characterization. Stock cultures of Mn (II) oxidizing strains isolated from the biofilter were kept at −80°C in MSVP in the presence of 20% glycerol. The morphological and physiological characterization of this isolate obtained from the biofilter water via MSVP plus added manganese sulfate was done by following standard procedures [23]. The size and motility of the isolate was determined via the hanging drop method by phase contrast microscopy (Motic BA 310, China).

For morphological analysis of cells by electron microscopy, the selected isolate was cultivated in MSVP medium plus manganese sulfate on a shaker incubator (25°C, 150 rpm, 48 h). A 1 mL sample was centrifuged (5 min, 25°C, 14 000 *g*), the cell pellet obtained was resuspended in 500 μL sterilized tap water, again centrifuged, and the cell pellet resuspended in 100 μL of sterile distilled water. One droplet (i.e. about 20 μL) of this suspension was placed on carbon tape mounted on a specimen stub and viewed using a Philips XL 30 environmental scanning electron microscopy (ESEM, Netherlands) at low vacuum mode. Samples were subsequently sputter coated with gold palladium using a Polaron E5100 sputter coater (Eiko IB-3, Japan) and viewed in high vacuum mode. Negative staining using uranyl acetate was used to check for the presence of flagella via transmission electron

microscopy (TEM; Philips CM 120 Biotwin, Netherlands). In addition, the isolate was analyzed using the BioMérieux Vitek-2 compact system (France) with a Gram-negative identification card.

2.3 16S rRNA gene sequence analysis

A single colony of the selected strain from MSVP plates with 1 mM manganese sulfate was suspended in 100 μL of sterile distilled water and treated by five freeze–thaw cycles (i.e. 5 min at 95°C followed by 10 min in liquid nitrogen). A 2 μL sample was amplified by PCR using the published primer pair fd1 and rp2 and the corresponding PCR protocol [24]. The amplification product (1400 bp) was sequenced (Inqaba Biotec, Pretoria, South Africa). The sequence obtained was deposited under accession number JN315799 with GenBank (NCBI) and compared to 16S rRNA gene sequences deposited in GenBank using the NCBI Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov). A phylogenetic tree using type strain sequences for 16 species within the genus *Acinetobacter* deposited within RDP (ribosomal database project, rdp.cme.msu.edu) was generated, based on sequence alignment established with clustal w and the neighbor joining method using MEGA 5 [25] with resampling for 1000 replicates.

2.4 Manganese oxidation in MSVP

Manganese oxidation tests using the selected isolate were carried out in order to verify its ability to oxidize manganese. A volume of 30 mL sterile MSVP medium containing 1 mM manganese sulfate was added to sterile 100 mL Erlenmeyer flasks. Flasks were inoculated using bacterial cells pregrown in liquid MSVP to an initial cell density of about 5×10^7 cells/mL of MSVP medium and growth was followed over time using a Helber-type bacterial counting chamber. Abiotic control flasks contained MSVP medium with 1 mM manganese sulfate in the presence of heat-inactivated cells. The flasks were incubated in a shaker incubator (MRC orbital shaker incubator, Israel) at 25°C and 150 rpm for 8 days. The sodium pyruvate concentration was lowered in our experiments 10 times (i.e. to a final concentration of 0.1 g sodium pyruvate per liter MSVP) from the original recipe [21] as the higher pyruvate concentration seemed to mask manganese (II) oxidation in the system [26]. Sets of three individual flasks for pH, ORP, and cell counts including control flasks with inactivated cells and flasks with active cells were analyzed once every 2 days over an 8-day period. Samples taken were centrifuged (14 000 *g*, 15 min, 25°C) and the supernatant was used for pH and ORP analysis using a calibrated electrode (Hach sension1, Germany). Crystals formed in flasks over the 8-day period were detected via phase contrast microscopy (Motic BA310) and analyzed by an Oxford (X-MAX) energy dispersive X-ray (EDX) detector coupled to scanning electron microscopy (Zeiss Evo LS 15, Germany).

2.5 Detection of manganese oxidation using leucoberbelin blue

Leucoberbelin blue was employed to check for the presence of oxidized manganese in bacterial cultures after incubation [19]. Hence, the selected bacterial isolate was cultivated in the medium (i.e. LBB medium, containing 3 g bacto-peptone and 1 g yeast extract per liter) suggested by the same authors specifically for the leucoberbelin blue assay. However, it contained 1 mM $MnSO_4$, 1 mg/L of Fe (II), and distilled water was used as a substitute for the aged sea water in the original medium. Erlenmeyer flasks (100 mL) containing 30 mL of the above medium were inoculated with 3×10^7 cells (pregrown in LBB medium) per milliliter and incubated for 5 days in a shaker incubator at 25°C and 150 rpm.

Controls were incubated in the absence of bacterial cells with and without 1 mg/L Fe (II). As it is known that certain proteins or other biomolecules involved in manganese (II) oxidation processes are heat stable [27], an additional control was run with heat-inactivated (20 min at 121°C) bacterial cells in the presence of 1 mg/L Fe (II). For the qualitative detection of manganese oxidation, 1 mL of 0.04% (w/v) leucoberbelin blue solution in 45 mM acetic acid was added to flasks after incubation and visually analyzed for the formation of a blue color in comparison to abiotic controls. For the spectrophotometric quantification of manganese (II) oxidation, 1 mL of culture supernatant (diluted with LBB medium prior to measurement if required) was added to 2 mL of the above leucoberbelin blue solution, vortexed for 30 s and incubated in the dark for 5 min at ambient temperature. Thereafter the absorbance was measured at 628 nm [26] using a Bio-Rad Smartspec Plus (USA). Calibration curves were generated using culture medium containing known quantities of Mn (IV) in a linear range from 0 to 80 μM.

2.6 Biofilm formation assay

Biofilm formation assays were carried out according to Maldonado et al. [28] with the following modifications. The isolate was cultivated in 75 mL LBB medium (as specified under 2.5) in 250 mL Erlenmeyer flasks until the late logarithmic phase. Triplicate samples of 15 mL were aseptically dispensed into sterile 90 mm Petri dishes and incubated for 24, 48, 72, and 96 h, respectively. Controls contained only sterile, noninoculated LBB medium. Additional controls employed the known manganese-oxidizing bacterial species *Leptothrix mobilis* (DSM 10617). After incubation, the bacterial suspension was discarded from the Petri dish and the Petri dish gently rinsed twice with distilled water to remove unattached cells. Thereafter, 20 mL of a 0.1% (w/v) crystal violet (CV) solution was added to each Petri dish that was incubated for 20 min at room temperature with occasional mild shaking. The CV solution was discarded and the plates gently rinsed twice with distilled water to remove any excess dye not absorbed. Thirty milliliters of 95% ethanol was added to each plate. These were then incubated at room temperature for 5 min with manual, occasional mild shaking to extract the bound CV. The ethanol solution was collected and the absorbance measured at 540 nm in centrifuged samples (samples were diluted with 95% ethanol where necessary) using 95% ethanol as blank.

2.7 Chemicals

Leucoberbelin blue was obtained from Sigma-Aldrich (USA). Unless otherwise stated, all other chemicals used were of the highest purity commercially available.

3 Results and discussion

3.1 Quantification of aerobic oligotrophic and manganese-oxidizing bacteria in biofilter water samples

After 2 weeks incubation, MSVP agar (with 1 mM added manganese sulfate) gave rise to 1.35×10^6 cfu/mL while R2A agar (with 1 mM added manganese sulfate) gave rise to 8.8×10^4 cfu/mL for biofilter water samples. In most freshwater aquatic environments, microbial cell densities fall in a range of 10^5 to 10^6 cell/mL [29, 30]. The values determined are in a range reported for other biofiltration systems used to eliminate manganese from groundwater. Vandenabeele et al. [31] reported cell counts ranging from 2×10^3 to 7.9×10^4 cfu/g of biofilter sand and of 1.4×10^3 cfu/mL for the influent water treated using PYM agar, while Burger et al. [32] reported heterotrophic counts of 10^6–10^8 cfu/g of biofilter sand using MSVP and R2A. The count reported by Vandenabeele et al. [31] for the biofilter sand is lower than the number established for the biofilter water analyzed in our study, which might be due to a low recovery of cells from the sand and the fact that a different medium (PYM) was employed in this study.

3.2 Microbiological characterization of the selected isolate

The isolate selected for further characterization was Gram negative with cells appearing as plump short rods ($1 \times 1.5\ \mu$m) and cells typically showed a substantial amount of clumping (Fig. 1). Cells had capsules and were nonmotile, oxidase negative, and catalase positive. The above characteristics of the strain are typical features of species within the genus *Acinetobacter* [33, 34]. In order to further verify the taxonomic affiliation of the unknown isolate, a commercial test system (Vitek 2) and sequence analysis of the 16S rRNA gene were employed. The results of the Vitek analysis confirmed that strain LB1 belongs to the genus *Acinetobacter* based on the biochemical reaction pattern obtained. In addition, the sequence obtained for strain LB1 showed high similarity ($\geq$ 99%) to 16S rRNA gene sequences of environmental isolates identified as *Acinetobacter calcoaceticus* (DQ187381.1, JF683591.1). This result was confirmed by phylogenetic analysis depicting a close relationship between the isolated strain LB1 and *A. calcoaceticus* (Fig. 2).

3.3 Manganese oxidation by the isolated strain *Acinetobacter* sp. LB1 in MSVP

The pH of the MSVP medium containing 1 mM manganese sulfate remained fairly constant in heat-inactivated controls over the 8-day incubation period (Fig. 3B). However, in replicate Erlenmeyer flasks with the isolated *Acinetobacter* sp. LB1 present, the pH dropped between day 6 and 8 from about 7 to below 4 (Fig. 3B) This was accompanied by an increase in ORP from about 0 mV to a value of +198 mV in the presence of strain LB1, while the ORP in controls remained virtually constant over the 8-day period (Fig. 3A). At the same time, the cell number had increased to about 8×10^8 cells/mL between day 6 and day 8, thus indicating that the pH and ORP changes were triggered by the presence of increased cell numbers indicating that stationary phase was reached (Fig. 3C). Adams and Ghiorse [35] reported that the highest Mn (II) oxidizing activity of the well known β-proteobacterial species *L. discophora* SS1 was present in early stationary-phase cultures. Similarly, Tebo et al. [36] concluded that most bacterial strains known to oxidize Mn (II) perform this oxidative process in the stationary growth phase.

The ORP of the medium is directly related to the pH [13] and the pH in turn can drop due to the formation of both CO_2, manganese oxides as well as hydroxycarboxylic acids [37]. The hydroxycarboxylic acids of microbial origin exert a catalytic effect on Mn (II) oxidation, establishing the formation of an insoluble, brown manganese complex presumably containing Mn (III) [37]. The presence of oxygen in water is not sufficient for quantitative Mn (II) oxidation to take place at

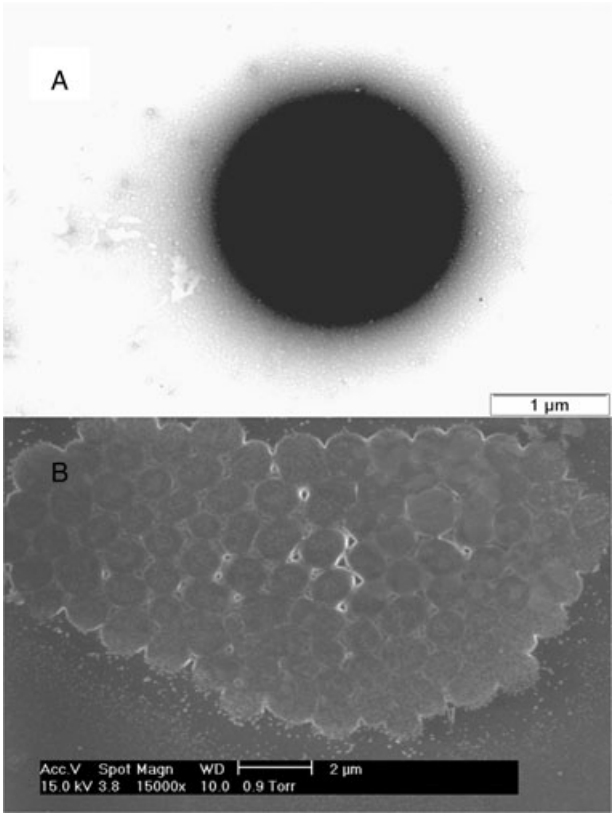

Figure 1. TEM (A) and ESEM (B) image of the manganese-oxidizing isolate *Acinetobacter* sp. strain LB1. (A) Negatively stained single cell. (B) Sputter coated cell aggregate.

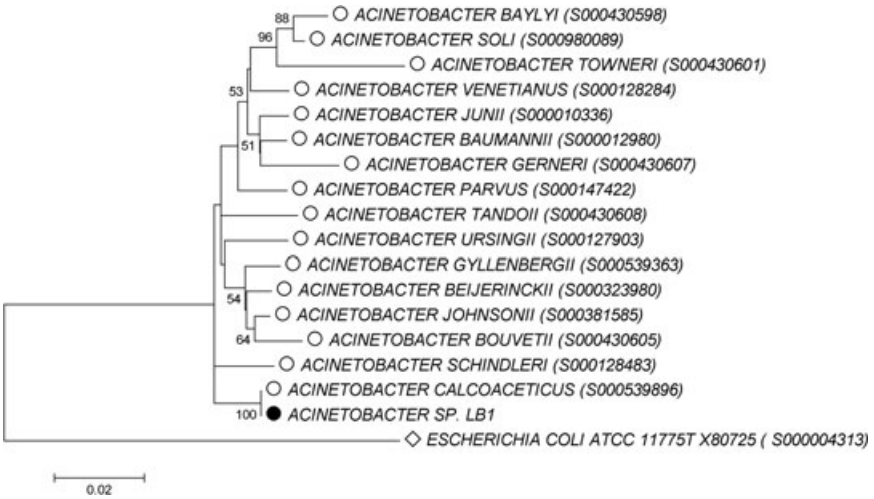

Figure 2. Phylogenetic affiliation of the strain LB1 (black circle) based on the comparison of its 16S rRNA gene sequence with 16 selected 16S rRNA gene sequences for type strains of the genus *Acinteobacter* (open circles). The alignment of selected sequences and the construction of the tree are specified in section 2. The scale bar represents two estimated changes per 100 nucleotides. Numbers shown at nodes indicate calculated bootstrap values (only values > 50% are shown). *Escherichia coli* was used as an out-group (open diamond).

neutral pH [17], while the presence of previously oxidized manganese or Fe (II) allows the normally slow oxidation reaction to go to completion [13]. Brownish crystals were formed in MSVP in the presence of *Acinetobacter* sp. LB1 and in minute quantities even in control flasks containing Fe (II) and heat-inactivated cells while being virtually absent in controls, lacking both *Acinetobacter* sp. strain LB1 and Fe (II) (data not shown). This result is not unexpected on chemical grounds as Mn (II) can be oxidized abiologically—albeit slowly—in the presence of Fe (II) [17, 38]. The appearance of crystal complexes formed was analyzed by light microscopy and ESEM (Fig. 4A). EDX analysis verified that these crystals contained both manganese and oxygen thus suggesting that these crystals contained manganese oxides (Fig. 4B).

The above results indicated that the strain *Acinetobacter* sp. LB1 was able to oxidize manganese (II) as neither pH nor ORP changed in controls inoculated with inactivated cells. It is evident that with Fe (II) present and sufficient oxygenation, Mn (II) oxidation can occur to a limited extent, but in the presence of manganese-oxidizing bacteria such as *Acinetobacter* sp. LB1, this activity is clearly enhanced. Vandenabeele et al. [31] reported that the incubation of manganese-oxidizing bacterial consortia in the presence of manganese sulfate led to a pH drop of about 0.7 units over a 0.5 h incubation period. This pH drop is consistent with the stoichiometry of the bacterial manganese oxidation according to the reaction shown below (1), which is a proton-generating process [17, 31].

$$Mn^{2+} + 0.5\ O_2 + H_2O \rightarrow MnO_2 + 2H^+ \quad (1)$$

3.4 Detection of manganese (II) oxidation using leucoberbelin blue

In comparison to controls with heat-inactivated cells, the qualitative leucoberbelin blue assay clearly demonstrated an enhanced manganese (II) oxidation in the presence of *Acinetobacter* sp. strain LB1. Flasks with *Acinetobacter* sp. strain LB1 showed a deep blue color formation after incubation while flasks without active bacteria present displayed at most a pale blue color (see Supporting Information, Fig. S1). This confirmed that manganese (II) oxidation had taken place in *Acinetobacter* sp. LB1 containing flasks and only to a much lesser extent even in abiotic controls containing Fe (II). Similarly, MSVP plates containing added manganese sulfate showed blue color formation after staining with leucoberbelin blue demonstrating that *Acinetobacter* sp. strain LB1 and *L. mobilis* (DSM 10617) are able to oxidize Mn (II) while controls without added manganese sulfate present or plates inoculated with *Escherichia coli* (ATCC 8739) showed no color formation due to Mn (II) oxidation (see Supporting Information Fig. S2). In addition, Fig. S2 illustrates a principal difference between the two Mn (II) oxidizing proteobacterial strains. In case of *Acinetobacter* sp. strain LB1, the oxidation of leucoberbelin blue by Mn (III/IV) species to its blue oxidation product was evident throughout the medium, while this reaction was in case of *L. mobilis* (DSM 10617) apparently limited to the proximity of the bacterial cells. This verifies that oxidized manganese species, as reported previously [39], attach preferably to the sheath of *Leptothrix* spp. This appears not to be the case in *Acinetobacter* sp. strain LB1 to the same degree, which might be due to a lesser affinity of oxidized manganese species to attach to the cell surface or excretion of Mn (II) oxidizing agents into the surrounding medium.

Using the medium outlined in section 2.5, spectrophotometric analysis using leucoberbelin blue demonstrated that in flasks containing *Acinetobacter* sp. strain LB1 in the presence of Fe (II), about 11% of Mn (II) present (i.e. 1 mM) had been oxidized after 5 days incubation. In the absence of added Fe (II), still about 7% of Mn (II) had been oxidized by the bacterial strain (Fig. 5). However, in all three different controls employed (no bacteria, no added Fe (II), heat-inactivated cells with Fe (II) present), less than 1% of Mn (II) initially present had been oxidized (Fig. 5). As leucoberbelin blue is quickly oxidized by Mn (III) and Mn (IV) but neither by Mn (II) nor other metals when present at small concentrations [19], our data confirmed that Mn (II) was oxidized by active cells of *Acinetobacter* sp. strain LB1. Similar to our results, El Gheriany et al. [26] monitored manganese (II) oxidation over time in the presence of *L. discophora* SS-1 cells using the leucoberbelin blue colorimetric assay and found that Fe (II) is essential for this particular strain to achieve maximum oxidation rates for Mn (II).

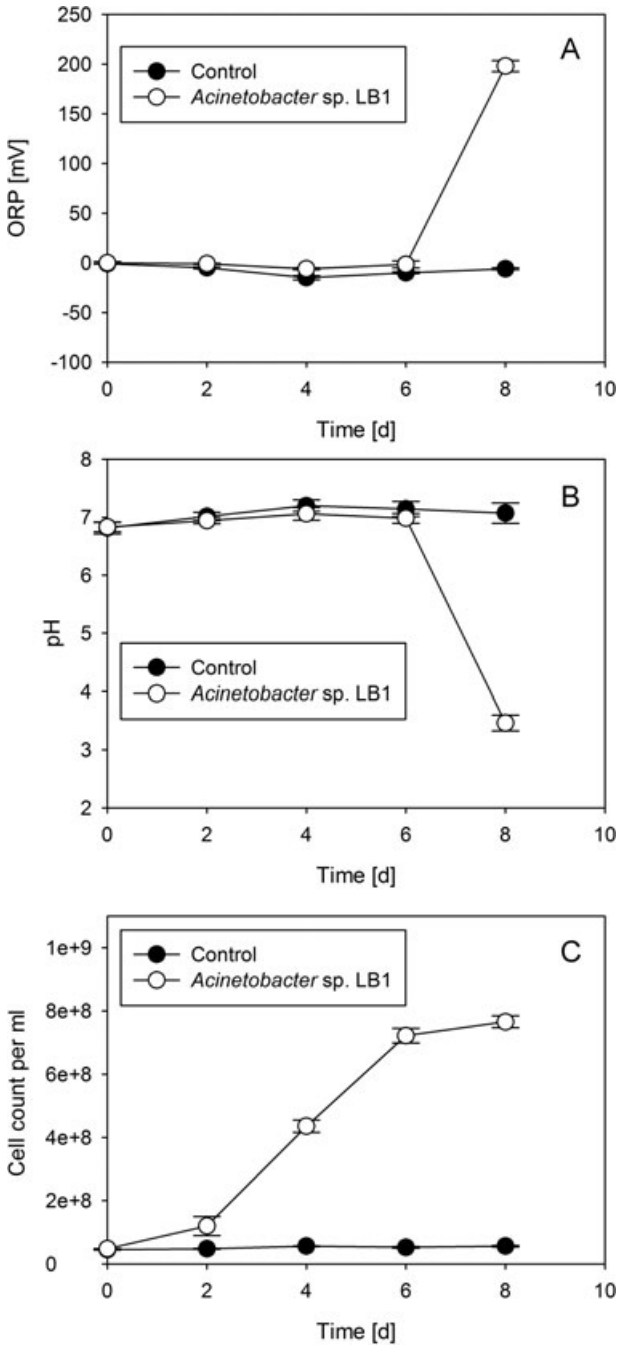

Figure 3. Impact of Mn (II) oxidation on ORP (A) and pH (B) and correlation with growth (C) over time. Data shown are the means of experiments performed in triplicate in the presence of actively growing cells of the manganese-oxidizing strain *Acinetobacter* sp. strain LB1 (open symbols) in comparison to controls (heat-inactivated cells, filled symbols). Error bars indicate the standard deviation.

3.5 Microscopical detection of an EPS-like structure surrounding cells of *Acinetobacter* sp. strain LB1

Quantifying the oxidation of Mn (II) to brownish manganese oxides is not always straightforward as Mn (II) can adsorb to

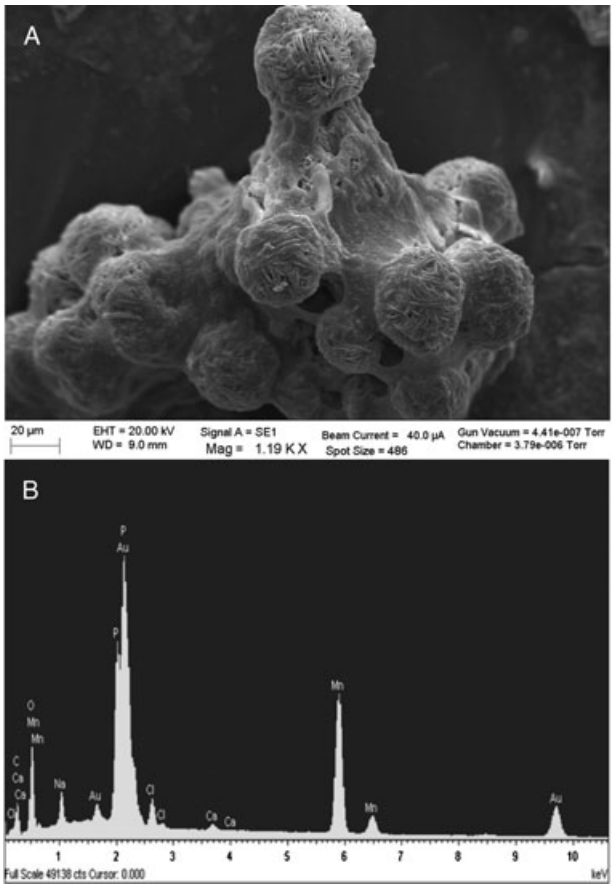

Figure 4. ESEM image (A) of a representative crystal formed in MSVP medium with 1 mM manganese sulfate in the presence of *Acinetobacter* sp. strain LB1 after 8 days incubation. Corresponding EDX spectrum of the crystal analyzed (B).

cellular materials such as sheaths or extracellular polymeric substances (EPS) layers or even MnO_2 produced at an earlier stage [40]. In addition, it was reported that the proteinaceous compound responsible for the catalysis of Mn (II) oxidation is co-precipitated proportionally to the amount of manganese dioxide produced [41]. Lastly, manganese dioxide produced during the early stationary growth phase of the microorganisms catalyzing the oxidation of Mn (II) may be partly reduced by respiring cells, thus somewhat masking part of the manganese-oxidizing activity [41].

Light microscopy showed the presence of an EPS-like capsule for *Acinetobacter* sp. strain LB1. As indicated in Fig. 1A, TEM analysis of negatively stained individual cells of *Acinetobacter* sp. strain LB1 revealed the presence of an EPS-like layer surrounding individual cells. This structure could be assisting in the oxidation of manganese (II) as it is known that extracellular polysaccharides can contribute to the bacterial oxidation of Mn (II) and their production is directly related to the growth of the bacteria [39]. Such exopolysaccharides may be found as a tightly attached capsule or a slime layer surrounding the cell or may even be released into the surrounding environment [18]. Previous studies conducted on the exopolysaccharide layer of

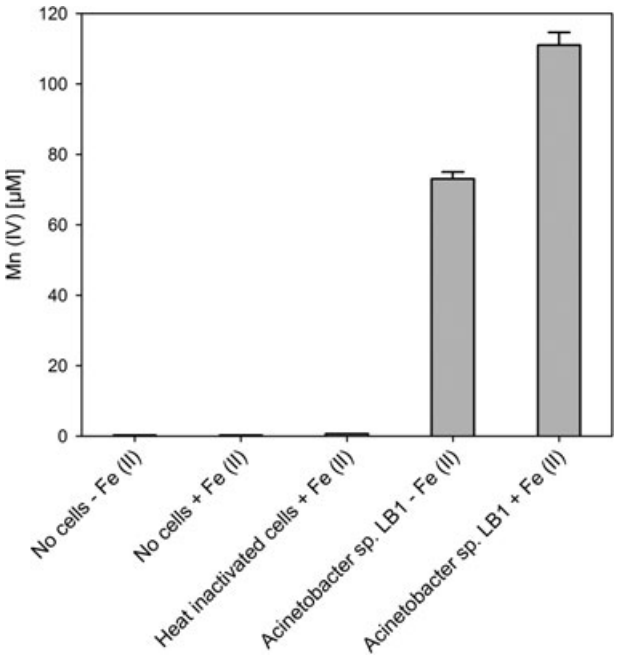

Figure 5. Oxidation of 1 mM Mn (II) after 5 days incubation in the presence and absence of 1 mg/L Fe (II) by *Acinetobacter* sp. strain LB1 in LBB medium in comparison to controls without cells in the presence and absence of 1 mg/L Fe (II) and heat-inactivated cells in the presence of 1 mg/L Fe (II). Error bars indicate the standard deviation.

A. calcoaceticus BD4 revealed a high rhamnose content of the polysaccharide layer, which was a function of the carbon source and the growth rate [18].

The association of manganese oxides with the surface of microbial cells is well known in aquatic and terrestrial habitats [36, 42] and has been shown to develop through microbe-mediated oxidation of Mn (II) both in the environment [43] and in pure cultures [44]. Binding of MnO_2 to an EPS layer could involve formation of specific manganese complexes with carbohydrate groups of EPS layers and is enhanced by an increase in pH, suggesting that surface charges and ionic attraction may also be involved [45].

3.6 Biofilm formation by *Acinetobacter* sp. strain LB1

The production of extracellular polymers enabling adhesion to surfaces and subsequently leading to biofilm formation is a feature protecting bacterial cells from gradual desiccation and is frequently encountered in water treatment systems in the presence of metals [46, 47]. In metal-rich environments, the biofilm can act as a chemical buffer at the cell's surface, immobilizing metals and thus protecting against metal accumulation within the cell to toxic levels [46].

Under conditions supporting biofilm formation, the isolated manganese-oxidizing strain *Acinetobacter* sp. LB1 has the ability to form a biofilm, apparently to a greater capacity than a species from the well-known iron and manganese-oxidizing genus *Leptothrix* (Fig. 6). Similar to studies reporting the ability of

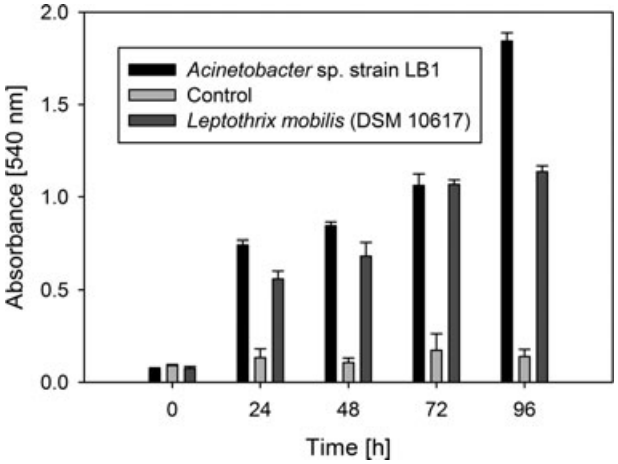

Figure 6. Biofilm-formation capacity of *Acinetobacter* sp. strain LB1 in comparison to the manganese (II) oxidizing strain *L. mobilis* (DSM 10617) and noninoculated controls. The data shown are the means obtained from measurements done in triplicate. Error bars indicate the standard deviation.

L. discophora biofilms to remove manganese [48], the ability to form a biofilm indicates that the Mn (II) oxidizing strain *Acinetobacter* sp. LB1 can assist in the biotechnological removal of manganese from the treated borehole water by being able to attach to the biofilter matrix. The metals present can attach to the surface of the biofilm and are subsequently removed from the water via precipitation reactions.

4 Concluding remarks

A Gram-negative bacterial strain isolated from a biofiltration plant treating borehole water in KwaZulu-Natal was able to oxidize Mn (II). This was demonstrated via detection of a decreasing pH and concomitantly increasing ORP in MSVP medium containing 1 mM manganese sulfate in the presence of *Acinetobacter* sp. LB1 and confirmed with EDX analysis of crystals formed therein. Employing the leucoberbelin blue dye assay confirmed that the bacterial oxidation of Mn (II) was more important in quantitative terms than purely chemical oxidation. To the best of our knowledge, this is the first report demonstrating the isolation and characterization of a Mn (II) oxidizing species of the genus *Acinetobacter* from a biofiltration system in South Africa. This study highlights the potential role of *Acinetobacter* species for the biotechnological elimination of Mn (II) and is an additional example demonstrating the important role of heterotrophic proteobacterial species in the biogeochemical cycling of manganese.

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The authors have declared no conflict of interest.

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Supplemental figures

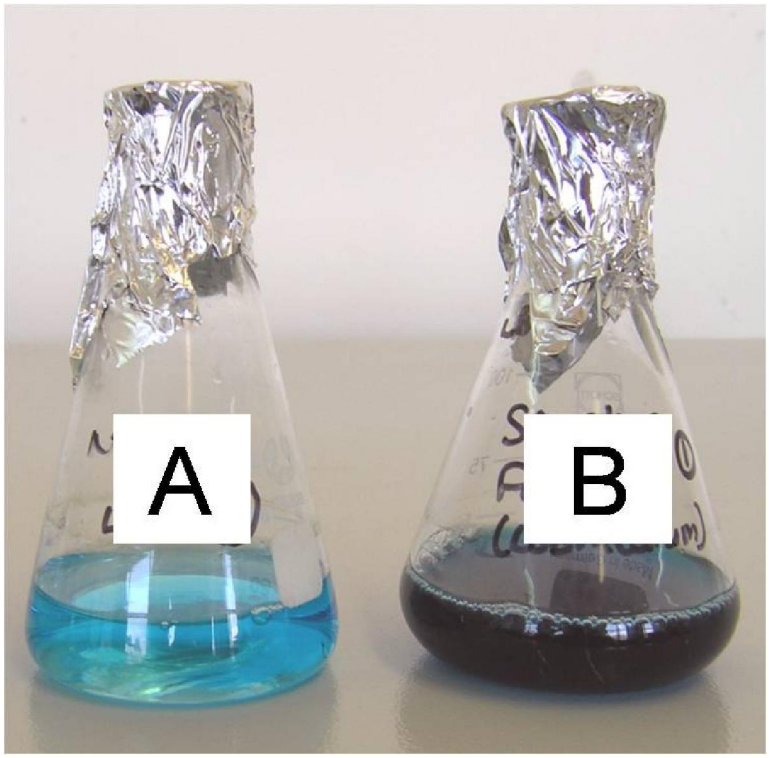

Figure S1: Qualitative detection of Mn (II) oxidation by the manganese (II) oxidizing isolate *Acinetobacter* sp. strain LB1 using leucoberbelin blue.

[A] Non-inoculated LBB medium with 1 mM manganese sulfate after 2 weeks incubation.

[B] LBB medium with 1 mM manganese sulfate inoculated with *Acinetobacter* sp. strain LB1 after 2 weeks incubation.

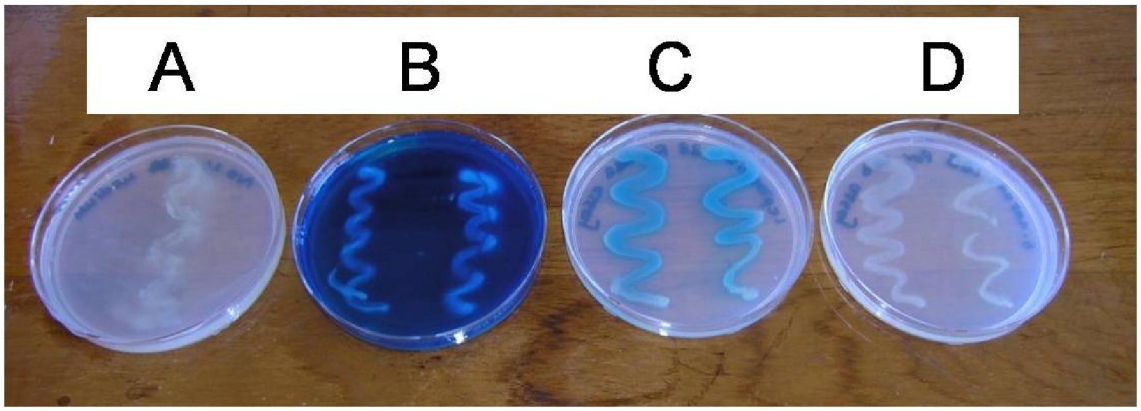

Figure S2: Qualitative detection of Mn (II) oxidation by the manganese (II) oxidizing isolate *Acinetobacter* sp. strain LB1 using leucoberbelin blue.

[A] LBB agar without 1 mM manganese sulfate inoculated with *Acinetobacter* sp. strain LB1 after 2 weeks incubation.

[B] LBB agar with 1 mM manganese sulfate inoculated with *Acinetobacter* sp. strain LB1 after 2 weeks incubation.

[C] LBB agar with 1 mM manganese sulfate inoculated with *Leptothrix mobilis* (DSM 10617) after 2 weeks incubation.

[D] LBB agar with 1 mM manganese sulfate inoculated with *E. coli* (ATCC 8739) after 2 weeks incubation.

Chapter 3 – Appendix

MALDI-TOF MS analysis of *Acinetobacter* sp. LB1

Introduction

In addition to 16S rRNA gene sequence analysis and biochemical tests, *Acinetobacter* sp. LB1 was further characterized by MALDI-TOF MS (matrix assisted laser desorption ionization time of flight mass spectrometry) analysis of whole cells and cell extracts using a Bruker Microflex MALDI-TOF MS bench top system (Bruker Daltonics, Bremen, Germany). The proteobacterial genus *Acinetobacter* contains Gram-negative, strictly aerobic, oxidase negative, nonmotile coccobacilli. Members of the genus *Acinetobacter* are widely distributed in both clinical and environmental settings and are currently comprised of 33 genomic species (Dijkshoorn *et al*., 2007; Nemec *et al*., 2010). MALDI-TOF MS has the capacity for high throughput identification of microorganisms in both clinical (Carbonnelle *et al*., 2011) and environmental settings (Ruelle *et al*., 2004). This method has previously been reported to enable an accurate, quick and cost-effective identification of bacterial isolates for example *Acinetobacter baumannii* as well as species from other genera (Böhme *et al*., 2010; Šedo *et al*., 2011; Krishnamurthy and Ross, 1996). In clinical settings MALDI-TOF MS of whole cells and cell extracts identified over 84% of isolates correctly at species level and over 95% of isolates correctly at genus level (Degand *et al*., 2008; Eigner *et al*., 2009). MALDI-TOF MS was first employed for the identification of small ribosomal proteins released from bacterial cells (Anhalt and Fenselau, 1975). During MALDI-TOF MS analysis, these target proteins (either released via laser induced lysis of the bacterial cells or by being present in the form of cell extracts) become ionized and each protein or protein fragment gains a unique m/z value thereby providing a specific fingerprint for the bacterium under investigation (Koubek *et al*., 2012; Ryzhov and Fenselau, 2001).

Materials and methods

Bacterial cultivation

The isolate was cultivated in LBB medium as initially specified by Krumbein and Altmann (Krumbein and Altmann, 1973), modified as reported by Beukes and Schmidt (2012). A 100 µl sample from an overnight culture of *Acinetobacter* sp. LB1 grown in LBB medium (late logarithmic growth phase) was spread plated onto LBB agar followed by incubation for 24 h at 25°C to obtain single colonies.

Sample preparation

A single colony of *Acinetobacter* sp. LB1 was selected and further processed for whole cell analysis or analysis of extracts according to standard protocols (Freiwald and Sauer, 2009; Maier and Kostrzewa, 2007). For whole cell analysis, a colony of *Acinetobacter* sp. LB1 grown for 24h was spotted at 2 positions on the MALDI-TOF MS analysis plate and allowed to air dry. *E. coli* (ATCC 8739, using a colony from an overnight nutrient agar plate grown at 37°C) was analysed in parallel. Thereafter 1 µl of saturated α- cyano-4-hydroxy-cinnamic acid (HCCA) matrix solution was applied to each spot and air dried before analysis. For cell extract analysis, the bacterial cells were initially suspended in 300 µl of sterile distilled water, mixed thoroughly, and then treated with 900 µl of 75% ethanol. The mixture was then centrifuged and the pellet obtained was thoroughly dried to remove all ethanol. Thereafter, 50 µl of 70% formic acid was added to the pellet which was vortexed at 2000 rpm for 1 min to disrupt the cells. An equal volume of acetonitrile was then added to the bacterial/formic acid solution, centrifuged (2 min, 14000 g) and 1 µl of the supernatant was spotted on 10 target positions on the MALDI-TOF MS analysis plate; the spots were allowed to dry at room temperature. Thereafter each spot was overlain with 1 µl saturated α-cyano-4-hydroxy-cinnamic acid (HCCA) matrix solution (Bruker) and allowed to air dry. Measurements were done at 3 times per spot to generate a sum spectrum using cell extracts.

MALDI-TOF MS analysis

Ionization was done using a 337 nm nitrogen laser and cell extract spectra were established as the average of 300 laser shots (30 shots, 10 spot positions) in positive linear mode at 60.0 Hz, analysed in the mass range from 2 to 20 kDa. Analysis and processing of the spectrum was done using the Bruker software (FlexControl, V.2.4 and Biotyper V. 3). Identification was done using the Bruker Biotyper 3.0 Reference Library 1.0 (V. 3.1.2, 2011, 3395 entries). The system was calibrated using *E. coli* DH5α as a bacterial standard (Bruker Daltonics). MALDI-TOF MS was also used to establish main spectra projections (MSP) of *Acinetobacter* strain LB1 protein extracts as described by Barbuddhe *et al*. (2008). The spectrum was created using mMass Version 5.3 (Strohalm *et al*., 2010).

Results and discussion

Using whole cells, *Acinetobacter* sp. LB1 was successfully identified to genus level, with the best match obtained for *Acinetobacter* genomospecies_3 [Tab. 1] with a score value of 2.02. This score value reveals a secure genus and possible species identification [Tab. 2]. The result obtained was in agreement with results obtained by Beukes and Schmidt (2012) for 16S rRNA gene sequence analysis and analysis of the isolate using a commercial test system (Vitek 2). *E. coli* (ATCC 8739) was used as a control for the MALDI-TOF MS system and was correctly identified to species level [Tab. 1].

Table 1. Score values generated on the Bruker Microflex MALDI-TOF MS bench top system for *Acinetobacter* sp. LB1 and *E. coli* (control) using whole cells in comparison to the best matched organism in the database.

Sample Name (score symbol)	Best Match	Score Value
F1 (++)	*Acinetobacter* genomospecies_3	2.02
F2 (+++)	*Escherichia coli* (ATCC 8739, control)	2.324

Table 2. Description of the score ranges generated on the Bruker Microflex MALDI-TOF MS bench top system.

Score Range	Description	Symbols
2.300 - 3.000	Highly possible species identification	(+++)
2.000 - 2.299	Secure genus identification, possible species identification	(++)
1.700 - 1.999	Possible genus identification	(+)
0.000 - 1.699	Not reliable identification	(-)

Table 3. Main spectra projections for cell extracts of *Acinetobacter* sp. LB1 in relation to other species within the same genus from the literature*

Organism	Genus specific signature peak masses	Probable protein
Acinetobacter sp. LB1	3718, 5177	unknown/50S ribosomal protein L34
Acinetobacter baumannii ATCC 15308	5172	50S ribosomal protein L34
Acinetobacter beijerinckii NIPH 2111	3724, 5176	unknown /50S ribosomal protein L34
Acinetobacter beijerinckii NIPH 838	3725, 5176	unknown /50S ribosomal protein L34
Acinetobacter haemolyticus NIPH 510	3725, 5176	unknown /50S ribosomal protein L34

*Peak masses for *Acinetobacter beijerinckii* NIPH 2111, *Acinetobacter beijerinckii* NIPH 838 and *Acinetobacter haemolyticus* NIPH 510 were obtained from Šedo *et al*. (2011) and data for *Acinetobacter baumannii* ATCC 15308 was obtained from Böhme *et al*. (2010).

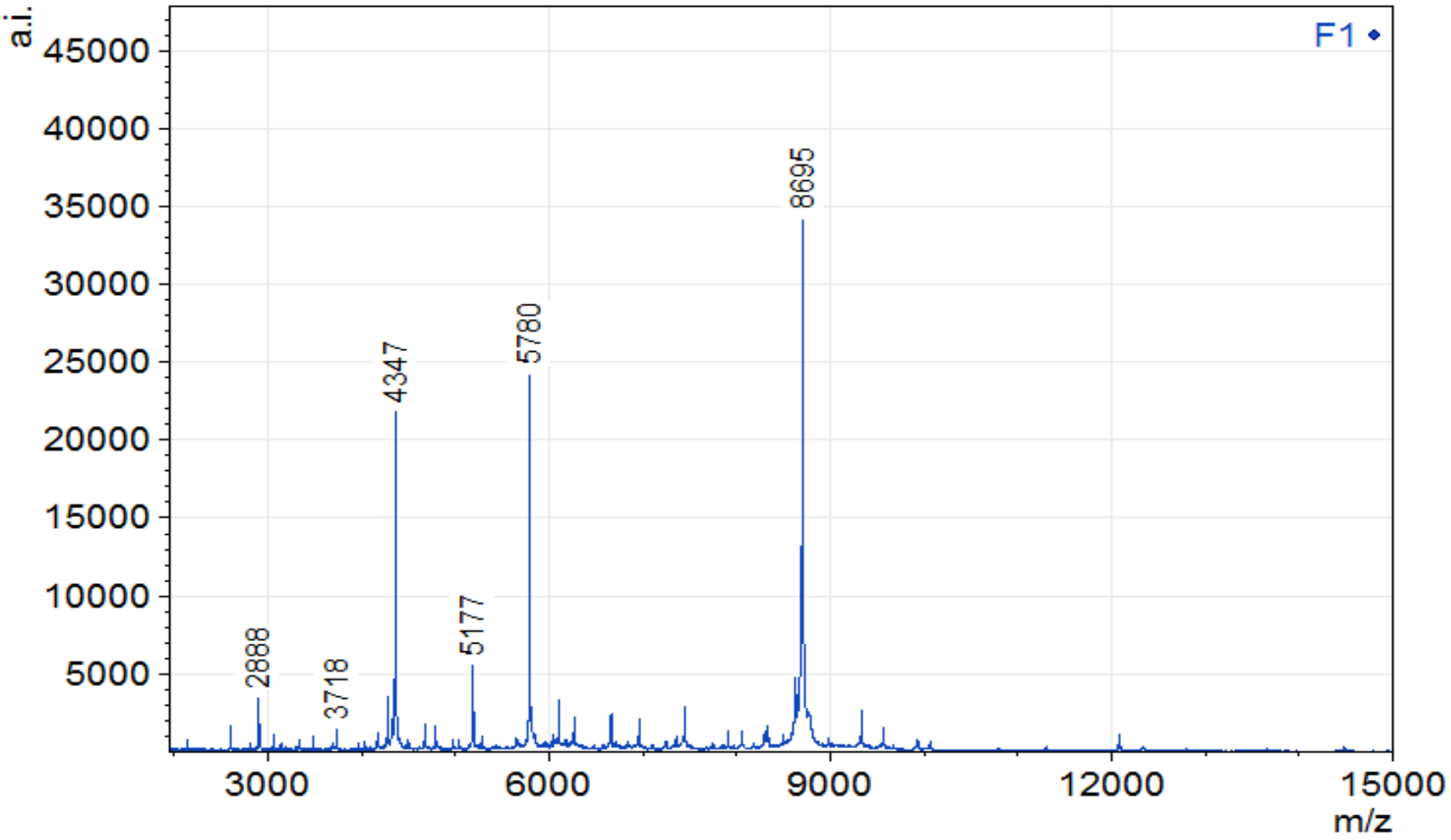

Figure 1: MALDI-TOF MS profile for cell extracts of *Acinetobacter* sp. LB1 with selected m/z peak values

The MS profile obtained for cell extracts of *Acinetobacter* sp. LB1 (Figure 1) was compared to published MS profiles for species from the same genus (Šedo *et al*., 2011; Böhme *et al*., 2010). A peak in the m/z range of 5172-5177, probably resembling the protein L34 of the large subunit of the bacterial ribosome, present in extracts of *Acinetobacter* sp. strain LB1, was common amongst three other *Acinetobacter* species (*Acinetobacter baumannii* ATCC 15308, *Acinetobacter beijerinckii* NIPH 2111, *Acinetobacter beijerinckii* NIPH 838, *Acinetobacter haemolyticus* NIPH 510) [Tab. 3]. Similarly, a peak in the range of 3718-3725 was found in *Acinetobacter* sp. LB1 and all of the above species except *Acinetobacter baumannii* ATCC 15308 [Tab. 3].

Bacterial identification using MALD-TOF MS analysis is based on the generation of bacterial protein profiles, the majority being ribosomal proteins (Ryzhov and Fenselau, 2001). In comparison to 16S rRNA gene sequence analysis and biochemical profiling such as Vitek 2, the time taken to obtain results using MALDI-TOF MS was much shorter. Analysis of proteins using MALDI-TOF MS does not require lengthy biochemical reactions thus making it a more rapid technique (Carbonnelle *et al*., 2011). Given that pure culture material is available, the entire analysis procedure using MALDI-TOF can take 30 minutes from the time of sample/smear preparation to the final results (He *et al*., 2010). Conventional biochemical tests can take up to 48 h for identification, depending on the system employed for the analysis and the bacterial species analysed (Klein *et al*., 2012). Biochemical tests are limited in that two microorganisms belonging to the same genus can have fairly different biochemical profiles (Koubek *et al*., 2012). In a study conducted by Klein *et al*. (2012), MALDI-TOF MS correctly identified 31% more Gram-negative bacterial isolates than a separator gel tube-based method typically employed in blood culture diagnostics. However, both methods fell short in identifying clinical isolates of Gram-positive grape-like clustered cocci. In another study, the Bruker Biotyper correctly identified 33% more Gram-negative *bacilli* to the genus level and 22% more to species level than a conventional biochemical test system (Saffert *et al*., 2011).

16S rRNA gene sequence analysis has been established as a reliable and accurate technique for the identification of bacterial isolates although it does have disadvantages which include a high price for reagents, a somewhat longer analysis period and that it is a labour - intensive process (Koubek *et al*., 2012). The MALDI-TOF mass spectrometry also has limitations in that spectra obtained might contain a small number of peaks, thereby not allowing proper

taxonomic identification or grouping according to spectral similarities. This problem can be attributed to various experimental factors as well as to the components of the bacteria (e.g., composition of the cell wall) (Koubek *et al*., 2012). Another limitation to MALDI-TOF MS analysis is that identifications of microorganisms are limited to the spectral profiles available in a compiled database generated by the MALDI-TOF system used. However, optimization of the sampling procedure and sample preparation, can allow the identification of bacteria to the strain level using MALDI-TOF MS analysis given a reliable spectral database is available (Arnold and Reilly, 1998; Vargha *et al*., 2006; Grosse-Herrenthey *et al*., 2008).

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Chapter 4

Do microorganisms enhance Fe (II) oxidation in the Nottingham biofiltration system at circumneutral pH?

The potential contribution of microorganisms to the oxidative removal of Fe (II) in a biofiltration system at neutral pH was determined by measuring the difference in Fe (II) oxidation rates in the presence and absence of selected metabolically active microorganisms at neutral pH, with controls run at acidic pH and alkaline pH, in the presence of the biocide formaldehyde and in the presence of heat inactivated bacterial cells. Two strains, *Acinetobacter* sp. LB1 and *Burkholderia* sp. strain LB2, isolated from the biofiltration system and able to oxidize Fe (II) were used to evaluate the potential contribution of microorganisms to Fe (II) oxidation. Initially, Fe (II) toxicity tests were carried out to determine the concentration of Fe (II) inhibiting the growth of two microorganisms, *Acinetobacter* sp. LB1 and *E.coli* (ATCC 8739). A nominal concentration of Fe (II) between 2 and 4 mg/L inhibited the growth of both microorganisms. The ferrozine colorimetric Fe (II) assay was employed in a test comparing the biological and abiotic Fe (II) oxidation rates using saline solution and in batch tests using borehole water. The comparative test employing saline solution confirmed that the rate of Fe (II) oxidation in the presence of active microorganisms at neutral pH was not very different to that in the abiotic controls. Surprisingly, the rate of Fe (II) oxidation in the heat inactivated control took place at a faster rate than in all other flasks. In the batch tests employing borehole water at neutral pH, Fe (II) oxidation took place at a faster rate in flasks containing *Acinetobacter* sp. LB1 than in flasks containing the reference Fe (II) oxidizing strains and EDX (energy dispersive X-ray analysis) analysis of crystals formed in this flask further verified the ability of this strain to oxidize Fe (II). As expected, at acidic pH, very little oxidation of Fe (II) took place whilst at alkaline pH a large amount of Fe (II) was removed from the flasks after 60 minutes. These results demonstrate that the presence of metabolically active microorganisms does not per se enhance Fe (II) oxidation at neutral pH under fully aerobic conditions.

Keywords: Fe (II) oxidation, biofiltration system, abiotic and biological, *Acinetobacter* sp. LB1, Fe (II) toxicity, batch tests, ferrozine colorimetric Fe (II) assay, EDX analysis

Abbreviations: EDX (energy dispersive X-ray analysis), EC_{50} (half maximal effective concentration), ESEM (environmental scanning electron microscopy)

1. Introduction

Iron often occurs in the same environment as manganese and is one of the most widely distributed metals in the earth's crust and (Katsoyiannis and Zouboulis, 2004). At circumneutral pH iron exists primarily in the form of insoluble, solid-phase minerals in the divalent [Fe (II)] or trivalent [Fe (III)] oxidation states (Cornell and Schwertmann, 2003). Fe (III) is more soluble at low pH (Stumm and Morgan, 1996) and at a pH below 4, even in the presence of oxygen, Fe (III) exists as an aqueous species (Weber *et al*., 2006). Iron is an essential cofactor for bacteria but is also responsible for oxidative stress, as it generates reactive oxygen species (ROS) such as superoxide ions (O_2^-), hydrogen peroxide (H_2O_2) and the destructive hydroxyl radical ($^{\bullet}OH$), via the Fenton reaction (1). In order to prevent oxidative stress, bacteria must control the uptake and storage of iron so as to avoid the buildup of reactive oxygen species (Cornelis *et al*., 2011).

$$\begin{aligned} Fe^{3+} + O_2^- &\longrightarrow Fe^{2+} + O_2 \\ Fe^{2+} + H_2O_2 &\longrightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \\ \hline O_2^- + H_2O_2 &\longrightarrow {}^{\bullet}OH + OH^- + O_2 \end{aligned} \qquad (1)$$

Reactive oxygen species can cause damage to Fe-S clusters, induce protein carbonylation, bring about Cys/Met-residue oxidation and membrane lipid peroxidation and cause DNA damage in bacterial cells (Imlay and Linn, 1988; Winterbourn, 1995). High concentrations of Fe (II) are not desired in drinking water as the excess accumulation of this metal can result in or contribute to the development of diseases such as hemochromatosis, thalassemia and/or chronic liver disease (Beaton and Adams 2007; Kohgo *et al*., 2008). The biological process used to remove iron from water involves the use of naturally or genetically engineered microbes which oxidize Fe (II) to Fe (III), producing an insoluble product that can easily be removed (Haferburg and Kothe, 2007). Abiotic oxidation involves the chemical oxidation of Fe (II) and/or the surface-mediated oxidation on abiogenic iron oxides (Rentz *et al*., 2007).

The efficiency of abiotic oxidation depends on the physical and chemical properties of the water being treated (Søgaard *et al*., 2000). These properties have to be adjusted in order for the process to function optimally and is facilitated by the reaction with oxidized manganese [Mn (IV)] or by the diffusion of aqueous Fe (II) into an aerobic environment where it reacts with molecular oxygen (O_2) (Weber *et al*., 2006). At anoxic-oxic transition zones the oxygen concentrations are very low, causing the chemical oxidation of Fe (II) to proceed at a slow rate (Emerson and Moyer, 1997). It is at this stage that biological Fe (II) oxidation at neutral pH becomes more appreciable.

Biological oxidation of Fe (II) is a consequence of lithotrophic metabolism and oxidation catalyzed by biogenic iron oxides (Rentz *et al*., 2007). Biogenic iron oxides consist of bacterial cells in combination with variable amounts of hydrous ferric oxide minerals, which form as a result of chemical or bacterial Fe (II) oxidation, resulting in Fe (III) (Hansel and Lentini, 2011; Ferris, 2005). Fe (III) undergoes hydrolysis to precipitate in the form of iron hydroxides in association with bacterial cells, allowing bacteria to compete kinetically by exploiting steep redox gradients (Hansel and Lentini, 2011; Ferris, 2005). Due to the very reactive surface properties of these oxides, they are considered good sorbents of metal ions (Ferris, 2005; Ferris *et al*., 1999). At circumneutral pH, chemical Fe (II) oxidation is rapid (Liang *et al*., 1993) but in acidic environments were chemical Fe (II) oxidation is slow; bacteria such as *Acidithiobacillus* or their archael, thermophilic counterparts such as *Ferroplasma* spp., accelerate the reaction (Ferris, 2005; Hedrich *et al*., 2011). The aerobic microbial oxidation of Fe (II) has been recognized for many years and the detection of anaerobic Fe (II) bio-oxidation has closed a gap in the iron redox cycle (Widdel *et al*., 1993).

Some of the common bacteria found in iron containing aquatic environments at neutral pH are *Leptothrix ochracea*, *Gallionella* spp., members of the *Sphaerotilus/Leptothrix* group and members of the *Siderocapsaceae* group. Fe (II) oxidizing bacteria can be beneficial in the biological removal of Fe (II) in engineered systems that treat water used for human consumption (Rentz *et al*., 2007). The role that these bacteria play in the oxidation of Fe (II) at circumneutral pH has been enigmatic for both geochemical and microbiological reasons (Emerson and Moyer, 1997). The negative net charge of the bacterial cell envelope enables them to accumulate metal cations from the environment (Haferburg and Kothe, 2007). Whilst iron bacteria are beneficial for the removal of Fe (II), they are also problematic. Through

their oxidation activities they corrode metal surfaces, particularly carbon steel (Hamilton, 2003). These bacteria consume oxygen creating microaerobic/anaerobic niches which are colonized by sulfate-reducing bacteria that are actively involved in metal-surface corrosion (Hamilton, 2003). In addition these bacteria discolour the water through the release of iron from within the corrosion scales and also contribute to the odour that accompanies the discoloured water (Hamilton, 2003). The objective of this study was to determine if microorganisms like the previously isolated *Acinetobacter* sp. LB1 (Beukes and Schmidt, 2012) are able to enhance the oxidation of Fe (II) under fully aerobic conditions at neutral pH like in the case of Mn (II) oxidation.

2. Materials and methods

2.1 Bacterial strains used for Fe (II) oxidation tests

Acinetobacter sp. LB1 and an isolated strain of *Burkholderia* sp. strain LB2 (identity confirmed via API20 NE analysis (see supplementary data) were previously isolated from a biofiltration system treating borehole water and routinely maintained and cultivated in MSVP medium (minimal salts medium containing vitamins and pyruvate) as reported by Beukes and Schmidt (2012). *Leptothrix mobilis* (DSM 10617) was grown and maintained in a medium suggested by Spring *et al*. (1996) and *Sphaerotilus natans* (DSM 565) (Stokes, 1954) was grown and maintained using beef extract agar as suggested by the supplier's instructions. *Sphaerotilus natans* was inoculated into tap water covered beef extract agar slants and incubated between 20-25°C for at least 48 hours before further use in experiments. Stock cultures of the aforementioned strains were kept at -80°C in their respective culture media in the presence of 20% glycerol.

2.2 Fe (II) toxicity tests

The impact of varying concentrations of Fe (II) on the growth of two selected proteobacterial strains was used to establish the minimum Fe (II) concentration potentially inhibiting microbial growth in the biofiltration system. *E. coli* (ATCC 8739) was used as a well-known non-iron oxidizing reference strain representing the proteobacterial group of microorganisms.

Acinetobacter sp. LB1 was used to determine the toxicity of Fe (II) on an organism isolated from the biofiltration system. The assay was carried out in LBB (Leucoberbelin blue) medium suggested by Krumbein and Altmann (1973) and modified according to Beukes and Schmidt (2012). The nominal concentrations of Fe (II) used were: 0, 2, 4, 6, 8 and 10 mg/L. Erlenmeyer flasks (100 mL) containing 30 mL of the above medium were inoculated with 3×10^5 cells (pregrown in LBB medium) per mL and incubated in a shaker incubator (MRC orbital shaker incubator, Israel) at 25°C and 150 rpm. To monitor cell growth, 1 mL samples from each flask were sampled at 2hr intervals and the density of biomass formed was measured at OD_{600}. One milliliter of sterile LBB medium was used as a blank.

2.3 Ferrozine based Fe (II) quantification

A ferrozine assay suggested by To *et al*. (1999) was used to test for the presence of Fe (II) and total iron within samples. The assay was carried out in 25 mL volumetric flasks. Reagents used for the assay were as follows: ferrozine [4.9 mM - stock solution in distilled water], Fe (II) supplied in the form of ammonium ferrous sulfate [100 mg/L in acidified (6M HCl, pH 2) distilled water for the standard samples - stock solution], hydroxylamine hydrochloride [10% w/v - stock solution in distilled water] and ammonium acetate buffer (pH 7-7.5) [30% ammonium hydroxide - 467 mL, glacial acetic acid - 230 mL, made up to 1L with distilled water - stock solution]. The presence of Fe (II) was detected by a violet color resulting from the interaction of Fe (II) ions and ferrozine. Two standard curves were prepared, one employing hydroxylamine hydrochloride and the other without. The addition of hydroxylamine hydrochloride served to reduce Fe (III) to Fe (II), in order to measure the total iron in the system. Fe (III) values were obtained by subtracting Fe (II) from the total iron. For the standard curve, standard Fe (II) samples were prepared from the ammonium ferrous sulfate stock solution as follows: 0, 2, 4, 6, 8 and 10 mg/L Fe (II).

Analysis of experimental and standard samples for Fe (II) without the addition of hydroxylamine hydrochloride included an initial centrifugation step (3 minutes, 25°C, 14000 g) to remove any Fe (III) formed in the flasks. Samples with hydroxylamine hydrochloride for total Fe (II) measurements were measured directly. The Fe (II) standard and experimental samples were prepared by adding 0.5 mL ferrozine reagent, 20 mL of the respective Fe (II) standard (diluted appropriately using acidified distilled water, pH 2) or experimental sample,

0.5 mL hydroxylamine hydrochloride (when measuring total iron), 1.25 mL ammonium acetate buffer and the volume was made up to 25 mL in a volumetric flask with distilled water. The samples were thoroughly mixed and incubated in the dark at room temperature for 15 minutes to allow for color development. For the spectrophotometric quantification of Fe (II) in the system, the absorbance of a 2 mL sample was measured at 562 nm using a BIO-RAD Smartspec™ Plus. A sample without the addition of iron but only 20 mL of distilled water was used as a blank.

2.4 Comparison of biological and abiotic Fe (II) oxidation rates

Acinetobacter sp. LB1 was employed for the biological Fe (II) oxidation test with the intention to determine whether this Mn (II) oxidizing isolate has the ability to oxidize Fe (II) and to determine the potential contribution of microorganisms to Fe (II) oxidation at neutral pH under aerobic conditions. Iron oxidation tests were carried out in separate sets of 100 mL Erlenmeyer flasks containing 30 mL of 0.85% saline at pH 7 with additional controls run at pH 2.42 (acidified with HCl) and at pH 8.76 (5 mM HEPES buffer). Tests were conducted in the absence of bacteria (abiotic), in the presence of resting cells (*Acinetobacter* sp. LB1) (biological), in the presence of heat inactivated bacterial cells (*Acinetobacter* sp. LB1, 121°C for 15 minutes) or bacterial cells poisoned with 3.5% v/v formaldehyde. Resting cells were prepared by growing an overnight culture of *Acinetobacter* sp. LB1 in LBB medium and then washing and resuspending the cells in 0.85% saline. The tests were conducted over a 120 minute period for the test conducted at neutral pH and for 60 minutes for the low and high pH tests. The samples were incubated in a shaker incubator at 25°C and 250 rpm (MRC orbital shaker incubator, Israel). Flasks used for the test conducted at neutral pH were spiked with 4.50 mg/L Fe (II) and 5.70 mg/L for the low and high pH tests. Flasks containing bacterial cells were inoculated with 1×10^{8} cells/mL. The ferrozine assay was employed to measure Fe (II) concentrations in samples before and after incubation, using the sample analysis procedure outlined in 2.3.

2.5 Fe (II) tests simulating biofilter conditions

This test was divided into two parts, quantitative and qualitative analysis of Fe (II) oxidation. For the qualitative analysis, flasks (500 mL) containing borehole water (100 mL) and filter sand (10 g) (manganese greensands - glauconite with manganese oxides of various Mn valence states - intended to specifically remove Mn (II), Fe (II), hydrogen sulfide, and arsenic (Casale *et al*., 2002)) were set up. The sand granules were washed three times with distilled water and autoclaved at 121°C for 15 minutes prior to experimental analysis. Both control and experimental flasks were set up. Separate sets of flasks (500 mL Erlenmeyer flasks) were set up, employing borehole water (100 mL) and filter sand, spiked with the previously isolated *Acinetobacter* sp. LB1, an additional proteobacterial isolate from the biofiltration system - *Burkholderia* sp. strain LB2 (isolated in the same manner as *Acinetobacter* sp. LB1 as specified in Beukes and Schmidt (2012)) and two known manganese and iron oxidizing reference strains, *Leptothrix mobilis* (DSM 10617) and *Sphaerotilus natans* (DSM 565). Flasks containing 100 mL of borehole water and filter sand were spiked with 1×10^3 cells/mL of the respective bacterial strains to determine whether the addition of these microorganisms had any effect on the oxidation of Fe (II) in these flasks. A control containing borehole water and filter sand with native microorganisms (7.04×10^5 cfu/mL for the iron oxidizers, iron oxidizing bacteria were quantified in a similar manner to MOB although the MSVP (minimal salts vitamins pyruvate) medium used to quantify the iron oxidizers contained 2 mg/L iron sulfate instead of manganese sulfate (Beukes and Schmidt, 2012)), without the addition of any other microorganisms was also established.

Samples were incubated in a shaker incubator (MRC orbital shaker incubator) at 25°C and 150 rpm for 7 days. As particulate matter from the filter sand affected the detection of Fe (II) in the system, only flasks without the addition of filter sand were analyzed using the ferrozine assay (2.3) to determine the amount of Fe (II) present in samples. Flasks with filter sand were therefore only used to visually demonstrate the effects of Fe (II) oxidation in the flasks, simulating conditions in the biofiltration system. The flask spiked with *Acinetobacter* sp. LB1 was checked for the presence of Fe (II) oxidation products formed over the 7-day period, using an Oxford (X-MAX) energy dispersive X-ray (EDX) detector coupled to a scanning electron microscope (Zeiss Evo LS 15, Germany). For the quantitative analysis, batch tests were conducted using freshly collected borehole water (pH 7) from the borehole water tank at

the biofiltration system to somewhat simulate the biofilter conditions. This test was used to determine the difference in Fe (II) oxidation rates in the presence of native microorganisms and in the presence of iron oxidizing reference strains. The same sets of experimental and control flasks were set up like in the qualitative analysis except only borehole water was used in the quantitative analysis.

2.6 Chemicals

Ferrozine and hydroxylamine hydrochloride were obtained from Sigma-Aldrich (South Africa). Unless otherwise stated all other chemicals used were of the highest purity commercially available.

3. Results and Discussion

3.1 Fe (II) toxicity tests

The initial concentration of Fe (II) in the borehole water used in this study - as determined by the ferrozine assay - was 5.27 mg/L and after biofiltration the value dropped to < 0.02 mg/L in the effluent. Normal concentrations of Fe (II) in this biofiltration system range between 2-8 mg/L (Personal communication, Dudu Gwebu, Collin Van Der Merwe and Peter Thompson, Umgeni Water, Durban). *E. coli* grew with a doubling time of approximately 2 hours [pH 7.0, 25°C] in flasks containing up to 2 mg/L Fe (II) while growth was completely inhibited at concentrations ≥ 4 mg/L Fe (II) [Fig. 1A]. *Acinetobacter* sp. LB1 grew with a doubling time of approximately 40 minutes [pH 7.0, 25°C] in flasks containing up to 2 mg/L Fe (II) and, similar to *E. coli*, growth was completely inhibited at concentrations ≥ 4 mg/L Fe (II) [Fig. 1B]. *Acinetobacter* sp. LB1 reached the stationary growth phase at about 10 hrs while *E. coli* reached the stationary growth phase at about 16 hrs. An EC_{50} value for inhibition of biomass formation via OD_{600} at 16 hr was determined as approximately 2.9 mg /L nominal Fe (II) for *E. coli* and for *Acinetobacter* sp. LB1.

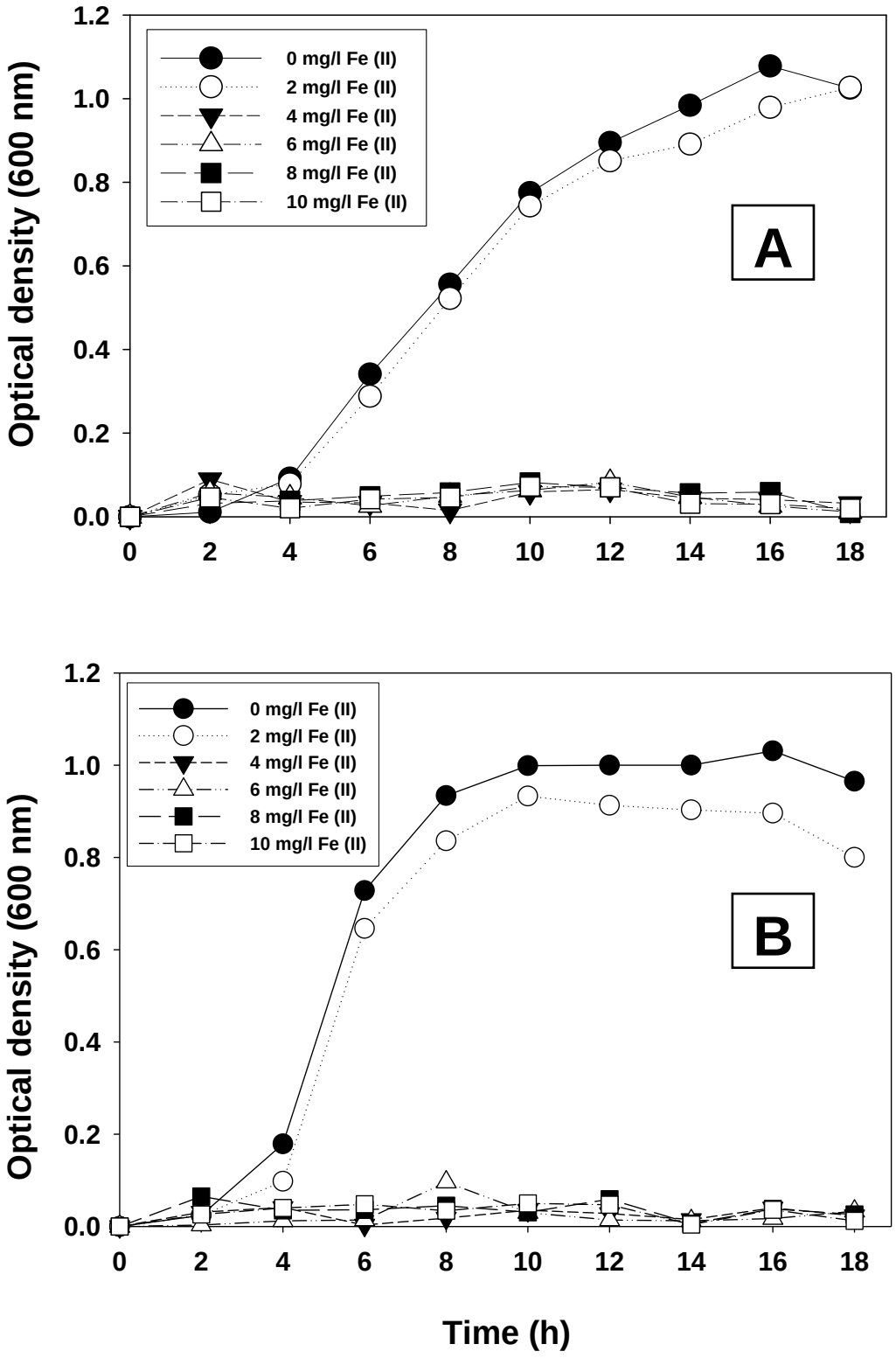

Figure 1: Growth of *E. coli* (ATCC 8739) [A] and *Acinetobacter* sp. LB1 [B] in LBB medium in the presence of 0-10 mg/L nominal Fe (II) concentrations over an 18hr period. All data shown are the average of duplicate flasks.

The complete inhibition of cell growth at concentrations ≥ 4 mg/L proved that Fe (II) becomes potentially toxic at this level. According to figure 1B, the strain isolated from the biofilter, *Acinetobacter* sp. LB1 grew much better than *E.coli* in LBB medium at 25°C with a doubling time of approximately 40 minutes at ≤ 2 mg Fe (II)/L. It must be noted that these values for toxicity were established on a small scale in the laboratory to determine the level of Fe (II) these microorganisms can tolerate. However, the bacteria in the biofiltration system are able to survive concentrations of iron exceeding 5.27 mg/L which can be attributed to the formation of biofilms containing high numbers of bacterial cells that are known to limit the toxic effect of metals in environmental systems (Trevors, 1989). In previous studies it was found that the large iron oxidizing bacteria, *Gallionella* spp. and *Leptothrix* spp. grew under static conditions in water with Fe (II) concentrations of up to 12 mg/L and growth of these bacteria was inhibited at l4 mg/L iron (Hasselbarth and Ludemann, 1972). These bacteria are able to grow at circumneutral pH but unlike *Acinetobacter* sp. LB1 and *E. coli*, they prefer environments where there are high concentrations of Fe (II) (Hanert, 1992). Whilst iron can be toxic to cells at high concentrations, it still forms a vital role in controlling and stimulating the growth of microorganisms (Emerson and Moyer, 1997).

3.2 Comparison of biological and abiotic Fe (II) oxidation rates

In order to measure Fe (II) oxidation over time the ferrozine assay was employed, without hydroxylamine hydrochloride in order to determine the amount of Fe (II) present in flasks before and after incubation. The addition of hydroxylamine hydrochloride was employed to determine the total iron concentration after incubation. Fe (II) oxidation took place in all experimental samples at neutral pH spiked with 4.50 mg/L Fe (II), however the time required to oxidize Fe (II) differed to some degree [Fig. 2A]. After 30 minutes incubation at neutral pH (7.0), approximately half of the initial Fe (II) present was oxidized in both the abiological control and resting cells flasks, thereafter oxidation took place at a slower rate [Fig. 2A]. Whilst *Acinetobacter* sp. LB1 did not clearly enhance Fe (II) removal or oxidation at neutral pH, it must be noted that iron oxidizing bacteria like *Gallionella ferruginea* (Hanert, 1992) have been frequently reported to enhance iron oxidation at neutral pH and it was also found that the sheath present on these microorganism was responsible for the enhanced iron oxidation in previous experiments (Rentz, *et al*., 2007; Katsoyiannis and Zouboulis, 2004; de Vet *et al*., 2011).

The *Sphaerotilus/Leptothrix* group of sheathed bacteria has also been previously found to oxidize iron at circumneutral pH and has been found in surface water environments in the presence of encrustations of iron (Dondero, 1975). Neubauer *et al*. (2002) found that between 70-80% of the iron oxides present in bacterial cultures isolated from a wetland-plant rhizosphere were present in the form of sheaths or stalks. The same authors found that biological oxidation accounted for 18-53% of the total iron oxidized. Katsoyiannis and Zouboulis (2004) found that 50% of the total iron removal in groundwater was due to abiotic iron oxidation. In the current study it was also determined that approximately 50% of the iron oxidized was due to abiological activity. Also, the abiotic iron oxidation rates in this study were slightly higher than in the biological control. This indicated that the abiotic oxidation of iron largely contributes to the overall oxidation of iron in these experiments

Surprisingly, almost all of the Fe (II) present was removed in the heat inactivated control after 30 minutes and thereafter the concentration of Fe (II) in the saline solution seemed to remain constant [Fig. 2A]. Also, the formation of Fe (III) was highest in the heat inactivated control, indicating that Fe (II) was not merely bound by the cells but was in fact oxidized as indicated in figure 2B. To date, only limited literature is available on the use of heat inactivated cells for Fe (II) oxidation experiments, to determine the contribution of biology to iron oxidation at circumneutral pH. Presumably, this is because of the difficulty in explaining the results obtained. In a study conducted by Ghiorse and Hirsch (1979), cells of Pedomicrobium-like budding bacteria were grown in the presence of 10 mg/L Fe (II) in the form of iron sulfate and in separate experiments these bacterial cells were incubated in the presence of manganese. To establish abiotic controls the bacterial cultures were treated with the addition of 0.05% (w/v) glutaraldehyde, 1mM $HgCl_2$, or were heat treated at 93^{o}C for 15 minutes and later incubated at 30 ^{o}C for up to 30 days (Ghiorse and Hirsch, 1979). The viability of the formaldehyde treated bacterial cells was checked after incubation and results indicated that the cells were not viable as judged by the absence of colonies formed on PYGV agar (Ghiorse and Hirsch, 1979). Results of these experiments indicated that iron removal was not inhibited by the bactericidal treatment as reported by Ghiorse and Hirsch (1979). After incubation, microscopic analysis of the cultures in these treatments revealed a dense layer of material attached to the outer surface of the bacterial cells which was found to be iron oxides and was extracted using oxalic acid (Ghiorse and Hirsch, 1979).

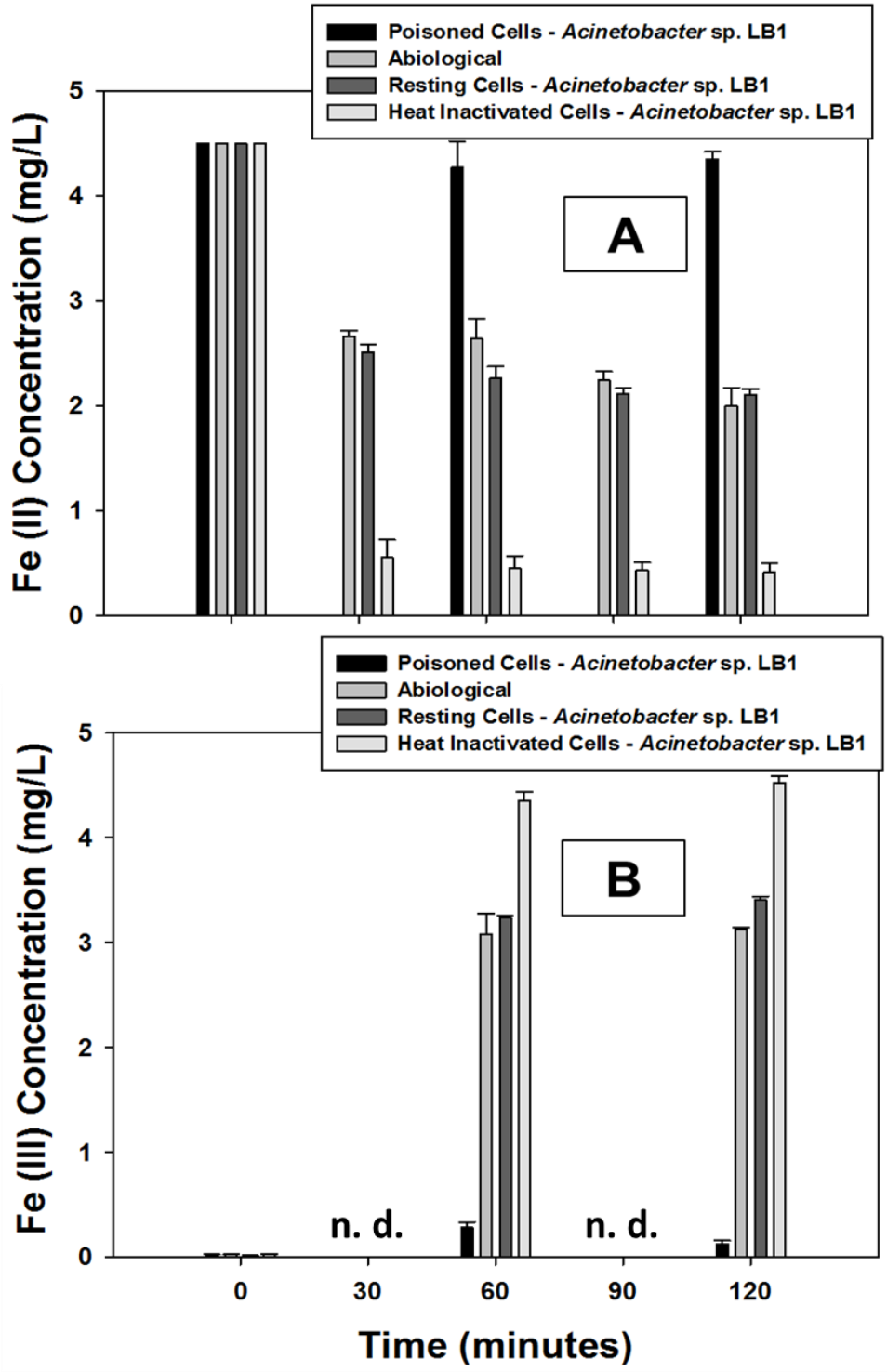

Figure 2: Fe (II) oxidation at neutral pH [A] in the presence of resting cells (*Acinetobacter* sp. LB1, 1×10^8 cells/mL), heat inactivated bacterial cells (*Acinetobacter* sp. LB1, 1×10^8 cells/mL), poisoned bacterial cells (*Acinetobacter* sp. LB1, 1×10^8 cells/mL) and in the absence of bacteria. The subsequent formation of Fe (III) is shown in [B]. Error bars indicate the standard deviation. n.d. = not determined.

After extraction of the dense material, staining of the cells with ruthenium red revealed the presence of a residual polymer layer surrounding the cells. This indicates that the polymer surrounding the cells was not completely destroyed by any of the bactericidal treatments and that it was somehow involved in the oxidation of Fe (II) (Ghiorse and Hirsch, 1979). Similarly, in this study, in the dead cell control, Fe (II) oxidation was highest as compared to the other flasks even after the cells were heat treated. This could possibly indicate that components within the EPS (extracellular polymeric substances) layer of *Acinetobacter* sp. LB1 were activated via heat treatment allowing more Fe (II) oxidation to take place at a faster rate, not forsaking the contribution of abiotic oxidation of Fe (II) in this flask. *Acinetobacter* spp. are known to contain a polysaccharide capsule which consists of L-rhamnose, D-glucose, D-glucuronic acid, and D-mannose (Kaplan *et al*., 1985), which contributes to the hydrophilic nature of the surface of these microorganisms**.**

The level of Fe (III) produced in the abiological control and in the presence of resting cells correlated with the amount of Fe (II) removed in these flasks [Fig. 2B]. In the flask containing *Acinetobacter* sp. LB1, poisoned with formaldehyde, very little oxidation of Fe (II) took place [Fig. 2A] and similarly very little Fe (III) was produced [Fig. 2B]. The use of formaldehyde to poison cells was based on the fact that this compound is an efficient biocide almost quantitatively killing heterotrophic bacteria such as *Pseudomonas fluorescens* (Whistler and Sheldon, 1989). The use of formaldehyde for poisoning cells was motivated by the study conducted by Ghiorse and Hirsch (1979) were 0.05% (w/v) glutaraldehyde was used as a bactericidal treatment and by a study conducted by Lies *et al*. (2005) were 20% formaldehyde was used to kill cells by exposure for 1 hour. However, at the same time formaldehyde is a reducing agent and caused a drop in pH in the saline to approximately 4 which could have also contributed to the reduction of Fe (III) in the saline solution.

Under aerobic conditions at neutral pH in the abiological flasks, flasks with resting cells and the heat inactivated control, Fe (II) was not merely precipitated out of the solution but oxidized as confirmed by the formation of Fe (III) over time [Fig. 2B]. Iron can exist in the divalent [Fe (II)] or trivalent [Fe (III)] oxidation states at circumneutral pH (Cornell and Schwertmann, 2003). Overall, Fe (II) oxidation in the presence of active bacterial cells seemed to only appear slightly faster than in the abiological control. This difference was not sufficient to confirm that microorganisms such as *Acinetobacter* sp. LB1 can speed up Fe (II)

oxidation at neutral pH and aerobic conditions like in the case of manganese oxidation (Beukes and Schmidt, 2012). However, it must be noted that the lab experiments using saline and *Acinetobacter* sp. LB1 do not reliably simulate conditions in the biofiltration system. There was very little Fe (II) removal in all experimental and control flasks at acidic pH (2.42) after 60 minutes incubation [Fig. 3A] and only very small quantities of Fe (III) were produced in these flasks, which confirmed that Fe (II) is very stable at acidic pH [Fig. 3B].

At acidic pH ≤ 4, even in the presence of oxygen, Fe (II) will exist in an aqueous form and the solubility of Fe (III) increases with a decrease in pH (Weber *et al*., 2006; Stumm and Morgan, 1996). At alkaline pH (8.76) almost all Fe (II) was removed from all experimental and control flasks [Fig. 4A]. However, very little Fe (III) was formed [Fig. 4B], indicating that the Fe (II) was basically removed via precipitation and apparently very little oxidation of Fe (II) took place. At acidic pH under aerobic conditions, the contribution of bacteria to the oxidation of Fe (II) was negligible. At acidic pH almost all Fe (II) is kept in solution thus preventing Fe (III) formation. It is at this pH that the presence of bacteria like *Acidithiobacillus ferrooxidans* becomes appreciable (Cullimore and Mc Cann, 1977). This bacterium obtains energy from the oxidation of inorganic sulphur and iron simultaneously (Temple and Colmer, 1951) and has been previously cultivated in a medium with a pH as low as 1.3 (Tuovinen *et al.*, 1971a). *A. ferrooxidans* also showed a high tolerance to sulphur and iron, which is an important ecological feature of this microorganism in comparison to other microbial competitors (Tuovinen *et al.*, 1971b).

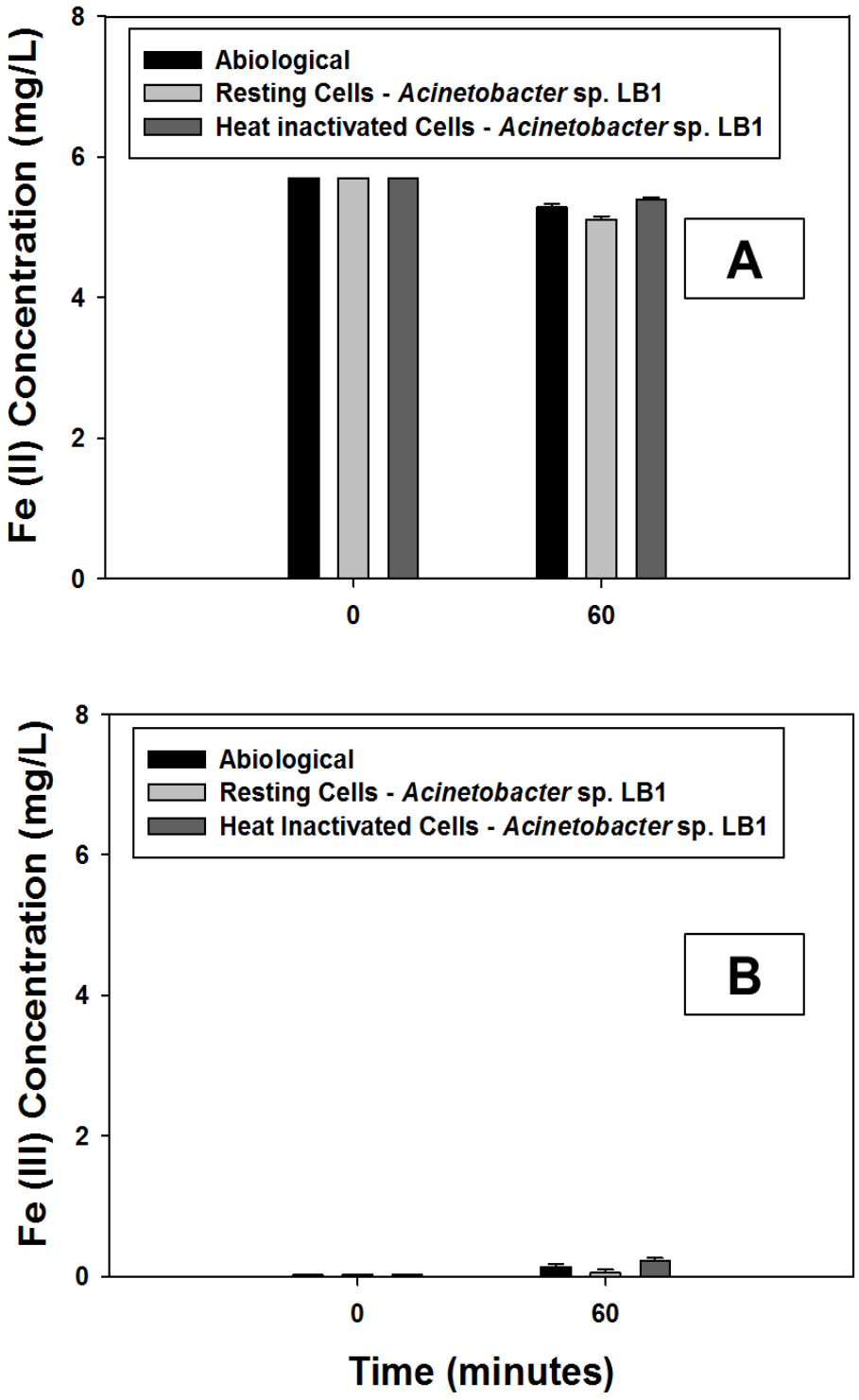

Figure 3: Fe (II) oxidation at low pH (2.42) [A] in the presence of resting cells (*Acinetobacter* sp. LB1, 1×10^8 cells/mL), heat inactivated bacterial cells (*Acinetobacter* sp. LB1, 1×10^8 cells/mL) and in the absence of bacteria. The subsequent formation of Fe (III) is shown in [B]. Error bars indicate the standard deviation.

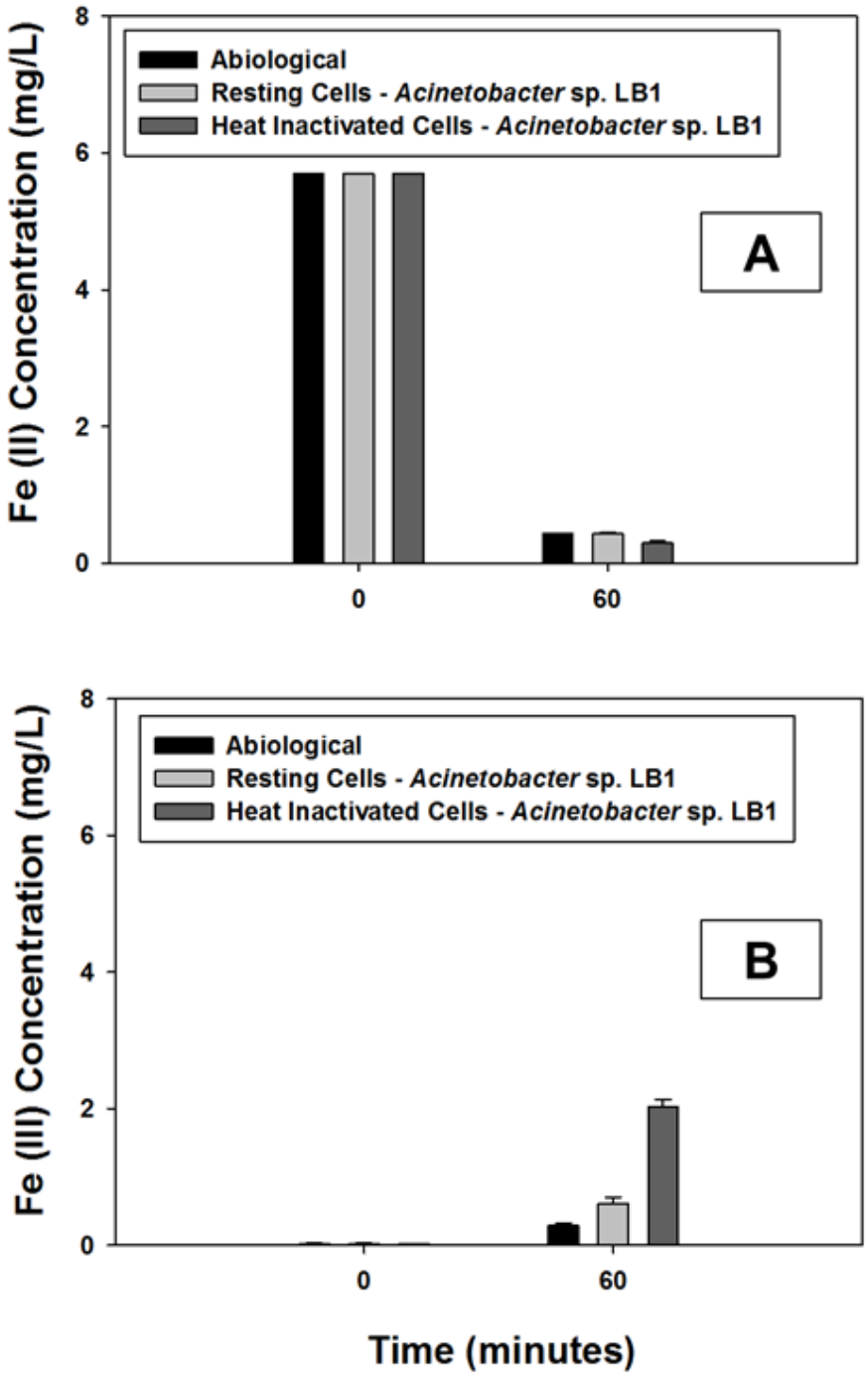

Figure 4: Fe (II) oxidation at alkaline pH (8.76) [A] in the presence of resting cells (*Acinetobacter* sp. LB1, 1×10^8 cells/mL), heat inactivated bacterial cells (*Acinetobacter* sp. LB1, 1×10^8 cells/mL) and in the absence of bacteria. The subsequent formation of Fe (III) is shown in [B]. Error bars indicate the standard deviation.

The flasks incubated at high pH contained HEPES buffer, which was used to establish a pH of 8.76. A high level of Fe (III) was expected given the rapid removal of Fe (II) after 60 minutes, however HEPES buffer is also known to chelate/bind iron and probably therefore only very little Fe (III) was formed as shown in figure 4B. Welch *et al*. (2002) measured the rate of Fe (II) removal in different buffers such as HEPES (50 mM) and phosphate buffer (50 mM), and found that with an increase in pH in the buffer, the rate of Fe (II) removal increased. The same authors also compared ultra-pure HEPES to regular grade HEPES (containing micromolar concentrations of iron, copper and other transition metals) and found that Fe (II) removal was slower in the ultra-pure HEPES as compared to the regular grade HEPES and it was also found that phosphate buffers and bicarbonate buffers were much stronger Fe (II) chelators than HEPES.

The redox potential and pH of the water being treated are the main factors that determine to which degree Fe (II) oxidation is going to take place either biologically or abiotically, with a shift to the latter being the most favourable due to an 80% reduction in operational costs (Mouchet, 1992). In experiments conducted on wetland-plant rhizospheres by Neubauer *et al*. (2002), Fe (II) oxidation in the presence of actively respiring cells accounted for 18-53% of the total Fe (II) oxidized, whilst in the presence of inactive cells (poisoned with sodium azide), the oxidation rate dropped by only 6%. This indicates that biological oxidation of Fe (II) does occur at neutral pH but at a very slow rate. The results from the study conducted by Neubauer *et al*. (2002), by Ghiorse and Hirsch (1979) and this study therefore indicate that it is not sufficient to demonstrate that the presence of microorganisms speeds up the oxidation of Fe (II) at neutral pH under aerobic conditions as demonstrated in this study in batch tests employing saline

3.3 Fe (II) batch tests simulating biofilter conditions

A. Qualitative test for iron oxidation using borehole water in the presence of filter sand

The batch culture test employing freshly collected borehole water aimed to simulate Fe (II) oxidation as possibly occurring within the biofiltration system. Four bacterial strains were employed, *Acinetobacter* sp. LB1, *Leptothrix mobilis, Sphaerotilus natans* and *Burkholderia* sp. strain LB2. All flasks before incubation were free of turbidity [Fig. 5B] and the initial Fe

(II) concentration in the borehole water used in the flasks was determined as 5.27 mg/L (pH 7 and ORP > 300 mV). The poisoned control flask served to demonstrate the removal of iron in the presence of inactive cells. Two flasks poisoned with 3.5% formaldehyde were set up, one which contained just the borehole water and formaldehyde and one which contained borehole water, formaldehyde and 10g filter sand. These flasks were incubated for seven days and thereafter checked for the presence of viable cells via streaking out samples on LBB agar. The turbidity in the flask containing filter sand and formaldehyde was not due to bacterial growth but due to particulate matter from the sand particles during incubation [Fig. 5C]. In the first set of experimental flasks used for the qualitative analysis of Fe (II) oxidation, flask A spiked with *Acinetobacter* sp. LB1 developed a black colour, flask C spiked with *Leptothrix mobilis* and flask F containing normal borehole water with no added bacteria developed an orange colour [Fig. 5A].

These colour developments in the above mentioned flasks are indicative of manganese (black colour) and iron (orange colour) oxidation (vanVeen, 1972) and are similarly present in the manganese and iron filters in the biofiltration system. The rest of the flasks did not show any colour development except for turbidity formation. The colour formation in flask A spiked with *Acinetobacter* sp. LB1 was a result of the co-precipitation of both iron and manganese as determined by EDX analysis. EDX analysis of the black precipitate formed in this flask revealed the presence of crystals which contained iron and manganese with a higher proportion being iron [Fig. 6] as would be expected given the high concentrations of Fe (II) in the borehole water. It was previously established that the strain *Acinetobacter* sp. LB1 used in flask A was capable of oxidizing Mn (II) (Beukes and Schmidt, 2012); these results indicate that the isolate is also able to contribute somewhat to the oxidation of Fe (II) at neutral pH under aerobic conditions.

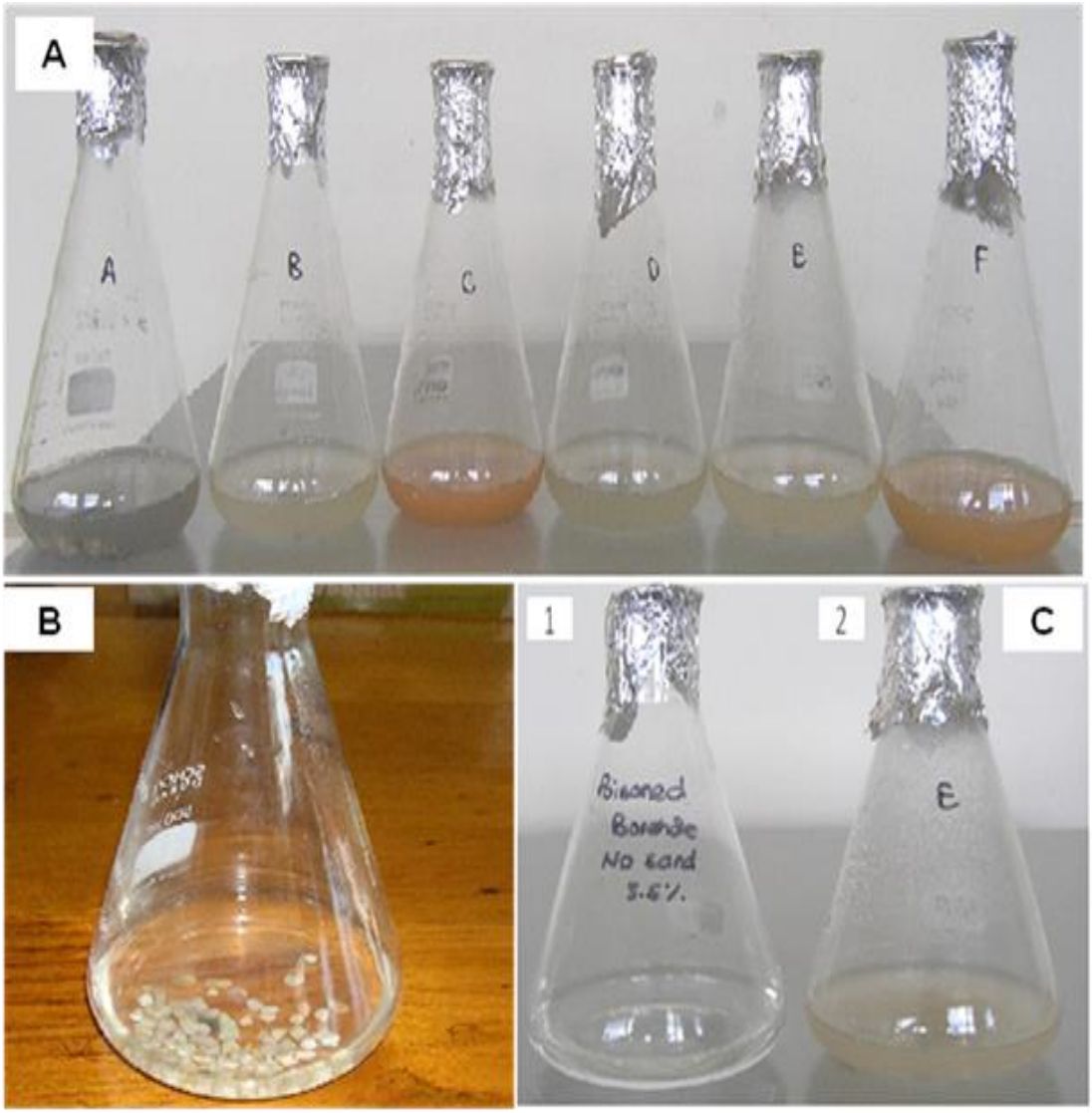

Figure 5: Fe (II) oxidation in batch culture tests before and after the 7 day incubation [A] and the contribution of the filter sand to the turbidity formed in the flask poisoned with 3.5% formaldehyde [B+C].

Constituents of flasks in image [A]:

[A] Borehole water + filter sand spiked with *Acinetobacter* sp. LB1 (1×10^3 cells/mL)

[B] Borehole water + filter sand spiked with *Burkholderia* sp. strain LB2 (1×10^3 cells/mL)

[C] Borehole water + filter sand spiked with *Leptothrix mobilis* (1×10^3 cells/mL)

[D] Borehole water + filter sand spiked with *Sphaerotilus natans* (1×10^3 cells/mL)

[E] Borehole water with native bacteria poisoned with 3.5% formaldehyde

[F] Normal borehole water with native bacteria (7.04×10^5 cfu/mL - iron oxidizers)

Flasks containing *Burkholderia* sp. strain LB2 and *Sphaerotilus natans* did not develop any distinct colour, which does not mean iron oxidation did not take place in these flasks [Fig. 5], but could have been taking place at a slower rate than in the other flasks. The other possibility for the lack of colour development could be that these two strains produce chelating agents like siderophores, which bind the metal and prevent it from being oxidized, thus the lack in the orange colour formation which should have formed due to Fe (II) oxidation. The largest class of compounds known to bind and transport Fe (II) are called siderophores (Gadd, 2010). These compounds are highly specific Fe (II) ligands which are released by a wide variety of bacteria and fungi to assist in iron absorption (Gadd, 2010). Siderophores can also complex other metals but iron is the only known essential element for which these shuttles operate because Fe (II) is needed in large amounts by cells and also because the solubility of ferric hydroxides at neutral pH is low, thus the free Fe (III) concentration is too low to support microbial growth (Gadd, 2010). Bacteria present in environments containing iron and manganese are capable of producing and releasing siderophores into the extracellular milieu to chelate these metals (Salomons and Förstner, 1984; Babich and Stotzky, 1985). *Pseudomonas fluorescens* in particular, is capable of producing siderophores to chelate metals such as Fe (II) (Dhanya and Potty, 2007). As particulate matter released from the filter sand during incubation interfered with the ferrozine assay, the quantification of Fe (II) oxidation was only employed in the second set of flasks which did not contain any filter sand. However, the flasks containing filter sand did provide a good visual demonstration of the oxidation processes occurring within the biofiltration system.

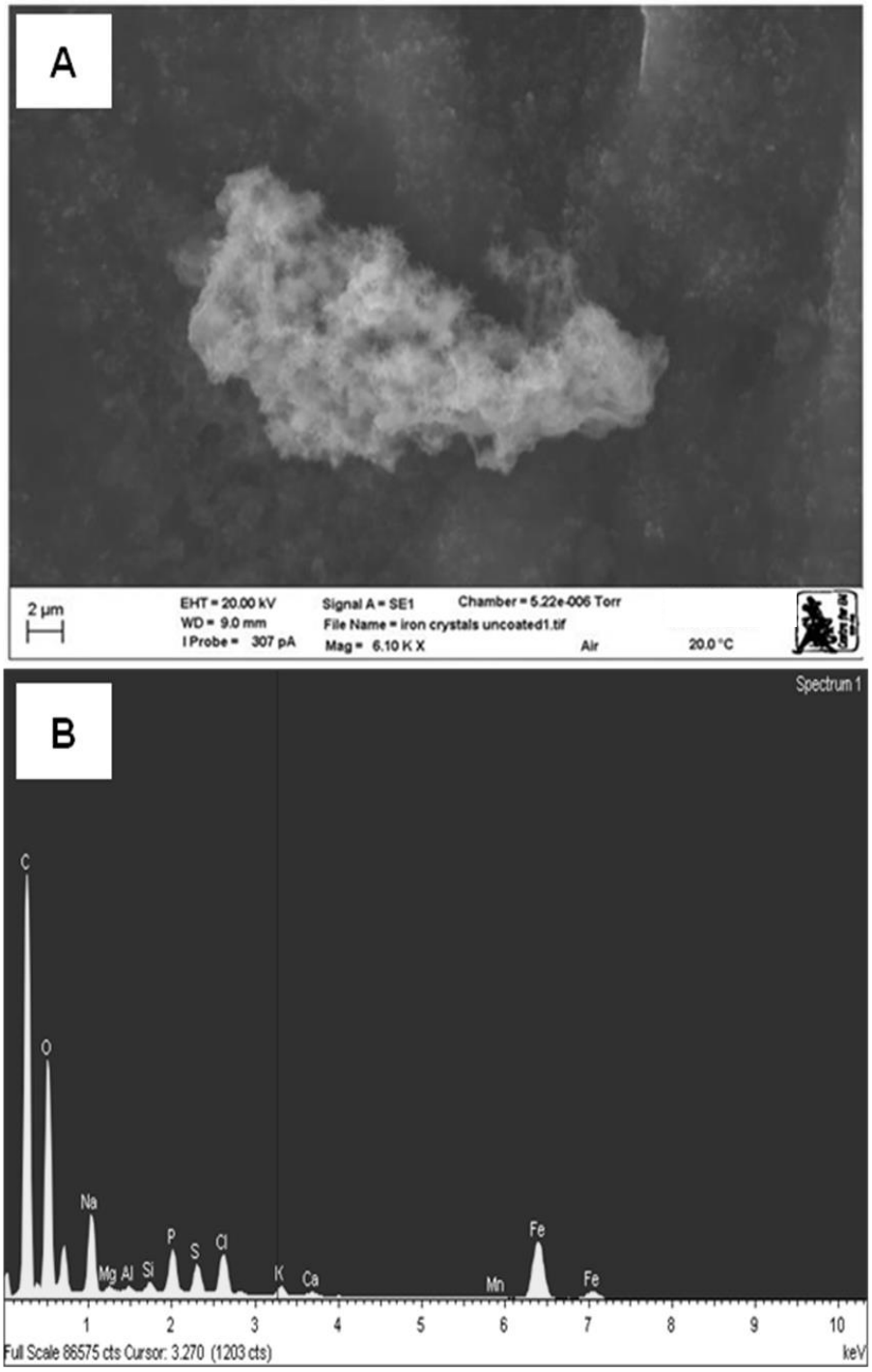

Figure 6: ESEM image [A] of crystals formed in the flask with the black precipitate in borehole water containing 5.27 mg/L Fe (II) in the presence of *Acinetobacter* sp. LB1 after 7 days incubation and the corresponding EDX spectrum of the crystals [B].

B. Quantitative test for iron oxidation using borehole water in the absence of filter sand

In the second set of flasks without filter sand, more than 97% of the Fe (II) was removed after 7 days incubation from the borehole water in the presence of *Acinetobacter* sp. LB 1, while about 90% was removed from the borehole water flasks spiked with *Leptothrix mobilis* and *Sphaerotilus natans* [Fig. 7]. More than 95% of Fe (II) was removed in flasks spiked with *Burkholderia* sp. strain LB2 and in the normal borehole water flask (with native microorganisms). Although the number of bacterial cells inoculated into these flasks was low, the addition of these microorganisms did seem to have a marked effect on the removal of Fe (II) in the borehole water [Fig. 7]. The borehole water contains trace quantities of nutrients which might support the growth of the native microorganisms, like *Burkholderia* sp. strain LB2 and *Acinetobacter* sp. LB1, whilst the other microorganisms like *Leptothrix mobilis* and *Sphaerotilus natans* which are not native to the system might struggle to grow in this environment and therefore may not be able to remove Fe (II) as efficiently as the native microorganisms. The flask containing formaldehyde showed the least amount of Fe (II) removal which verifies that actively metabolizing bacteria are necessary for the efficient removal of Fe (II). However the effect of formaldehyde on the rate of Fe (II) removal in the saline solution was not the same as in the borehole water. This is possibly due to the longer incubation period in the borehole water flask which could have also caused the pH of the medium to change, allowing more of the abiotic removal of Fe (II) to take place. The results confirm that the presence of active microorganisms is necessary for the efficient removal of Fe (II) in biofiltration systems like the system under investigation.

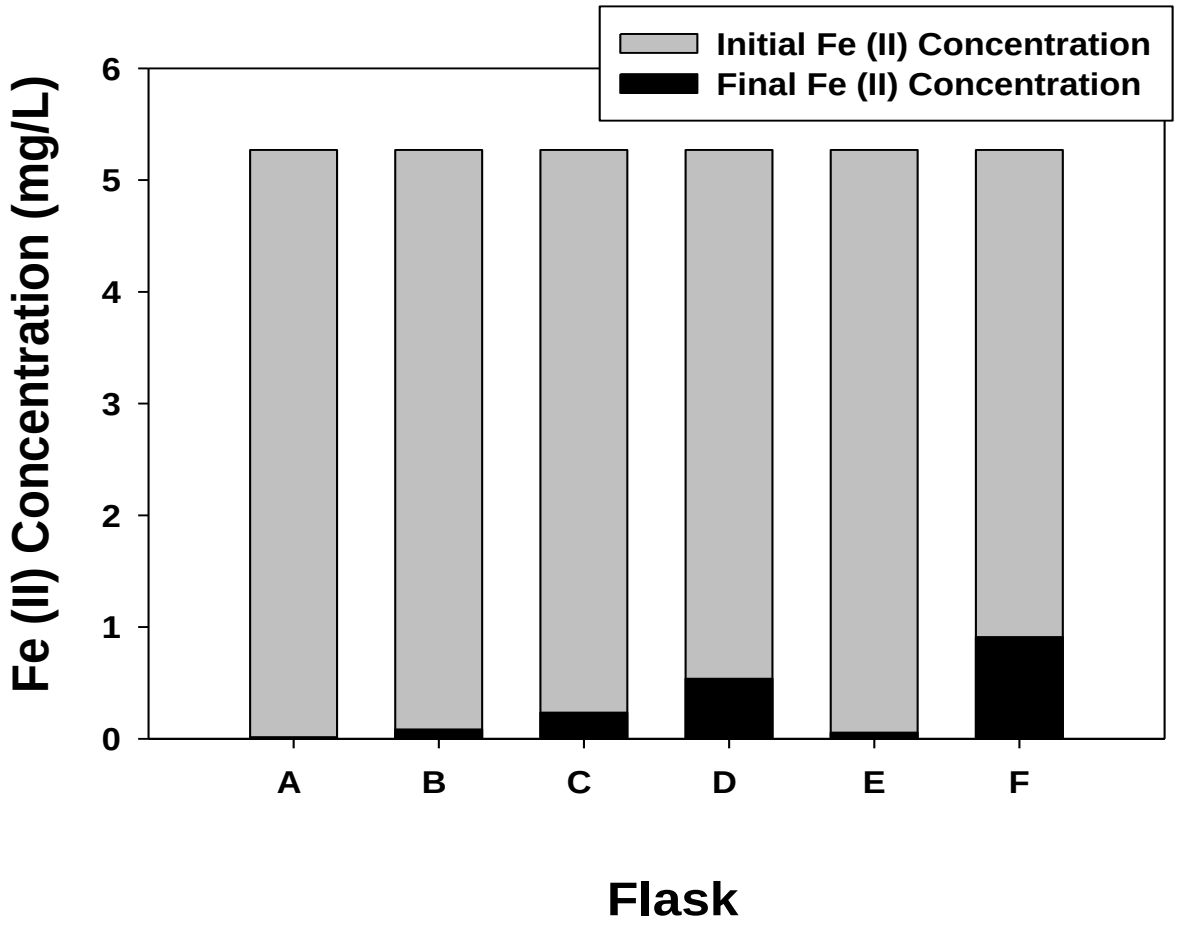

Figure 7: Initial and final Fe (II) concentrations (after a 7 day incubation period) in borehole water in batch culture flasks. All data shown are the average of duplicate flasks.

[A] Borehole water spiked with *Acinetobacter* sp. LB1 (1×10^3 cells/mL)

[B] Borehole water spiked with *Burkholderia* sp. strain LB2 (1×10^3 cells/mL)

[C] Borehole water spiked with *Leptothrix mobilis* (DSM 10617) (1×10^3 cells/mL)

[D] Borehole water spiked with *Sphaerotilus natans* (DSM 565) (1×10^3 cells/mL)

[E] Normal borehole water with native bacteria (7.04×10^5 cfu/mL - iron oxidizers)

[F] Borehole water containing native bacteria poisoned with 3.5% formaldehyde

There are a number of challenges that are faced by microorganisms that gain energy from the oxidation of iron at circumneutral pH. The first problem is the low level of energy obtained from iron oxidation at circumneutral pH (Ehrlich *et al*., 1991), secondly, under fully aerobic conditions the half-life of iron in circumneutral freshwaters is approximately 2-10 minutes depending on the pH (Stumm and Morgan, 1981) and under the above conditions, the shortest reported doubling time for a lithotrophic iron oxidizer was reported to be 8h (Emerson and Moyer, 1997). The circumneutral lithotrophic microorganisms are thus limited to microaerophilic zones because abiotic iron oxidation rates in these zones are much lower due to the lower oxygen levels (Liang *et al*., 1993**;** Stumm and Morgan, 1981). Finally the competition for Fe (II) at circumneutral pH is enhanced by the fact that iron oxides as well as the surfaces of microorganisms are able to catalyze the abiotic oxidation of Fe (II). Separating these two mechanisms in order to determine the contribution of bacterial cells to Fe (II) oxidation is therefore challenging.

Total iron oxidation includes abiotic and biological oxidation. Abiotic oxidation is comprised of the chemical oxidation of aqueous Fe (II) including the surface-mediated oxidation of Fe (II) on abiogenic iron oxides, which is also known as autocatalysis (Stumm and Morgan, 1981). The biological oxidation of Fe (II) consists of catalysis that is directly mediated by metabolism of lithotrophic Fe (II) oxidizers and surface-mediated oxidation on biogenic iron oxides (autocatalysis) (Stumm and Morgan, 1981). Another form of autocatalysis is through the passive adsorption of Fe (II) to bacterial cells (Chatellier and Fortin, 2004, Warren and Ferris, 1998) and then the facilitation of iron oxide formation (Konhauser, 1997). Surface-mediated Fe (II) oxidation on biogenic iron oxides would not take place in the absence of iron oxidizing microorganisms even though it is not directly linked to bacterial metabolism (James and Ferris, 2004). The rate at which these processes occur and the contribution of microorganisms to these processes is not clearly understood.

4. Conclusion

The results in this study show that there is apparently very little difference in the rate of abiological and biological Fe (II) removal in batch tests performed at neutral pH under aerobic conditions in saline solution. However, it was confirmed that the presence of active microorganisms is necessary for the efficient removal of Fe (II) at neutral pH under aerobic

conditions when compared to flasks containing poisoned bacterial cells in batch tests with borehole water. The results also confirmed that the previously isolated manganese oxidizing strain - *Acinetobacter* sp. LB1- can contribute to Fe (II) removal from borehole water. Whilst estimating biological and abiological Fe (II) oxidation rates at neutral pH presents a challenge, the above results demonstrate that microorganisms can play a role in Fe (II) oxidation at neutral pH under aerobic conditions although apparently only to a limited extent.

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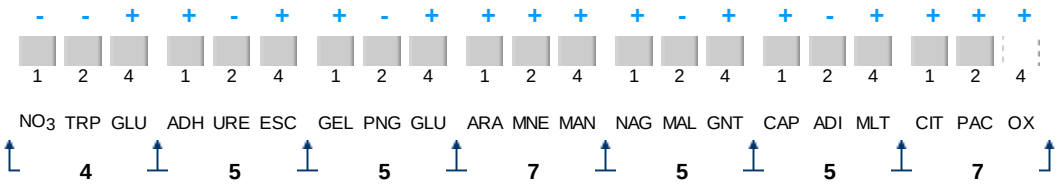
Supplementary data



REFERENZ

Lorika24h

KOMMENTAR

GUTE IDENTIFIZIERUNG

Streifen	API 20 NE V7.0
Profil	4 5 5 7 5 5 7
Hinweis	MÖGLICHKEIT VON Burkholderia gladioli

Signifikante Taxa	**% ID**	**T-Wert**	**Widersprechende Tests**
Burkholderia cepacia	**97.3**	**0.33**	**GLU 24% ADH 1% ADIa 93%**

Nächstes Taxon	**% ID**	**T-Wert**	**Widersprechende Tests**
Pseudomonas fluorescens	**2.5**	**0.02**	**GLU 0% ESC 1% PACa 16%**

Zusatztest(s)	**CELac**	**SUCROSEac**
Burkholderia cepacia	**+**	**+**
Burkholderia gladioli	**-**	**-**

Chapter 5

The detection of active biofilms and the assessment of the bacterial diversity in a biofiltration system treating borehole water in KwaZulu-Natal (South Africa)

In order to assess the microbiological status of a biofiltration system used to treat borehole water, filter matrix samples were initially analyzed for the presence of active biofilms using confocal laser scanning microscopy (CLSM). CLSM revealed the presence of biofilms on the filter matrix with actively metabolizing microbial cells being present. Thereafter, heterotrophs and manganese oxidizing bacteria (MOB) and iron oxidizing bacteria (IOB) present in the biofilms on the filter matrix in both manganese and iron filters were quantified in the combined filter matrix sample. For heterotrophs a count of 2.9×10^7 cfu/g was established using R2A agar. Counts for MOB and IOB were established as 2.4×10^7 cfu/g and 3.1×10^7 cfu/g respectively. In addition, a clone library was established using DNA extracted to from a pooled filter matrix sample from both manganese and iron filters to assess the diversity of bacteria present within the biofilter matrix in both filters. A total of 100 randomly selected clones were further separated into 15 unique OTU's (operational taxonomic unit) based upon restriction patterns of amplified partial 16S rRNA genes. Seventy three percent of clones analyzed had sequence similarity scores of ≥ 94% to the closest related genus. The remaining 27% of the clones had sequence similarity scores of < 94% to the closest related genus. The majority of the clones were closely related to the genera *Nitrospira* and *Lactococcus*. Overall, 42% of the clones were assigned to the phylum *Proteobacteria*, 13% to the phylum *Actinobacteria*, 24% to the phylum *Firmicutes* and 21% to the phylum *Nitrospirae*.

Keywords: Biofiltration system/ biofilm/ CLSM image analysis/ microbial counts/ 16S rRNA gene clone library

Abbreviations: ARDRA (amplified "rDNA" restriction analysis), OTU (operational taxonomic unit), MOB (manganese oxidizing bacteria), IOB (iron oxidizing bacteria), PCR (polymerase

chain reaction), rRNA (ribosomal RNA), CTAB (cetyltrimethylammoniumbromide), SDS (sodium dodecyl sulfate), TBE (Tris-borate EDTA), CTC (5-cyano-2,3-ditolyltetrazolium chloride), DMSO (dimethyl sulfoxide), CLSM (confocal laser scanning microscopy)

1. Introduction

One of the top priorities in civil society with regards to human health concerns is the provision of safe drinking water (Kormas *et al.*, 2010; WHO/UNICEF, 2012). Iron and manganese are natural constituents in the earth's crust and are found in surface and groundwater in varying concentrations, whilst even at low levels they remain a problem from an aesthetic, health, technical and economic point of view (Bouchard *et al*., 2011; Hamilton, 2003; Lovley, 2000). Biological filtration of groundwater is one of the methods employed in successfully removing these metals from water (Gage *et al.*, 2001). This process involves passing aerated water free of chlorine through a column of filter sand, thereby allowing microorganisms to multiply as biofilms on interfaces within the column (Gage *et al.*, 2001). The majority of microorganisms in aquatic environments grow in the form of biofilms which are considered a prominent mode of microbial colonization and mode of survival in metal-rich environments (Harrison *et al.*, 2007), yet their identity and physiology remain poorly understood (Stein *et al.*, 2002).

Biofilms typically consist of a large variety of microorganisms which exist in a highly organized community were nutrients are continuously recycled (Harrison *et al*., 2007). Bacteria in these biofilms produce capsular material which contributes to the stability of the biofilm (Sheng *et al*., 2010). This capsular material also acts as a chemical buffer at the cell's surface were essential ions are accumulated and toxic substances immobilized when a critical level is reached within the cell (Sheng *et al*., 2010; Trevors, 1989). The microorganisms commonly involved in iron and manganese sequestration belong to genera such as *Leptothrix*, *Crenothrix*, *Hyphomicrobium*, *Metallogenium* and *Siderocapsa* (Takeda *et al*., 2012; Hedrich *et al*., 2011; Mouchet, 1992). Previous studies on a water distribution system in the Southeastern USA revealed the presence of over fourteen separate MOB (manganese oxidizing bacteria) species, indicating that multiple organisms are involved in the oxidation of this metal within water distribution systems (Cerrato *et al.*, 2006). Similarly, large numbers of bacteria such as *Gallionella ferruginea* and *Leptothrix*

orchracea - responsible for oxidizing iron in water distribution systems - were found in backwash sludge in a filtration system in Canada (Gage *et al.*, 2001).

Monitoring and assessing microbial communities in biotechnological systems involved in water purification can be useful in controlling potential microbial risks associated with these systems (Kormas *et al.*, 2010). Culture based techniques used to analyze microorganisms present in such systems typically target a specific selection of relevant hygiene indicator organisms and do not usually target all possible potentially pathogenic and biofilm forming bacterial species (Kormas *et al.*, 2010). Therefore, an approach targeting bacterial DNA via PCR based amplification of target genes can assist in detecting waterborne pathogens such as *Legionella pneumophila* which are not usually detected by commonly employed culture based techniques targeting selected fecal indicator bacteria (Colwell *et al.*, 1985). RNA is unstable outside of bacterial cells due to the abundance and stability of RNases in the environment and is also rapidly degraded in stressed cells (Keinänen-Toivola *et al.*, 2006), which make DNA targeting approaches more useful in characterizing active bacterial communities (Spiegelman *et al.*, 2005; Morgan *et al.*, 2002). The following study aimed to assess the microbial diversity of the biofilter matrix within the manganese and iron filters of a biofiltration system, by initially analyzing the filter matrix for the presence of active biofilms in the manganese and iron filters and thereafter quantifying viable heterotrophs as well as MOB and IOB in these biofilms. Finally, a snapshot of the bacterial population present on the biofilter matrix was determined via the establishment of a 16S rRNA gene based clone library from a pooled sample of filter matrix from both the manganese and iron filters.

2. Materials and Methods

2.1 Biofilm detection on the biofilter matrix

Two staining methods were employed for the detection of biofilms on the filter matrix, namely acridine orange (AO) and 5-cyano-2,3-ditolyltetrazolium chloride (CTC) staining. Staining of filter matrix samples collected from the manganese and iron biofilters was carried out using AO according to procedures suggested by Kepner and Pratt (1994) with the following modifications.

A pooled filter matrix sample of 5 g wet weight and matrix particle size in the range of 3-5 mm was gently rinsed three times with 20 mL sterile distilled water before staining. Thereafter a 2 ng/mL solution of acridine orange was prepared in distilled water containing 1% DMSO (dimethyl sulfoxide), for staining of the filter matrix. The filter matrix was covered with 20 mL of the stain solution in a sterile 90 mm petri dish and stained for one minute in the dark. Thereafter, the stained sample was directly analyzed via confocal laser scanning microscopy (Zeiss LSM 710, Germany) with excitation at 488 nm and green fluorescence detection with a long pass filter between 520-560 nm. Red fluorescence was detected after excitation at 568 nm with a long pass filter at 590 nm.

The CTC staining procedure was carried out in accordance with procedures reported by Bartosch *et al.* (2003), with the following modifications. The pooled filter matrix sample of 5 g wet weight was stained with 20 mL of a 15 mM CTC solution in a sterile 90 mm petri dish at room temperature for 24 hours in the dark. After staining, samples were rinsed with sterile distilled water to remove any unbound stain followed by CLSM visualization. The formazan of CTC, CTF, formed upon enzymic reduction of CTC was detected via its emitted red fluorescence after excitation at 568 nm using a long pass filter at 590 nm. A sample of the filter matrix before the biofiltration plant started operation was stained with AO and CTC and used as a control to verify that AO and CTC were not staining any material other than the biofilm established during filtration. An additional control using a filter matrix sample before the biofiltration plant started operation and without any stain was employed to determine whether the surface of the filter matrix emitted any background fluorescence during CLSM analyses.

2.2 Quantification of bacteria in biofilms associated with the biofilter matrix

Filter matrix samples - so called manganese greensand (Casale *et al.*, 2002) - for the quantification of bacteria within the biofilter were collected from 5, 20 and 35 cm (top, middle and bottom layers) within the manganese and iron filters of the biofiltration system. A pooled filter matrix sample from both filters was used to quantify MOB, IOB and heterotrophs. Manganese oxidizing bacteria were quantified according to procedures recently reported by Beukes and Schmidt (2012). Iron oxidizing bacteria and heterotrophs were quantified in a similar

manner to MOB although the MSVP (minimal salts vitamins pyruvate) medium used to quantify the iron oxidizers contained 2 mg/L iron sulfate instead of manganese sulfate. Filter matrix samples were processed by mixing 5 g wet weight of the pooled matrix sample into 45 mL of sterile MSVP medium followed by 3 minutes vortexing at maximum speed to displace the biofilm from the filter matrix. Thereafter decimal dilutions of samples were prepared, using R2A or MSVP plus added manganese sulfate or iron sulfate as a diluent. One hundred microlitres of the appropriate decimal dilutions (typically in a range from 10^{-2} to 10^{-6}) were spread plated in triplicate using R2A or MSVP agar with added manganese sulfate or iron sulfate. Plates were incubated at 25°C for seven days in the dark to provide adequate time for the growth of slow-growing bacteria.

2.3 DNA extraction and PCR based amplification of 16S rRNA fragments

A pooled filter matrix sample from the manganese and iron filters was used for the establishment of the clone library. DNA was extracted from biofilm material attached to the filter matrix, using a CTAB (cetyltrimethylammoniumbromide) and SDS (sodium dodecyl sulfate) based procedure which is known to be effective in reducing humic acid contamination (Zhou *et al.*, 1996). Prior to DNA extraction, 5 g of the pooled filter matrix sample was washed three times with 20 mL of sterile saline to remove non-biofilm planktonic cells. The sample was then added to a 50 mL Nalgene tube and an initial vortexing step for 3 min at maximum speed was employed using 13.5 mL DNA extraction buffer (100 mM Tris-HCl, 100 mM sodium EDTA, 100 mM sodium phosphate, 1.5 M NaCl and 1% CTAB, pH 8) to displace the biofilm from the filter matrix. Thereafter, DNA was extracted from the biofilm material. The pellet of crude nucleic acids obtained after extraction was washed with cold 70% ethanol, and resuspended in sterile distilled water to give a final volume of 500 µl. Amplification of the isolated DNA was carried out using Hot Start PCR in a Labnet Multi GENE II Cycler with the following quantities of reagents per 25 µl reaction: 2 µl $MgCl_2$ [25mM], 3 µl of PCR Buffer (Fermentas Hot Start PCR Buffer), 3 µl of a 2 mM dNTP mix, 0.25 µl of forward and reverse primers (100 µmol), 0.2 µl Maxima Taq polymerase (5 u/µl), 1µl of the DNA template and 15.3 µl of nuclease free water.

The primer pair Eub 338 (forward primer 5′-GCTGCCTCCCGTAGGAGT-3′) (Amann *et al*., 1990) and Eub 907 (reverse primer 5′-CCGTCAATTCCTTTRAGTTT-3′) (Muyzer *et al*., 1995) was used to amplify a 16S rRNA gene fragment used in the establishment of the clone library. Parameters used for PCR were as follows: an initial denaturing cycle was carried out at 95°C for 3 minutes, followed by a total of 35 cycles of: denaturing at 95°C for 1 minute, annealing at 65°C for 1 minute and extension at 72°C for 3 minutes. An additional extension cycle was carried out at 72°C for 7 minutes and holding at 4°C for ∞. The yield and purity of the 16S rRNA gene amplicons was analyzed by gel electrophoresis of 5 µl amplification reaction sample in a 2% agarose gel [1x TBE (Tris-borate EDTA) buffer (10 mM, pH 8), run time 45 minutes at 100V] after post-staining with ethidium bromide. The size of amplification products was verified by using a 100-1000 bp DNA ladder (Fermentas). *E.coli* (ATCC 8739) served as a positive and sterile MilliQ water as a negative control for the PCR.

2.4 Clone library construction and analysis of recombinant clones

The 16S rRNA amplicons were cloned using a CloneJET™ PCR cloning kit (Fermentas, Lucigen Corporation) and transformed into *E.cloni*® 10G chemically competent cells (Stratagene) according to the manufacturer's specifications. After transformation, the competent cells were spread plated on LB agar containing ampicillin (50 mg/mL) and incubated at 37°C overnight. One hundred clones were randomly selected from the established clone library and checked for the correct insert via colony PCR. For this purpose one colony was placed in 100 µl of sterile distilled water and treated by five freeze-thaw cycles (i.e. 5 minutes at 95°C followed by 10 minutes in liquid nitrogen). Samples were then centrifuged at 14,000 × g for 5 minutes and a 2 µl sample was either directly used for PCR or samples were stored at -20°C for further use. The presence and correct size of inserts was determined using the supplied primer pair (pJET 1.2 forward primer, 5′-CGACTCACTATAGGGAGAGCGGC-3′, pJET 1.2 reverse primer, 5′-AAGAACATCGATTTTCCATGGCAG-3′) flanking the cloning site on the pJET 1.2 blunt cloning vector.

After amplification of inserts according to the manufacturer's instructions, the PCR products were checked on a 2% agarose gel and further screening of PCR fragments was done via ARDRA (amplified "rDNA" restriction analysis) to select clones representing phylotypes for subsequent sequence analysis. A double digestion was done at 37°C for 5 minutes using the following restriction enzyme reagents per 30 µl reaction: water, 16 µl; 10x Green Buffer, 3 µl; FastDigest® (Fermentas) Hin P1l, 0.5 µl; FastDigest® (Fermentas) Hae lll, 0.5 µl; PCR product, 10 µl. Restriction fragments were then separated on a 2% agarose gel (1x TBE buffer) at 90 V for 1 hr 15 minutes [Fig. S1], the fragment size was verified as described in 2.3. Band patterns on DNA restriction gels were analyzed using GeneSnap version 7.09.06 (SynGene, Cambridge, United Kingdom). Enzymes were selected based on the expected number of restriction sites present on the amplified fragment as checked via NCBI (http://www.ncbi.nlm.nih.gov).

2.5 Phylogenetic analysis of the clone library

For each OTU, one representative clone containing an insert of the correct size was analyzed by sequencing (Inqaba Biotec, Pretoria, South Africa). The partial 16S rRNA gene sequences obtained were compared to 16S rRNA gene sequences deposited in RDP (ribosomal database project, rdp.cme.msu.edu). A phylogenetic tree using sequences deposited within RDP, was generated based on sequence alignment established with Muscle and the tree was constructed using the maximum likelihood method in MEGA 5.1 (Tamura *et al.*, 2011) with resampling for 1000 bootstrap replicates. Rarefaction analysis was carried out using Estimate S (Version 9.0, R. K. Colwell, http://viceroy.eeb.uconn.edu/estimates) as a means to assess the species diversity covered by the clone library established using DNA isolated from biofilm material within the manganese and iron biofilters. In addition, Chao-1 values were calculated to estimate the total species richness expected for the clone library.

2.6 Chemicals

Acridine orange and CTC were obtained from Merck (South Africa). Unless otherwise stated, all other chemicals used were of the highest purity commercially available.

3. Results and Discussion

3.1 Biofilm detection on the biofilter sand

Filter matrix samples were analyzed for biofilm formation approximately three weeks after the biofiltration system started operating. It was found that the filter matrix provided a suitable support matrix enabling biofilm formation within the biofilters. CLSM (confocal laser scanning microscopy) analysis proved to be a useful tool in the detection of biofilm material on the matrix. According to Quader and Bock (1995), CLSM allows for the in situ visualization of microorganisms within the pore system of mineral materials. The results obtained from AO and CTC staining clearly demonstrated the presence of active biofilms on the filter matrix. The unused biofilter matrix (filter sand - manganese greensand (Casale *et al.*, 2002) - before use in the biofiltration system) showed some degree of green fluorescence [Fig. 1A] after staining with AO while no red fluorescence was detected [Fig. 1B] after staining with CTC. This indicates that a metabolically active biofilm was not present on the filter matrix before operation started and the biofilm only developed during biofiltration of the borehole water, as demonstrated by the difference in fluorescence intensities between figures 1A and 2A and between figures 1B and 3. It also indicates that AO is not entirely specific in staining biofilm material, as a slight green fluorescence on the washed and autoclaved filter matrix was detected even before biofiltration.

This green fluorescence could have been emitted from the rough surface of the matrix upon excitation due to interaction with AO. However, no fluorescence was emitted from a matrix particle used before biofiltration, without any stain. The AO staining revealed the presence of metabolically active and metabolically inactive microorganisms with metabolically inactive microorganisms shown in areas with green fluorescence [Fig. 2A] and metabolically active microorganisms in areas with red fluorescence [Fig. 2B]. The interaction of acridine orange with double stranded DNA, usually predominant in inactive bacterial cells, emits fluorescence at a wavelength of 530 nm whilst the interaction of acridine orange with single-stranded RNA, usually predominant in active bacterial cells, results in fluorescence at a wavelength of 640 nm (Hobbie *et al*., 1977; Darzynkiewicz *et al*., 1975).

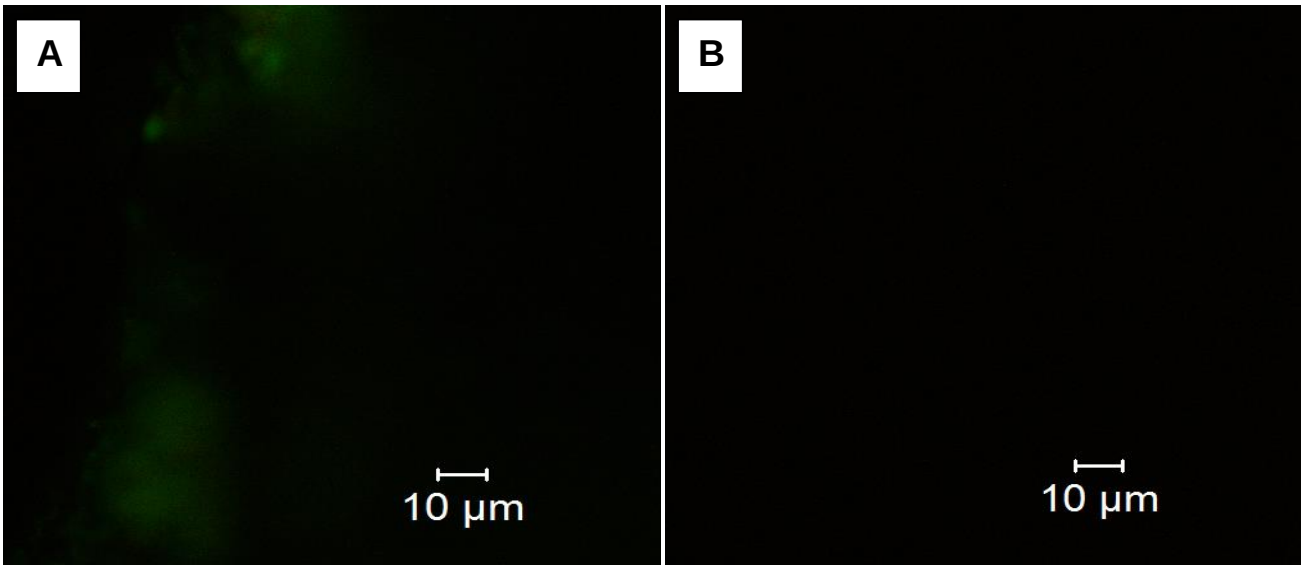


Figure 1: Confocal laser scanning micrographs of a filter matrix particle before use in the biofiltration system stained with acridine orange (A) and CTC (B).

Acridine orange binds to DNA and RNA, emitting orange-red fluorescence when bound to RNA and green fluorescence when bound to DNA (Kasten, 1981). Similarly, Pettipher *et al*. (1980) found that active microorganisms emitted orange fluorescence and inactive microorganisms emitted green fluorescence. The orange fluorescence is believed to be due to high dye/nucleotide ratios (Back and Kroll, 1991). Although useful in determining the presence of microorganisms on the filter matrix, AO staining falls short in reliably distinguishing metabolically active from metabolically inactive microorganisms as both can in fact emit green fluorescence (Bartosch *et al.*, 2003). This is due to DNA retaining its staining properties even in the presence of nonviable bacterial cells (Kepner and Pratt, 1994). Another problem associated with AO staining is that the rough surface of the filter matrix apparently interacts with the stain thereby emitting green fluorescence upon excitation in the same manner as the inactive cells [Fig. 2B]. Thus living cells present within the biofilm cannot be reliably differentiated when using the filter matrix employed in the biofiltration system analyzed in this study.

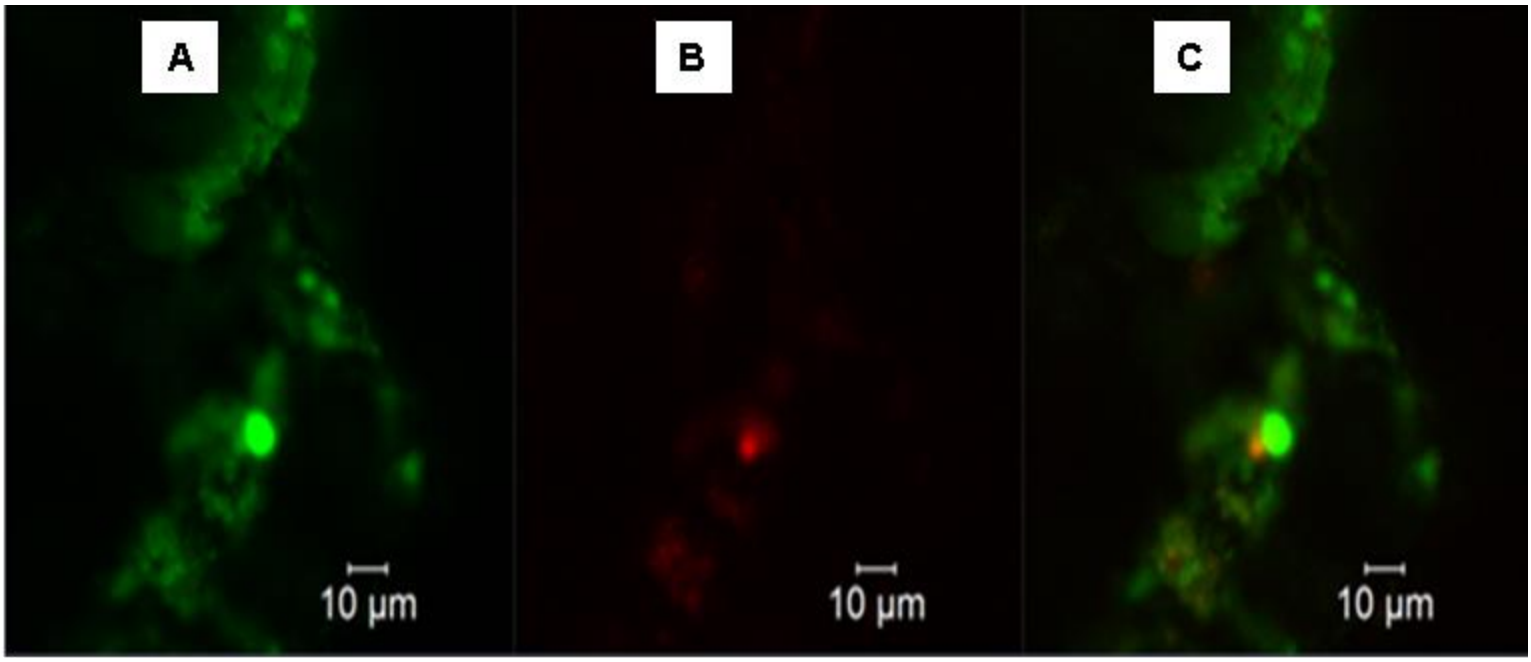


Figure 2: Confocal laser scanning split micrograph of the filter matrix after 3 weeks biofiltration showing the presence of apparently inactive cells in the biofilm (A), active cells (B), and a combination of the two (C), after staining with acridine orange.

In a study conducted by Rapposch *et al*. (2000), it was found that the direct epifluorescent filter technique (DEFT) using acridine orange was not successful in determining the bacterial counts of Gram-negative microorganisms when compared with the standard plate counting method. Overall, the same authors also found that the detection of microorganisms, when using acridine orange, depended on the degree of cell metabolism, which was influenced by environmental conditions, the Gram reaction of the microorganism and the species. Mason and Lloyd (1997) found that in bacterial cell suspensions exceeding 10^6 cells/ml, the differential staining of nucleic acids with acridine orange did not occur. The alternative approach using CTC in combination with CLSM proved useful in detecting actively metabolizing bacteria within the biofilm. The CTC reduction product - CTF (formazan crystals) formed by actively metabolizing bacteria - emits red fluorescence upon excitation [Fig. 3] and is therefore suitable for the visualization of active microorganisms on the filter matrix using CLSM (Bartosch *et al.*, 2003). Only actively respiring microorganisms on the filter matrix reduce CTC to CTF. This was evident on a matrix particle used before biofiltration, without any stain and on a matrix particle used before

biofiltration, with CTC stain were no emitted red fluorescence was detected by CLSM (data not shown). Tetrazolium salts make it possible to visualize and quantify actively respiring microorganisms in situ by acting as artificial electron acceptors within functional electron transport systems or for certain active dehydrogenases (Bartosch *et al.*, 2003). To date only limited data is available using CTC on stone/sand material while CTC was mainly applied previously to quantify active bacteria in aquatic environments (Servais *et al*., 2001; del Giorgio *et al*., 1997).

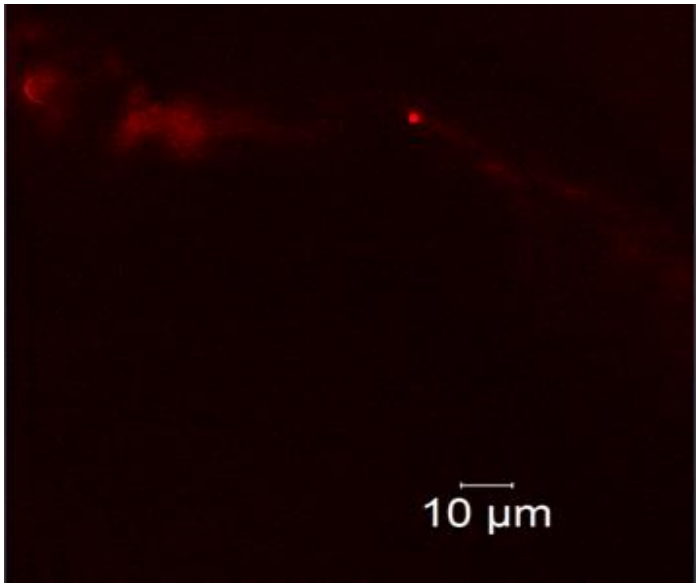


Figure 3: Confocal laser scanning micrograph of a CTC stained filter matrix particle showing actively metabolizing cells in red.

3.2 Quantification of bacteria in biofilter sand samples

MSVP agar with added iron sulfate gave rise to 3.1×10^7 cfu/g for the presumptive iron oxidizers and MSVP agar (with added manganese sulfate) gave rise to 2.4×10^7 cfu/g for the presumptive manganese oxidizers while counts of 2.9×10^7 cfu/g were established for the heterotrophs using R2A agar. The values determined are in a range reported for other biofiltration systems used to eliminate manganese from groundwater. Burger *et al.* (2008) reported heterotrophic counts of 10^6-10^8 cfu/g for biofilter sand/matrix using R2A similar to the heterotrophic counts reported in this study when using R2A agar. Using PYM agar supplemented with manganese sulfate

Vandenabeele *et al*. (1992) reported counts of 10^3 - 10^4 cfu/g for Mn-oxidizing bacteria and for heterotrophs after two weeks incubation from sand material used in biofilters treating groundwater from two different locations. More recently, Cerrato *et al*. (2010) reported counts for manganese oxidizers in biofilms isolated from drinking water systems using Mn-oxidation agar supplemented with manganese sulfate, in the range of 5×10^1 to 5×10^4 per gram of biofilm material. In a study conducted on groundwater seeps at neutral pH, Fe (II) oxidizing microorganisms accounted for 10^3-10^5 cells/ml using the MPN tube method (Blöthe and Roden, 2009). Hirsch and Rades-Rohkohl (1988) established counts of 7.3×10^3 cfu/g of iron oxidizers and 1.5×10^3 cfu/g of manganese oxidizers using PYGV and PM medium.

3.3 Phylogenetic analysis of the clone library

The clone library was established from the filter matrix samples with the assumption that most of the bacteria found in the water flowing through the system would ultimately colonize the filter matrix during biofiltration, thus forming active biofilms with high microbial densities as confirmed by confocal imaging [Fig. 2A, Fig. 3]. One hundred randomly selected clones were divided into 15 different OTU's based on ARDRA analysis [Tab. 1]. In a previous study, De Santis *et al*. (2007) determined that a 16S rRNA gene sequence similarity of ≥ 94% allowed for a reliable assignment of the clones to genus level and a similarity of ≥ 97% for species level assignment. Similarly, Revetta *et al*. (2011) used 97% as the taxonomic unit cut off threshold. These thresholds were therefore used for the 15 OTU's analyzed in this study.

Based on the analysis of sequences representing the 15 OTU's established, seventy three percent of the clones had sequence similarities equal to and above 94% and were divided into eleven groups as defined by ARDRA analysis, in order of descending predominance - *Lactococcus*, *Corynebacterium*, *Enterobacter*, *Staphylococcus*, *Bradyrhizobium*, *Legionella*, *Methylobacter*, *Anaeromyxobacter*, *Bacteriovorax* and uncultured alpha- and gamma- *Proteobacteria*. The remaining OTU's had sequence similarity scores of < 94% to the closest related genus. The phylogenetic tree [Fig. 4] confirmed the results summarized in table one. OTU BFS-6 contained the highest number of clones (21 representing *Nitrospira*) while OTU BFS-7 contained only one clone closely related to uncultured bacteria. Similarly, in previous studies, *Nitrospira* and

Nitrobacter have been the dominant nitrogen oxidizing bacteria (NOB) commonly found in wastewater treatment effluents, rivers and sediment environments (Cébron and Garnier, 2005; Dionisi *et al*., 2002). The potential disadvantages of PCR-based methods are due to the inefficient and preferential extraction of intact DNA from samples (Head *et al*., 1998; Niemi *et al*., 2001; von Wintzingerode, 1997), differences in bacterial 16S rRNA gene copy numbers (Crosby and Criddle, 2003), sensitivity to the template concentration (Chandler *et al*., 1997), primer specificity (Nocker *et al*., 2007) and the amplification bias and efficiency (Liesack *et al*., 1991), all of which limit the reliability of PCR-based methods (Polz and Cavanaugh, 1998). Advantages of cloning and sequencing include a high phylogenetic resolution and the identification of species or the determination of the closest phylogenetic neighbour within samples (Singleton *et al*., 2001; Tyson *et al*., 2004).

Whilst sequencing is an automated procedure, cloning can be time-consuming (Nocker *et al*., 2007). Despite the limitations of clone libraries to reflect diversity, they are still highly considered the "gold standard" for primary microbial diversity assessments and surveys (Spiegelman *et al*., 2005). The ideal size of 16S rRNA sequences for phylogenetic analysis is considered ~1,500 bp (Spiegelman *et al*., 2005). A study conducted by Schmeisser *et al*. (2003) using a small insert library from biofilms in water distribution systems revealed that approximately half of the sequences obtained were of poor quality due to short lengths of reads or vector contamination. However clone libraries based on inserts of 400-600 bp have been used successfully in analyzing mixed microbial populations (Amann *et al*., 1990) and to determine the genetic diversity of hydrothermal vent bacterial populations (Muyzer *et al*., 1995).

Table 1: Partial 16S rRNA gene sequences representing OTU clones established from the biofilm material on the filter matrix and the closest related taxa from RDP for each phylotype.

Phylotypes	No. of clones	Size (bp)	Closest related Genus	RDP acc. no. of closest related Genus	Putative Affiliation	Sequence similarity %
BFS-1	4	467	*Methylobacter*	S001990601	Gammaproteobacteria	97
BFS-2	4	442	*Unclassified bacteria*	S001077809	Deltaproteobacteria	89
BFS-3	10	489	*Enterobacter*	S000398862	Gammaproteobacteria	99
BFS-4	20	497	*Lactococcus*	S000591889	Firmicutes	99
BFS-5	3	492	*Uncultured bacteria*	S001225280	Alphaproteobacteria	95
BFS-6	21	494	*Nitrospira*	S001021547	Nitrospirae	92
BFS-7	1	545	*Uncultured bacteria*	S001115490	Gammaproteobacteria	94
BFS-8	3	545	*Bacteriovorax*	S002205007	Deltaproteobacteria	97
BFS-9	3	546	*Anaeromyxobacter*	S000401094	Betaproteobacteria	97
BFS-10	2	546	*Acidithiobacillus*	S002337221	Gammaproteobacteria	92
BFS-11	4	544	*Legionella*	S002871182	Gammaproteobacteria	98
BFS-12	13	546	*Corynebacterium*	S000358606	Actinobacteria	99
BFS-13	4	547	*Bradyrhizobium*	S001224760	Alphaproteobacteria	99
BFS-14	4	545	*Unclassified Gammaproteobacteria*	S001167827	Gammaproteobacteria	91
BFS-15	4	545	*Staphylococcus*	S000765688	Firmicutes	99

ARDRA does not provide information about the type of microorganisms present in environmental samples but is useful for assessing the microbial diversity in environmental samples (Smit *et al*., 1997). It is a procedure commonly employed to identify unique clones and for estimating the diversity of OTU's in environmental samples (Smit *et al*., 1997). ARDRA does not require the use of specialised equipment but does require several restrictions for adequate genotypic resolution (Moyer *et al*., 1996) and can be time and labour-intensive (Nocker *et al*., 2007). However, Ramos *et al*. (2010) analysed a clone library of 162 clones and found 28 unique OTU's using only one restriction enzyme, indicating the usefulness of the ARDRA approach in reducing the sequencing effort when assessing microbial diversity. A major limitation of this procedure is that the restriction patterns produced by complex microbial communities are often problematic to resolve on agarose gels (Dunbar *et al*., 1999). However, a second agarose gel with initially sorted clones can allow for a more accurate sorting of OTU's, to determine the most dominant groups in the library and to detect small differences in RFLP (restriction fragment length polymorphisms) patterns that might be missed by commonly employed image analysis programs (Vergin *et al*., 2001). Krakat *et al*. (2010) determined the detection limit of ARDRA by spiking an autoclaved fermentor matrix with various cell concentrations of five different strains of methanogenic *Euryarchaeota* and established the detection limit of ARDRA as approximately 10^5 cells/mL, a value similar to the detection limit of epifluorescence microscopy, thus validating the reliability and sensitivity of ARDRA.

Some of the identified taxa in the biofilter matrix clone library are typically associated with or are present in human beings, some of which are even pathogenic. The majority of rural communities in South Africa do not have access to clean water or proper sanitation facilities such as toilets (WHO/UNICEF, 2012). If toilets are present they may be situated upstream from a drinking water source, which increases the risk of fecal contamination in the water which can have adverse health effects on those who consume it. The groundwater in turn can also become contaminated. This is a possible explanation for the presence of bacteria such as *Legionella* spp., *Lactococcus*, *Enterobacter* or *Staphylococcus* that might be of human origin in the current biofiltration system. In the current study common MOB and IOB representatives like the previously isolated manganese oxidizing bacterium - *Acinetobacter* sp. LB1 (Beukes and Schmidt, 2012) - were however not detected via the establishment of the clone library.

This could be due to the fact that *Acinetobacter* sp. LB1 was originally isolated from water samples of the manganese filter while the clone library was established from DNA extracted from the filter matrix. The detection limit of ARDRA is about 10^5 cells/mL and individually, *Acinetobacter* sp. LB1 comprised about 10^5 cells/mL of a mixed microbial community in water samples in the manganese filter. The pooled filter matrix sample contained $\approx 10^7$ presumptive MOB/IOB from a mixed microbial population. In order for a reliable detection of *Acinetobacter* sp. LB1, individual representatives must be present at about 10% of the viable count. Thus individual representatives might not be detected via ARDRA if they are close to the reported detection limit of this method. This is further complicated by the fact that DNA extractions will not quantitatively capture the DNA of the less abundant groups from biofilter material. It must also be noted that before DNA was extracted from the biofilm material for the construction of the clone library, planktonic cells were washed away using saline, these cells could have constituted a major proportion of the genus *Acinetobacter*.

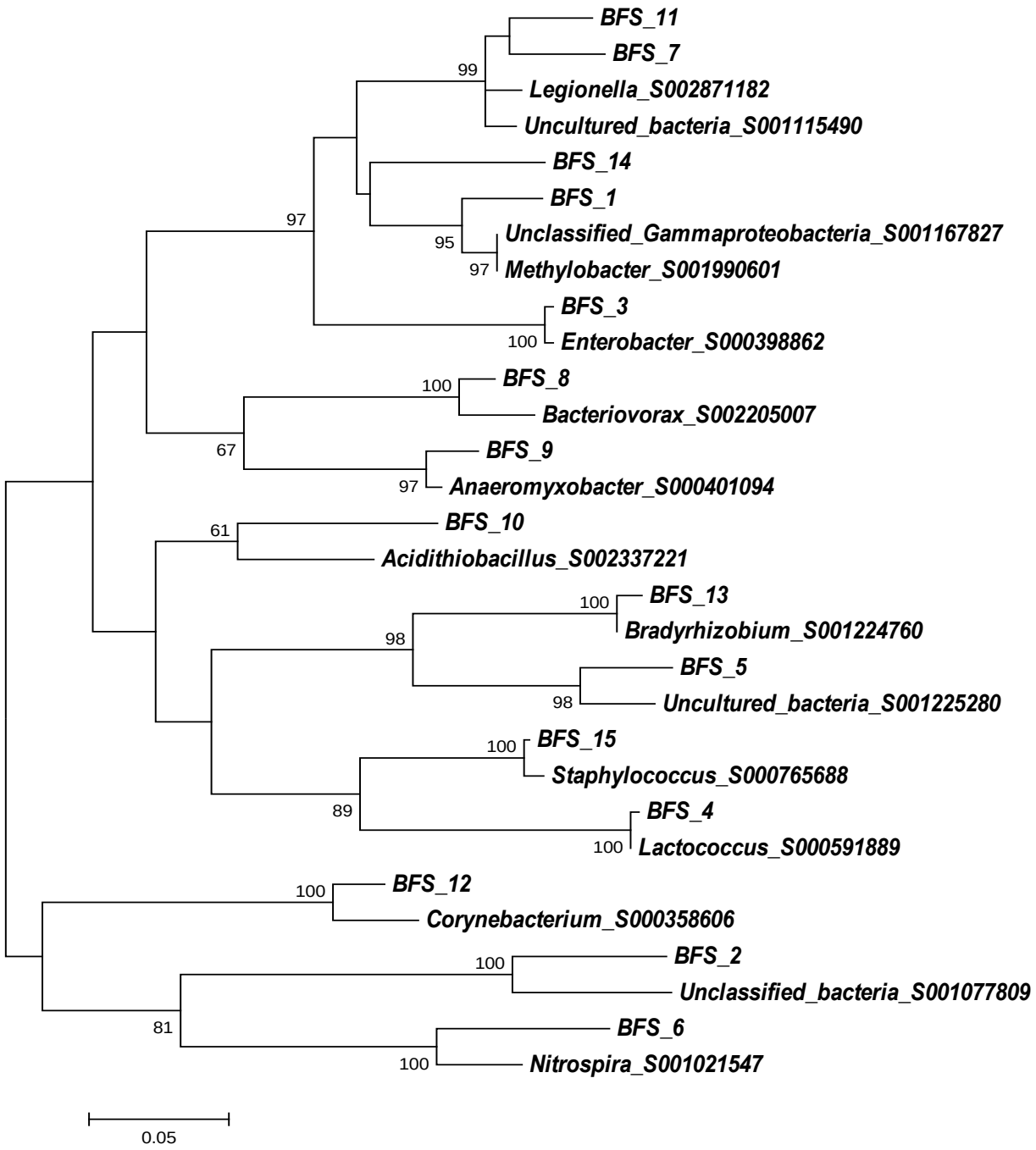


Figure 4: Phylogenetic affiliation of 16S rRNA genes of 15 OTU representatives based on sequence comparisons to environmental strains in RDP. Numbers shown at nodes indicate calculated bootstrap values (only values of > 50% are shown). The alignment of selected sequences and the construction of the tree are specified in section 2. The scale bar indicates five estimated changes per 100 nucleotides.

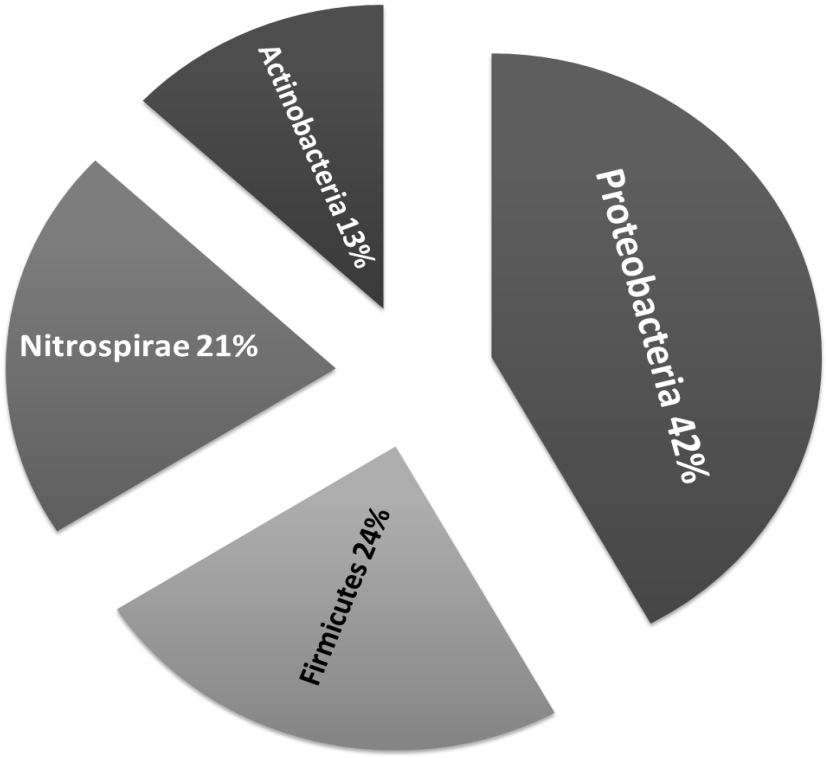


Figure 5: Quantitative assignment of the 15 OTU's from the 16S rRNA gene clone library to their closest phylogenetic groups.

The phylum *Proteobacteria* has been shown to be the most dominant group of microorganisms in water distribution systems around the world (Schmeisser *et al.*, 2003). Other studies based on sequences obtained from both bulk water and biofilm material indicated that alpha-, beta-, and gamma-*Proteobacteria* were the most predominant microorganisms in bacterial communities inhabiting water distribution systems (Santo Domingo *et al*., 2003; Williams *et al*., 2004). Similarly, a study conducted on biofilm communities on copper pipes revealed that dominant members of the clone library were closely related to the phyla gamma- and beta- *Proteobacteria,* such as *Acinetobacter* and *Pseudomonas* species respectively, and the less abundant groups were closely related to the phyla alpha- and delta- *Proteobacteria*, *Flavobacteria*, *Sphingobacteria,* and uncultured bacteria (Pavissich *et al*., 2010). The results obtained in this study are in agreement with previous studies were the dominant group of bacteria was most closely related to the phylum *Proteobacteria* which comprised 42% of the clone library in this study.

The rest of the clone library was comprised of the following phyla: *Nitrospirae* - 21%, *Firmicutes* - 24% and *Actinobacteria* - 13% [Fig. 5]. OTU BFS-6 contained the highest number of clones which belonged to the genus *Nitrospira*. The level of nitrite in the borehole water was < 0.050 mg/L (Personal communication, Dudu Gwebu, Collin Van Der Merwe and Peter Thompson Umgeni Water, Durban) which is much lower than the recommended level (3 mg/L as nitrite ion or 0.9 mg/l as a nitrite-nitrogen couple) (WHO, 2011). Thus the high levels of *Nitrospira* could be due to contamination of the borehole water with urine. A study by Cébron and Garnier (2005) found that large amounts of nitrogen present in river water were due to contamination from agricultural activities and from urban effluents. The urine contains urea which can be hydrolysed to NH_3 which undergoes oxidation by AOB's (ammonium oxidizing bacteria) to form nitrite; the oxidation of which to nitrate is carried out by NOB's (nitrogen oxidizing bacteria) (Bock et *al*., 1992). This is possible, as there was a toilet situated upstream from the biofiltration system.

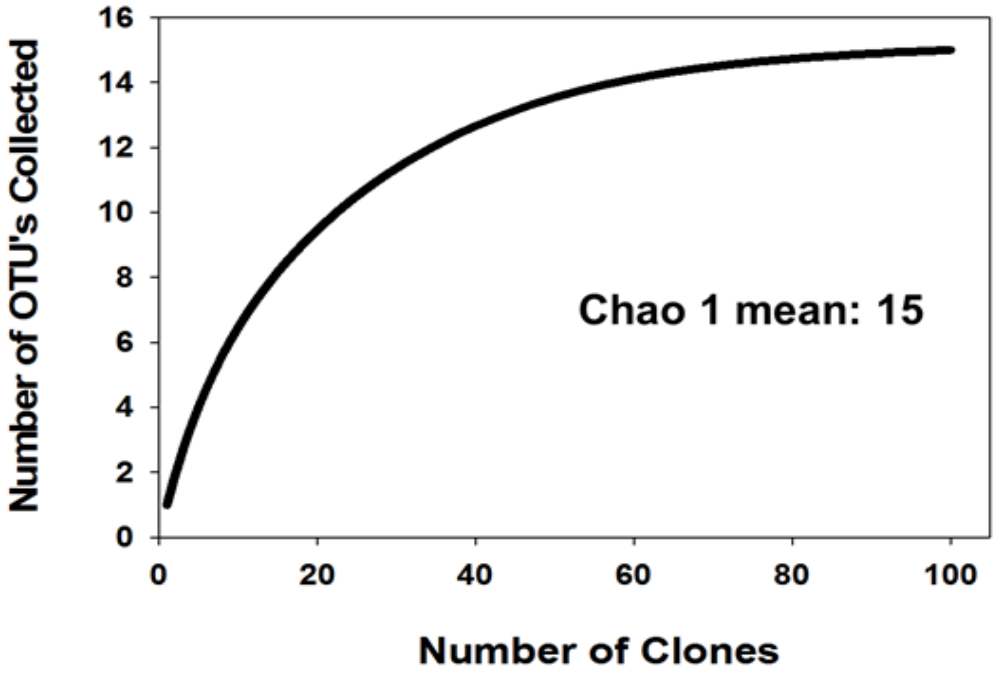


Figure 6: Rarefaction curve depicting the relationship between the number of clones collected and the number of OTU's (phylotypes) detected, computed using Estimate S.

Typical clone libraries of 16S rRNA genes contain less than 1000 sequences and would therefore display only a partial view of the microbial diversity in such samples (Dunbar *et al*., 2002). For the clone library analyzed in this study, the rarefaction curve indicated that the number of clones analyzed only partially covered the diversity of bacteria present on the filter matrix in the population [Fig. 6]. However, Chao-1 calculations matched the established number of OTU's.

Conclusion

The results indicate that about 10^7 MOB and IOB and heterotrophs were present per gram of filter matrix of the manganese and iron filters respectively. As confirmed by CLSM, actively metabolizing cells were present within the biofilm formed on the filter matrix, demonstrating that the filter sand is a suitable support matrix enabling biofilm formation. Via the establishment of a clone library, it was determined that the majority of OTU's belonged to the *Proteobacteria* group and that 27% percent of clone sequence similarities fell below the 94% sequence similarity threshold. Molecular techniques pose a challenge in environmental technology in that the important microorganisms detected via these techniques have often not been identified, cultured or sequenced (Rittmann, 2010). Nevertheless these techniques provide a deeper understanding into the microbial community that needs to be controlled or manipulated in order to ensure that systems like the one under investigation are able to function efficiently.

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Supplementary figures

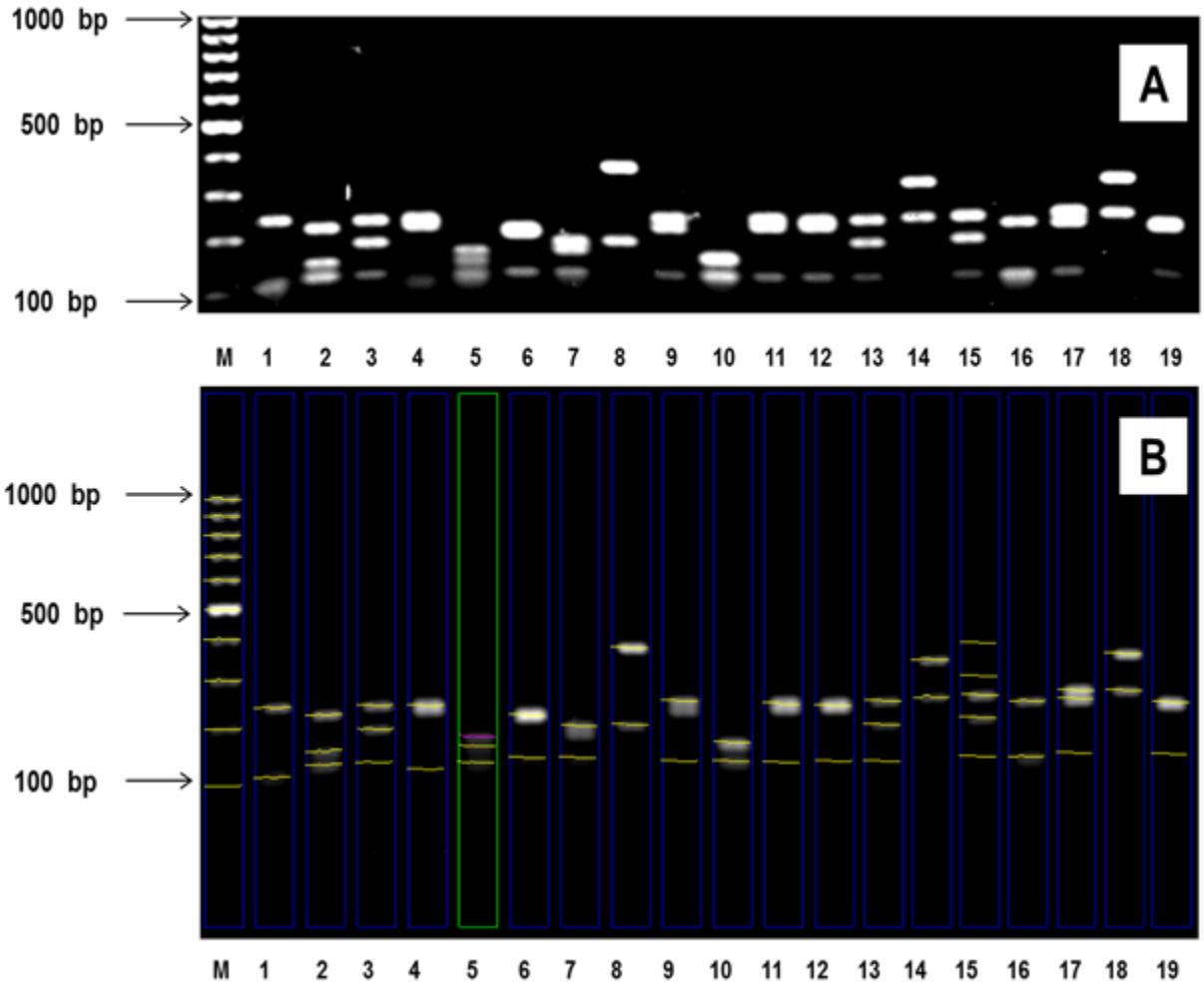

Figure S1: [A] Representative 2% agarose gel depicting unique banding patterns after ARDRA of 19 out of 100 randomly selected clones. Lane M - Molecular weight marker, lane 1-19 - samples 59 – 87. [B] Representative 2% agarose gel as for image A, highlighting band regions analysed using GeneSnap program version 7.09.06 (SynGene, Cambridge, United Kingdom).

Chapter 6

Concluding Remarks

Analyses of the biofilter water using selective media revealed the presence of moderately high numbers of MOB (manganese oxidizing bacteria) and IOB (iron oxidizing bacteria) which subsequently led to the isolation and characterization of a manganese and iron oxidizing bacterial strain belonging to the genus *Acinetobacter*. Species within this genus are classified as strict Gram negative aerobes, incapable of carbohydrate fermentation, denitrification, or reduction of nitrate to nitrite (Baumann *et al.*, 1968). This group of microorganisms is ubiquitous in the environment occurring in soil, water, on human skin (causing opportunistic and nosocomial infections) (Wagner *et al.*, 1994) and plays a vital role in the aerobic mineralization of organic matter in nature (Baumann, 1968). Previous studies have linked these organisms to the biological removal of phosphate in the environment (EBPR) (Fuhs and Chen, 1975; Kämpfer *et al.*, 1992). They are also able to thrive at 30^{o}C and have the ability to grow at the expense of a wide range of organic compounds as sole carbon sources (Baumann, 1968). The metabolic versatility of this group suggests that these organisms may play an important role in the solution of pollution problems associated with petroleum, as well as in with the decomposition of motor oils, due to their ability to grow in the presence of a variety of carbon compounds (Juni, 1978). These are some unique properties of the genus *Acinetobacter* that make it applicable to tackle pollution by aliphatic compounds. However, so far there is no information concerning the ability of members of the genus *Acinetobacter* regarding metal oxidation.

However, in this study, evidence is provided regarding the ability of a member of the genus *Acinetobacter* for the oxidative removal of Mn (II) and to a lesser extent Fe (II) from groundwater. Manganese oxidation tests conducted in the presence of appropriate abiotic controls at neutral pH showed that manganese oxidation was invariably enhanced in the presence of metabolically active *Acinetobacter* sp. LB1 cells. These results clearly link manganese oxidation in this biofiltration system to microbial processes. Not only was the isolate effective in the oxidation and subsequent removal of manganese but also, albeit to a lesser extent, able to oxidatively remove Fe (II). Although the oxidation of iron in the biofiltration system is very

rapid in the presence of oxygen, experimental analysis confirmed that the presence of metabolically active microorganisms is necessary for the more efficient removal of iron in the system when compared to poisoned controls. Batch tests employing borehole water and saline further confirmed the ability of *Acinetobacter* sp. LB1 to oxidize iron. The manganese and iron oxidation tests demonstrated that the auto catalytic removal of manganese and iron is not sufficient to explain the oxidation rates observed within the system. Analysis of the biofilter matrix revealed the presence of active microbial consortia attached to the filter matrix in the form of biofilms as confirmed by confocal laser scanning microscopy (CLSM) when stained with either CTC (5-cyano-2,3-ditolyltetrazolium chloride) or acridine orange. CTC proved to be more reliable at detecting actively metabolizing bacterial cells within the biofilm. A snapshot of the bacterial population present on the biofilter matrix was determined via the establishment of a partial 16S rRNA gene based clone library from a pooled sample of filter matrix from both the manganese and iron filters. Based on ARDRA (amplified "rDNA" restriction analysis) analysis of 100 randomly selected clones, 15 unique OTU's were detected with the majority of the clones analyzed closely related to the genera *Nitrospira* and *Lactococcus*. However, *Proteobacteria* dominated the microbial population (42% of all clones) and the rest of the population was comprised of 13% of clones falling into the phylum *Actinobacteria*, 24% belonging to the phylum *Firmicutes* and 21% assigned to the phylum *Nitrospirae*.

A recent study in Durban (South Africa) confirmed the need for removing manganese from groundwater thus highlighting the importance of the research conducted (Batterman *et al*., 2011). Whilst an essential element for nutrition in human beings, manganese (Mn (II)) has been reported to have toxic effects (neurobehavioral development problems) on children after the intake of water containing high levels of Mn (II) that exceed the US EPA recommended level (Woolf *et al.*, 2002; Bouchard *et al.*, 2007). Similarly, high concentrations of Fe (II) can result in or contribute to the development of diseases such as hemochromatosis, thalassemia and/or chronic liver disease (Beaton and Adams 2007; Kohgo *et al*., 2008). Not only is the removal of these metals important for health reasons but also for the aesthetic appeal of water (Cullimore and Mc Cann, 1977) and to avoid the corrosion of stainless steel (Rao *et al*., 2000) in water systems as a result of bacterial oxidation activities. For application purposes, an understanding of environmental conditions that favour or inhibit the growth of native microorganisms responsible

for manganese and iron oxidation is a prerequisite for the design of a successful technology that uses microorganisms for removal of metals from groundwater sources.

It can be concluded that in the presence of bacteria like the isolated *Acinetobacter* sp. LB1, the oxidative removal of manganese from borehole water is enhanced and, to a smaller degree, the removal of Fe (II). The use of biofiltration systems for the treatment of borehole water provides a cost effective way of providing rural communities with potable water in South Africa. Biofiltration systems like the system currently under investigation require basic process control and operator intervention unlike the conventional processes that require chemical dosing, advanced oxidation processes, sophisticated process control and operator skills which are a challenge in rural communities. In the current study, a total of 99% iron and 96% manganese were typically removed from the borehole water after biofiltration (Personal communication, Dudu Gwebu, Umgeni Water, Durban). The current research highlights that the high removal rate of manganese in this system is attributed to the presence of metabolically active microorganisms and the high removal of iron is attributed in part to physiochemical reactions within the biofiltration system and in part to biological processes.

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Thesis supplementary figures

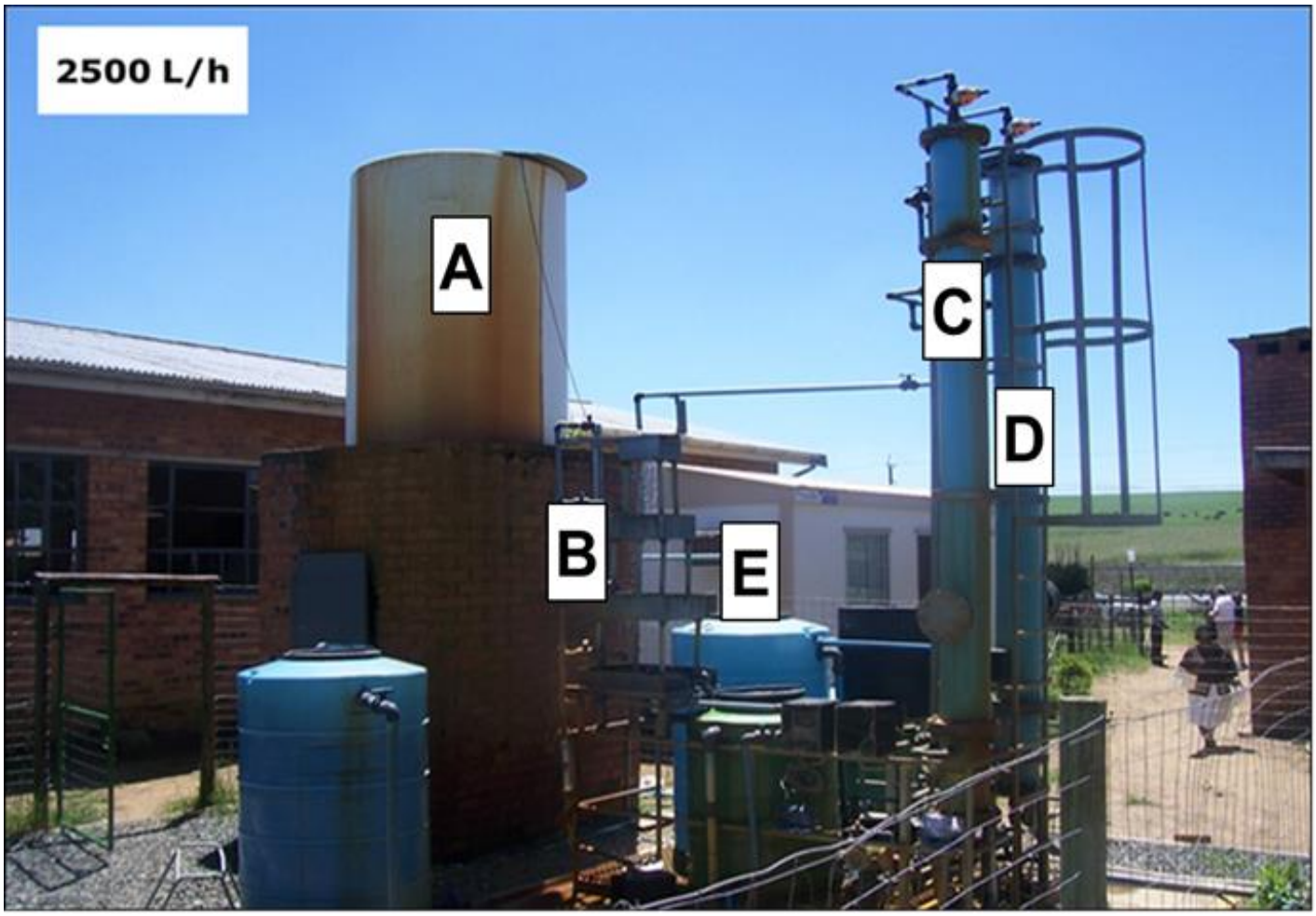

Figure S1: The biofiltration system employed to remove manganese and iron from borehole water in Nottingham road outside the Nottingham combined school, KwaZulu Natal, South Africa.

[A] Borehole water tank
[B] Aeration cascade
[C] Iron biofilter
[D] Manganese biofilter
[E] Final receiving tank

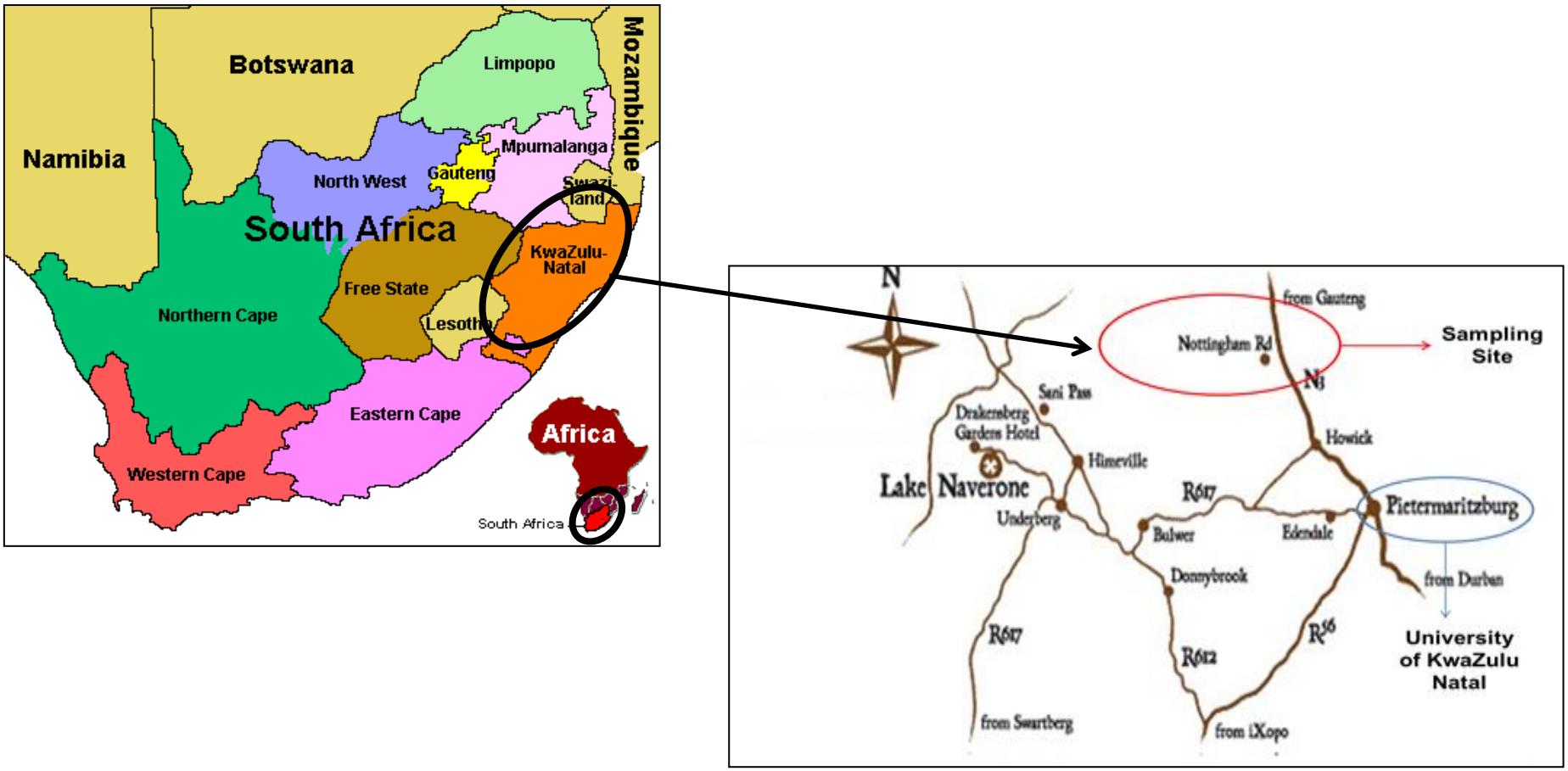

Figure S2: Location of the biofiltration system (sampling site) in Nottingham road outside the Nottingham combined school, KwaZulu Natal, South Africa. Maps adapted from http://www.maps-africa.blogspot.com.