Microbial community study of the process- and groundwater of the Sishen Iron-Ore Mine, South Africa

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Abstract

Investigating the microbial community of the Sishen Iron-Ore Mine in South Africa has become a topic of interest. Micro-organisms could prove to be useful in bioleaching processes, resulting in the minimisation of the negative impact that certain substances, such as phosphorus (P) and potassium (K), have on the economic functioning of the mine. The objective of this investigation was, therefore, to determine which micro-organisms were indigenously present in the process- and groundwater systems of the mine. Groundwater samples and three different process water samples were collected from the mine, followed by chemical- and microbial community analyses. Microbial inhibition was observed in all the process water samples due to the relatively high levels of copper, chromium and zinc present. *Aeromonas hydrophila* proved to be the dominant bacterial species in all the process water samples, whereas *Pseudomonas aeruginosa* and *Herbaspirillum* spp. were observed in the groundwater of the mine. None of the isolated micro-organisms have been implicated in bioleaching practices, and therefore these organisms will not be included as candidates for the removal of P and K from the iron-ore of the Sishen Iron-Ore Mine.

Keywords: Sishen Iron-Ore Mine, microbial community, process water, groundwater, bioleaching

Introduction

The depletion of high-quality iron-ore (>60% Fe; <0.24% K) deposits necessitates the processing of lower-quality iron-ore (<60% Fe; >0.24% K) (Jian and Sharma, 2004; Taljaard, 2005). Impurities, such as P and K contained within the lower-quality iron-ore have a detrimental effect on the steel-making process, and steel-making plants charge penalties when purchasing iron-ore with P and K levels exceeding 0.24% (Yusfin et al., 1999). In the past, low-quality iron-ore concentrate has been blended with high-quality iron-ore, in an attempt to 'dilute' the P and K contained within the export iron-ore concentrate of the mine (Dukino et al., 2000). However, the low-quality iron-ore stockpiles of the Sishen Iron-Ore Mine are increasing, and therefore it is essential to develop an economically and environmentally friendly process to reduce the high P and K concentrations of the iron-ore concentrate.

Micro-organisms could prove to be useful in the removal of the P and K from the iron-ore, as they may have metabolic properties, which could enable them to produce acids that may prove invaluable when applied in industrial practice (Gupta and Sharma, 2002; Lesniak et al., 2002). It is essential to determine which micro-organisms are indigenous to the mine environment before strategising how best to employ them to industrial advantage. Therefore, there has been an increasing interest in the microbial community of the Sishen Iron-Ore Mine environment.

It is hypothesised that indigenous micro-organisms already living in the Sishen Iron-Ore Mine environment are capable of using the P and K in the iron-ore as structural components for their cell walls and membranes, as well as many other metabolic processes, such as organic acid production, since the environment selects for them to do so. The purpose of this investigation was to determine which micro-organisms are indigenously present in the process- and groundwater of the Sishen Iron-Ore Mine, as well as to determine the microbial diversity. To date no information regarding the microbial community of the Sishen Iron-Ore Mine's aquatic environment exists.

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Experimental design

Sample selection and processing

Process water samples (10 ℓ) were collected in sterile containers at three different sampling points of the Sishen Iron-Ore Mine. The sampling points included water from the process dam, water flowing into the slimes dam, and water flowing from the slimes dam. In addition, a groundwater sample was collected from a borehole located within the Sishen Iron-Ore Mine. The samples were stored at 4°C until processing.

Chemical analysis of the process- and groundwater

The pH and turbidity, as well as the levels of ammonium, hydrogen sulphide, nitrates, nitrites, total phosphorus, potassium, free chlorine, fluoride, copper, chromium, iron, manganese and zinc were determined for all water samples by spectrophotometry using the Spectroquant® Photometer SQ 118 (Merck, Darmstadt, Germany). Spectroquant® test kits (Merck) for each of the abovementioned parameters were used according to the manufacturers instructions. The pH of the water samples was measured using a Beckman Φ34 pH meter (Beckman Coulter, Inc., Fullerton, CA, USA).

Total plate counts of the process- and groundwater

Heterotrophic plate counts of the process- and groundwater of the mine were conducted using the pour plate method (Health

Protection Agency, 2004a). A dilution series for each water sample was prepared in sterile test tubes using distilled water (dH_2O) (Health Protection Agency, 2004b). One millilitre of each dilution was pipetted into a 90 mm Petri dish (Concorde Plastics, Johannesburg, South Africa), followed by the addition of 20 mℓ of liquid (50°C) standard nutrient agar to each Petri dish. Once the agar had solidified, the agar plates were incubated for 48 h at 28°C. Each process- and groundwater sample was analysed in triplicate. Following the incubation period, the bacterial colonies were enumerated and the Simpson's index of diversity ($1\text{-}D = 1\text{-}\ \Sigma pi^2$), as well as the Equitability Index ($E_D = D/D_{max}$) (D: Simpson's diversity index; pi: proportion of species made up of the ith species; D_{max}: the maximum value D could assume if individuals in the community were completely evenly distributed).

Preparation of pure cultures

Pure cultures of each morphologically distinct bacterial colony, which was isolated on the standard plate-count agar plates, were prepared. Each colony was inoculated separately onto agar plates containing solidified standard nutrient agar. The agar plates were incubated for 48 h at 28°C in order to obtain single bacterial colonies. The procedure was repeated, followed by the bacterial identification. Suspensions from the pure cultures isolated from the groundwater sample were prepared, using sterile dH_2O, for molecular analysis.

Bacterial identification of the bacteria isolated from the process water

Bacteria isolated from the process water samples were Gram-stained according to the method described by the Health Protection Agency (2007). Oxidation-fermentation (OF) analysis was performed by the Hugh-Leifson Test (Health Protection Agency, 2004c), using OF basal medium, supplemented with a 10% filter-sterilised solution of D (+) glucose (Merck), lactose (Merck) and sucrose (Merck). The oxidase test using *N*, *N*, *N*', *N*'-tetramethyl-*p*-phenylenediamine (Aldrich Chemical Co, Milwaukee, Wisconsin) was performed on all isolated bacteria (Health Protection Agency, 2004d). Finally, the bacterial species were identified using the API 20E and 20NE identification systems as described by the manufacturer (Analytab Products, Plainview, NY).

16S polymerase chain reaction for the amplification of bacterial DNA from the groundwater sample

A 16S polymerase chain reaction (PCR) was performed by amplifying a portion of the 16S eubacterial gene from the bacterial suspensions prepared from the pure cultures of bacteria isolated from the groundwater sample. The following primers were used for DNA amplification:

PRUN518r: 5'-ATT-ACC-GCG-GCT-GCT-GG-3' (Siciliano et al., 2003),
PA8f-GC: 5'-CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG-3' (Fjellbirkeland et al., 2001).

All PCR reagents were manufactured by Bio-Rad Laboratories (Hercules, CA, USA), unless otherwise stated. The PCR reaction was performed in a reaction volume of 25 µℓ containing 150 mM KCl, 30 mM Tris-HCl (pH 9.0), 0.3% Triton X-100, 50 mM $MgCl_2$, 10 µM PCR nucleotide mix, 5 pmol primer PRUN518r (Whitehead Scientific, Cape Town, South Africa), 5 pmol primer PA8f-GC (Whitehead Scientific), 1.5 units of Taq polymerase and 0.5 µℓ bacterial suspension. Denaturation of extracted DNA at 95ºC for 10 min was followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 51ºC for 30 s, and extension at 72ºC for 1 min (Bio-Rad Thermal Cycler, Bio-Rad Laboratories). A final extension at 72ºC for 10 min concluded the PCR amplification of the DNA. A reaction containing no DNA template was included as a negative control. The amplified PCR products were separated using 1% agarose gel electrophoresis in tris-acetate-EDTA (TAE) buffer.

Sequence analysis of the bacterial DNA from the groundwater sample

Sequences of the 16S eubacterial gene of the rDNA operon were obtained using primer PRUN518r. The sequences reported in this study were compared to 16S eubacterial gene sequences present in the GenBank database by using the BLAST program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Matching hits with e-values closest to 0.0 were chosen for alignment. Reported and reference sequences were aligned using CLUSTAL X version 1.8 (ftp://ftp-igbmc.u-stras-bg.fr/pub/ClustalX/) (Thompson et al., 1997) and inserted gaps were treated as missing data. Ambiguously aligned regions were excluded from the data set before analysis. Phylogenetic analysis was based on parsimony using PAUP 4.0b8 (Phylogenetic Analysis Using Parsimony) (Swofford, 2000). Heuristic searches were made with random addition of sequences (1 000 replicates), tree bisection-reconnection (TBR), branch swapping and MULPAR effective and MaxTrees set to auto-increase. Evaluating tree length distributions over 100 randomly generated trees assessed phylogenetic signal in the data sets. The consistency (CI) and retention indices (RI) were determined for all data sets. Characters were re-weighted to the CI, and only informative characters were included, while missing, ambiguous and constant characters were excluded. Phylogenetic trees were rooted with *Bacillus subtilis* as out-group to the remaining taxa. Bootstrap analyses were conducted, retaining groups with 70% consistency, to determine confidence in branching points (1 000 replicates) for the most parsimonious trees generated.

Results and discussion

The chemical analysis of the process- and groundwater samples of the Sishen Iron-Ore Mine is reported in Table 1. The pH of all water samples ranged between 7.25 and 7.80. The water flowing to the slimes dam contained the highest concentrations of copper (0.72 mg·ℓ$^{-1}$), chromium (0.24 mg·ℓ$^{-1}$) and zinc (0.44 mg·ℓ$^{-1}$). This water also contained ammonium (0.23 mg·ℓ$^{-1}$), hydrogen sulphide (0.34 mg·ℓ$^{-1}$), high levels of nitrates (>90.0 mg·ℓ$^{-1}$), nitrites (>3.0 mg·ℓ$^{-1}$), phosphorus (2.4 mg·ℓ$^{-1}$), free chlorine (0.6 mg·ℓ$^{-1}$), fluoride (0.89 mg·ℓ$^{-1}$), iron (0.52 mg·ℓ$^{-1}$) and manganese (0.8 mg·ℓ$^{-1}$). The water collected from the process dam contained lower concentrations of copper (0.29 mg·ℓ$^{-1}$), chromium (0.20 mg·ℓ$^{-1}$) and zinc (0.31 mg·ℓ$^{-1}$), compared to the water flowing to the slimes dam, while it contained the highest concentrations of ammonium (0.39 mg·ℓ$^{-1}$), hydrogen sulphide (0.41 mg·ℓ$^{-1}$), phosphorus (3.4 mg·ℓ$^{-1}$), free chlorine (0.8 mg·ℓ$^{-1}$), fluoride (0.97 mg·ℓ$^{-1}$), iron (0.66 mg·ℓ$^{-1}$) and manganese (1.3 mg·ℓ$^{-1}$), as well as high levels of nitrates (>90.0 mg·ℓ$^{-1}$) and nitrites (>3.0 mg·ℓ$^{-1}$).

In contrast, the water collected from the slimes dam con-

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tained only high levels of nitrates (>90.0 mg·ℓ$^{-1}$), nitrites (2.0 mg·ℓ$^{-1}$) and fluoride (0.74 mg·ℓ$^{-1}$). These results indicate that the slimes dam is functioning properly by precipitating elements such as copper, chromium, iron, manganese and zinc. The groundwater, however, only showed traces of nitrates (10.5 mg·ℓ$^{-1}$) and high levels of nitrites (>3.0 mg·ℓ$^{-1}$) in the water, with all other elements below the detection limits, indicating that the groundwater table remains isolated from contamination with heavy metals and other chemical compounds and elements, which may arise from the mining process.

TABLE 1
Chemical analyses of the process- and groundwater samples of the Sishen Iron-Ore Mine

Parameters	Process dam	Water to the slimes dam	Water from the slimes dam	Groundwater
pH	7.54	7.61	7.80	7.25
Turbidity	54 NTU	14 NTU	1 NTU	23 NTU
Ammonium	0.39 mg·ℓ$^{-1}$	0.23 mg·ℓ$^{-1}$	ND	ND
Hydrogen sulphide	0.41 mg·ℓ$^{-1}$	0.34 mg·ℓ$^{-1}$	ND	ND
Nitrates	>90.0 mg·ℓ$^{-1}$	>90.0 mg·ℓ$^{-1}$	>90.0 mg·ℓ$^{-1}$	10.5 mg·ℓ$^{-1}$
Nitrites	>3.0 mg·ℓ$^{-1}$	>3.0 mg·ℓ$^{-1}$	2.0 mg·ℓ$^{-1}$	>3.0 mg·ℓ$^{-1}$
Total phosphorus	3.4 mg·ℓ$^{-1}$	2.4 mg·ℓ$^{-1}$	ND	ND
Potassium	ND	ND	ND	ND
Free chlorine	0.8 mg·ℓ$^{-1}$	0.6 mg·ℓ$^{-1}$	ND	ND
Fluoride	0.97 mg·ℓ$^{-1}$	0.89 mg·ℓ$^{-1}$	0.74 mg·ℓ$^{-1}$	ND
Copper	0.29 mg·ℓ$^{-1}$	0.72 mg·ℓ$^{-1}$	ND	ND
Chromium	0.20 mg·ℓ$^{-1}$	0.24 mg·ℓ$^{-1}$	ND	ND
Iron	0.66 mg·ℓ$^{-1}$	0.52 mg·ℓ$^{-1}$	ND	ND
Manganese	1.3 mg·ℓ$^{-1}$	0.8 mg·ℓ$^{-1}$	ND	ND
Zinc	0.31 mg·ℓ$^{-1}$	0.44 mg·ℓ$^{-1}$	ND	ND

**ND – Not detected*

Table 2 illustrates the average plate counts obtained for each dilution of the process- and groundwater samples. When the bacterial counts for each dilution of the process water samples were compared to one another, it became evident that the bacterial growth was inhibited in all the undiluted samples. However, no inhibitory effect was observed in the groundwater collected from the mine. The inhibitory effect that heavy metals have on bacterial growth is well documented (Gordon et al., 1994; Yenigün et al., 1996), and therefore, it can be assumed that the inhibitory effect observed in this study is most likely as a result of the copper, chromium and zinc contained in the process water of the mine.

TABLE 2
The average bacterial counts (cfu·mℓ$^{-1}$) obtained for the different dilutions of the process- and groundwater samples of the Sishen Iron-Ore Mine

Sample	Undiluted	1 log	2 log	3 log
Process dam	1.1×10^2	5.13×10^3	2.17×10^3	2.0×10^3
To slimes dam	0	5.53×10^2	4.67×10^2	1.33×10^3
From slimes dam	2.97×10^2	9.23×10^2	6.0×10^2	0
Groundwater	2.66×10^1	0	0	0

As the copper, chromium and zinc were diluted in the dilution series, the inhibitory effect decreased in all the process water samples. The inhibitory effect was diminished at a 3-log dilution in the water flowing to the slimes dam, compared to a 1-log dilution in the water from the process dam and the water flowing from the slimes dam. This indicates that the level of the substance(s) responsible for the inhibitory effect must have been significantly higher in the water flowing to the slimes dam than in the other two water sources. The total amount of copper, chromium and zinc in the water flowing to the slimes dam (1.4 mg·ℓ$^{-1}$) was significantly higher than observed in both the water from the process dam (0.8 mg·ℓ$^{-1}$) and the water flowing from the slimes dam (~0.0 mg·ℓ$^{-1}$), as well as the groundwater (~0.0 mg·ℓ$^{-1}$), confirming that these heavy metals were indeed responsible for the bacterial inhibitory effect observed during this study.

Bacteria isolated and identified by API analysis from the different process water samples collected at the Sishen Iron-Ore Mine, are listed in Table 3. *Aeromonas hydrophila* was found to be the dominant bacterial species in all the process water samples from the mine.

TABLE 3
Bacteria isolated and identified from the different process water samples collected at the Sishen Iron-Ore Mine

Identification of isolated bacteria	Average bacterial count (cfu·mℓ$^{-1}$)
Water from the process dam	
Aeromonas hydrophila	4.67×10^3
Alcaligenes faecalis	3.21×10^2
Brevundimonas vesicularis	1.39×10^2
Acinetobacter junii	0.33×10^1
Water flowing from the slimes dam	
Aeromonas hydrophila	7.99×10^2
Pantoea spp.	7.01×10^1
Flavobacterium meningosepticum	5.35×10^1
Water flowing to the slimes dam	
Aeromonas hydrophila	1.08×10^3
Chryseomonas luteola	1.72×10^2
Enterobacter sakazakii	8.60×10^1

Comparing the Simpson's Index of Diversity (1-*D*) calculated for the three process water samples, it is evident that the bacterial diversity is greatest in the water flowing to the slimes dam (0.3279), followed by the water flowing from the slimes dam (0.2415) and water from the process dam (0.1677). Although the species richness of the water from the process dam is the highest (4), the population is dominated by *A. hydrophila*. The species richness of both the water flowing to and from the slimes dam was found to be 3, and the bacterial population was dominated to a lesser extent by *A. hydrophila* compared to the water from the process dam. This suggests that the species are more evenly distributed in both the water flowing to and from the slimes dam, compared to the water from the process dam.

Aeromonas hydrophila is a Gram-negative ubiquitous aquatic bacterium, which has been isolated from a wide range of water sources, such as river water, drinking water, as well as water distribution pipe biofilms (Havelaar et al., 1990; Chauret et al., 2001; Lynch et al., 2002; Bomo et al., 2004; Canals et al., 2006). *A. hydrophila* has been found to persist in chlorinated drinking water supplies as a result of biofilm production within distribution pipe systems (Fernandez et al., 2000; Bomo et al.,

2004). *A. hydrophila* is able to produce cytotoxins and enterotoxins that are often associated with acute gastroenteritis, as well as wound infections in humans, and less commonly associated with septicaemia of immunocompromised patients (Janda and Abbott, 1998; Fernandez et al., 2000). In addition, *A. hydrophila* could also be pathogenic to fish, reptiles and amphibians, causing haemorrhagic septicaemia (Fernandez et al., 2000). The pathogenicity of *A. hydrophila* has been associated with toxins, proteases, outer membrane proteins, lipopolysaccharides and flagella (Merino et al., 1996; Negueras et al., 2000; Rabaan et al., 2001; Canals et al., 2006). *Alcaligenes faecalis* is a heterotrophic nitrifying bacterium, which is commonly found in wastewater treatment systems where it is used for the removal of nitrogen from wastewater (Nishio et al., 1998; Kim et al., 2004; Joo et al., 2007). The phenol-degrading ability of *Alcaligenes faecalis* in wastewater sediments has also been documented (Tong et al., 1998). *Brevundimonas* (formerly *Pseudomonas*) *vesicularis* is an aerobic, non-sporulating and non-fermenting Gram-negative bacillus (Segers et al., 1994; Gilligan et al., 2003), which has been isolated from soil, bottled mineral water and hydrotherapy pools (Aspinall and Graham, 1989; Morais and Da Costa, 1990; Davis et al., 1994). *Brevundimonas vesicularis* is an opportunistic human pathogen, which has mostly been associated with infections due to the immunocompromised state caused by underlying diseases, such as autoimmune disorders associated with long-term steroid use, end-stage renal disease treated by haemodialysis, and sickle cell anaemia with functional asplenia (Gilad et al., 2000; Chi et al., 2004; Choi et al., 2006; Sofer et al., 2007). *Pantoea* spp. belong to the *Enterobacteriaceae* family and are important pathogens causing intestinal and systemic illness in humans and animals, and are commonly found in domestic wastewater sludge (Vacca et al., 2005; Chale-Matsau and Snyman, 2006). *Chryseobacterium* (formerly *Flavobacterium*) *meningosepticum* is an opportunistic pathogen often responsible for nosocomial infections associated with water systems in hospitals (Squier et al., 2000). *Chryseobacterium meningosepticum,* which is typically found in water and soil, generally has low pathogenicity, but may be of clinical importance in immunocompromised patients, often causing neonatal meningitis with a high mortality rate (Ratner, 1984; Sheridan et al., 1993). *Chryseomonas luteola* has been implicated in the biosorption of chromium from industrial wastewater (Ozdemir and Baysal, 2004). *Chryseomonas luteola* was isolated from the water flowing to the slimes dam, containing the highest levels of chromium (0.24 mg·ℓ$^{-1}$) (Table 1). Therefore, the possibility exists to use *C. luteola* during water treatment to remove the chromium from the wastewater of the mine.

DNA was successfully extracted from all pure cultures isolated from the groundwater samples collected from the Sishen Iron-Ore Mine. The PCR of total DNA extracted from the pure cultures isolated from the groundwater samples yielded a ca. 500bp fragment of PCR product on a 1% TAE agarose gel. No protein contamination in the walls of the gel or RNA contamination smears below the DNA bands was observed. The phylogenetic analysis of the 16S eubacterial gene of the rDNA operon of the bacteria isolated from the groundwater of the mine is illustrated in Fig. 1. The phylogenetic analysis indicated that 5 of the bacterial isolates from the groundwater samples have a strong sequence comparison with *Pseudomonas aeruginosa* (Verce et al., 2000) and a further 7 bacterial isolates a strong sequence comparison with a *Herbaspirillum* species (Probian et al., 2003).

Herbaspirillum species have previously been isolated from groundwater systems (Connon et al., 2005). These micro-

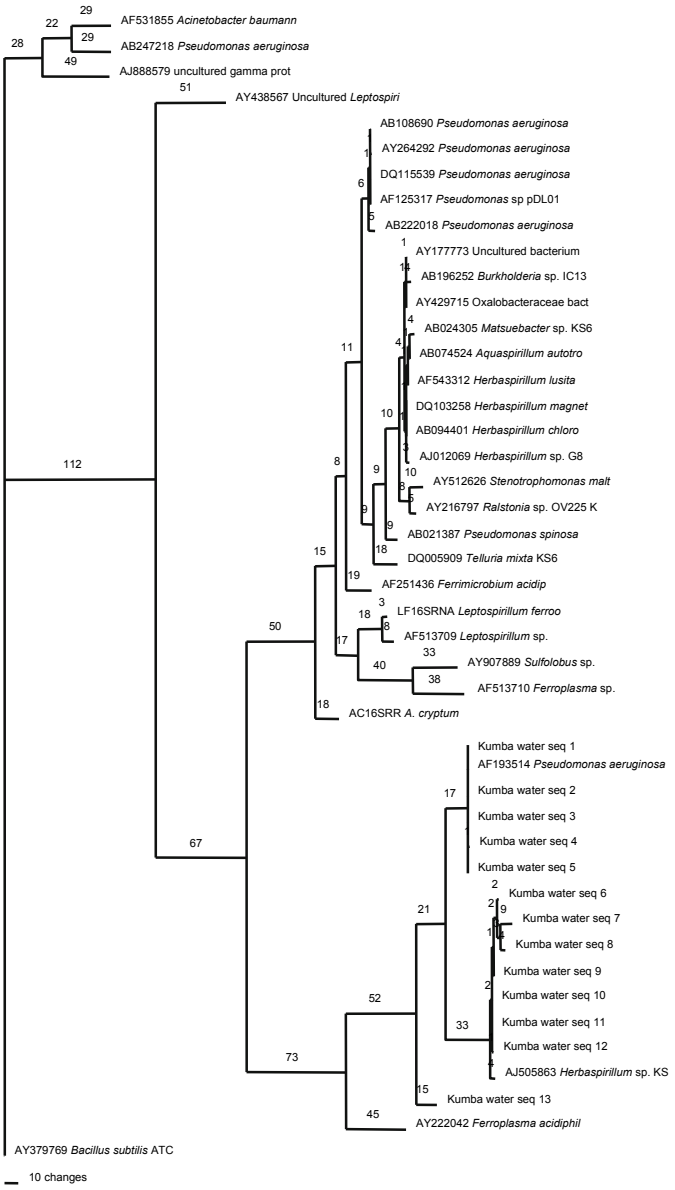

Figure 1
Phylogenetic analysis of the 16S Eubacterial gene of the rDNA operon of the bacteria isolated from the groundwater of the Sishen Iron-Ore Mine

organisms are able to exist in groundwater systems where nitrogen limitation prevails, due to their ability to fix nitrogen (Elbeltagy et al., 2001; Kirchhof et al., 2001; Connon et al., 2005). The fact that *Herbaspirillum magnetovibrio* was identified as surviving in the iron-ore (Williams and Cloete, 2008), and that nitrogen (~11 mg·ℓ$^{-1}$) is limited in the groundwater, may explain why this bacterium is able to exist in the groundwater of the Sishen Iron-Ore Mine.

Pseudomonas aeruginosa is a Gram-negative rod-like bacterium, which is ubiquitous in soil and water, and commonly detected in great amounts in sewage contaminated by humans and animals, although its main habitat remains controversial (Pellett et al., 1983; Römling et al., 1994; Todar, 2004). In nature, *P. aeruginosa* may be found in surface biofilms, or in a planktonic form, actively swimming by means of a single polar flagellum (Sauer et al., 2002; Todar, 2004). Taking into account the low nutritional content in the groundwater of the Sishen Iron-Ore Mine (Table 1), it is not surprising to isolate

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P. aeruginosa, as this bacterial species has very simple nutritional requirements (Todar, 2004). *Pseudomonas aeruginosa* is an opportunistic pathogen in humans and a major cause of nosocomial infection (Khan and Cerniglia, 1994; Römling et al., 1994), where it may cause urinary tract infections, acute respiratory illness (ARI), dermatitis, soft tissue infections, bacteraemia, bone and joint infections, acute gastrointestinal illness (AGI) and a variety of systemic infections, particularly in immunocompromised patients (Fegan et al., 1990; Hirarkata et al., 1991; Furuya et al., 1993; Todar, 2004; US Dept of Health and Human Services, 2006).

Conclusions

Except for *C. luteola* which may be used for the biosorption of chromium from wastewater, there is no indication of bioleaching properties for any of the micro-organisms isolated in the process- and groundwater systems, and therefore, they should be excluded as bioleaching candidates for the removal of undesirable substances from the iron ore of the Sishen Iron-Ore Mine.

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