Bioremediation of Arsenic Contaminated Groundwater

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

By

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January 2008

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Abstract

Sulphate-reducing bacteria (SRB) mediate the reduction of metals/metalloids directly or indirectly. Bioremediation of arsenic contaminated water could be a cost-effective process provided a cheap carbon source is used. To this end, molasses was tested as a possible source of carbon for the growth of sulphate-reducing bacteria (SRB). Its chemical composition and the tolerance of SRB toward different arsenic species [As (III) and As (V)] were also investigated. Batch culture studies were carried out to assess 1, 2.5 and 5 g l^{-1} molasses as suitable concentrations for SRB growth. The results indicate that molasses does support SRB growth, the level of response being dependent on the concentration; however, growth on molasses was not as good as that obtained when lactate, the usual carbon source for SRB, was used.

The molasses used in this study contained several metals including Al, As, Cu, Fe, Mn and Zn in concentrations ranging from 0.54-19.7 μg g^{-1}, but these levels were not toxic to the SRB. Arsenic tolerance, growth response and sulphate-reducing activity of the SRB were investigated using arsenite and arsenate solutions at final concentrations of 1, 5 and 20 mg l^{-1} for each species. The results revealed that very little SRB growth occurred at concentrations of 20 mg l^{-1} As (III) or As (V). At lower concentrations, the SRB grew better in As (V) than in As (III).

Batch cultures of sulphate-reducing bacteria (SRB) in flasks containing pine bark, sand and polystyrene as support matrices and Postgate medium B were used to study formation of biofilms. The effects of the support matrices on the growth of the organisms were evaluated on the basis of pH and redox potential change and the levels of sulphide production and sulphate reduction. Characterisation of the matrix surfaces was done by means of environmental scanning electron microscopy (ESEM).

A consortium of SRB growing on polystyrene caused a 49% of original sulphate reduction whereas on sand a 36% reduction occurred. Polystyrene was further examined for its durability as a long-term support material for the growing of SRB in the presence of As(III) and/or As(V) at concentrations of 1, 5 and 20 mg l^{-1}. Both sulphate reduction and sulphide

production were greater in this immobilised system than in the matrix-free control cultures. With pine bark as support matrix no significant sulphate reduction was observed. The kinetics of sulphate reduction by the immobilised cells were compared with those of planktonic SRB and found to be superior.

The leaching of organic compounds, particularly phenolic substances, from the pine bark had a detrimental effect on the growth of the SRB. Different proportions of pine bark extract were used to prepare media to investigate this problem. Growth of SRB was totally inhibited when 100% pine bark extract was used. Analysis of these extracts showed the concentration of phenolics increased from 0.33 mg l^{-1} to 7.36 mg l^{-1} over the extraction interval of 15 min to 5 days. Digested samples of pine bark also showed the presence of heavy metals.

The effects of nitrate, iron and sulphate and combinations thereof were investigated on the growth of a mixed culture of sulphate-reducing bacteria (SRB). The addition of 30 mg l^{-1} nitrate does not inhibit the production of sulphide by SRB when either 50 or 150 mg l^{-1} sulphate was present. The redox potential was decreased from 204 to -239 mV at the end of the 14 day batch experiment in the presence of 150 mg l^{-1} sulphate and 30 mg l^{-1} nitrate. The sulphate reduction activity of the SRB in the presence of 30 mg l^{-1} nitrate and 100 mg l^{-1} iron was about 42% of original sulphate, while if no iron was added, the reduction was only 34%. In the presence of 20 mg l^{-1} either As(III) or As(V), but particularly the former, growth of the SRB was inhibited when the cells were cultured in modified Postgate medium in the presence of 30 mg l^{-1} nitrate.

The bioremoval of arsenic species [As(III) or As(V)] in the presence of mixed cultures of sulphate-reducing bacteria was investigated. During growth of a mixed SRB culture adapted to 0.1 mg l^{-1} arsenic species through repeated sub-culturing, 1 mg l^{-1} of either As(III) or As(V) was reduced to 0.3 and 0.13 mg l^{-1}, respectively. Sorption experiments on the precipitate produced by batch cultured sulphate-reducing bacteria (SRB-PP) indicated a removal of about 77% and 55% of As(V) and As(III) respectively under the following conditions: pH 6.9; biomass (2 g l^{-1}); 24 h contact time; initial arsenic concentration,

1 mg l^{-1} of either species. These results were compared with synthetic iron sulphide as adsorbent. The adsorption data were fitted to Langmuir and Freundlich isotherms. Energy dispersive x-ray (EDX) analysis showed the SRB-PP contained elements such as sulphur, iron, calcium and phosphorus. Biosorption studies indicated that SRB cell pellets removed about 6.6% of the As(III) and 10.5% of the As(V) from water containing an initial concentration of 1 mg l^{-1} of either arsenic species after 24 h contact. Arsenic species were precipitated out of synthetic arsenic-contaminated groundwater by reacting it with the gaseous biogenic hydrogen sulphide generated during the growth of SRB. The percentage removal of arsenic species was dependent on the initial arsenic concentration present.

Lastly, laboratory scale bioreactors were used to investigate the treatment of arsenic species contaminated synthetic groundwater. A mixed culture of SRB with molasses as a carbon source was immobilised on a polystyrene support matrix. The synthetic groundwater contained either As(III) or As(V) at concentrations of 20, 10, 5, 1 or 0.1 mg l^{-1} as well as 0.1 mg l^{-1} of a mixture with As(III) accounting for 20, 30, 40, 60 and 80% of the total. More that 90% and 60% of the As(V) and As(III) respectively were removed by the end of the 14-day experiment. At an initial concentration of 0.1 mg l^{-1} total arsenic had been reduced to below the WHO acceptable level of 10 μg l^{-1} when the proportion of As(III) was 20 and 30%, while at 40% As(III) this level was reached only when the treatment time was increased to 21 days. The efficiency of As(III) removal was increased by first oxidising it to As(V) using MnO_2.

Declaration

I hereby certify that this thesis is the result of my own investigations unless specifically indicated in the text and has not been submitted for a higher degree at any other institution.

Signed ……………………..…………………

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We hereby certify that this statement is correct.

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January, 2008

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Literature Review

1.1   Introduction

The availability of clean drinking water around the world is decreasing due to the increasing human population. Surface waters cannot alone satisfy the demands for pure drinking water. This leads to the use of groundwater resources in many parts of the world. The extensive use of groundwater as drinking water is causing a global epidemic of arsenic poisoning (e.g., in Bangladesh, India, Chile and other countries) (Nordstrom, 2002).

A situation that occurred in Bangladesh with regard to arsenic poisoning is worth mentioning here. In order to control waterborne diseases brought about by drinking contaminated surface water, the World Bank and UNICEF (United Nations Children's Fund) provided funds to sink tube-wells into the aquifers that lie beneath the Bangladeshi Delta. As many as 10 million wells were sunk in the 1990s (Clarke, 2003). But, the assumed clean drinking water turned out to be arsenic tainted. Some of the wells contain about 400 times the WHO (World Health Organisation) maximum permissible level of arsenic for safe drinking (Clarke, 2003).

Arsenic contamination levels in Africa are not shown in scientific papers except for Ghana; however, this does not mean that there is no arsenic contamination of groundwater in Africa. At the 8th World Congress on Environmental Health held in Durban, South Africa, it was noted that many African countries see environmental issues as "non-issues", relative to their other pressing problems (Carnie, 2004). Professor Jerome Nriagu, a researcher from the University of Michigan, said at the conference that the scientific literature is silent on arsenic contamination of groundwater in Africa and predicts that many communities on the continent are ingesting arsenic from groundwaters containing well above the safety threshold level.

A survey by Sami and Druzynski (2003) on the distribution of naturally occurring arsenic, selenium and uranium in South Africa's groundwater found that little documentation exists on the geological occurrence of arsenic.

Arsenic is a trace metal that ranks 20th in abundance in the Earth's crust (Jolly, 1966), and is found associated with igneous and sedimentary rocks (Léonard, 1991). Arsenic can be easily mobilised in groundwater, depending on pH, redox conditions, temperature, bedrock type, and solution composition. There have been several different mechanisms suggested for the mobilisation of arsenic. One is the oxidation and dissolution of arsenic pyrite (FeAs) and arsenopyrite (FeAsS). The oxidation is facilitated by infiltrating oxygenated water, or through the lowering of the groundwater table caused by irrigation, or because of climatic variations. Reductive dissolution of arsenic-rich, iron oxyhydroxides (FeOOH) can also give rise to high arsenic concentrations in groundwater. There is also a growing body of research on the mobilisation of arsenic that is mediated by microbial activity. Conversely, bioremediation of arsenic contaminated water by microbes may also provide us with a solution to the problem.

Arsenic is a human carcinogen. Consuming drinking water that contains arsenic at high levels has been found to increase the risk of skin cancer and tumours of the bladder, kidneys, liver and lungs (NRC, 1999). Both long- and short-term exposure to high arsenic levels have their own health implication, e.g., thickening and discoloration of the skin, numbness in the hands and feet, muscular cramping or pain, and other health problems. The WHO has set an upper limit for the concentration of arsenic in drinking water at 10 μg l^{-1} (WHO, 1993).

Different techniques that have been used for the treatment of arsenic contaminated water include co-precipitation, sorption and membrane separation (USEPA, 2000b). These techniques have their respective advantages and disadvantages. Major disadvantages include high cost, technologically complex operation and maintenance, and generation of toxic sludges. It is thus imperative in Third World countries that are at risk, to develop an

efficient and low cost bioremediation technique that can remove arsenic species to the level of the WHO standard.

1.2 Arsenic

Arsenic (As) is a metalloid with atomic number 33 and atomic mass 74.9216 that belongs to group 15 of the periodic table, below phosphorus and above antimony. Arsenic is a ubiquitous element present in soil, water, air and in all living matter (Tamaki & Frankenberger, 1992). Arsenic ranks 20th in abundance in the Earth's crust, 14th in seawater and 12th in the human body (Mandal & Suzuki, 2002).

Common sources of arsenic in nature include volcanic activity, rock weathering, biological activity (Cullen & Reimer, 1989), marine sedimentary rocks (Smedley & Kinniburgh, 2002) and fossil fuels, including coal and petroleum (Smedley & Kinniburgh, 2002). In terrestrial environments arsenic is also associated with igneous and sedimentary rocks (Cullen & Reimer, 1989) and in sulphidic minerals in the form of arsenides of nickel, cobalt, copper and iron. Common arsenic-bearing ores are arsenopyrite (FeAsS) [most common and widespread (O'Neil, 1995)], enargite (Cu_3AsS_4), orpiment (As_2S_3), realgar (As_4S_4) (Tamaki & Frankenberger, 1992) and also lolingite ($FeAs_2$), chloanthite ($NiAs_2$), niciolite (NiAs), cobalite (CoAsS), gersdorffite (NiAsS), tennantite ($Cu_{12}As_4S1_3$) and proustite (Ag_3AsS_3) (Ferguson, 1990).

Arsenic occurs in the environment in the oxidation states –3 (arsine), 0 (semi-elemental arsenic), +3 (arsenite) and +5 (arsenate) (Léonard, 1991). The chemical nature of arsenic is dominated by its behaviour of changing its oxidation states or chemical form due to chemical or biological reactions that are common in the environment.

The source of arsenic in groundwater is usually geogenic, although anthropogenic arsenic pollution does occur. The following chemical structures show the differences in molecular structure between arsenite and arsenate that are the most common forms of arsenic in groundwater.

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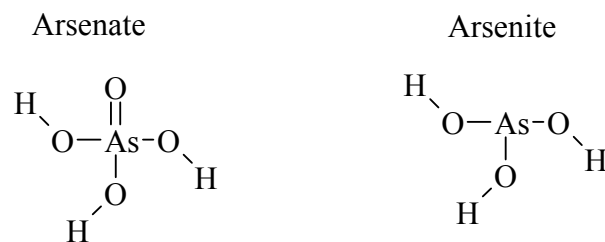
Arsenate   Arsenite

Figure 1.1   Molecular structures of arsenate and arsenite.

Under reducing conditions, arsenite is the dominant form while arsenate is generally stable in oxygenated environments.

Figure 1.2 shows the input of arsenic to aquatic ecosystem environment and the global arsenic cycle (Langdon *et al*., 2003; Matschullat, 2000).
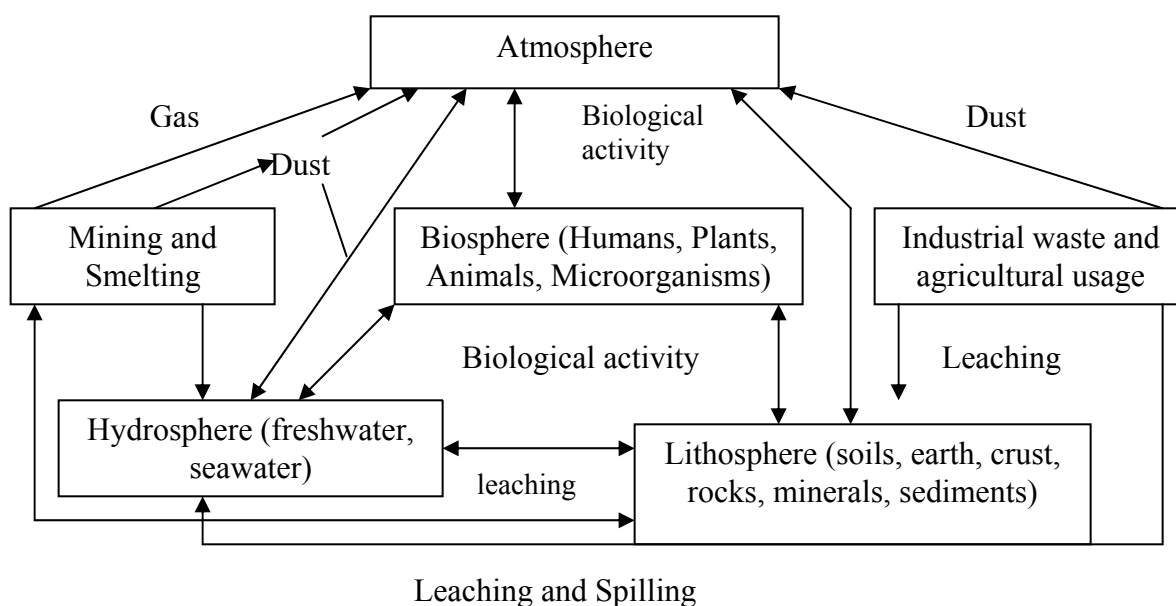
Figure 1.2   The global arsenic cycle (Langdon *et al*., 2003; Matschullat, 2000).

Several analytical methods are available for the determination of arsenic species. These include: atomic absorption spectrometry (AAS); atomic fluorescence spectrometry (AFS); and inductively coupled plasma–atomic emission spectrometry (ICP-AES). These can be coupled to chromatographic separation techniques, e.g., high performance liquid

chromatography (HPLC) and gas chromatography (GC). A hydride generation technique can also be used for the determination of arsenic speciation.

1.2.1   Chemical and Physical Properties of Arsenic

Arsenic is a bright silver-gray metal which forms trigonal crystals and has an average hardness of 3-4 on the Mohs scale. Modification variations include yellow arsenic and three amorphous forms. Some of the physical constants of arsenic are as follows (Ullman, 1985):

Density: Metal arsenic 5.72 g cm^{-3} at 20°C, yellow arsenic 2.03 g cm^{-3}

Melting point: 1090 K (817°C) at 3.7 MPa

Sublimation point: 886 K (613°C) at 0.1 MPa

Potential of arsenic with respect to hydrogen gas: 0.24 V

1.3   Arsenic in Groundwater

1.3.1   Introduction

As mentioned previously, the presence of arsenic in groundwater often arises from geogenic sources mainly in the forms of arsenite and arsenate. Anthropogenic sources may also have an impact on the level of arsenic which can take any form including organic arsenic species. The anthropogenic and natural sources of arsenic in groundwater are discussed separately below.

1.3.2   Occurrence Due to Anthropogenic Sources

Anthropogenic sources of arsenic in the environment include the manufacturing of arsenic based compounds, the application of arsenic compounds in agriculture as pesticides and insecticides; mining and smelting of arsenic containing ores; waste discharging from industries (e.g., tanneries), combustion of fossil fuels, landfilling of industrial wastes, and disposal of chemical warfare agents (Goh & Lim, 2005). It was suggested (Peryea, 1991) that arsenic in topsoil could move into the subsoil and contaminate groundwater where the water table is shallow.

In soil contaminated with lead arsenate pesticides, the potential exists for release of arsenic into the groundwater if phosphate fertiliser is applied (Davenport & Peryea, 1991; Woolson *et al*. 1973). Moreover, the application of phosphate fertiliser to uncontaminated water could release adsorbed arsenic into the groundwater (Welch *et. al*., 2000).

Swine and poultry wastes, where the feed contained arsenic, might contaminate groundwater. Other anthropogenic sources that are responsible for arsenic contamination of groundwater are the co-disposal of arsenical wastes with municipal wastes (Blakey, 1984) and arsenic in phosphate detergents (Angino *et al*., 1970).

1.3.3 Occurrence due to Natural Sources

Arsenic in groundwater may be due to atmospheric precipitation, surface water and aquifer materials. In the absence of anthropogenic sources, precipitation contributes little to arsenic levels in groundwater. Geothermal waters contain higher concentrations of arsenic (Stauffer & Thompson, 1984) than do non-thermal waters (Welch *et al*., 1988).

1.4 Distribution of Arsenic

1.4.1 World

It has been estimated that about one-third of the world's population depends on groundwater for drinking (UNEP, 1999).

The level of arsenic in groundwater differs from country to country worldwide. In Europe, several countries such as Greece, Finland, Italy and Hungary have to deal with arsenic contaminated groundwater that is used as drinking water; however, in Bangladesh and India, arsenic concentrations in the groundwater have been reported to reach levels as high as 1 mg l^{-1} (Nordstrom, 2002). The European Commission (EC) has set the maximum contamination level (MCL) of arsenic in drinking water at 10 μg l^{-1} (down from 50 μg l^{-1} in 2003) to be complied to by all European Union countries (EC, 1998). Similarly, USEPA (United States Environmental Protection Agency) decided that the permissible level of arsenic in drinking water supply systems in the US be reduced to 10 μg l^{-1} in 2006

(USEPA, 2002a). The World Health Organisation has also recommended the same level (WHO, 1996).

Bangladesh and India

The presence of arsenic in the Bangladesh groundwater is considered one of the most serious environmental disasters in the world (Ahmed *et al*., 2004). Arsenic in the sedimentary aquifers of the Bengal Delta Plain in Bangladesh and West Bengal in India is mobilised by natural processes (Kinniburgh & Smedley, 2001). A population of more than 150 million people uses groundwater from various Bengal Delta Plain aquifers for drinking. Moreover, groundwater in this region is extensively used for cultivation of rice and other crops so people living in the region ingest arsenic from both their food and drink (Roychowdhruy *et al*., 2002; Huq *et al*., 2000). The high concentrations of arsenic in this region is not from anthropogenic sources, but are due to geogenic factors related to the geological environment of the Holocene Bengal Delta Plain aquifer system (Nickson *et al*., 1998; Bhattacharya *et al*., 1997).

Argentina

Most people in rural areas of Argentina depend on groundwater that contains high arsenic concentrations that exceed the Argentine drinking water standards of 0.05 mg l^{-1} (Bundschuh *et al*., 2004). The most affected areas within the country are found in the Chaco-Pampean Plain of Robles County in the province of Santiago Del Estreo.

Mexico

Different regions in Mexico (e.g., Zimapán, Andoctiun) have groundwaters that are contaminated with arsenic (Rodríguez *et al*., 2004). The increased arsenic concentration in the Mexican groundwater may be due to oxidation conditions induced by local rainfall. When water flows through fractures in the rock, it will increase oxidation thereby enhancing the incorporation of arsenic into the groundwater.

U.S.A.

High arsenic concentrations (>10 μg l^{-1}) in groundwater have been documented in large areas of the the United States where the aquifers are influenced by geochemical sources and evapoconcentration of surface water (Welch *et al.*, 2000). In some areas, groundwater from private wells has arsenic concentrations more than 100 times greater than the drinking water standard of 10 μg l^{-1} (Shiber, 2005).

1.4.2   Africa

No regions in Africa are identified as having high concentrations of arsenic in their groundwater and its associated health problems (Smedley, 1996). In Africa, arsenic may be associated with sulphide mining activity and can cause a localised arsenic problem. But few studies have been carried out on arsenic in groundwater from areas with mineralised basement rocks, several of which have been subjected to metalliferous mineralisation (Smedley *et al.*, 2007).

The widespread use of arsenic-containing herbicides in Africa (e.g., monosodium methanearsonate (MSMA), Masamar, cacodylic acid) and wood treatment chemicals [e.g., chromated copper arsenate (CCA)] that contain arsenic, together with mining activities, could be a source of arsenic in groundwater (Prof. M.D. Laing, Personal Communication, September 08, 2004).

Since groundwater is the major source of drinking water in many parts of Africa (areas with crystalline basement rock), especially in arid areas, the quality of groundwater in these aquifers is of paramount importance to human life (Smedley *et al.*, 2007). The following country-specific information details the contamination levels and the danger of arsenic poisoning in some African countries. Except for South Africa, the study was conducted by the British Geological Survey.

Burkina Faso

Even though the extent and scale of arsenic contamination in groundwater is not well defined, the problem has been identified in some parts of the country. Smedley *et al.* (2007)

analysed groundwater samples from hand-pumped boreholes and dug wells close to the town of Ouahigouya in northern Burkina Faso. Most samples analysed had arsenic concentrations of <10 µg l^{-1}, with concentrations ranging from <0.5 µg l^{-1} to 1630 µg l^{-1}. The highest values were obtained in borehole waters.

Ghana

Smedley (1996) investigated the concentration of arsenic in rural groundwater of Obuasi (Ashanti region) and Bolgatanga (Upper East region) in Ghana. The concentrations of arsenic were in the range <1 to 64 µg l^{-1} for the Obuasi area and <1 and 141 µg l^{-1} for the Bolgatanga region. The arsenic in these areas results from sulphide mineralisation of pyrite and arsenopyrite.

Mozambique

Areas where mineralisation associated with gold-bearing ores has occurred are prone to high levels of arsenic contamination. Areas containing young alluvium, river valleys, the Zambezi Delta and the coastal marshes may also be at increased risk.

South Africa

Groundwater use in South Africa is widespread with ⅔ of the rural community being highly dependent on groundwater. Some of the groundwater sources are situated in geological units known, or suspected, to contain trace elements including arsenic which has affinity to gold, copper, nickel, zinc, lead, cobalt, silver and other metals and their ores. Therefore, the dissolution of sulphide minerals containing arsenic could be the source of this element in South African waters.

The existence of high levels of uranium, arsenic and fluoride in groundwater taken from aquifers in the Pofadder area, North Western Cape was reported by Tones *et al.*, 1998. The study further showed the positive correlation between elevated levels of uranium and arsenic in groundwater and haematological anomalies related to leukaemia.

Until recently, South African drinking water sources have not been routinely analysed for arsenic. In the Northern Cape and Limpopo provinces arsenic concentrations of more than 1000 μg l^{-1} have been recorded. Hence, a large-scale monitoring program is needed (Sami & Druzynski, 2003).

Tanzania

The groundwaters in most areas of Tanzania have low arsenic concentrations; however, in the Rift zones where groundwater pH values, alkalinity and fluoride concentration are high and also in areas where groundwaters interact with hot springs, elevated levels of arsenic may occur. Unfortunately, no reliable data are available.

Uganda

Little data is available for arsenic concentrations in Ugandan groundwater. It is becoming imperative that testing of groundwater from the East African Rift be carried out to assess if an arsenic problem exists.

Zambia

High concentrations of arsenic may be found in areas where sulphide mineralisation is prominent and mining activities exist (e.g., the copper belt).

Table 1.1 shows the worldwide distribution of arsenic with human populations potentially exposed thereto and the environmental conditions responsible for the elevated arsenic concentrations detected.

Table 1.1 Global levels of arsenic contaminations in groundwater (after Nordstrom, 2002)

Country/region	Potential exposed population	Concentration ($\mu g\ l^{-1}$)	Environmental conditions
Bangladesh	30 000 000	< 1 to 2 500	Natural; alluvial/deltaic sediments with high phosphate, organics
West Bengal, India	6 000 000	< 10 to 3 200	Similar to Bangladesh
Vietnam	> 1 000 000	1 to 3 050	Natural; alluvial sediments
Xinjiang, Shanxi	> 500	40 to 750	Natural; alluvial sediments
Argentina	2 000 000	< 1 to 9 900	Natural; loess & volcanic rocks
Mexico	400 000	8 to 620	Natural & anthropogenic; volcanic sediments, mining
Germany		< 10 to 150	Natural; mineralised sandstone
Ghana	< 100 000	< 1 to 175	Anthropogenic & natural; gold mining
USA and Canada		< 1 to > 100 000	Natural & anthropogenic

1.5   Uses of Arsenic

The demand for elemental arsenic is limited. The main use for metallic arsenic is in the manufacture of nonferrous alloys; high purity arsenic is also used in electronic and semiconductor devices. The addition of about 0.5% arsenic to the lead grid in lead-acid storage batteries increases endurance and corrosion resistance. Similarly, the same amount of arsenic in copper alloys improves high temperature stability and corrosion resistance

(Ullmann, 1985). Diodes (LED), infrared detectors, and lasers can be produced using high purity arsenic (about 99.9999%) in combination with gallium or indium (Brooks, 2002).

Most arsenic is used in the form of compounds. Arsenic trioxide is usually the starting material for arsenical compounds. The following list gives some of the uses of arsenic compounds.

1. Forestry – the production of wood preservatives is the main application of arsenic compounds. The product is chromated copper arsenate (CCA) which is an effective wood preservative that is hard to replace. A number of countries have banned its use (e.g., USA), but, it is still widely used in different regions, including Africa.

2. Agriculture – Arsenical compounds are used as herbicides and insecticides on agricultural land. Monosodium methanearsonate (MSMA) was the primary form of arsenic applied to cotton, coffee and rice plantations (Adriano, 1986; Ullmann, 1985). Relatively small quantities of disodium methanearsonate (DMSA) and dimethyl arsinic acid (cacodylic acid) (DMAA) have also been used. Lead arsenate (PbHAsO$_4$) was used as an insecticide in fruit orchards before the introduction of DDT in 1947 (Shepard, 1951).

3. Glass industry – Arsenic compounds can be used as fining agents and decolourisers. As$_4$S$_4$ gives a red colour to glass.

4. Feed additives – Arsanilic acid and roxarsone (3-nitro-4-hydroxyphenyl arsonic acid) were added to increase rate of weight gain and improve feed efficiency in chickens and swine, and to control swine dysentery.

5. Organoarsenic compounds are used in the treatment of diseases (sleeping sickness), amebiasis and non-parasitic skin diseases in animals (Smith, 1973).

6. Arsenic oxide is also used as a depilatory in the production of fine leather.

The estimated worldwide production of arsenic trioxide from 1998 to 2002 is shown in Table 1.2.

Table 1.2 Estimated world production of arsenic trioxide in metric tons[1,2] (after Brooks, 2002)

Country[3]	1998	1999	2000	2001	2002
Belgium	1 500	1 500	1 500	1 000	1 000
Bolivia	284[4]	437[4]	318[4]	846[r,4]	850
Canada	250	250	250	250	250
Chile	8 400[4]	8 000	8 000	8 000	8 000
China	15 500	16 000	16 000	16 000	16 000
France	2 000	1 000	1 000	1 000	1 000
Germany	200	200	200	100	100
Ghana[5]	5 000[4]	7 000	3 000	--	--
Iran	323[4]	300	400	400	400
Japan	40	40	40	40	40
Mexico	2 573[4]	2 419[4]	2 522[4]	2 381[r,4]	2 300
Namibia	175[5]	--[5]	--	--	--
Peru[6]	624[4]	1 611[4]	2 495[4]	1 958[r,4]	2 000
Portugal	50	50	50	50	50
Russia	1 500	1 500	1 500	1 500	1 500
Total	**38 400**	**40 200[r]**	**37 200[r]**	**33 400[r]**	**33 400**

[r]Revised. -- Zero.
[1]World totals and estimated data have been rounded to no more than three significant digits; may not add to totals shown.
[2]Table includes data available through April 1, 2003.
[3]Austria, Hungary, the Republic of Korea, Serbia and Montenegro, South Africa, Spain,the United Kingdom, and Zimbabwe produced arsenic and/or arsenic compounds in previous years, but information is inadequate to make estimates of output levels, if any.
[4]Reported.
[5]Production ceased in mid-2000. Ashanti Goldfields Ltd. Obuasi roaster closed.
[6]Output of Empresa Minera del Centro del Perú (Centromín Perú) as reported by the Ministerio de Energía y Minas.

1.6   Speciation of Arsenic

Speciation of arsenic in environmental samples is important, as the toxic effects of arsenic depend on its oxidation state (Jain & Ali, 2000). Hence, the total concentration of arsenic does not reflect its toxicity, mobility, bioavailability or accumulation (Magnuson *et al*., 1996). The various arsenic species follow different metabolic pathways that affect their toxicity (Cornelis & Kimpe, 1994); therefore, it is important to identify arsenic species in the environment.

Speciation of an element may be defined as the analytical activities of identifying and/or measuring the quantities of one or more individual chemical species (Templeton *et al*., 2000) that make up the total concentration of the element in a given sample (Florence, 1982); however, the identification of element species presents many analytical challenges (Beauchemin *et al*., 1989). Some of the challenges include contamination and loss of the species during sample preparation (Burguera & Burgurea, 1997).

Figure 1.3 shows the chemical formulae for some of the different arsenic species occurring in the environment.
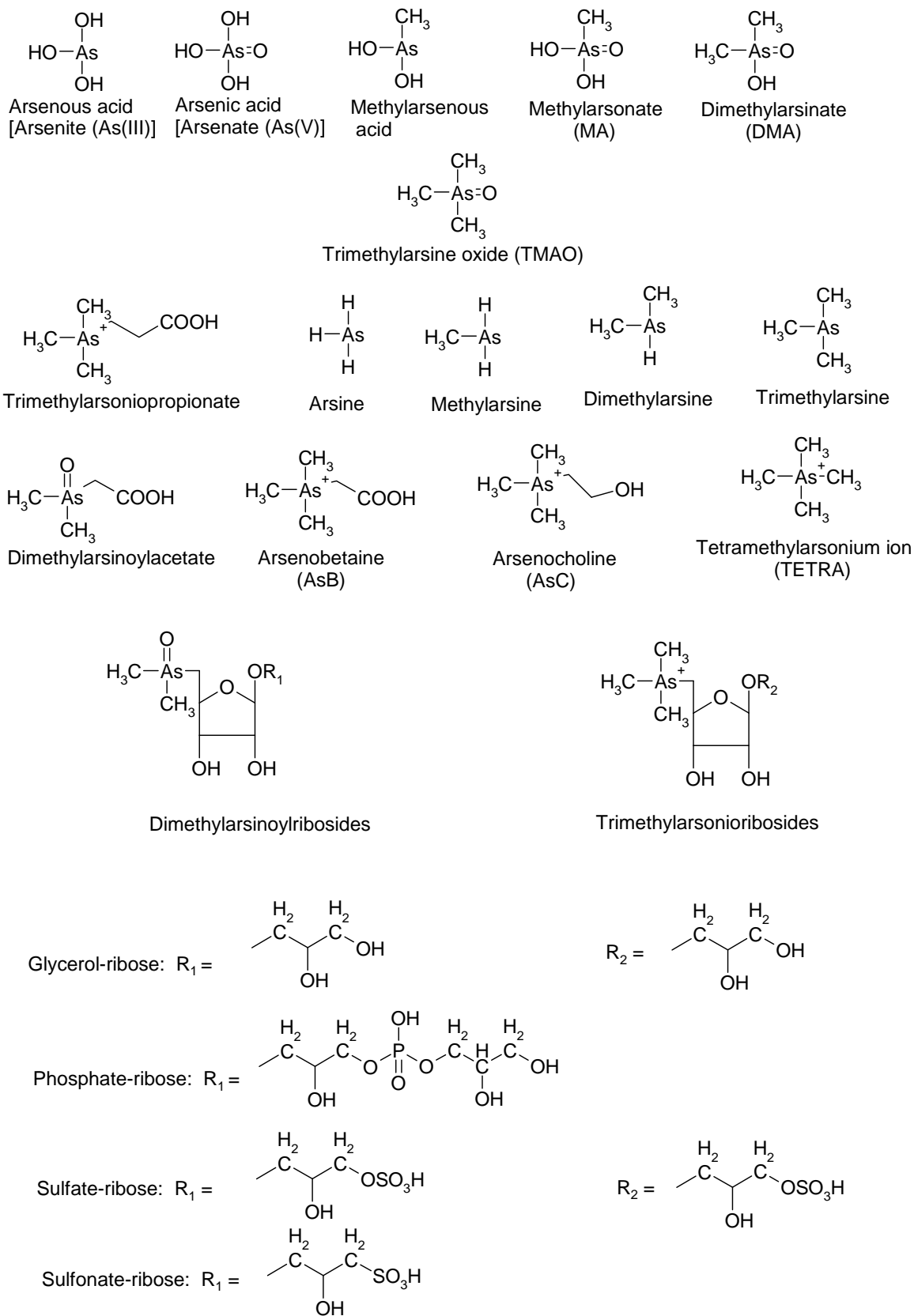
Figure 1.3 Environmental arsenic compounds (after Frankenberger, 2002).

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The double bond in arsenate (Figure 1.1) influences its ability to be ionised through the loss of hydrogen ions. The pK_a constants (tendency for ionisation) for arsenate and arsenite are as follows (O'Neil, 1995):

Arsenate: H_3AsO_4 $pK_1 = 2.2$ $pK_2 = 7.0$ $pK_3 = 11.5$

Arsenite: H_3AsO_3 $pK_1 = 9.2$ $pK_2 = 12.1$ $pK_3 = 13.4$

These ionisation steps occur at different pH values for arsenate and arsenite. The following diagrams show the occurrence of arsenate and arsenite as a function of pH.
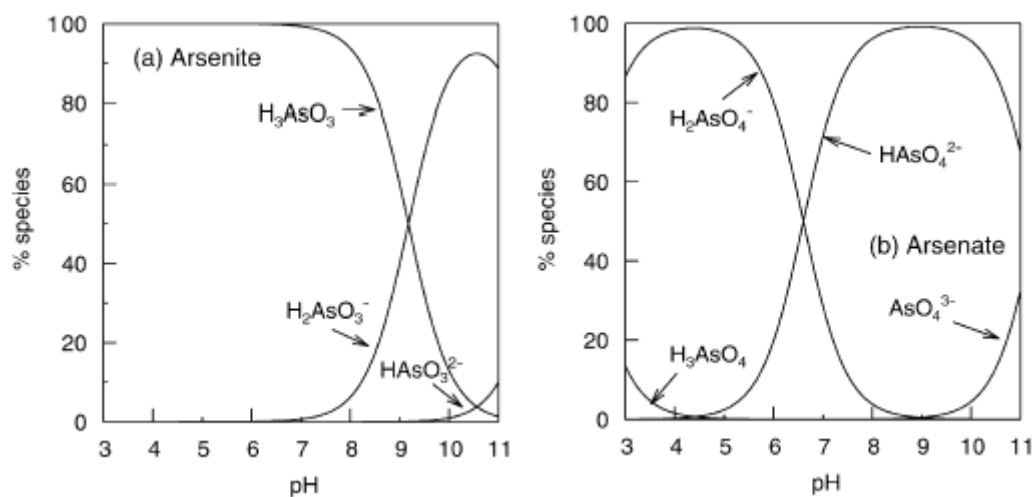
Figure 1.4 Occurrence of arsenite and arsenate as influenced by pH (after Sami & Druzynski, 2003).

From Figure 1.4 it can be seen that dissolved arsenite occurs mainly as the H_3AsO_3 compound at the near neutral pH of most groundwaters and consequently undergoes no sorption or exchange processes (Sami & Druzynski, 2003).

The controls for the distribution and speciation of arsenic in the environment can be identified using geochemical modeling (Cullen & Reimer, 1989). Even though it gives useful predictions of the occurrence, absence, or fate of various dissolved and solid arsenic species in the environment, the use of geochemical modeling is hampered by the lack of full thermodynamic data for the different arsenic species. Anthropogenic sources and

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biological intervention could cause deviation of the predicted distribution of arsenicals (Cullen & Reimer, 1989).

The presence of particular species under specific environment conditions is controlled by several factors particularly pH and redox potential. The predominant soluble species and solid forms in an environment can be identified by drawing redox potential (Eh) versus pH diagrams. In preparing the diagrams, various factors must be considered. The stability diagram for arsenicals in the environment in the presence of oxygen and water, oxygen alone, and water and sulfur are shown below.

A   B
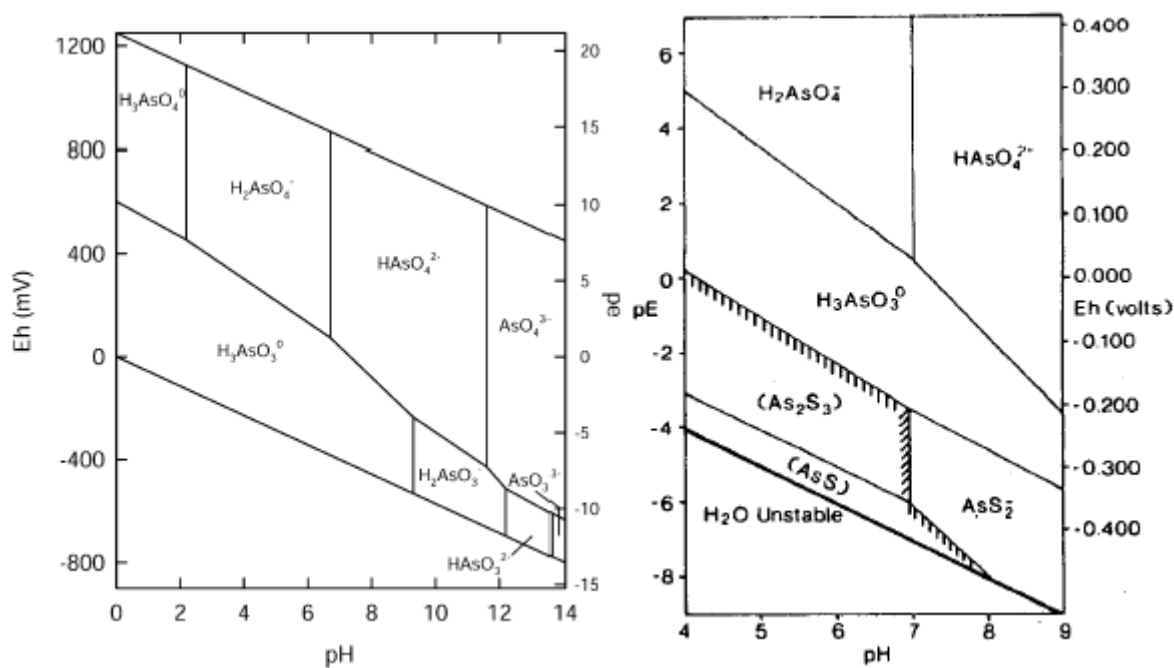
Figure 1.5 (A) pE-pH diagram for the As-H_2O system at 25°C. Total dissolved As species set at 50 μg l^{-1}. (B) pE-pH diagram for the As-S-H_2O system at 25°C with total dissolved As and S species set at 50 μg l^{-1} and 32 mg l^{-1}, respectively. (after Cullen & Reimer, 1989).

From the above diagram, it can be seen that redox potential and pH are the most important factors controlling arsenic speciation (Smedley & Kinniburgh, 2002). At low pH (<6.9) and under oxidising conditions, $H_2AsO_4^-$ is dominant whereas at higher pH, $HAsO_4^{2-}$ will be dominant. At extremely acidic and alkaline conditions, H_3AsO_4 and AsO_4^{3-} may be present,

17

respectively. Under reducing conditions and lower pH, arsenic (III) acid becomes stable, mainly as H_3AsO_3 (Cullen & Reimer, 1989). The transition from arsenite to arsenate occurs at near neutral pH and at an Eh of approximately 0 mV.

In the presence of dissolved sulfur and under stronger reducing conditions, dissolved arsenic-sulphide species may be significant. For example, the formation of orpiment (As_2S_3), realgar (AsS) or other sulphide minerals containing co-precipitated arsenic is favoured under reduced and acidic conditions (Cullen & Reimer, 1989).

1.7   Mobilisation of Arsenic

The unique character of arsenic with respect to its mobilisation as compared to other heavy metalloid oxyanions is its sensitivity to mobilisation over the range of pH found in groundwater (6.5-8.5). The mobilisation of arsenic at any given area could be affected by: redox condition, pH, biological activity, solid-phase precipitation and dissolution reactions; adsorption/desorption reactions, presence of competing anions and complexing ions, salinity, clay content, grain size and composition of the soil and sediment, presence of other metal ions and non metals such as sulphur and phosphorus (Pandey *et al*., 2002). Moreover, the concentration of arsenic can be affected by the interactions between groundwater and rock mass (Sami & Druzynski, 2003). In summary, the mobilisation of arsenic is very complex.

Dissolved arsenate ($H_2AsO_4^-$ and $HAsO_4^{2-}$) compounds sorb readily onto iron, manganese and aluminum oxides or hydroxides, as well as onto clay minerals and organic matter. As a result, free arsenate may be found at low concentrations in groundwater. Conversely, arsenite exists mainly as the neutral compound (H_3AsO_3) and does not undergo sorption or exchange processes (Sami & Druzynski, 2003) and is, therefore, more mobile than arsenate.

Different mechanisms have been suggested for the mobilisation of arsenic in groundwater. The mobilisation and transport of arsenic depends on its speciation since the different forms differ in their aqueous solubility (Cullen & Reimer, 1989).

Generally, mobilisation of arsenic depends on the prevailing redox geochemistry that has importance in the release and subsequent transport of arsenic in groundwater (Bhattacharyya *et al.*, 2003).

Different studies have been conducted to investigate the mechanisms by which arsenic is released into groundwater. Studies on the aquifers of West Bengal and Bangladesh (Bose & Sharma, 2002) and other groundwater sources indicate the following probable mechanisms for arsenic mobility:

1. Arsenic mobilisation by reductive dissolution of iron hydroxides

Arsenic could be derived through the reductive dissolution of arsenic-rich iron oxyhydroxides due to change in Eh-pH conditions (Sami & Druzynski, 2003; Bhattacharya *et al.*, 1997). Iron oxyhydroxides are produced from weathering of a base-metal sulphide (Nickson *et al.*, 1998). Sedimentary iron oxyhydroxides are known to scavenge arsenic (Mok & Wai, 1994). Hence, reduction of iron oxyhydroxide will release the scavenged arsenic, the process being driven by the concentration (up to 6%) of sedimentary organic matter (Nickson, 1997).

The reduction of organic matter under reducing conditions could involve reduction or dissolution of iron oxyhydroxide and ferric hydroxide ($Fe(OH)_3$) to release Fe^{2+} and As^{3+} to the groundwater using the following chemical reactions (Sami & Druzynski, 2003):

$$4Fe(As)OOH + CH_2O + 7H_2CO_3 \rightarrow 4Fe^{2+} (As^{3+}) + 8HCO_3^- + 6H_2O \qquad (1.1)$$
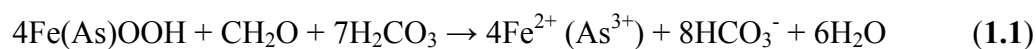
The above reaction shows the positive correlation of arsenic and bicarbonate in anoxic water (McArthur, 1999).

2. Mobilisation of arsenic due to the oxidation of arsenic-bearing pyrite minerals

It has also been reported that arsenic may be mobilised by the oxidation of arsenic-rich pyrite. Oxygen infiltrates the aquifer as a result of lowering of the water level by abstraction (irrigation) or climate variations (Schreiber *et al.*, 2000); however, research

conducted on 46 wells in Bangladesh showed that most oxic (shallow) wells contained below 50 μg l^{-1} arsenic whereas in anoxic waters arsenic concentrations were ≤ 260 μg l^{-1} (Nickson *et al.*, 1998).

3. Arsenic mobilisation by sulphide oxidation

Arsenic is found in many sulphide minerals, and oxidation of these minerals could lead to high concentrations of arsenic in groundwater (Sami & Druyznski, 2003). As stated previously, oxygen may infiltrate to groundwater due to lowering of the water table and thus raises the Eh. The following chemical reaction shows the oxidation of arsenopyrite which releases H$_3$AsO$_4$, Fe^{2+} and sulphate (Sami & Druyznski, 2003).

$$FeAsS + 7/2O_2 + 4H_2O \rightarrow Fe(OH)_3 + H_3AsO_4 + SO_4^{2-} + 2H^+ \tag{1.2}$$
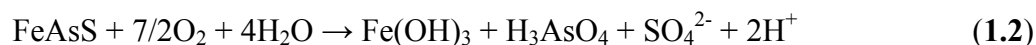
The H$_3$AsO$_4$ will dissociate to H$_2$AsO$_4^-$ at low pH (<6.9) and to HAsO$_4^{2-}$ at higher pH. In the absence of oxygen, nitrate can oxidise pyrite using a microbially mediated reaction. High arsenic concentrations (exceeding 1 000 μg l^{-1}) have been recorded in groundwater where sulphide minerals are present (Schreiber *et al.*, 2000). In South Africa, the mobilisation of arsenic is likely to occur due to mineral sulphide dissolution (Sami & Druyznski, 2003).

4. Acharya *et al.* (1999) suggested release of sorbed arsenic from aquifer minerals by the competitive exchange with phosphate ions that have percolated to the groundwater by application of fertilisers to the soil.

It is possible that the above processes may have been initiated by microbial activities; however, data from the studies of Bose & Sharma (2002) indicate that the redox reactions involving iron and arsenic are predominantly through abiotic pathways. These results do not necessarily mean that abiotic redox processes are dominant, but it is possible for transformations involving iron and arsenic to occur in anoxic surface environments.

Guo *et al.* (2003a) discussed the anomalies related to arsenic groundwater chemical characteristics and enrichment in the groundwater. High pH, high concentrations of phosphate and organic matter, and an anoxic environment were the main factors responsible for the release of adsorbed arsenic in aquifers. In another study by Guo *et al.* (2003b) on the mechanism of arsenic release to shallow groundwater, it was found that groundwaters with high arsenic levels contained low concentrations of sulphate and nitrate. This was due to the microbial metabolism of sedimentary organic matter, which provides as much as 1% organic carbon. The microbial activity decreases Eh and produces CO_2, which helps the dissolution of carbonates and increases pH; therefore, the clay minerals and colloids in groundwater that had previously scavenged arsenic, release the absorbed arsenic to the groundwater at lower Eh and higher pH.

Anawar *et al.* (2004) investigated samples of subsurface sediments from Bangladesh for arsenic leaching by bicarbonate ions. The following arsenic leaching efficiency with different carbonate and bicarbonate ions was reported:

$$Na_2CO_3 > NaHCO_3 > BaCO_3 > MnCO_3 \qquad\qquad (1.3)$$

From the above order, it can be seen that sodium carbonate and sodium bicarbonate leach arsenic most efficiently. The kinetic study showed that the leaching of arsenic increased with the reaction time of the bicarbonate solution. Leaching of arsenic by bicarbonate can occur under either oxic or anoxic conditions. One of the suggested mechanisms is that the carbonate ion may form complexes on the surface sites of iron hydroxide and thereby replace arsenic from the surface of minerals and sediments, which results in the release of arsenic to the groundwater.

In summary, the mechanisms causing release and mobility of arsenic to groundwater are complex and may involve different pathways under different conditions. The next sub-section discusses the treatment technologies commonly employed to remove arsenic from contaminated groundwaters.

1.8   Analytical Methods

As mentioned previously, the speciation of arsenic is critically important due to the differences in toxicity of the various species (Burguera & Burguera, 1997). Since the concentration of arsenic in water is very low, a sensitive (μg l^{-1} level) and selective method is required for its analysis (Jitmanee *et al*., 2005). The report by Braman and Foreback (1973) of a hydride generation procedure able to determine several individual inorganic and methylated arsenic compounds at low concentrations marked the beginning of a comprehensive study of environmental arsenic speciation. There is no standard method for determining arsenic speciation and there are no certified reference materials available. The only reference material is certified for total element content (Anderson *et al*., 1986).

There are several analytical methods reported for the quantification of arsenic species in biological, environmental and industrial samples. The basic steps in determining arsenic speciation include: sample pretreatment, derivatisation (e.g., hydride generation), separation (GC or HPLC) and detection (e.g., AAS, ICP, MS) (Quevauviller *et al*., 1996). One of the most widely used analytical procedures for speciation analysis couples the separatory power of chromatography with the detection ability of atomic spectrometry. Some of the interfaced techniques such as HPLC-ICP-AES (Morita *et al*., 1981), HPLC-ICP-MS (Dean *et al*., 1987), GC-ICP-MS (Szpunar *et al*., 2000), HPLC-HG-AAS (Chana & Smith, 1987) and HPLC-ETAAS (Larsen, 1991) are some of the combinations used in identifying arsenic species.

The analytical methods used to determine the uptake of arsenic by microbes (due to intracellular accumulation or adsorption on the cell surface) can be analysed using transmission electron microscopy (TEM), scanning electron microscopy (SEM), and/or energy dispersive x-ray (EDX) analysis. It is important to measure the arsenic content of any precipitate formed and calculate a balance for total arsenic used in the experiment. It is not sufficient to determine arsenic concentrations in the supernatant solution as some arsenic may have precipitated or may have been removed by sorption to the walls of flasks and bioreactors.

TEM is an effective way of locating and visualising metal deposits associated with microorganisms. When the high energy beam of TEM passes through matter, the path of the electrons are deflected by high atomic number elements. It is difficult to visualise cells of microorganisms by TEM as they are composed of low atomic number elements such as C, H, N, O (and small amounts of P and S). Therefore, specimens must be coated with "pools" of heavy metal salts to stain them negatively or with chemically complex heavy metal ions to stain them positively. Hence, a microorganism that actively accumulates arsenic can be easily visualised by TEM.

Transmission electron microscopy alone cannot give compositional analyses of elemental deposits, so must be coupled to EDX/SEM. The EDX technique relies on the capture of "signature" x-rays that are emitted when the electron beam interacts with metals in/on a specimen. Scanning electron microscopy operates at high vacuum and so preparation of biological samples requires fixation, dehydration, and air-drying or critical-point drying. Non-conducting materials should be coated with a conductive film of metal; otherwise a build up of electrons (charging) will occur and prevent the formation of clear images (Habold *et al*., 2003).

A new development in SEM techniques, ESEM, uses unique secondary electron detectors capable of forming high-resolution images at pressures in the range 0.1 to 20 torr. Hence, direct observations of uncoated, non-conductive specimen can be made.

1.9 Treatment Technologies for Removal of Arsenic Species

There are different treatment technologies for arsenic species that can reduce their concentrations to levels that comply with the regulations for arsenic in drinking water (USEPA, 2001). The treatment technologies chosen will depend on the scale of the system, level of arsenic in the water and other water quality requirements. Arsenic cannot be destroyed; but it can be changed into different forms or it can form insoluble compounds with other elements (Shih, 2005). As already discussed, the mobility of arsenic can be predicted depending on pH and Eh; hence, an effective treatment system can be designed.

Inorganic arsenic (arsenite and arsenate) is found in natural waters whereas organic arsenic species (MMA and DMA) occur in marine and biological materials (Jitmanee *et al*., 2005). It is the inorganic arsenic compounds that generally are treated. The neutrality of arsenite in the pH range 4-9 causes it to interact to a lesser extent with most solid surfaces; as a result arsenite is difficult to remove by conventional treatment technologies such as adsorption and precipitation (Kartinen & Martin, 1995). Consequently, pre-oxidation of arsenite is necessary. Many authors suggest the oxidation of arsenite using air, but the oxidation reaction is very slow (Kim & Nriagu, 2000; Pierce & Moore, 1982). Hence, strong oxidising agents like chlorine, potassium permanganate, hydrogen peroxide or manganese oxides must be employed to actively oxidise arsenite to arsenate (Kim & Nriagu, 2000; Driehaus *et al*., 1995). Unfortunately, some of these oxidising agents may cause several secondary problems such as by-product formation, thereby increasing the treatment cost (Katsoyiannis & Zouboulis, 2004). Some of the treatment technologies also use large amounts of chemicals and create sludge which must then be disposed of in an environmentally safe manner (Leist *et al*., 2000). Different treatment technologies are discussed briefly below with an emphasis on the chemistry and process technology.

1.9.1   Physico-Chemical Treatments

Precipitation/Coagulation

Several studies suggest that a precipitation/coagulation technique is effective in the removal of arsenic (see, e.g., Fuller *et al*., 1993). Co-precipitation of arsenate with the floc formed from ferric and aluminum salts (alum) is a practical method of arsenic removal. Ferric iron co-precipitation can also remove arsenate with an efficiency of 50% at pH 7.0 as a result of van der Waals bonding (Pierce & Moore, 1982).

The chemistry of precipitation/co-precipitation is usually complex and depends on the speciation of arsenic, the chemicals used for precipitation and their concentrations, the pH of the water, and the presence of other elements in the water. As a result, the mechanism for precipitation/co-precipitation is process-specific (USEPA, 2002b). For example, the presence of sulphate may decrease arsenic removal using ferric chloride as a coagulant, but

the presence of iron or calcium may increase the removal efficiency of arsenic (USEPA, 2000b).

Disadvantages:

1. The sludge generated from ferric iron coagulation can remobilise arsenic if disposed of under unsuitable Eh/pH conditions.
2. The technique requires the use of multiple chemicals adding to the cost of treatment and environmental pollution.

Adsorption

Adsorption is a treatment technology that uses materials that have a strong affinity for soluble arsenic, i.e., arsenic is attracted to the sorption sites on the adsorbent surface. This technology is efficient in removing arsenic from solution (Lorenzen *et al.*, 1995). Sorbents used in the adsorption of arsenic species include aluminum oxide (Anderson *et al.*, 1976), activated carbon (Chuang *et al.*, 2005), iron-based media (Gupta *et al.*, 2005) and greensand filtration (New Mexico State University, 1999). Even though it is expensive, activated carbon is by far the most widely used of the adsorbents (Shih, 2005).

The removal of arsenate in the pH range 5.5-6 by activated alumina is inefficient due to the nonionic nature of arsenic in this pH range. Adsorption of arsenite occurs initially due to van der Waals bonding; however, factors such as pH, arsenic oxidation state and the presence of competing ions all affect removal of arsenic. The following adsorption selectivity sequence have been shown for activated alumina (Clifford & Lin, 1995):

$$OH^- > H_2AsO_4^- > Si(OH)_3O^- > F^- > HseO_3^- > SO_4^{2-} > H_3AsO_3 \qquad \textbf{(1.4)}$$

Ion Exchange

Ion exchange is a process by which arsenical anions are exchanged for chloride or other anions bound in a resin bed (Shih, 2005). Ion exchange has been suggested as the best technology for arsenic removal from drinking water (Clifford, 1995). Nonionic arsenite is significantly adsorbed by weak-base anion exchangers due to van der Waals bonding.

Clifford *et al.* (1998) have shown that when sulphate levels are low (about 40 mg l^{-1}), the number of breakthrough volumes (BV) to exhaustion is limited by nitrate breakthrough. If the sulphate level is high (about 100 mg l^{-1}), however, the number of BV to exhaustion is limited by arsenic breakthrough. In other words, sulphate competes with both nitrate and arsenic, but competes more aggressively with arsenic than with nitrate.

Disadvantages of ion exchange as a treatment technology include: generation of toxic chemical reagents and their release into the environment (Shih, 2005). Efficiency can be reduced if there are competing ions present, and arsenic can be released to the treated water if the system fails.

Membrane Technology in Arsenic Removal

Membranes have billions of microscopic holes that selectively allow passage of some constituents while retaining others (Shih, 2005). Generally, the mechanism by which membrane filtration works depends on:

1. Exclusion based on particle size, and
2. Exclusion depending on electrostatic repulsion of arsenical ions.

Brandhuber and Amy (1998) noted that the negatively charged membranes of ultra and nano filters repelled the anionic arsenate in natural water.

The movement of particles across a membrane needs a driving force. Usually, this driving force is pressure. Membrane filtrations are classified in two categories according to the pressure used (Shih, 2005):

1. High pressure membranes such as reverse osmosis and nanofiltration (50-1 000 psi)
2. Low pressure membranes (5 psi to 100 psi), such as those used in standard membrane filtration and in ultra filtration

The pore sizes of high pressure membranes is small compared to those of low pressure membranes. Contaminants are removed by chemical diffusion (Aptel & Buckley, 1996) in the case of high pressure, while physical sieving removes contaminants in low pressure membrane processes (Shih, 2005). The four types of membrane filtration (reverse osmosis, nanofiltration, microfiltration and ultrafiltration) are discussed briefly below.

Reverse Osmosis

Reverse osmosis is the oldest and best technology for removal of arsenic in small-scale processes (Shih, 2005). But, a recent economic study shows that reverse osmosis is expensive (USEPA, 2000a). The membranes in reverse osmosis contain extremely small pores (<0.001 μm) (Schneiter & Middlebrooks, 1983). A solvent transports through the free volume of the segments in the polymer of the membrane through the action of osmotic pressure (high when compared to other processes). Clifford and Lin (1991) have reported 100% and 28% removal of arsenate and arsenite respectively; however, in the 1980s during the evolution of cellulose-acetate reverse osmosis, it was reported that a 90% and 70% removal of arsenate and arsenite respectively, was achieved using 400 psi operational pressure (Clifford *et al*., 1986). Oxidation of arsenite is not recommended in reverse osmosis because the use of an oxidising agent may damage the membrane.

The use of reverse osmosis in developing countries is hampered due to the expense and the high consumption of energy by the technology. Oh *et al*. (2000) devised a bicycle pump based energy-generator for the removal of arsenate and arsenite; the efficiencies were 95% for arsenate and 55% for arsenite. The advantage of reverse osmosis is that it does not use chemicals, and it is an effective means of removing arsenic, particularly arsenate. The production of only small amounts of treated water, its high cost and large energy consumption are some of its disadvantages.

Nanofiltration

Nanofiltration is also a high-pressure process and has the ability to remove dissolved arsenate due to the small pore size of the membrane (Shih, 2005). USEPA's report (USEPA, 2000b) showed that dissolved arsenate as well as arsenite was removed from

water due to size exclusion. The mechanism of nanofiltration is removal of uncharged components by size exclusion and separation of ionic species due to the nature of the membrane.

Saitúa *et al*. (2005) studied the effects of operating conditions on the removal of arsenic species from synthetic and surface waters. They found that arsenic rejection was independent of trans-membrane pressure, cross flow velocities and temperature. The effect of pH on arsenic species removal by nanofiltration was studied by Vrijenhoek and Waypa (2000) who found the removal efficiency of arsenate increased significantly with increasing pH. This was due to the change in speciation from monovalent arsenate to divalent arsenate; divalent ions have larger hydrated radii than monovalent ions. The removal efficiency of arsenite is unaffected in the pH range 4-8 due to the uncharged nature of arsenite in this pH range. Nanofiltration processes need less pressure for operation than does reverse osmosis. This has an advantage of saving energy.

Microfiltration/Ultrafiltration

Microfiltration is a low-pressure separation technique. The pore size in microfiltration is so large that it cannot easily remove dissolved arsenic or colloidal arsenic species; however, particulate forms of arsenic can be removed by microfiltration (Shih, 2005). As a result, microfiltration is highly dependent on the size distribution of arsenic-bearing particles. Since the presence of particulate arsenic in water is very limited, coagulants and flocculants (Han *et al*., 2002) are often added to increase the arsenic particle size. These processes increase the efficiency of the microfiltration technique for the removal of arsenic species. The coagulant $FeCl_3$ hydrolyses in water to form a $Fe(OH)_3$ precipitate which has a positive charge on its surface. The negatively charged arsenate adsorbs to the positively charged $Fe(OH)_3$ precipitate by surface complexation; however, the uncharged arsenite (in the pH range of 4-8) is poorly removed. Therefore, for efficient arsenite removal by microfiltration, a complete oxidation of arsenite to arsenate is needed (Shih, 2005).

Like microfiltration, ultrafiltration is also a low-pressure separation technique and removes constituents through physical sieving (Shih, 2005). Since the pore size of the membrane is

large and the percentage of particulate arsenic in water is low, ultrafiltration is not an efficient arsenic removal technique. Some researchers (e.g., Brandhuber & Amy, 2001) showed increased arsenic removal efficiency when ultrafiltration was coupled with electric repulsion as compared to the process when only pore size dependent sieving was employed.

1.9.2 Biological Treatment

Biological treatment is currently receiving attention for the removal of arsenic species from contaminated waters. The advantage of biological treatment physico-chemical treatment methods is that it uses microorganisms instead chemicals to reduce/oxidise or remove contaminants. Biological treatment can be used alone or in combination with adsorption, filtration and other physico-chemical procedures. The basic principle governing biological treatment is a change in oxidation-reduction (redox).

Katsoyiannis and Zouboulis (2004) studied the removal mechanism of arsenite from contaminated groundwater during biological iron oxidation while attempting to optimise conditions for the efficient removal of arsenate and arsenite to comply with the 10 μg l^{-1} permissible level. They found that both inorganic forms of arsenic in the concentration range of 50-200 μg l^{-1} could be efficiently treated and the oxidation of As(III) was found to be catalysed by bacteria, leading to enhanced overall arsenic removal.

A variety of microorganisms may be involved in biological treatment. One such group of microorganisms is the sulphate-reducing bacteria (SRB) that have been used for the treatment of contaminated mine waters (Chang *et al*., 2000; Elliott *et al*., 1998). Sulphate-reducing bacteria generate sulphide and alkalinity. Rittle *et al.* (1995) studied the precipitation of arsenic during bacterial sulphate reduction to sulphide. Arsenic was precipitated as an iron-arsenic-sulphide as shown by EDS analysis. Moreover, the sulphide formed can react with arsenic to form a low solubility complex (Ksp = -11.9) As_2S_3 (Eary, 1992). Kirk *et al.* (2004) showed the presence of sulphate-reducing bacteria in groundwater could reduce the level of arsenic.

1.9.3 Phytoremediation

Phytoremediation has been proposed as a cost effective methods for removal of arsenic from soils (Lasat, 2002). The application of phytoremediation depends on plant biomass production and uptake of metals (Reeves & Baker, 2000). Plants and/or genotypes that accumulate metals above specified concentrations in the above-ground biomass are called hyperaccumulators (Kabata-Pendias & Pendias, 1984).

A fern, *Pteris vittata* also known as Chinese brake fern, was the first identified arsenic hyperaccumulator. It was found to grow on CCA-contaminated soils in central Florida (Ma *et al.*, 2001). It can tolerate arsenic levels as high as 1 500 μg g^{-1} in soils and has a bioaccumulation factor of 193 (Zhang *et al.*, 2002). Moreover, it transports arsenic efficiently from its roots to the fronds (Tu & Ma, 2002; Tu *et al.*, 2002). Fayiga *et al.* (2004) demonstrated the ability of *Pteris vittata* to accumulate arsenic in the presence of heavy metals (Cd, Ni, Pb or Zn) and found that most of the arsenic in the plant was present as arsenite.

Although physical/chemical and biological treatment technologies can efficiently remove arsenic from contaminated waters, it is important to note that arsenic cannot be destroyed; therefore, the waste generated must be properly disposed of, otherwise other problems will arise. Arsenic in waste sludge can be mobilised by microorganisms using arsenate as an electron acceptor during anaerobic anoxic respiration (Oremland & Stolz, 2003). These microorganisms, known as dissimilatory arsenate-reducing prokaryotes (DARPs), have been isolated from different sources and transform arsenate to arsenite, thereby enhancing the mobility of arsenic in the environment (Oremland & Stolz, 2003).

1.10 Health Effects of Arsenic

Arsenic has long been known to cause cancer (Smith *et al.*, 2002). The growing demand for underground sources of drinking water exacerbates the problem of epidemic arsenic poisoning in different regions of the world, e.g., Bangladesh (Hadi & Parveen, 2004), China (Sun, 2004), Mexico (Meza *et al.*, 2004) and others.

Exposure of humans to arsenic is mainly through the consumption of food and water containing high levels of arsenic. People may also be exposed to arsenic via industrial sources (USEPA, 2000b).

The toxicity of arsenical compounds increases from the least toxic organic forms to the most toxic arsine form (Sami & Druzynski, 2003). The following list gives the order of toxicity of arsenic species:

$$\text{Arsine} > \text{MMAA} > \text{DMAA} > \text{As(III)} > \text{As(V)} > \text{Organic arsenic} \qquad (1.5)$$
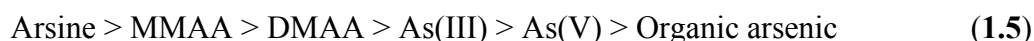
It has been reported that the LD_{50} for dimethylarsinic (cacodylic) acid in rats ranges from 700 to 2 600 mg kg^{-1}, methanearsonic acid 700 to 1 800 mg kg^{-1} calcium arsenate 20 mg kg^{-1} and potassium arsenite 14 mg kg^{-1} (Craig, 1986). The volatile arsine gas (AsH$_3$) is highly toxic to mammals (LD_{50} in rats 3 mg kg^{-1}), but it is unstable and not usually found in nature (Tamaki & Frankenberger, 1992). Choline and betaine (organic arsenic compounds) are non-toxic and can be fed to animals at high levels. They are excreted rapidly via the kidneys.

The toxic effects of arsenic depend on its speciation, its solubility and physical form. Arsenate is more readily absorbed than arsenite, and inorganic forms are more readily absorbed than organic forms. Moreover, arsenate is excreted more readily than arsenite and organic arsenic compounds; and organic arsenic compounds are excreted more readily than inorganic forms (USFDA, 1993). Also, bioavailability and toxicity of arsenic can be affected by retention of arsenic species in the body.

Elemental arsenic is considered to be of low toxicity on ingestion due to its poor absorption and rapid elimination from the body in an unchanged form (Duker *et al*., 2005). Soluble arsenic compounds are absorbed from the gastrointestinal tract (Hindmarsh & McCurdy, 1986) and eliminated as arsenate or organic arsenic through the kidneys (Luten *et al*., 1982). Human tissues and fluids contain different arsenic concentrations (Underwood,

1977) depending on the level of arsenic intake, the organ investigated and the age of the individual (Anke, 1986).

Contaminated groundwater contains arsenic species (arsenite and arsenate) that are highly toxic to humans. The affinity of arsenite for sulfhydryl groups in enzymes and tissue proteins such as keratin, skin, nail and hair (Knowles & Benson, 1983) is what makes arsenite more toxic than arsenate. Arsenite can also denature proteins and enzymes (Gebel, 2000; Graeme & Pollack, 1998) and can damage cells due to increased levels of reactive oxygen species (Ahmad *et al*., 2000; Nies, 1999; Chen *et al*., 1998; Wang *et al*., 1996). Around 200 enzymes in the body are also inhibited by arsenite (Abernathy *et al*., 1999).

The toxicity of arsenate is due to its structural similarity to phosphate, so that it can substitute for phosphorus in the body (Ellenhorn & Barceloux, 1988; Arena & Drew, 1986). For example, arsenate may replace phosphate in substituted monosaccharides such as glucose-6-phosphate resulting glucose-6-arsenate (Craig, 1986). In the cell, arsenate is easily hydrolysed which prevents phosphate for being transferred to adenosine diphosphate (ADP) to form adenosine triphosphate (ATP) and as a result depletes the energy of the cell (Winship, 1984). The most toxic of the arsenicals, arsine (Léonard, 1991) causes haemolysis of red blood cells (Fowler, 1977).

There are two processes by which inorganic arsenic can be removed from the human body. The first process is the rapid absorption and assimilation in the blood with subsequent removal in the kidneys and passage from the body in the urine. The second process is the slow detoxification into methylated forms (Crecelius, 1977). But both *in vivo* and *in vitro* studies show that the so-called "detoxified" arsenic species (MMAA & DMAA) are toxic to animals and humans (Styblo *et al*., 2000; Ochi *et al*., 1996; Kaise *et al*., 1989). Therefore, the efficacy of methylation as a detoxification process has been challenged through studies that have shown the methylation products MMAA and DMAA to be more toxic than the original inorganic arsenic species (e.g., Del Razo *et al*., 2001; Aposhian *et al*., 2000).

Farmer and Johnson (1990) reported that some authors (ATSDR, 2000) have shown that about 40-60% of ingested arsenic might be retained in hair, nails, skin, muscles and small amounts in teeth and bones. Hence, analysis of hair and/or nails can be used as a diagnostic feature of arsenic poisoning (Underwood, 1977).

Arsenic effects on terrestrial and fresh water plants were studied by Nissen and Benson, (1982). It was shown that terrestrial plants do not transform arsenic into arsonium phospholipids while freshwater plants do. This shows that freshwater plants have developed a mechanism to detoxify arsenic.

Chronic Effects of Arsenic

Arsenic is known to affect a variety organisms, including humans (Cervants *et al.*, 1994). Chronic effects of arsenic in humans have been documented and reviewed (e.g., Pershagen, 1983; Webb, 1966). Organs that are associated with the absorption, accumulation, and/or excretion of arsenic species such as the gastrointestinal tract, circulatory system, liver, kidneys, skin and some body tissues are very sensitive to arsenic. Other parts of the body are secondarily affected by arsenic exposure (Duker *et al.*, 2005). Signs of chronic arsenic toxicity may include dermal lesions (e.g., hyperpigmentation, hyperkeratosis, desquamation and loss of hair (Zaloga *et al.*, 1985), peripheral neuropathy, skin cancer and peripheral vascular disease (Sams *et al.*, 2007). These symptoms are observed in populations where arsenic concentrations in the drinking water are high (Smith *et al.*, 2000; Tseng, 1977). Dermal lesions are the most commonly observed symptoms and might occur within a period of five years. The skin has a high keratin content which contains several sulfhydryl groups causing it to localise and store arsenic (Kitchin, 2001). As a result, skin is highly sensitive to the toxic effects of arsenic. Hypertension and cerebrovascular disease (i.e., cerebral infection) has been linked to long-term arsenic ingestion (Chen *et al.*, 1995). Arsenic also decreases DNA repair processes (Brochmőller *et al.*, 2000) and, as a result, enhances the possibility of cancer, e.g., skin cancer (Wei *et al.*, 1994) and non-cancer related diseases (Feng *et al.*, 2001).

1.11 Microorganisms and Arsenic

Microbial processes mediate the cycling of metals in the environment. These processes can include redox reactions, increasing or decreasing the solubility using different complexation reactions, changing pH, and adsorption or uptake of a substance from the environment (Smith *et al.*, 1994).

Although many metals are essential for microbial metabolism in trace amounts, the biological activity of microorganisms can be inhibited by high concentrations of these same metals and their salts. As a result, some microorganisms have developed a mechanism whereby the toxic effects of the substances are reduced or eliminated. These mechanisms include adsorption, oxidation, reduction or methylation (Smith *et al.*, 1994). In the following five sub-sections the interactions of microorganisms with arsenic will be discussed.

1.11.1 Oxidation of Arsenite to Arsenate

As stated previously, the changing of arsenite to arsenate is regarded as a detoxification mechanism as arsenate is less toxic than arsenite. In 1909, Brűnnich noted the oxidation of arsenite to arsenate in cattle dipping fluids. This was confirmed by Green in 1918 who isolated the bacterium *Bacillus arsenoxydans*, (subsequently lost) from arsenical cattle dips in South Africa. The bacterium grew in 1% arsenic trioxide medium. Then in 1949, Turner investigated the spontaneous oxidation of arsenite to arsenate in cattle dips in Australia by five different bacterial species (15 strains) characterised as three *Pseudomonas*, one *Xanthomonas*, and one *Achromobacter*.

In other studies, 34 different strains of arsenite-oxidising organisms were isolated from sewage (Phillips & Taylor, 1976; Osborne & Ehrlich, 1976).

Mokashi and Parknikar (2002) studied the oxidation of arsenite to arsenate using a culture of *Microbacterium lacticum* isolated from municipal sewage by an enrichment culture technique and used for the removal of arsenic from contaminated groundwater. The oxidation of arsenite in groundwater by indigenous microorganisms has also been reported

by Hambsch *et al.* (1995) who isolated a very active arsenite-oxidising bacterium from a largely inorganic mining environment. Battaglia-Brunet *et al.* (2002) found an arsenite oxidising bacterial population (CASO1) that exhibits the following properties:

- autotrophic growth with a low nutrient requirement
- the ability to oxidise arsenite over a wide range of pH, temperature and arsenite concentrations
- High arsenite oxidising rates in a fixed-bed reactor

1.11.2 Reduction of Arsenate to Arsenite

The reduction of arsenate to the more toxic arsenite has been described by many researchers (e.g., Rensing *et al.*, 1999; Ji & Silver, 1995; Nies & Silver, 1995). Arsenate reduction to arsenite is an energetically favourable reaction which various anaerobic bacteria take advantage of by using arsenate as the electron acceptor during respiration (Oremland & Stolz, 2005; Liu *et al.*, 2004; Oremland & Stolz, 2003; Newman *et al.*, 1997; Ahmann *et al.*, 1994).

1.11.3 Biomethylation of Arsenic

The linking of an alkyl group (e.g., $-CH_3$) to a metal or metalloid mediated by microbes forms an organometal(loid) compound via a process known as bioalkylation. Methylation, the linking of a methyl group to a metal(loid), is widespread in nature (Herman & Maier, 2000) occurring in microorganisms, algae, plants and humans. But, several monkeys, chimpanzees, and guinea pigs do not methylate arsenic to any extent (Aposhian, 1997). Methylation affects the physical and chemical properties of the metal(loid), its fate and biological impact on the environment, and the toxicity of the element (Herman & Maier, 2000). Biomethylation of arsenic can form volatile methylated compounds like $(CH_3)_n AsH_{3-n}$; for (n=1,2 and 3, the products are mono-, di- and trimethyl arsine, respectively) and nonvolatile methylated compounds, e.g., methyl arsonate and dimethyl arsinate (Bentley & Chasteen, 2002). *Candida humiculus* methylates the widely known wood preservative chromated copper arsenate (CCA) to trimethyl arsine (Cullen *et al.*,

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1984). A survey of methylating agents shows that there are three co-enzymes available for the transfer of a methyl group (Ridley *et al*., 1977). These are:

i) S-adenosyl methionine

ii) N^5-methyl tetrahydrofolate derivatives and

iii) Vitamin – B_{12} (methyl corrinoid) derivatives

Both fungi and bacteria can mediate methylation of arsenic. Based on the work of du Vigneaud *et al*. (1941), Challenger (1945) studied the action of four strains of the mold *Scopulariopsis brevicaulis* on methylating arsenic compounds, especially As_2O_3, and supported the hypothesis that the methylation of arsenic involved the transfer of a methyl group from betaine, methionine, or a choline derivative. In subsequent work (Challenger *et al*., 1954), a significant transfer of $^{14}CH_3$-labeled methionine to arsenite was observed. This result suggested that methylation of arsenic was caused by the transfer of active methionine, also called S-adenosyl methionine (SAM) (Cantoni, 1953) in fungi. McBride and Wolfe (1971) showed the formation of arsine from arsenate by the *Methanobacterium* strain MOH growing anaerobically on hydrogen and carbon dioxide.

Cheng and Focht (1979) reported the formation of arsine when two soil bacteria (a *Pseudomonas* sp. and an *Alcaligenes* sp.) were incubated anaerobically with arsenite and arsenate.

1.11.4 Sulphate-Reducing Bacteria (SRB)

Sulphate-reducing bacteria are a group of prokaryotes that grow in anoxic fresh or salt waters with the ability to reduce sulphate to sulphide where sulphate is used as the electron acceptor during oxidation of organic compounds or hydrogen (Bridge *et al*., 1999). In the favoured microenvironment of sulphate-reducing bacteria, the combination of neutral pH, low Eh and high sulphide concentration makes the availability of soluble metals extremely low. This allows for the growth of sulphate-reducing bacteria in environments containing high levels of toxic elements. Hence, there is great interest in the use of SRB for bioprecipiation of toxic metals from contaminated environments (White & Gadd, 1996;

Gadd & White, 1993). The solubility of most heavy metal sulphides is very low (Chang, 1993) and, therefore, a small amount of S^{2-} can effectively precipitate out toxic metals to a safe level (Crathorne & Dobbs, 1990).

The term SRB conventionally represents a heterogeneous group of anaerobic bacteria which conducts dissimilatory SO_4^{2-} reduction (Postgate, 1979). Owing to the discovery of some archael sulphate reducers, the use of the term "sulphate-reducing bacteria" has been replaced to "sulphate-reducing prokaryotes" in some literature (Brüser *et al*., 2000; White *et al.*, 1997). SRB can be classified according to their morphology. Table 1.3 shows a key to the classification of SRB.

Table 1.3   Classification of SRB (after Levett, 1990)

Genus	Morphology	Motility	Flagella	Desulfoviridin	Cytochrome	Physiological group
Desulfovibrio	Vibrios	+	Single, polar	+	c_3	1
Desulfotomaculum	Rods	+	peritrichous	-	b, c	1
Desulfobacter	Rods/cocci-bacilli	-	-	-	b, c	2
Desulfococcus	Cocci	-	-	-	b, c	3-2
Desulfosarcina	Irregular, in packets	-	-	-	*	3-2
Desulfobulbus	Citron-shaped cocci	-	-	-	b, c	3-1
Desulfonema	Filaments	+	-	-	b, c	3-1

* Not determined

Campbell & Postgate (1965) and Postgate & Campbell (1966) classified SRB into two genera: viz *Desulfovibrio* and *Desulfotomaculum*. These are the well-established (Postgate, 1979), largest and most frequently encountered genera among the SRB (Levett, 1990).

Except for *Desulfonema*, all SRB are gram-negative (Levett, 1990). The genus *Desulfovibrio* is the best known because members are easy to isolate and purify. *Desulfovibrio* are usually mesophilic and can be halophilic. They do not sporulate and contain higher percentages of guanine and cytosine in their DNA than *Desulfotomaculum* spp. (Pankhurst, 1971). Mesophilic *Desulfovibrio* have an upper temperature limit between 45 and 48°C, whereas, *Desulfotomaculum* spp. are either mesophilic or thermophilic [thermophilic *Desulfotomaculum* strains have optimal growth temperature around 60°C with lower limits of 35°C (Postgate, 1979) and sporulate, which *Desulfovibrio* cells do not (Pankhurst, 1971)]. Morphologically, most *Desulfovibrio* spp. are curved rods whereas most *Desulfotomaculum* spp. are straight rods (Postgate, 1979).

SRB can also be classified according to the organic substrates that each genus (or individual species) utilises (Levett, 1990). Group-1 is represented by organisms that oxidise

lactate or pyruvate in the presence of SO_4^{2-}. They also metabolise H_2 in the presence of CO_2 and acetate. Growth of this group is usually rapid. *Desulfovibrio* is an example of this group.

Group-2 consists of organisms that use a limited range of substrates, e.g., acetate is the preferred substrate for oxidative metabolism. Hydrogen is not utilised and growth of these organisms is slow. *Desulfobacter* and *Desulfotomaculum* spp, including *Desulfotomaculum acetoxidans,* are examples of this group.

The third physiological group represents the more metabolically active SRB. Group-3 organisms oxidise fatty acids higher than acetate. This group is subdivided into:

Group 3.1 – organisms that are not capable of utilising acetate (incomplete fatty acid oxidisers). *Desulfobulbus* spp. and *Desulfovibrio sapovorans* are included in this sub-group. Growth of these organisms is more rapid than that of members of group-2 but slower than that of group-1 organisms.

Group 3.2 – organisms in this group have the ability to oxidising acetate (complete fatty acid oxidisers). Fatty acids, H_2, formate, alcohol, succinate and aromatic carboxylic acids are substrates utilised by group 3.2 organisms. *Desulfococcus*, *Desulfosarcina* and *Desulfonema* are examples of group 3.2 organisms. The growth of group 3.2 members is slower than that of other SRB.

SRB can be isolated from both oxic-acidic and anoxic-neutral tailings (Wielinga *et al*., 1990). Anoxic and near-neutral pH are preferable conditions for SRB (Widdel, 1988); however, they have been shown to tolerate O_2 and low pH conditions (Canfield & De Marais, 1991). The survival of SRB under oxidising conditions has been reported (Rogers, 1940 & Zobell, 1958 cited by Pankhurst, 1971) and probably can be explained by the existence of anaerobic microenvironments (Postgate, 1979). SRB are difficult to grow and enumerate, and procedures for their cultivation can be tedious and time-consuming; however, under anaerobic and reducing conditions, both pure and mixed cultures are

relatively easy to grow and long lived (Pankhurst, 1971). Since SRB are found in most soils and waters, but are usually outnumbered by other microbes, except in special environments, it is necessary to enrich for them before attempting their isolation (Postgate, 1979).

Zelinsky (1893), Beijerinck (1895) and Van Delden (1903) (cited by Pankhurst, 1971) were the first to describe the isolation of dissimilatory SRB. These bacteria get energy by reducing of SO_4^{2-} which acts as the terminal electron acceptor in an anaerobic process. SRB can also grow with sulphite, thiosulphate and usually tetrathionate (Postgate, 1979).

There are different media used for isolating and growing SRB. Most of the media are liquid but can be solidified by adding agar if needed (Pankhurst, 1971). Most SRB require no special organic growth factors (Postgate, 1979). SRB can assimilate significant amounts of CO_2, but they cannot grow as strict autotrophs. Glycerol and certain simple alcohols are less suitable as substrates. Some strains can also use carbohydrates such as glucose or sucrose. Previously, it was assumed that carbohydrates were widely used by SRB but this is not correct (Postgate, 1979). The addition of organic materials such as yeast extract and mixtures of amino acids stimulate growth due to the effect on Eh (by cysteine) or to the chelating action of amino acids on Fe^{2+} (Postgate, 1979; Pankhurst, 1971). Grossman and Postgate (1953) showed that L-cysteine, HCl, Na_2S and thioglycollic acid did not function in a nutritive capacity but rather established the desired low Eh in the medium. Therefore, it is necessary to add one or more specific reducing agents to the medium to create Eh values low enough for the growth of SRB.

Butlin *et al*. (1949), Miller (1949) and Bunker (1939) (cited in Pankhurst, 1971) explained the stimulatory effect of yeast extract as partly due to the constituent amino acids. EDTA in media may increase the solubility of ferrous sulphide thereby making inorganic iron available (Postgate, 1951, 1953, 1965). SRB can obtain N_2 from the ammonium ion whilst several strains can fix gaseous N_2 (Postgate, 1970; Riederer-Henderson & Wilson, 1970).

A qualitative test for the presence of SRB is the blackening of the media as a whole, or the zone round a colony, due to the formation of Fe sulphide precipitate (Postgate, 1979). But,

sulphide can be liberated from cysteine by organisms that decompose this S-containing amino acid. Therefore, cysteine cannot be incorporated in media to be used for diagnostic purposes. Even though thioglycollic acid also contains a -SH group, there are no microorganisms that form H_2S from it (Postgate, 1979).

Different approaches have been used in the development of rapid and dependable methods for detection and enumeration of SRB in natural and industrial environments. SRB can be enumerated using:

(i)   Direct detection methods and

(ii)   Culture methods (Vester & Ingvorsen, 1998).

A culture method based on MPN has been used extensively for enumeration of SRB. The techniques for counting viable SRB include use of liquid media or colony counts in solid or semi solid media (Postgate, 1979).

The toxicity of different specific heavy metals towards SRB ranges from a few mg l^{-1} to 100 mg l^{-1}; conversely very low concentrations of heavy metals may stimulate the growth of SRB (Utgikar et al., 2002). Hao et al. (1994) found the toxic concentrations of several heavy metals for a mixed culture of SRB to be: Zn (25-40 mg l^{-1}), Pb (75-80 mg l^{-1}), Cu (4-20 mg l^{-1}), Cd (>4-20 mg l^{-1}), Ni (10-20 mg l^{-1}) and Cr (60 mg l^{-1}).

1.12   Mechanisms of Microbial Resistance to Arsenic Species

The resistance of some microorganisms to toxic elements may have evolved due to their exposure to such substances shortly after life began on earth. Another possibility is that microorganisms might have developed resistance to toxins in response to anthropogenic pollution (Roane & Pepper, 2000). Selected mechanisms by which organisms detoxify or resist arsenic toxicity will be presented briefly. As indicated in the preceding sub-sections, arsenic can exist in two biologically important oxidation states, i.e., As(III) and As(V). The difference in pKa of arsenic compounds is crucial for the type of transport system by which As(III) and As(V) are taken up by the cell. The other aspect of importance in the biological

activity of arsenic, especially of As(III), is its ability to form strong bonds with functional groups such as thiolates of cysteine residues and the imidazolium nitrogens of histidine residues (Rosen, 2002).

Generally, the resistance mechanisms to arsenic in microorganism include: genetically determined resistance, hydrogen sulphide production, production of organo-arsenical compounds, uptake and intracellular or extracellular accumulation and metal transformation. The genetically determined resistance to arsenic species in microorganisms will be discussed briefly.

Genetically Determined Resistance to Arsenic Species

Plasmids in bacterial cells encode genes that confer resistance to toxic elements (Summers & Silver, 1978; Novick & Roth, 1968). They remove toxic elements, including arsenic, using energy-dependent encoded efflux systems (Smith, 1978). Arsenate, which is a biochemical analogue of phosphate, enters the cell via a phosphate specific transport pathway (Silver & Nakahara, 1983). Some of the efflux systems involve ATPase, while others are chemiosmotic ion/proton pumps (Silver & Phung, 1996). Plasmid-mediated mechanisms giving resisitance towards arsenic species have been studied in both gram-positive (Novick & Roth, 1968) and gram-negative bacteria (Hedges & Baumberg, 1973).

Brőer *et al*. (1993) studied the arsenic resistance mechanism in the *Staphylococcus aureus* plasmid pI258. They compared the encoded efflux mechanism with that encoded by *E.coli* plasmid R773 with its *ars* operon comprising *arsR, arsD, arsA, arsB and arsC* genes (Silver & Walderhang, 1992; Chen *et al*., 1986;) and found only *arsR, arsB and arsC* in the *Staphylococcus* plasmids (Ji & Silver, 1992; Rosenstein *et al*., 1992). The *arsR* gene encodes a repressor protein (Rosenstein *et al*., 1992; Wu & Rosen, 1991), whilst the *arsB* protein is a single polypeptide functioning as a chemiosmotic transporter (Silver & Phung, 1996). The *arsC* gene encodes the intracellular conversion of arsenate to arsenite, which is subsequently transported out of the cell (Ji & Silver, 1992). The *arsA* gene product functions as an arsenite-simulated ATPase dependent transport system, and *arsD* encodes a secondary down-regulatory protein (Brőer *et al*., 1993).

Chromosomally encoded arsenic resistance can occur by selective phosphate uptake which blocks arsenate uptake (Rosenberg *et al.*, 1977). Concomitant accelerated removal of arsenate from the cell can be a consequence of plasmid-determined resistance (Mobley & Rosen, 1982). Chromosomally-mediated resistance is distinct from plasmid-mediated resistance, but the level of resistance conferred by each system is additive (Tamaki & Frankenberger, 1992).

1.13   Economic Considerations of the Technologies Designed to Remove Arsenic

Any process designed to remove arsenic from groundwater must be cost effective and economically viable.

Designing commercial bioreactors for arsenic removal must include process economics with the following general economic guidelines and financial considerations:

- Relative efficiencies of bioreactors
- Engineering economic guidelines
- Establishing economic analysis procedures for alternative processes
- Economic analysis for investment and operating costs
- Capital investment economics (start-up costs, depreciation guidelines, bioreactor utilities like pumps, electricity consumption, etc.)
- Ongoing operating and maintenance costs
- Sludge volumes generated and disposal costs thereof

The following table reproduces some of the data released by USEPA in December 2000 for the costs of treatment technologies capable of achieving a MCL of 10 μg l^{-1}, assuming an influent arsenic concentration of 50 μg l^{-1}. Even though the contamination level may be much higher than 50 μg l^{-1}, the data nonetheless gives an indication of the costs associated with different treatment technologies.

Table 1.4 Costs of selected arsenic treatment technologies in US dollars (from USEPA, 2000a)

Size (People)	Treatment	1	2	3	4	5	6
<100	Treatment Capital Costs	8 999	7 483	26 970	193 923	24 983	13 619
	Treatment O&M Costs	484	260	5 365	21 251	7 747	4 433
	Waste Disposal Capital Costs	-	-	3 955	36 236	3 955	-
	Waste Disposal O&M Costs	-	-	392	9 187	464	-
	Annual Costs (7%)	1 333	966	8 676	52 164	10 943	6 372
50 000-100 000	Treatment Capital Costs	455 707	315 625	43 632	5 074 043	1 942 521	3 362 537
	Treatment O&M Costs	61 149	32 533	5 365	76 604	128 791	1 216 748
	Waste Disposal Capital Costs	-	-	5 085	717 287	5 085	-
	Waste Disposal O&M Costs	-	-	6 967	110 698	16 683	-
	Annual Costs (7%)	104 165	62 326	16 930	733 963	329 314	1 695 498

Note: **1** = Lime Softening; **2** = Coagulation/filtration; **3** = Anion exchange (< 20 mg l^{-1} SO$_4$); **4** = Coagulation assisted microfiltration; **5** = Oxidation filtration; **6** = Reverse osmosis

The data suggest that none of these high technology processes are realistically applicable to the poor Third World countries of Africa, Asia and South America.

1.14   Project Objectives

Contamination of groundwater with arsenic from natural geochemical and/or anthropogenic sources is a serious health problem to millions of people (Smith *et al*., 2002), especially in the rural areas of developing countries. Therefore, there is a high level of interest in establishing methods aimed at cleaning or detoxifying arsenic contaminated water to levels below the WHO maximum allowable contamination limits (MCLs).

Arsenic comes in different ionic forms with arsenite [As(III) being far more toxic to living creatures than arsenate (As(V)]. Therefore, at the least it is important to oxidise the arsenite to the arsenate, but preferably both forms of arsenic should be removed from drinking water.

One of the most promising technologies to reduce arsenic levels in groundwater is a biological approach. Therefore every effort will be made to design a microbe-driven bioreactor which is cost effective, easy to operate, safe, generates a minimum of sludge, is

environmentally friendly, utilises a cheap, readily available source of organic carbon as a nutrient source for the microorganisms and uses a readily available solid matrix to support the biomass. An understanding of the mechanisms by which microorganisms remove arsenic from groundwater will be sought.

Specific objectives are:

1. To determine the chemical composition of molasses and assess its suitability as carbon/energy source for sustained SRB activity and to investigate the effect of arsenic species [As(III) and As(V)] on the growth of a mixed culture of SRB.

2. To evaluate the adhesion capability of a mixed culture containing SRB to pine bark, polystyrene and sand and to compare the arsenic removal capacity of this immobilised biomass with that of planktonic SRB populations.

3. To examine the effect of sulphate, nitrate and ferrous iron amendments on the growth of mixed SRB.

4. To investigate the bioremoval of arsenic species [As(III) or As(V)] during the growth of a mixed culture of SRB, sorption of the species on precipitates resulting from sulphate-reducing bacterial activity and precipitation as sulphide when the species react with gaseous hydrogen sulphide.

5. To investigate the removal of arsenic species in bioreactors inoculated with SRB, with molasses as carbon source, sulphate as electron acceptor and polystyrene as support matrix in the presence of various concentrations of either As(III) or As(V) and in the ratio of the species As(III):As(V) 0.25 to 4. Chemical oxidising reagents will be used in combination with the biological process in order to assess the efficiency of the removal of arsenic species, particularly As(III).

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Chapter 2
Materials and Methods

2.1   Source of Sulphate-Reducing Bacteria

The sulphate-reducing bacteria used in this study were isolated from anaerobic sediments from the Msunduzi River (Pietermaritzburg, South Africa). The sediments were sampled from approximately 10 cm below the surface of the sediment bed using a trowel.

2.2   Nutrient Media

The composition of the nutrient medium (Postgate medium B) for the growth of sulphate-reducing bacteria is given in Table 2.1. The pH of the medium was maintained to the required pH (around neutral) throughout the experiments by addition of NaOH. A small amount of precipitate formed when the pH of the medium was adjusted to within the specified pH range.

Table 2.1 Composition of Postgate medium B (g l^{-1}) for the isolation and culture maintenance of sulphate-reducing bacteria

Medium B	
KH_2PO_4	0.5
NH_4Cl	1
$CaSO_4$	1
$MgSO_4·7H_2O$	2
Sodium lactate	3.5
Yeast extract	1
Ascorbic acid	0.1
Thioglycollic acid	0.1
$FeSO_4·7H_2O$	0.5

The medium was boiled for a few minutes and flushed with nitrogen gas to drive off oxygen.

2.3   SRB Enrichment

About 50 g of wet sediment was added to a 1 l flask, which was then completely filled with Postgate medium B, sealed with a rubber bung and incubated in the dark at room temperature (25±2°C) for 7 days. After this, 200 ml of the cell suspension was sub-cultured into a new 1 l flask and incubated under identical conditions for a further 7 days. This procedure was repeated every 3 weeks to maintain the SRB culture.

The presence of sulphate-reducing bacteria was ascertained by the formation of a black precipitate (ferrous iron) that appeared a few days after the inoculation of the culture. Blackening of lead acetate impregnated filter paper, indicating the release of hydrogen sulphide, verified SRB activity.

2.4   Chemicals and Arsenic Species Solutions

All chemicals used were of analytical reagent grade purchased from either Sigma-Aldrich (Riedel de Haën, Fluka) or Merck.

Arsenic stock solutions [As(III) and As(V)] were prepared by dissolving sodium arsenite ($NaAsO_2$) or sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) in deionised water to a concentration of 1000 mg l^{-1} As. Arsenic species working solutions were freshly prepared by diluting the stock solutions with appropriate amounts of deionised water as needed.

2.5   Synthetic Groundwater

The arsenic-contaminated synthetic groudwater used in this study was prepared by spiking either tap or distilled water with As(III) and/or As(V). The range of concentrations of both forms of arsenic used were 20, 10, 5, 1 and 0.1 mg l^{-1}.

2.6   Immobilisation Substrates

The three materials evaluated as attachment surfaces for the immobilisation of SRB were pine bark, expanded polystyrene (packaging material) and sand. These substances were chosen because of their availability and low cost.

2.7   Source of Molasses

The molasses used as carbon source for the SRB, was obtained from Voermol Feeds (Pty) Ltd., South Africa.

2.8   Bioreactor Configuration and Experimental Set-up

The bioreactors used in this study were constructed from plastic containers in the Department's workshop. Each bioreactor had a capacity of 12 l. The inner containers had mesh at the bottom and top to disperse the upwards flow of the medium and were filled with either pine bark (mesh size 16-25 mm), sand (mesh size 50-90 μm) or polystyrene (cut into small pieces approximately (10-15) mm × (12-16) mm × (9-12) mm) as support matrices. The bioreactors were inoculated with a SRB culture containing ~3×10^5 cells ml^{-1} (20% vol vol^{-1}). The void volumes in the inner containers when filled with pine bark, sand and polystyrene were 4.8, 2.1 and 4.2 l respectively. Figure 2.1 shows a schematic diagram of a bioreactor and its dimensions. Photos of the actual bioreactors used in the project shown in Figure 2.2.
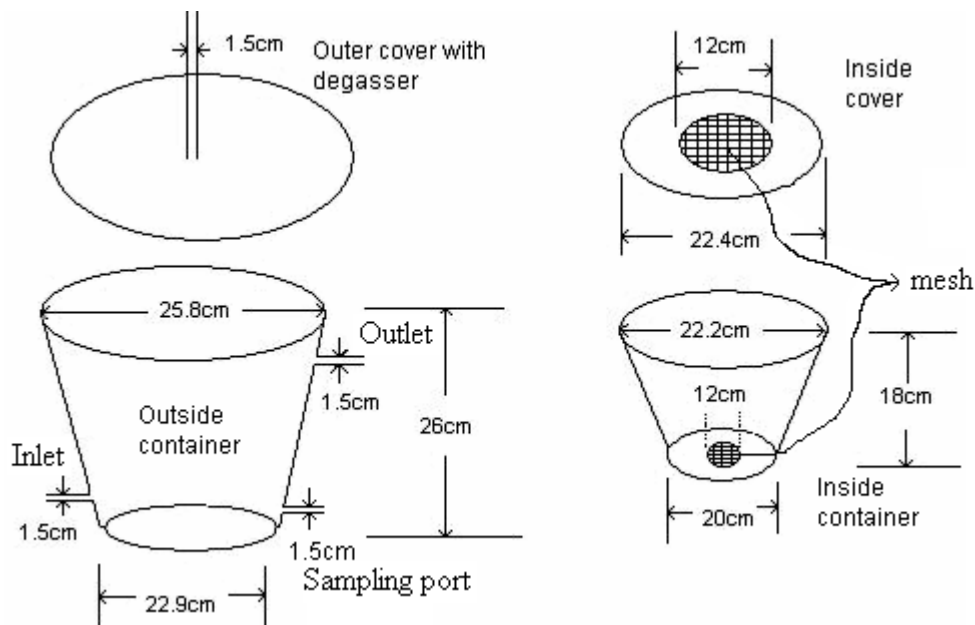
Figure 2.1 Schematic diagram of the plastic bioreactor. The inner container, which fits inside the outer one, is packed with the different biomass support matrices.

Figure 2.2   Photographs of SRB bioreactors.

Molasses served as the carbon source and sulphate as the electron acceptor. Water artificially contaminated with As(III) and/or As(V) at concentrations of 20, 10, 5, 1 mg l^{-1} and 0.1 mg l^{-1} [separately or in combinations of As(III) and As(V)] was fed into the bioreactors with a calibrated peristaltic pump (Watson Marlow). The bioreactors were operated batch-wise with regular monitoring of SRB activity. The parameters measured were SRB growth, pH, Eh, and concentrations of SO_4^{2-} and arsenic species. The effect of the support matrix on the performance of the bioreactors in terms of both SO_4^{2-} and arsenic reduction was assessed. Matrix-free bioreactors were established as positive controls and in each case, an appropriate negative control without SRB was used. The configuration of a single experimental bioreactor is shown in Figure 2.3.
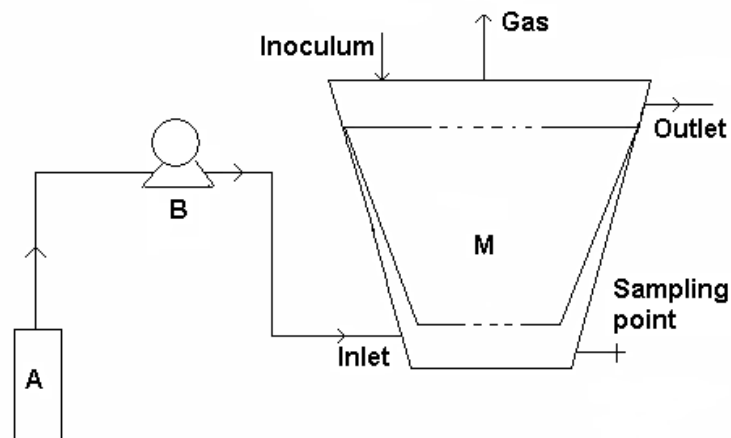
Figure 2.3 Configuration of a bioreactor (A – arsenic-contaminated water reservoir; B – peristaltic pump; M – support matrix) within the inner container.

2.9 Environmental Scanning Electron Microscopy (ESEM)

ESEM was used to study the biofilms and to characterise the surfaces of the pine bark, polystyrene and sand support materials. Samples of the different support matrices colonised by bacteria were fixed in 3% (v v^{-1}) glutaraldehyde, washed twice in 0.05 M cacodylate buffer (pH 7.1) for 10 min and dehydrated in an alcohol series (10 min each in 30%, 50%, 70%, 80%, 90%, and 3×10 min in 100%) in a fume cupboard. The specimens were then transferred into critical point drier baskets under 100% alcohol and placed in a pre-cooled Hitachi HCP-2 critical point drier. Following critical point drying (CPD) and gold-palladium sputter coating (Polaron Equipment Limited SEM, coating unit E5100), the samples were viewed in the ESEM (Philips, FEI XL 30) at an accelerating voltage of 15 keV.

2.10 Energy Dispersive X-Ray Analysis (EDX)

EDX is a chemical characterisation technique used in conjuction with scanning electron microscopy (SEM). An electron beam (10-20 keV) strikes a surface of a conductive sample and causes x-rays to be emitted from the point of the material under investigation. The energy of the emitted x-ray depends on the nature of the material. The constituent elements in the specimen can be determined by collecting and analysing the energy of the emitted x-rays. EDX provides information about elemental composition of the sample to a depth of 2 μm on the sample surface (Russ, 1984).

The precipitates deposited on the surface of the support matrices as well as the pellets collected after centrifuging a culture of SRB at 10 000 rpm for 20 min (Avanti J-26 XPI high-performance centrifuge, Beckman Coulter) were subjected to EDX analysis coupled to ESEM to quantify the elemental composition of the precipitates and to determine the amounts of metal ions, including arsenic, associated with the bacterial cells.

A Philips (FEI XL 30) environmental scanning electron microscope interfaced with EDAX digital controller was used. The specimen (mounted on an aluminium stub that had been covered by colloidal graphite and carbon tape) was placed in the ESEM and analysed under low vacuum mode by tilting to an angle of 15° toward the x-ray gun. A working distance of

10 mm and accelerating voltage of 15 keV were used for all EDX analysis, and the EDX spectra were collected over 270 s (live count time).

2-11   SRB Enumerations

SRB population sizes were determined by direct cell counting using a Neubauer counting chamber and phase contrast microscope (Zeiss).

2.12   Detachment of Bacteria from Support Matrices

Bacterial cells were detached from the different support matrices into sterile ultra pure water using an ultrasonic bath (Bandelin Sonorex TK 52, UK) for 15 min at 40 kHz and 50 watts.

2.13   Image Analysis for Surface Area

The total surface areas of the polystyrene, pine bark and sand used as support matrices for the immobilisation of the SRB were estimated using a microscope (Leica) fitted with a digital camera (JVC, model KY-F-1030U) and the software package analySIS.

2.14   Total Organic Carbon (TOC)

The organic carbon in water is composed of several compounds in various oxidation states. Measuring the quantity of organically bound carbon (TOC) can be done either by:

i)   Measuring both the amount of inorganic carbon (IC) present in an acidified aliquot and the amount of total carbon (TC) present separately. The IC is determined by lowering the pH (≤ 2) which liberates gaseous carbon from the sample which is then measured by the detector. The separate TC measurement involves no acidification, but only treatment to oxidise the carbon to release the gas from the sample which is again measured. TOC is calculated as the difference between the TC and IC.

ii)   The second most common method involves directly measuring TOC by acidifying the sample to pH 2 or lower. The resulting IC gas is vented to the air

and the remaining non-purgeable CO_2 in the liquid sample is oxidised to release the gas for measurement.

Methods utilised to convert organic carbon to CO_2 involve heat and O_2, ultraviolet irradiation, chemical oxidants, or combinations of these. The released CO_2 may be measured directly by a nondispersive infrared analyser, or it may be reduced to methane and measured with a flame ionisation detector, or the CO_2 may be titrated chemically (APHA, 1995). The total organic carbon (TOC) content of the molasses used in this study was analysed by diluting it to 1:1 000 with deionised water and measuring the TOC in a TOC-VCPN analyser (Shimadzu). Approximately 150 µl of the sample was injected into the instrument by the automatic autosampler and reacted with acid. The gaseous product of the reaction (CO_2) was purged using a carrier gas (compressed air) and transferred to the TC furnace. After passing through the dehumidifier/gas treatment chamber the gas was detected by a non-dispersive infrared detector (NDIR) and the data processed. Figure 2.4 shows the flow diagram for the measurement of TOC using the TOC-VCPN analyser.
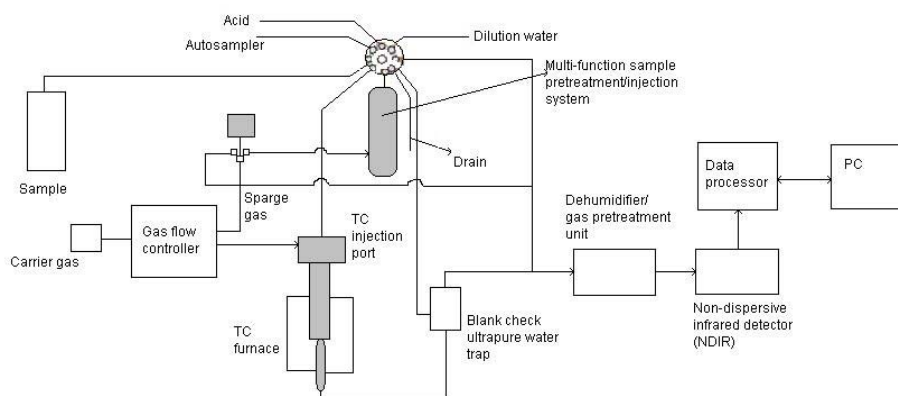
Figure 2.4   Line diagram of the (TOC-VCPN) instrument used to measure TOC.

2.15   pH

The pH of the water samples was measured using a Crison micro 2002 pH meter that was calibrated using pH 4.0 and pH 7.02 standards.

2.16   Temperature

The temperature of the synthetic groundwater in the reactors and experimental flasks and the ambient temperature in the laboratory were measured in degrees Celsius (°C).

2.17   Oxidation-Reduction Potential (Eh)

The redox potential of the samples (synthetic groundwater, media and SRB cultures) was measured with a platinum electrode system previously standardised against ZoBell's solution. The solution was prepared by dissolving 1.408 g potassium ferrocyanide, $K_4Fe(CN)_6 \cdot 3H_2O$, 1.098 g potassium ferricyanide, $K_3Fe(CN)_6$ and 7.456 g potassium chloride, KCl in 1000 ml deionised water at $25 \pm 2°C$.

2.18   Sample Preparation

2.18.1 Microwave Accelerated Reaction System

The microwave accelerated reaction system is designed for digestion and operates by hydrolysing different materials under laboratory conditions. The material (pine bark as used in this study) is heated using microwave energy at elevated pressure. Following treatment, the samples are ready for analysis by atomic absorption spectroscopy, inductively coupled plasma emission spectroscopy, gas or liquid chromatography. The procedure used when digesting pine bark for elemental analysis using the MARS-5 microwave (CEM Microwave Technology Ltd., U.S.A.) was as follows: approximately 0.2 g of pine bark were weighed into each of 6 reaction vessels (HP-500) followed by 5 ml of concentrated HNO_3 which were then microwave irradiated. The digestion was carried out under the following heating program: 5 min at 1200 W, 300 psi; 10 min at 1200 W, 130 psi and 15 min cooling. After cooling, each of the digested solutions were diluted to 100 ml with ultra pure water. Triplicate samples and reagent blanks without the addition of pine bark were digested and prepared in this way. The solutions were then analysed by ICP-OES.

2.18.2 Dry Ashing

Approximately 0.5 g of polystyrene was weighed onto porcelain crucible dishes previously cleaned by immersing in dilute HCl for 48 h and then rinsing in distilled water, and 10 ml of 20% $Mg(NO_3)_2 \cdot 6H_2O$ added. The samples were evaporated to dryness in an oven

(Gallenkamp, England) at 105°C overnight. The samples were then transferred to an electric muffle furnace (Gallenkamp, England) that was pre-heated to 100°C. The temperature was raised incrementally by 50°C and heated for 30 minutes at each temperature until a temperature of 400°C was reached. The samples were then ashed at 450°C for a further 4 hours. After the samples were converted to a white ash, the crucibles were left to cool. The same procedure was followed for a blank solution (without polystyrene) which served as a control. The ashed samples were dissolved in 2.5 ml concentrated HNO_3. The digests were filtered (Whatman number 41) into 50 ml volumetric flasks and made up to the mark with distilled water. All digestions were performed in duplicate.

2.18.3 Wet Digestion of Molasses

Molasses was tested for the presence of heavy metals using ICP-OES. Raw molasses was evaporated on a hot plate for about 1 h until a homogenous mixture was formed. About 4 g of the dried molasses was wet ashed using 40 ml concentrated HNO_3 in a beaker placed in a water bath until the characteristic brown gas stopped evolving (Mohamed, 1999). The solution was diluted to 100 ml using distilled water. A blank without molasses was prepared according to the same procedure and served as a control.

2.19   Nitrate and Ammonia Analysis using TrAAcs

The TrAAcs system is a continuous flow wet chemistry analyser that determines analyte concentrations in water or wastewater using a colorimeter to detect changes in colour produced by the presence of the analytes. It is capable of measuring different analytes, given the appropriate flow cell, filter and manifold set-up. The NH_4^+ and NO_3^- concentrations in the synthetic groundwater samples were analysed in the TrAAcs autoanalyser (Bran+Luebbe, Germany) using an adaption of the method proposed by Kamphake *et al*. (1967) for nitrate and nitrite and a method derived from Krom (1980) for ammonium. The following sub-sections give the detailed procedure for the analysis of nitrate and ammonia using the TrAAcs system.

2.19.1 Nitrate

The principle of the method is that nitrate (NO_3^-) is reduced to nitrite (NO_2^-) with hydrazine sulphate in alkaline solution in the presence of a copper catalyst. The NO_2^- (originally present) plus reduced NO_3^- is determined by diazotisation with sulphanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride (NEDD) ($C_{12}H_{14}N_2 \cdot 2HCl$) which form a pink azo dye compound that is measured colorimetrically at 520 nm. Phosphoric acid is added at the final stage to lower the pH thereby avoiding the precipitation of calcium and magnesium hydroxide. Moreover, the complexing of copper by organic material can be suppressed by adding zinc to the reducing agent. The method is applicable for potable and surface water and in domestic and industrial wastes the nitrogen content can be determined over a range of 0.01 to 10 mg N l^{-1} (APHA, 1995). The following diagram (Figure 2.5) shows the flow chart of the TrAAcs system for the analysis of nitrate.
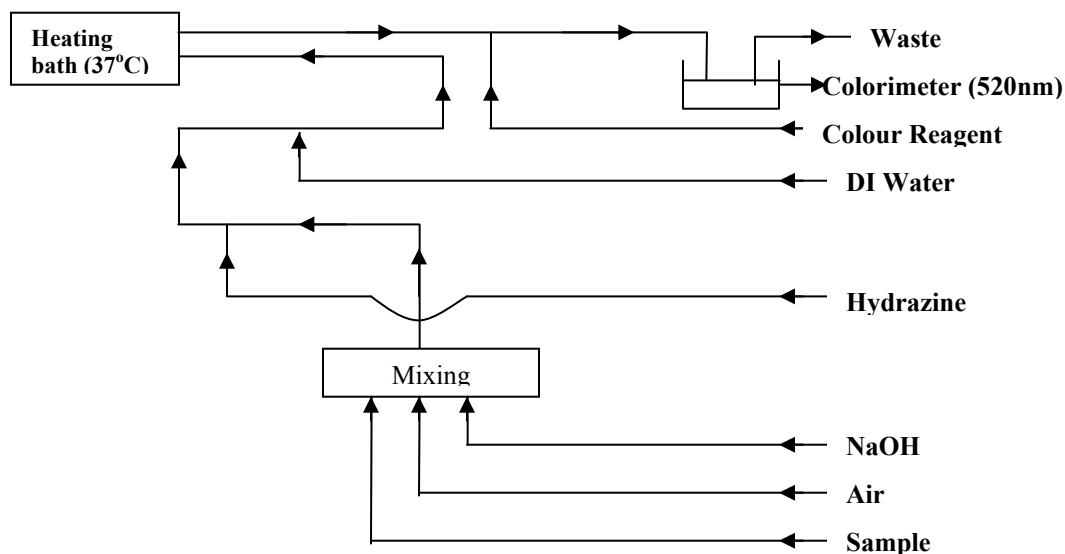
Figure 2.5   Flow diagram for analysis of nitrate by the TrAAcs system.

For details of the composition and preparation of the reagents see Appendix A1 and A2.

Samples (synthetic groundwater and SRB cultures) were run through the system set-up in Figure 2.4. The concentrations of the samples were calculated using the calibration curve that was plotted using known concentrations of the standards.

2.19.2 Ammonia

The method for the analysis of ammonia is based on the reaction of the sample with salicylate and dichloro isocyanuric acid to produce a blue compound measured at 660 nm (Figure 2.6). In the reaction, nitroprusside is used as a catalyst.
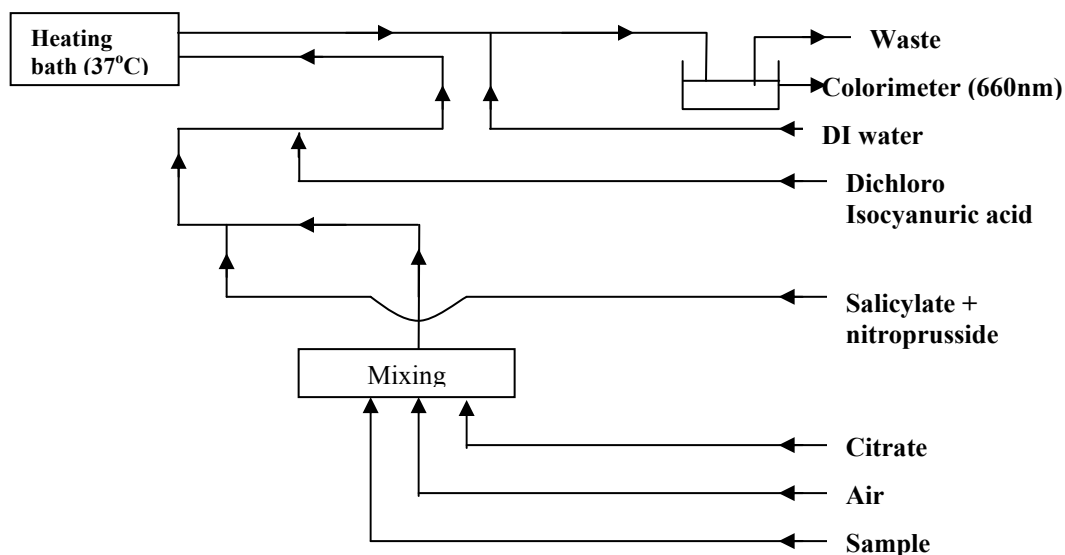
Figure 2.6   Flow diagram for analysis of ammonia by the TrAAcs system**.**

Samples (synthetic groundwater and SRB cultures) were run through the system as shown in Figure 2.6. The concentrations of the samples were calculated using the calibration curve that was plotted using known concentrations of the standards.

2.20   Sulphate Analysis

2.20.1 Photometric Spectroquant

Sulphate was measured photometrically using a SQ 200 photometer and the Spectroquant Sulphate test kit (Merck). The procedure followed was as follows.

A sample (synthetic groundwater and SRB cultures) of 2.5 ml, 2 drops of SO_4-1A solution (Merck) and the prescribed amount (manufacturer's protocol) of SO_4-2A (Merck) were mixed and shaken in a water bath at 40°C for 5 minutes. The solution was filtered and 4 drops of SO_4-4A reagent (Merck) were added. The resulting solution was heated in a

water bath (40°C) for 7 minutes. Finally, the sulphate concentration was measured in a photometer SQ 200 at 515 nm.

2.20.2 Modified Turbidimetric Method

A modified turbidimetric method was used to measure the residual sulphate concentration (Kolmert *et al*., 2000). Prior to sulphate determination, suspended solids were removed from the sample (synthetic groundwater and SRB culture) by centrifugation/filtration. One ml of conditioning reagent (150 g NaCl, 100 ml glycerol, 60 ml concentrated HCl and 200 ml 95% ethanol made up to 1 l with deionised water) and 1 ml of sample were mixed thoroughly in a test tube. After adding approximately 60 mg crushed barium chloride, the samples were stirred for 30 sec on a vortex mixer. The mixtures were then immediately poured into a cuvette and the absorbances measured at 420 nm. The mean values of the absorbances were compared against a standard curve. A blank containing the complete reaction mixture excluding sulphate served as a control.

2.21 Sulphide Analysis (Methylene Blue Method)

This method is based on the transformation of dimethyl-p-phenylene-diamine in the presence of hydrogen sulphide to the intermediate, leucomethylene blue. Leucomethylene blue is then oxidised to methylene blue by ferric iron which allows any sulphide present to be determined colorimetrically.

Since sulphide is volatile, 1.5 ml samples (synthetic groundwater and SRB cultures) were preserved with 0.6 ml 50 mM zinc acetate (dilution factor of 1.4). Following rigorous shaking aliquots were removed and 30 μl colour reagent (1.6 g N-N-dimethyl-1,4-phenylenediammonium dichloride dissolved in 100 ml 6M HCl) and 30 μl catalyser solution (1.6 g $FeCl_3 \cdot 6H_2O$ in 100 ml 6M HCl) were added, the mixture shaken well and left to react for 1 h. The absorbances of the resultant coloured solutions were measured at 660 nm. A washed crystal of $Na_2S \cdot 9H_2O$ was used to prepare a stock sulphide solution from which a series of standard sulphide concentrations was prepared. Sulphide concentrations in the samples were extrapolated from a standard calibration curve. Solubilisation of sulphide in the medium were not accounted during analysis of S^{2-}.

2.22   Metal Analysis

2.22.1 ICP-OES

All metal analyses were performed using a Varian 720-ES ICP-OES according to the operating conditions given in Table 2.2. The analytical wavelengths (nm) were set at the following spectral lines for each analyte: Al (396.152), As (193.696), Be (234.861), Ca (393.366), Cd (228.802), Co (238.892), Cr (267.716), Cu (324.754), Fe (259.940), K (766.490), Mg (279.553), Mn (257.610), Na (589.592, Ni (231.604), Pb (220.353), Si (251.611), Sn (189.926), Ti (334.941) and Zn (213.856).

Table 2.2   Experimental conditions for ICP-OES

ICP Spectrometer	Varian 720-ES ICP-OES
Power (kW)	1.00
Argon Plasma Flow (l min^{-1})	15.0
Auxiliary Argon Flow (l min^{-1})	1.50
Photomultiplier (V)	800
Intergaration time (sec)	1
Nebuliser (kPa)	240

2.22.2 Vapour Generation System (VG) for Arsenic Analysis and Speciation

Vapour generation is a system for atomic absorption (AA), inductively coupled plasma optical emission spectrometric (ICP-OES) and inductively coupled plasma spectrometric (ICP-MS) applications. Vapour generation is an extremely sensitive procedure to determine very low levels of arsenic.

Figure 2.7 is a schematic diagram of the vapour generation system used in this project. The VG-ICP-OES system is manufactured by Varian and comprises a VGA-77 hydride generation system coupled to a Varian ICP-OES. The chemicals and optimised experimental conditions used in the analyses are given in Table 2.3.
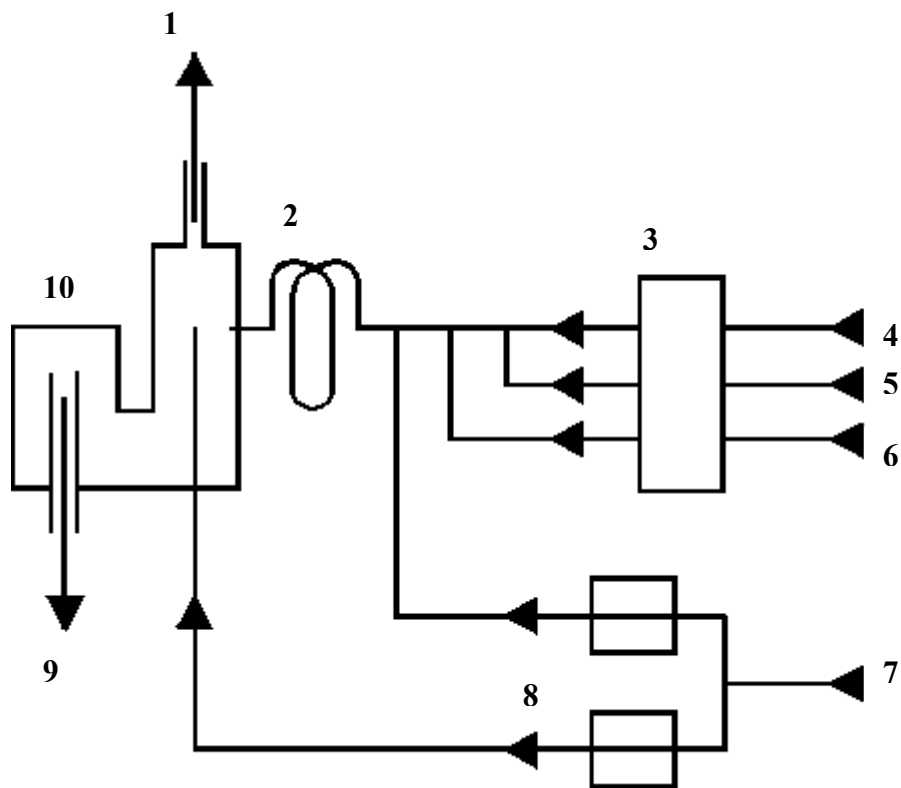
Figure 2.7 Schematic design of the VGA-77 and flow paths of the hydride generation system (1= to spectrometer; 2=reaction coil; 3=peristaltic pump; 4=sample; 5=acid; 6=reductant; 7=argon; 8=flow controller; 9=drain and 10=gas/liquid separator) (Reproduced from Varian Manual).

The sample and acid are allowed to merge first before the reductant, sodium borohydride (NaBH$_4$), enters the stream. Argon is then introduced into the liquid stream and the reaction proceeds while the mixture is flowing through the reaction coil. Vigorous evolution of hydrogen assists the stripping of the hydride (arsine) from the liquid into the argon. The gas is then separated from the liquid in the separator. The liquid drains away to waste. At this point, a second stream of argon is introduced to ensure that the gas stream is not saturated with water vapour and so does not condense in the sample introduction system. The gas containing the element of interest then passes out of the separator into the spectrometer where it is analysed.

Table 2.3 Operating conditions and chemicals used for arsenic analysis by ICP-VG-OES

ICP-OES		
Wavelength	[mV]	193.696
Gas		Argon
Power	[kW]	0.90
Plasma flow	[l min^{-1}]	15.00
Auxilary flow	[l min^{-1}]	1.50
Nebuliser flow	[l min^{-1}]	0.75
Replicate read time	[s]	10
Stablisation time	[s]	15
Sample uptake delay	[s]	60
Rinse time	[s]	60
Vapour Generation System		
Gas		Argon
Gas supply pressure	[kPa]	350
Sample flow	[ml min^{-1}]	8
HCl flow	[ml min^{-1}]	1
NaBH$_4$ flow	[ml min^{-1}]	1

Arsenic species were analysed using hydride generation coupled to an ICP detection system according to a modified method developed by Müller (1999). Total arsenic concentrations were determined by reducing As(V) to As(III) by reacting with 0.25% w v^{-1} L-cysteine for 12 h. Following this the solution was reacted with NaBH$_4$ at a concentration of 0.6% w v^{-1} in the presence of 0.5% NaOH and 32% HCl. As(III) was determined using NaBH$_4$ at a concentration of 0.05% w v^{-1} in 0.1M HCl. The amount of As(V) was calculated by subtracting the concentration of As(III) species from the total amount of arsenic present in the sample. Standard arsenic solutions with concentrations of 1, 5, 10, 25, 50, 100 and 150 μg l^{-1} were prepared to construct a calibration curve (See Appendix B1 and B2).

2.23   Analysis of Phenolic Compounds

Phenols in pine bark extracts, a class of aromatic compounds having a hydroxyl (- OH) group and other substituted groups on the benzene ring were determined using the USEPA Method 9065. The method involves the addition of 2 ml buffer solution (16.9 g NH_4Cl in 143 ml concentrated NH_4OH diluted to 250 ml with deionised water) to 100 ml pine bark extract followed by vigorous mixing. The pH should be in the range 10±0.2. Two ml of aminoantipyrine solution (2 g 4-aminoantipyrine in 100 ml deionised water) were added and mixed. Two ml of potassium ferricyanide solution (8 g $K_3Fe(CN)_6$ in 100 ml deionised water) were then added and mixed. After 15 min, the absorbance was read at 510 nm. A stock phenol solution (1000 mg l^{-1}) was prepared by dissolving 1.0 g phenol in freshly boiled and cooled deionised water and diluted to 1 l. Working solutions of an appropriate amount of standards in 100 ml volumetric flasks were prepared from the stock solution. The concentration of phenol in the pine bark extract was extrapolated from the calibration curve.

2.24   Ferrozine Assay for Ferrous Iron

Dissolved Fe(II) was determined according to Stookey (1970) with a Ferrozine [that binds only to Fe(II) in the presence of Fe(III) (Welch *et al.*, 2002)] solution (1 g l^{-1} of Ferrozine (3-(2-pyridyl)-5-6-bis(4-phenylsulfonic acid)-1,2,4-triazine) in 50 mM HEPES buffer [N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, pH 7.0)]. A 0.1 ml sample was diluted in 5 ml HCl (0.5 M) and after a 10-15 minutes, 50 μl were removed and mixed with 2.5 ml Ferrozine solution. After allowing time for colour to develop, the absorbance was measured at 562 nm on a spectrophotometer and the concentrations of Fe(II) was calculated from a calibration curve prepared from standard solutions treated in the same manner.

2.25   Transmission Electron Microscopy – Energy Dispersive X-ray Analysis (TEM-EDX)

The sulphate-reducing bacterial (SRB) cells grown in media that contained various arsenic species solutions were investigated for their ability to sequester (absorb) arsenic and other elements inside their cells using TEM-EDX analysis.

A small drop of the culture containing the cells to be studied was placed carefully using micropipette on copper grids (200 holes per 25 mm^2). TEM (Philips, CM 120, biotwin) analysis was then carried out at 100 kV. The microscope, equipped with an EDX spectrometer (DX4 system, EDAX microanalysis) was used to characterise the elemental composition within the cells. A spot size of 300 nm and 100 s live count time was used to collect the spectra.

2.26   Statistical Analysis

Data was analysed using analysis of variance (ANOVA) by Genstat (10th edition) program.

2.25 References

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Chapter 3

Determination of the Elemental Composition of Molasses and its Suitability as Carbon Source for Growth of Sulphate-Reducing Bacteria[*]

3.1 Introduction

The biological approach to bioremediation of arsenic-contaminated groundwater involves the use of sulphate-reducing bacteria (SRB) that can reduce sulphate to sulphide while oxidising a carbon source. The sulphide so generated can remove arsenic, precipitating it as arsenic sulphide. The effectiveness of SRB in removing arsenic from contaminated groundwater depends on the choice of an appropriate organic carbon source for use by the bacteria. The primary consideration when selecting a carbon source is its effect on the extent of microbial activity (biotreatment efficiency) and economic feasibility (Gibert *et al.*, 2004). A major problem associated with the treatment of arsenic-contaminated water using SRB could be the lack of an electron donor for the bacteria. The raw organic materials assessed in previous studies [especially in treatment of acid mine drainage (AMD)] cover a wide range of agricultural and food processing by-products (Gibert *et al.*, 2004;Chang *et al.*, 2000; Prasad *et al.*, 1999; Gross *et al.*, 1993). However, only a few studies have involved quantifying the biodegradability of the different organic carbon and cellulosic materials (Gibert *et al.*, 2004; Cocos *et al.*, 2002; Chang *et al.*, 2000; Gross *et al.*, 1993; Tuttle *et al.*, 1969a; Tuttle *et al.*, 1969b).

Among the main sources of carbon available to SRBs for biological sulphate reduction are volatile fatty acids and short-chain fatty acids. Long-chain fatty acids and certain aromatic compounds are occasional substrates (Hao *et al.*, 1994). Fermentation products such as methanol (Vallero *et al.*, 2003) and ethanol (Kaksonen *et al.*, 2003) are additional sources; as are other simple carbon substrates such as benzoate (Li *et al.*, 1996) and butyrate (Mizuno *et al.*, 1994). Polymers such as cellulose and hemicellulose are not good carbon sources, as cellulose is not known to be degraded by SRB (Chang *et al.*, 2000; Béchard *et al.*, 1994). Gibert *et al.*, 2004 assessed the degradability of different carbon substrates for

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[*] Portions of this chapter have been published in *Journal of Hazardous Materials,* In Press.

SRB and concluded that the lower the lignin contents of a substrate, the greater its degradability. Investigations by Kaksonen *et al*. (2007), Parshina *et al*. (2005) and Akagi and Jackson (1967) have shown the utilisation of sugars by SRB. Proteins, carbohydrates and lipids or even simple sugars are generally not utilisable by SRB (Tuttle *et al*., 1969a). But, other bacteria can metabolise the sugars making fermentation end-products such as lactate and acetate that can be used by SRB (Prasad *et al*., 1999). Researches have also shown that using mixtures of natural substrates rather than a single substrate can increase sulphate reduction (Amos & Younger, 2003; Cocos *et al*., 2002; Waybrant *et al*., 1998; Gross *et al*., 1993). An organic substrate for the growth of SRB, especially *Desulfovibrio* and *Desulfotomaculum,* (Cohen, 2005), can be supplied through mushroom compost.

SRB oxidise organic matter into bicarbonate anaerobically using sulphate as a terminal electron acceptor according to the reaction:

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^- \qquad \textbf{(3.1)}$$

where CH_2O represents the organic substrate. The hydrogen sulphide generated may form insoluble complexes with many heavy metals (Poulson *et al*., 1997; Rittle *et al*., 1995; Mueller & Steiner, 1992; Gadd & Griffiths, 1978). The energy substrates for SRB can thus range from hydrogen to aromatic compounds (Widdel, 1988); however, for economic reasons the choice of a carbon source for SRB should be a cheap and readily available substrate.

Molasses is a by-product of sugar processing and can be employed as a relatively cheap carbon source. The composition of molasses can be influenced by a number of factors, and Table 3.1 gives the charactersitic values of molasses found in many cane-producing countries (Paturau, 1989).

Table 3.1   Composition of molasses (after Paturau, 1989)

	% molasses	
Components	**Usual range**	**Characteristic average**
Water	17-25	20
Sucrose	30-40	35
Glucose	4-9	7
Fructose	5-12	9
Other reducing substances	1-5	3
Other carbohydrates	2-5	4
Ash	7-15	12
Nitrogenous compounds	2-6	4.5
Non-nitrogenous acids	2-8	5
Wax, sterols and phospholipids	0.1-1	0.4

Fermetative bacteria can easily change sucrose into carbon dioxide, hydrogen and short-chain fatty acids. SRB can use these fatty acids as a source of carbon for growth.

The activity of SRB can be adversely affected by the presence of heavy metals; hence, effective treatment of metal contaminated waters by SRB depends on a knowledge of their susceptibility to various heavy metals. Previous studies have shown that metal-microbe interactions occur with aerobic bacteria (Aiking *et al*., 1985; Foster, 1983), anaerobic consortia (White & Gadd, 1996) or sometimes to mesophilic SRB (Loka Bharathi *et al*., 1990). Studies by Capone *et al*. (1983), Saleh *et al*. (1964) and Booth and Mercer (1963) have shown the toxicity of heavy metals to SRB. Utgikar *et al*. (2003) have quantified the toxic and inhibitory impact of Cu and Zn on mixed cultures of SRB. The effects of copper amendments on bacterial sulphate reduction on bacterial consortia enriched from metal-contaminated and uncontaminated sediments were studied by Jin *et al*. (2007). Their results

showed that SRB from metal-contaminated environments have higher metal tolerance than those enrichment from uncontaminated environments.

Metal ion inhibition to SRB activity may occur with elevated dissolved metal concentrations (Velasco *et al*., 2008). Heavy metals exert a negative effect on bacterial communities by deactivating enzymes, denaturing proteins, and competing with essential cations (Mazidji *et al*., 1992; Mosey & Hughes, 1975). The effect of metal ions may result in a reduction in numbers and species diversity of a mixed SRB consortium or the development of strains capable of tolerating high concentrations of metal ions (White *et al*., 1997; Babich & Stotzky, 1985; Gadd & Griffiths, 1978). Sani *et al*. (2001) observed a negative effect of Pb on *Desulfovibrio desulfuricans* at concentrations greater than 3 mg l^{-1}, while a Cu(II) concentration of 0.85 mg l^{-1} caused a 50% inhibition in maximum specific growth rate. Poulson *et al*. (1997) reported inhibition of sulphate-reducing activity in *Desulfovibrio desulfuricans* by nickel and zinc at total ionic activities in excess of 1.6 mg l^{-1}. These differences in inhibitory concentrations of heavy metals to SRB are partly due to differences in the rates of precipitation and adsorption of the solubilised metals (Karri *et al*., 2006). The presence of sulphide can decrease the toxicity effect due to precipitation of metal sulphides.

Microbes protect themselves from the effects of heavy metals by complexation, extra-cellular precipitation, impermeability, or reduced transport of the metals across the cell membrane. Moreover, microorganisms can synthesise metal-binding metallothioneins (Atlas & Bartha, 1997). Biomethylation, volatilisation, biopolymerisation, bioprecipitation, biosorption and intracellular traps can also be employed by microorganism against the effects of heavy metals.

The objectives of this study were to determine the chemical composition of molasses and assess its capacity to sustain SRB activity and to investigate the effect of arsenic species [As(III) and As(V)] on the growth of a mixed culture of SRB in a molasses-containing medium.

3.2   Materials and Methods

3.2.1   Nutrient Medium and Source of Sulphate-Reducing Bacteria

The growth medium used was Postgate medium B (Postgate, 1979), the composition of which was described in **section 2.2**.

The culture of SRB was isolated from anaerobic sediments from the Msunduzi River (Pietermaritzburg, South Africa) as described in **sections 2.1** and **2.3**.

3.2.2   Source of Molasses and its Elemental Composition

The source of molasses used as carbon source in this study was described in **section 2.7**. In this study, the sugar content of molasses was not characterised. The elemental composition of the molasses was determined using ICP-OES under the experimental conditions given in Table 2.2. Raw molasses was digested following the procedure given by Mohamed (1999) which was described in **section 2.18.3**. A blank was also prepared following the same procedures but without the addition of molasses.

3.2.3   Experimental Cultures

Experimental cultures were grown with different concentrations of molasses (1, 2.5 and 5 g l^{-1}) as carbon source. The growth studies were performed in duplicate using a 20% (v v^{-1}) inoculum of log phase cells that had been sub-cultured 3 times. All cultures were incubated in the dark at room temperature (25±2°C) for 1-2 weeks. Growth of the SRB was monitored microscopically by direct cell counts and verified by measuring sulphate reduction levels. As a control, the same medium was used but with lactate as carbon source as most sulphate reducers can metabolise this compound. Also, lactate is known to support good growth of SRB in mixed culture (White & Gadd, 1996).

3.2.4   Arsenic Tolerance Study

The influence of different concentrations of As(III) and As(V) on growth of the SRB consortium was studied. Arsenite and arsenate solutions were prepared from $NaAsO_2$ (Ridel Riedel de Haën, AR) and $Na_2HAsO_4 \cdot 7H_2O$ (Fluka, AR), respectively. The arsenic solutions and the growth medium were sterilised separately and appropriate volumes of

either arsenate or arsenite were added to the culture medium from stock solutions to give final concentrations of 1, 5 and 20 mg l^{-1}. Controls contained the same growth media but without arsenic.

The effect of the two arsenic species on the growth of SRB grown on molasses as the carbon source, was evaluated according to the sulphate activity assay described in **subsection 3.2.5**. Molasses (1 g l^{-1}) was used as carbon-source throughout this experiment. Samples were collected daily to measure bacterial growth, pH, redox potential and sulphate concentrations.

3.2.5 Sulphate Reducing Activity

The sulphate activity assay, which involves sulphate reduction kinetics and measures maximum sulphate reduction level, was performed in triplicate 250 ml serum bottles. Sulphate-reducing bacteria cultures were transferred to the bottles containing 200 ml modified (molasses substituted) Postgate medium and incubated at 25±2°C. Samples for analysis were collected each day for 14 days.

3.2.6 Analytical Methods

Sulphate was measured photometrically using a SQ 200 photometer and the Spectroquant Sulphate test kit (Merck). The concentration of organic carbon (TOC) of molasses was analysed using a TOC-VCPN analyser (Shimadzu) as described previously.

3.2.7 SRB Enumerations

SRB cell counts were performed by direct counting using a Neubauer counting chamber and phase contrast microscopy (Zeiss).

3.3 Results

3.3.1 Growth of SRB on Molasses

At 1 g l^{-1}, cell numbers increased constantly from approximately 8×10^6 cells ml^{-1} to about 3.7×10^7 cells ml^{-1} after 96 h. When the concentration of molasses was increased to 2.5 g l^{-1} and 5 g l^{-1}, the number of cells ml^{-1} after 96 h were 3.7×10^7 and 3.8×10^7, respectively. This

showed that molasses is a potential carbon source for the growth of SRB; however, with lactate (3.5 g l^{-1}) as carbon source, SRB growth was slightly better than that on any of the three molasses concentrations (Figure 3.1).
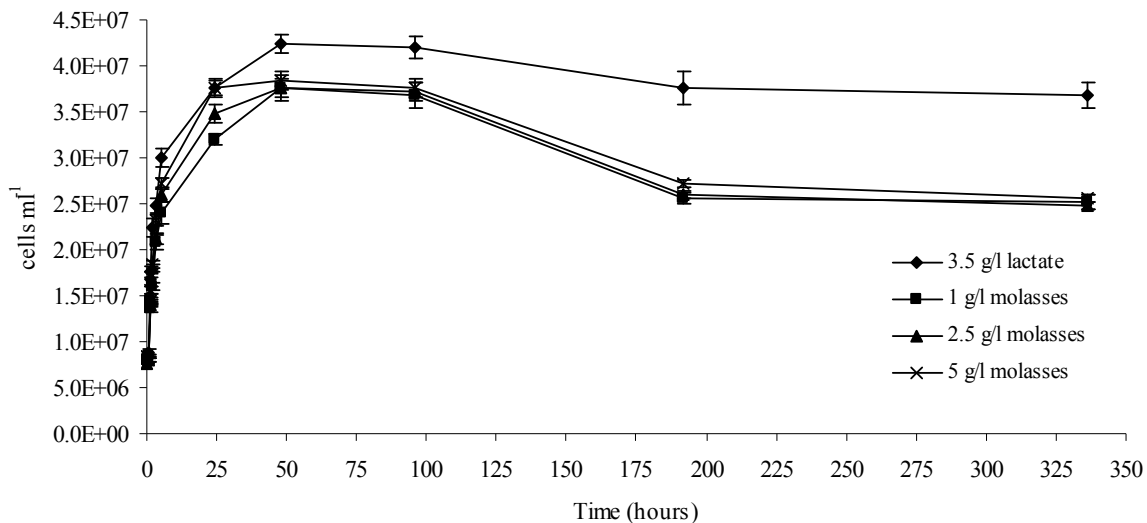
Figure 3.1 Cell number as a function of time following the growth of SRB in 1, 2.5 and 5 g l^{-1} molasses and 3.5 g l^{-1} lactate. Error bars represent standard deviation between 3 measurements. Statistically non-significant at P=0.05.

The changes in pH that occurred during the growth of SRB on the different concentrations of molasses are depicted in Figure 3.2. At a concentration of 1 g l^{-1}, the pH increased slightly from 6.4 to 6.9 over a 14-day period. For the same period, with molasses at 2.5 g l^{-1} and 5 g l^{-1} the pH increased to 7.0 and 7.1 respectively.
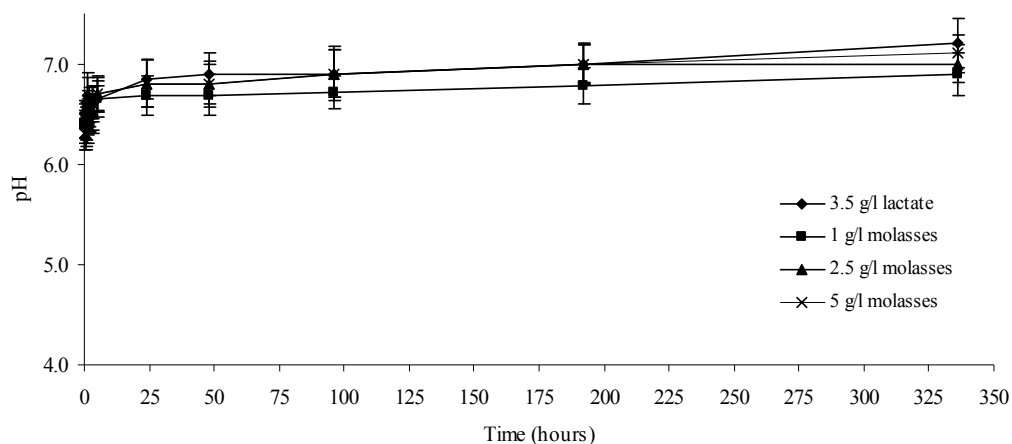
Figure 3.2 Changes in pH as a function of time during the growth of SRB on 1, 2.5 and 5 g l^{-1} molasses and 3.5 g l^{-1} lactate. Error bars represent standard deviation between 3 measurements.

There was no significance difference in all growth or medium pH at the various molasses concentrations investigated.

In parallel with the pH changes, the redox potential of the medium declined from 254 mV to -179 mV over 14 days for 1 g l^{-1} molasses; from 248 mV to -195 mV for 2.5 g l^{-1} molasses; and from 235 mV to -210 mV for 5 g l^{-1} molasses (Figure 3.3). However, the corresponding change when lactate was used as carbon source was from 245 to -269 mV (Figure 3.3). There was a slight difference in redox potential for the different molasses concentrations at the start of the experiments.
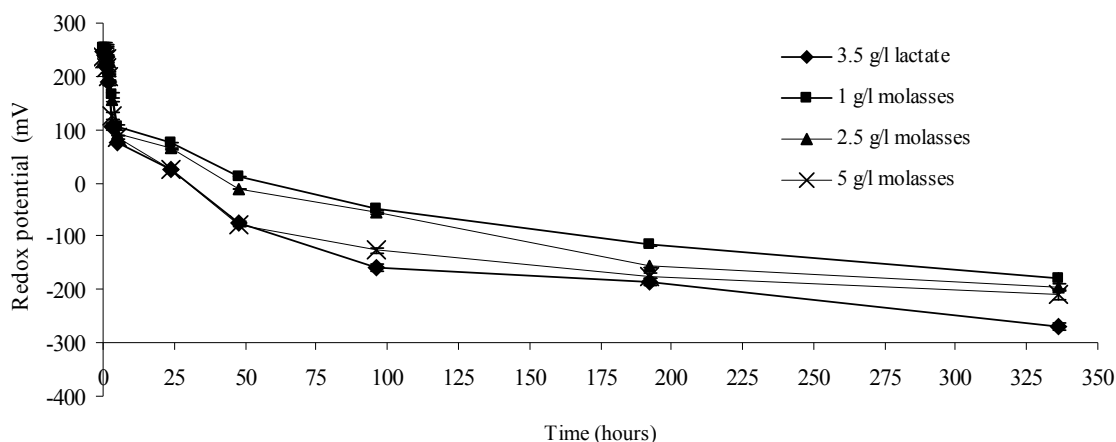
Figure 3.3 Changes in redox potential as a function of time during the growth of SRB on 1, 2.5 and 5 g l^{-1} molasses and 3.5 g l^{-1} lactate. Error bars represent standard deviation between 3 measurements. Statistically non-significant at P=0.05.

The percentage sulphate reduced during the 14-day batch culture experiments on SRB growth with different concentrations of molasses as carbon source is shown in Figure 3.4. The graphs show that the rate of sulphate reduction was higher in the control (lactate) bottles than in the bottles containing the three molasses concentrations. The percentage reduction was fairly similar in all the molasses-containing bottles.
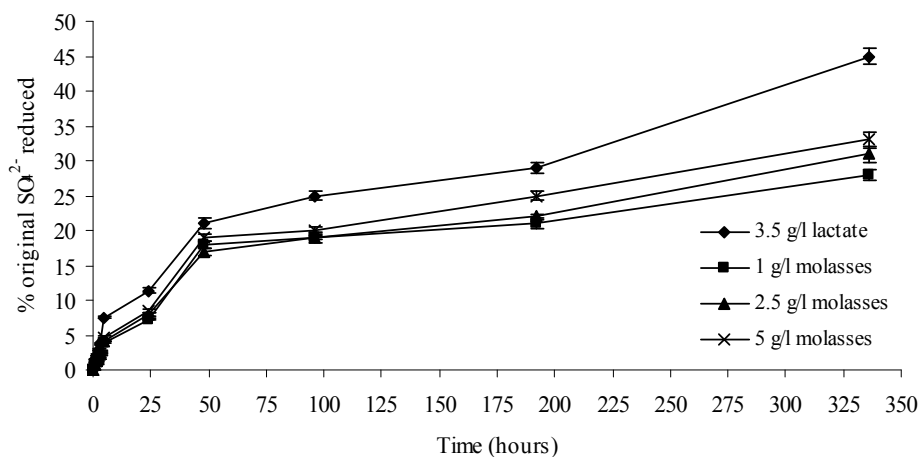
Figure 3.4 Percentage SO$_4^{2-}$ reduction as a function of time during the growth of SRB on 1, 2.5 and 5 g l^{-1} molasses and 3.5 g l^{-1} lactate. Error bars represent standard deviation between 3 measurements. Statistically non-significant at P=0.05.

The slight increase in pH and decrease in redox potential corresponded with the level of sulphate reduction during the same period of time for each of the molasses concentrations.

3.3.2   Effect of arsenic species on the growth of SRB

Arsenite and arsenate had a negative effect on the SRB, the growth rate being slower as the concentrations of the arsenic species increased from 1 mg l^{-1} to 20 mg l^{-1}. The duration of the lag phase also increased with increasing concentrations of each arsenic species, indicating that at high concentrations of arsenite and arsenate the growth of SRB was inhibited to some extent. Figure 3.5 shows the growth of SRB in the presence of increasing concentrations of arsenic species at the end of 14-days of batch culture.
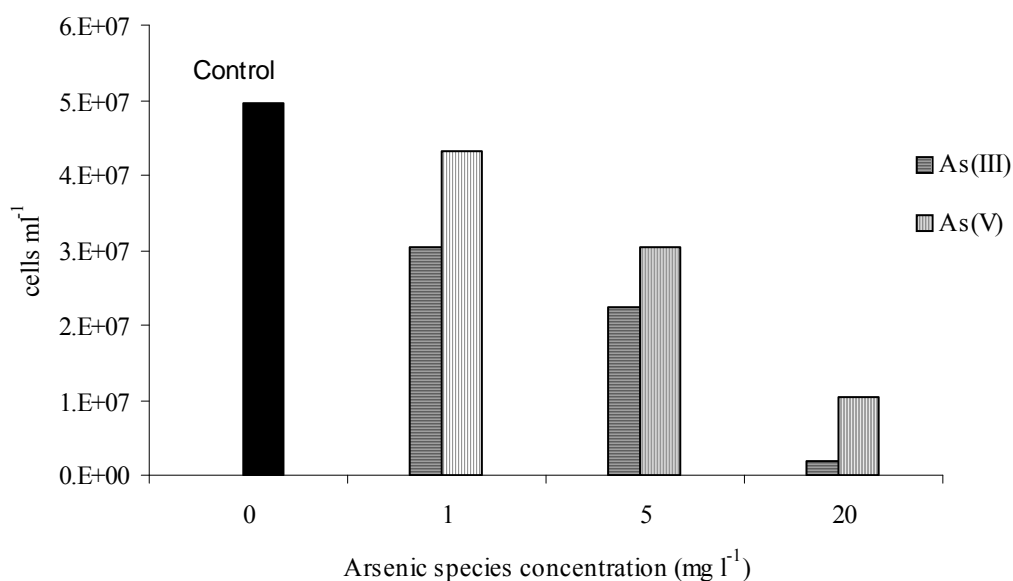
Figure 3.5 Cell number as a function of As(III) or As(V) concentrations after 14 days. Values are from a single measurement.

Figures 3.6 and 3.7 show the percentage sulphate reduction occurring in the presence of different concentrations of As(III) and As(V), respectively. For the control and for both arsenic species at 1 mg l^{-1}, the reduction of sulphate reached 5% on day 3 and thereafter increased at a roughly uniform rate. At 5 mg l^{-1} of either arsenic species the 5% reduction level was reached only on day 5. This indicated that at high concentrations of either arsenic species the ability of SRB to reduce sulphate to sulphide was greatly decreased.

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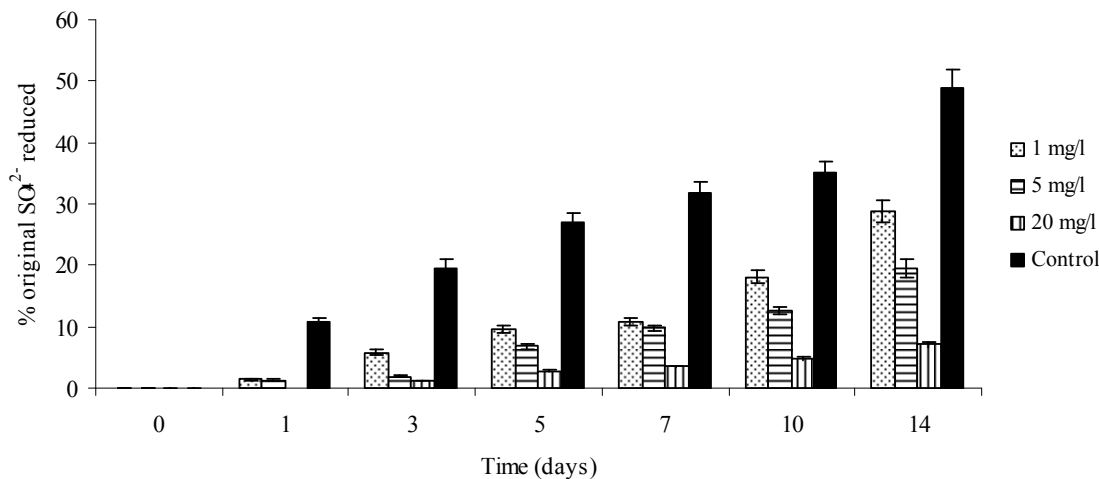
Figure 3.6 Percentage SO_4^{2-} reduction as a function of time during the growth of SRB in the presence of different As(III) concentrations. Error bars represent standard deviation between 3 measurements. Statistically significant at P=0.05.
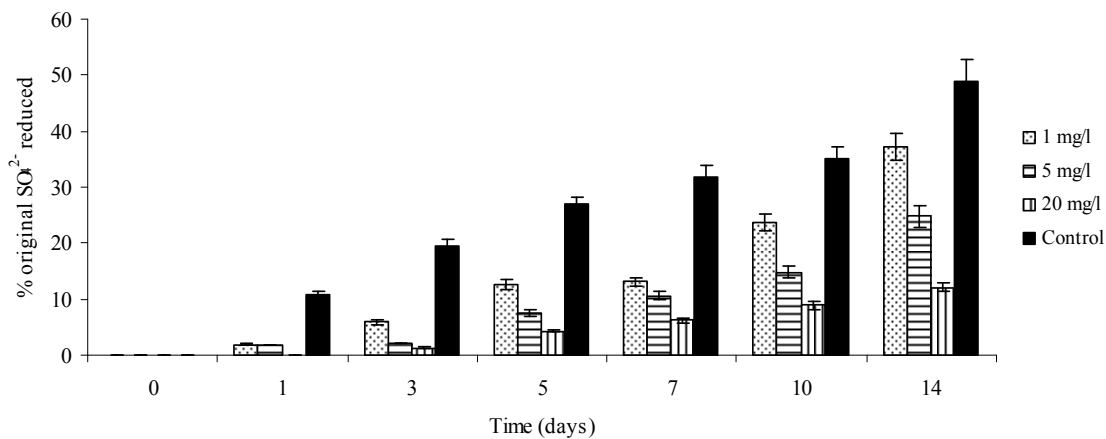
Figure 3.7 Percentage SO_4^{2-} reduction as a function of time during the growth of SRB in the presence of different As(V) concentrations. Error bars represent standard deviation between 3 measurements. Statistically significant at P=0.05.

Regardless of the initial concentration of arsenic, sulphate reduction was always greater in the presence of As(V) than in the presence of As(III). A comparison of the effects of the range of concentrations of the two arsenic species on the percentage sulphate reduction at the end of the 14-day experimental period is summarised in Figure 3.8.

Following adaptation to grow in the presence of arsenic species, higher sulphate reduction activities by SRB were recorded in the presence of both As(III) and As(V) as compared to the initial un-adapted cultures (data not shown).
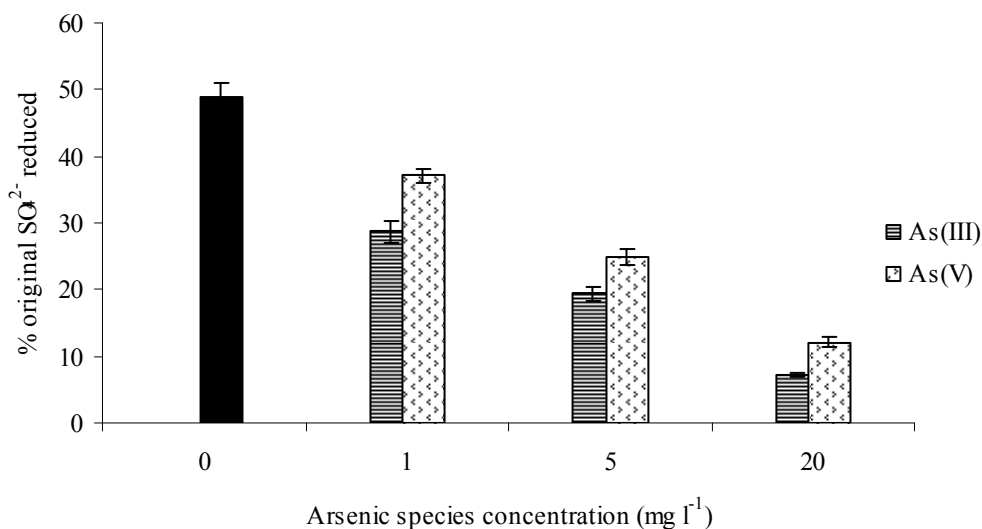
Figure 3.8 Percentage SO_4^{2-} reduction as a function of arsenic species concentrations (mg l^{-1}) for As(III) and As(V) after growth of SRB for 14 days. Error bars represent standard deviation between 3 measurements.

3.3.3 Chemical Characterisation of Molasses

The TOC concentration of molasses was 36.2±2.7 g l^{-1}. Digested molasses was analysed for various elements using ICP-OES. Table 3.2 shows the concentration of the metals detected.

Table 3.2   Elemental composition of molasses

Element	Concentration (µg g^{-1})	Limit of Detection (µg g^{-1})
Al	0.54±0.03	0.004
As	0.24±0.01	0.005
Cu	8.70±0.45	0.004
Fe	0.35±0.02	0.005
Mg	0.15±0.01	0.001
Mn	11.10±0.63	0.003
Zn	19.70±0.84	0.001

NB. Each value is the mean of three readings and standard deviations.

Cd, Pb, Hg and Ni were not detected in the molasses used in our experiments. The absence of toxic heavy metals in significant concentrations in molasses can make it a potentially useful carbon source for culturing SRB and other microorganisms.

3.4 Discussion

The increase in pH observed when either molasses or lactate were used as carbon source for growth of SRB reflects the oxidation of the organic carbon (electron donor) source into bicarbonate thereby increasing the alkalinity. Concomittantly, sulphate (the final electron acceptor) is reduced to hydrogen sulphide which combines with the metals present to form insoluble metal sulphides (Zagury *et al*., 2006). The increase in pH and accompanying decrease of redox potential during bacterial growth possibly indicate the establishment of anaerobic reducing conditions which are conducive to the growth of SRB. Sulphate reduction by SRB occurs when the redox potential is below -100 mV (Postgate, 1979).

A satisfactory level of sulphate reduction by SRB using molasses as electron donor has been reported previously (Gonçalves *et al*., 2005). This study showed that molasses at concentrations of 1, 2.5 and 5 g l^{-1} supported the growth of SRB; however, use of the higher concentrations of molasses can introduce additional, non-degradable materials (including products of caramelisation) that can have a deleterious effect on the growth of the bacteria (Annachhatre & Suktrakoolvait, 2001). Moreover, the presence of large amounts of volatile fatty acids when high concentrations of molasses are used can have a negative impact on the growth of SRB (Lo *et al*., 1990). At concentrations of 2.5 and 5 g l^{-1}, molasses imparted a brownish colour to the medium and this would have an aesthetically unacceptable effect on the visual quality of any water treated; therefore, use of the lower concentration of molasses (1% w v^{-1}) that supported the growth of SRB is recommended for the treatment of arsenic-contaminated waters.

In all our batch experiments, lactate was superior to molasses as a carbon source for SRB; however, due to the high cost of lactate, the operational costs of a large-scale operation would be prohibitive. A cheap source of carbon, such as molasses, is a prerequisite for treating large volumes of arsenic contaminated water with SRB. Zagury *et al*. (2006) have

characterised and assessed the reactivity of organic substrates for SRB in acid mine drainage treatment. They found that the mixture of organic materials (leaf compost, poultry manure and maple wood chips) were successful in promoting sulphate reduction and metal removal.

Molasses may have trace amounts of toxic heavy metals that can inhibit the growth of SRB. Heavy metals, even at concentrations as low as 5-10 mg l^{-1}, can adversely influence microorganisms by affecting their growth, morphology or biochemical activities. The impact of metals on microbial activity could be due to: (1) a decrease in viable cell numbers resulting from death of the less tolerant species due to toxicity; and (2) the metals could decrease the metabolic activity of the survivors in the population (Mazidji *et al*., 1992). However, as stated earlier, the potentially toxic metals Cd, Pb and Ni were absent and only very small amounts of Al, As, Cu, Fe, Mg, Mn and Zn were present in the molasses used in our experiments which could be beneficial since, in addition to serving as carbon source, it would also supply many of the essential trace elements required by the bacteria for balanced growth.

There are no reports in the literature concerning the maximum concentrations of arsenic that can be tolerated by growing cultures of SRB. In our experiments, arsenic at concentrations of 1 and 5 mg l^{-1} for both As(III) and As(V) did not affect the reduction of sulphate by our SRB culture; however, when the concentrations were increased to 20 mg l^{-1}, the level of sulphate reduction was greatly reduced. It is possible that the reduction of the toxic effects of arsenic species on SRBs may be due to precipitation and/or complexation of the arsenicals with chemicals present in the growth media. Utgikar *et al*. (2002) reported that the effect of heavy metals on the growth of sulphate-reducing bacteria can be stimulatory at lower concentrations and toxic/inhibitory at higher concentrations. The experimental culture used here comprised a mixture of SRBs so the quantification of its heavy metal tolerance could be difficult. Additional complications might include effects of metal hydroxides and sulphide precipitation, biosorption, and complexation with the constituents of the growth media (Utgikar *et al*., 2001). Hence, it is important to

characterise the dissolved heavy metals in the water to be treated since this could influence the design and operation of any bioremediation processes involving SRB.

3.5 Conclusions

Molasses provided as the source of carbon at concentrations of 1, 2.5 and 5 g l^{-1} supports the growth of SRB. With increased molasses concentration, growth also increased to some extent. This is accompanied by a slight increase in pH and decrease in redox potential. However, since at the higher molasses concentration the water turns brownish, lower levels (i.e., 1 g l^{-1}) are recommended for the SRB bioremediation of arsenic contaminated waters. The heavy metals occurring in molasses are not present in high enough concentrations to inhibit the growth of SRB.

At 20 mg l^{-1}, both arsenic species, but particularly As(III), were shown to reduce the growth of SRB. Likewise, sulphate reduction was reduced to less than 8% when this concentration of either As(III) or As(V) was present. At much lower concentration of both arsenic species, the growth of SRB is considerably better but a prolonged lag phase is evident.

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Chapter 4

Evaluation of Pine Bark, Polystyrene and Sand as Support Matrices for Immobilisation of Sulphate-Reducing Bacteria

4.1   Introduction

Immobilised microbial cells often outperform their planktonic counterparts in the treatment of metal polluted waters; thus, the immobilisation of SRB on solid support-matrices is likely to improve their performance during the bioremediation of arsenic contaminated waters. Arsenic removal from contaminated water using SRB in the presence of sulphate ions has to take into account different operational considerations for the process to be feasible when bioreactors are used. One such consideration is the support matrix for the growing biomass.

The attachment of microorganisms to solid surfaces is an important phenomenon in many environments, including aquatic habitats (Manly, 1970), soils (Marshall, 1976) and others. The attachment can be by special structures like pili or holdfasts, but in most cases it is through extracellular polymeric adhesives (Fletcher & Floodgate, 1973; Marshall *et al.*, 1971). Efficiency of collision between organisms and substratum surface, mass transport of microorganisms, and the reversibility of the process (continuous exchange of free and adhering organisms) determine the first stage of microbial adhesion to the substrate surface (Savage & Fletcher, 1985). Reversibility allows the microorganisms to leave the substratum surface when conditions become unfavourable (Busscher *et al.*, 1986; Rosenberg, 1986). Bacterial adhesion to surfaces can be inhibited in the presence of low-surface tension (<13 m J m^{-2}) caused by substances such as poly(methylpropenoxy fluoralkyl siloxane) or poly(perfluoroacrylate) polymers that may form relatively smooth surfaces (Tsibouklis *et al.*, 2000).

The formations of conditioning films that contain adsorbed organic components precedes the adhesion of bacteria. These films may either inhibit or promote bacterial adhesion (Schneider, 1996). The physicochemical properties of the underlying substratum surface (e.g., hydrophobicity and charge) control the final properties of the films (Taylor *et al.*,

1997). The initial bacterial attachment is a reversible adsorption process governed by electrostatic attraction and physical forces including van der Waals forces and hydrophobic attraction, but not chemisorption (Ong *et al*., 1999; van Oss *et al*., 1986). Parameters such as strength of cell-substratum interaction, the time of adhesive contact, the effect of pH, ionic strength, culture age and growth conditions all influence cell adhesion to surfaces (Bowen *et al*., 2000). This initial bacterial attachment is a crucial step in the process of biofilm development (Razatos *et al*., 1998).

It is important to select a suitable support-matrix for cell immobilisation, especially if sulphate reduction that is needed (Silva *et al*., 2006). The number and type of microorganisms adhering to the surface may differ from one support to another and as a result may affect the bioremediation efficiency of the system.

Sheng *et al*. (2007) studied the adhesion of two anaerobic sulphate-reducing bacteria (*Desulfovibrio desulfuricans* and a marine isolate) to four polished metal surfaces (stainless steel, mild steel, aluminium and copper) using a force spectroscopy technique with an atomic force microscope (AFM). The results showed that among the metals investigated the bacterial adhesion force was highest to aluminium and lowest to copper. In another study by Celis-Garcia *et al*. (2008) inorganic sulphur compounds were removed by a biofilm of sulphate reducing and sulphide oxidising bacteria over a plastic support in a down-flow fluidised bed reactor.

In this work, the adhesion of a mixed culture containing SRB to pine bark, polystyrene and sand was evaluated on the basis of sulphate reduction efficiency. ESEM was used to study biofilm formation in/on these support matrices. The results were compared with those obtained with planktonic SRB populations.

4.2 Materials and Methods

4.2.1 Nutrient Medium and Source of Sulphate-Reducing Bacteria

The growth medium used was Postgate medium B (Postgate, 1979) as described in **section 2.2,** and the bacteria were isolated from anaerobic sediments from the Msunduzi River (Pietermaritzburg, South Africa) as described in **sections 2.1** and **2.3**.

4.2.2 Immobilisation Substrates

The immobilisation substrates used in this study were described in **section 2.6**.

4.2.3 Experimental Cultures

The experimental cultures were grown in Postgate medium B with lactate as carbon source. SRB immobilisation and growth were performed in duplicate using a 20% (v v^{-1}) inoculum of cells sub-cultured three times on the same medium. All experiments were conducted in the dark at room temperature (25±2°C) for 1-2 weeks.

4.2.4 Immobilisation of SRB

The effects of cell immobilisation on pine bark, polystyrene and sand on the growth of SRB in terms of sulphate reduction, sulphide production and change in pH and redox potential throughout the experimental period were investigated by comparing these cultures with cells growing in a matrix-free system. Flasks containing Postgate medium B and either pine bark, polystyrene or sand were inoculated with a 20% inoculum of a pre-grown mixed culture of SRB. The nature of the surface area of each support matrix was determined before commencement of the batch experiments. Each support material was washed three times with distilled water and sterilised before use. Samples of each colonised support-matrix were collected at the end of the 14-day batch experiment to assess the extent of biofilm development using ESEM (Philips, FEI XL 30) (**section 2.9**). To determine the actual size of the population, bacterial cells were detached from the support matrices colonised by SRB into sterile ultra pure water using an ultrasonic bath (**section 2.12**) and enumerated by direct counting using a Neubauer counting chamber and phase contrast microscopy (Zeiss). Polystyrene immobilised cultures performed best and hence this support matrix was further investigated.

4.2.5 Growth of SRB in the Presence of Arsenic Species with Polystyrene as Support Matrix

The batch experiments were designed to study the effect of polystyrene on the growth of SRB in the presence of different concentrations of arsenic species. Arsenite and arsenate solutions were prepared according to **section 2.4**. The arsenic solutions and the growth medium were sterilised separately and appropriate volumes of either arsenate or arsenite were added to the culture medium to give final concentrations of 1, 5 and 20 mg l^{-1} in the presence of polystyrene as support matrix. Controls comprised the same growth media but without arsenic.

The effect of the two arsenic species on the growth of SRB in the presence of polystyrene was evaluated according to the sulphate activity assay and the production of sulphide in 250 ml serum bottles (in triplicate)according the procedures described in **sections 2.20** and **2.21**. All cultures were incubated in the dark at room temperature (25±2°C) for 2 weeks. Samples were collected to measure sulphate and sulphide.

4.2.6 Phenolic Extractions from Pine Bark

Additional experiments were conducted to investigate why pine bark gave different results when compared to sand and polystyrene as a support-matrix for SRB. About 25 g of pine bark were added to flasks with 150 ml distilled water at room temperature and extracts collected after 15 min, 6 hrs and 1, 3, 5 days. The flasks were static and open to the air. The extracts were filtered and analysed for phenolic compounds and pH. In addition, the extract from a prolonged pine bark extraction (~ 2 months) was analysed.

4.2.7 Growth of SRB on Pine Bark Extracts

Triplicate extracts of pine bark (100, 33 and 16% v v^{-1}) were used *in lieu* of or diluted with water to prepare Postgate medium B which was then inoculated with SRB. Growth of the bacteria in terms of cells ml^{-1} (determined according **section 2.11**) was compared to that in normal Postgate medium B.

4.2.8 Analytical Procedures

Culture samples were analysed for pH and redox potential using a Crison micropH 2000 meter. Sulphate was measured photometrically according to the method described in **section 2.20.1**, and sulphide was determined colorimetrically using the method described in **section 2.21**. Elemental composition of the pine bark used in this experiment was determined on microwave digisted samples (Mars 5, CEM microwave, US) using ICP-OES. The phenols in pine bark extracts were determined using the USEPA Method 9065 (**section 2.23**).

4.3 Results

The fine structures of the support matrices investigated are shown in Figure 4.1. In addition to differences in surface topography the three materials also differed in their specific surface area (estimated according to **section 2.13**) and void volumes (Table 4.1). The micrographs shows the colonisation of the support matrices with the bacteria (Figures 4.1

Table 4.1 Specific surface area and void volume in flasks (330 ml) packed to the capacity with the different support matrices

Support matrix	Specific surface area (cm^2 g^{-1})	Void volume (ml)
Pine bark	80	175
Sand	3 400	75
Polystyrene	30	180
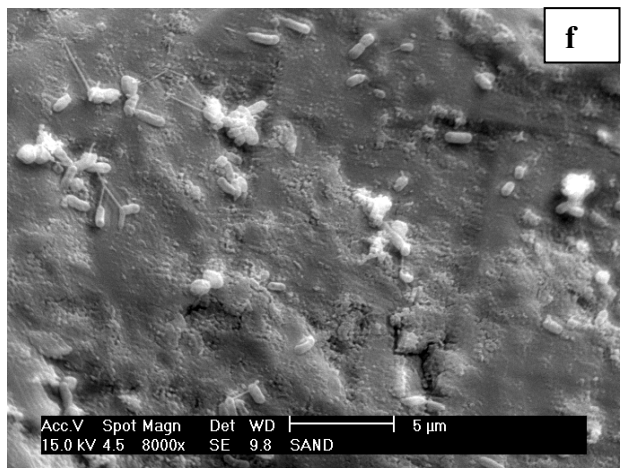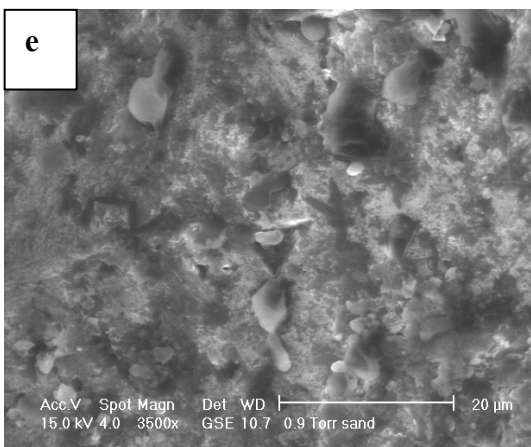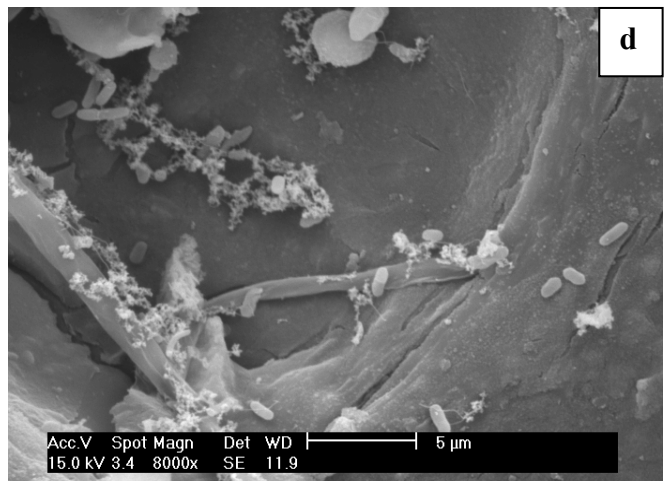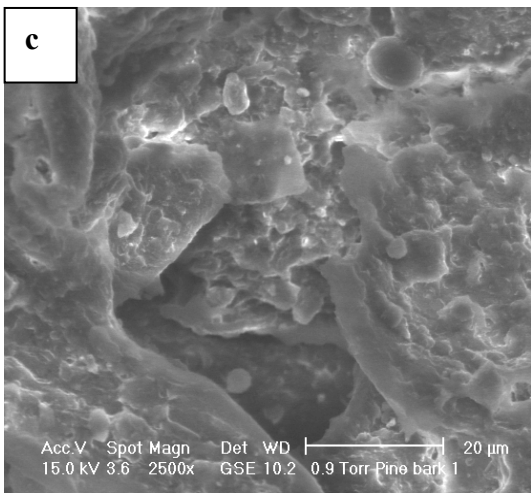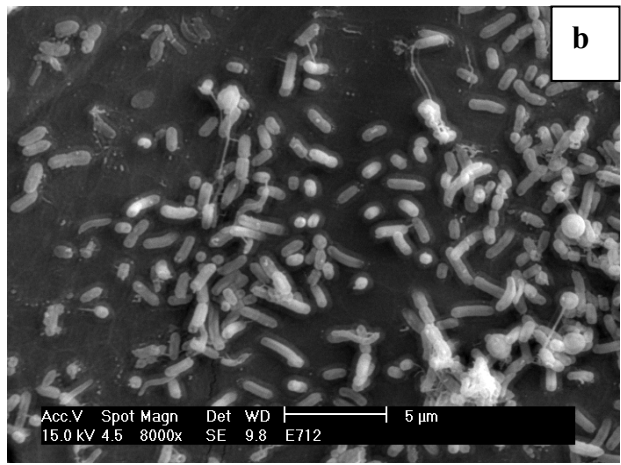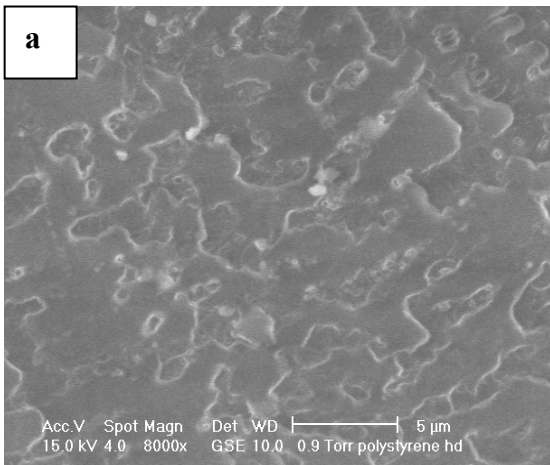
Figure 4.1 ESEM micrographs of control (**a**) polystyrene; (**c**) pine bark; (**e**) sand showing surface topography and respective SRB-colonised counterparts b, d and f.

pH and Redox Potential

Initially, the pH remained constant in all SRB-inoculated flasks except those containing pine bark as the support material The pH of the SRB culture used as inoculum was about 7.5 and this may have affected the pH. A very slow increase in pH occurred in the flasks containing sand and polystyrene and in the matrix-free control flasks after day one, whereas with pine bark the pH decreased fairly dramatically throughout the experiment (Figure 4.2). After 10 days, the pH in the polystyrene, sand and matrix-free system was approximately 7.2, 7.2 and 7.1 respectively whereas with pine bark it was 4.6.
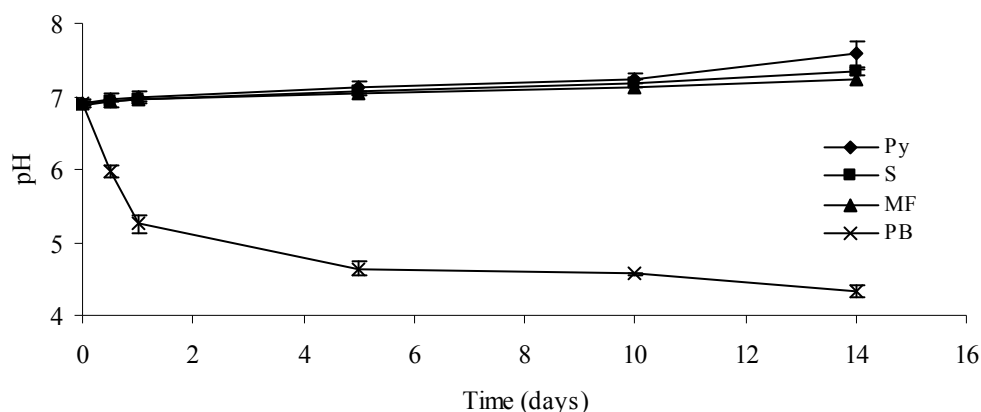
Figure 4.2 Changes in pH as a function of time (days) in flasks with polystyrene (Py), sand (S) and pine bark (PB) as support matrix and in the matrix-free (MF) control system. Error bars represent standard deviation between 3 measurements.

Similarly, the redox potential started to decrease from day one in the polystyrene -and sand-immobilised and in the free-living SRB cultures (Figure 4.3). At day 10, the redox potential in the polystyrene, sand and matrix-free cultures was –227, -205 and –195 mV respectively. In contrast the redox potential in the pine bark immobilised culture at day 10 was -19 mV.

Between days 10 and 14 the decrease in redox potential was gradual, reaching -295, -265, -235 and -50 mV in the polystyrene, sand, matrix-free and pine bark containing flasks respectively (Figure 4.3).
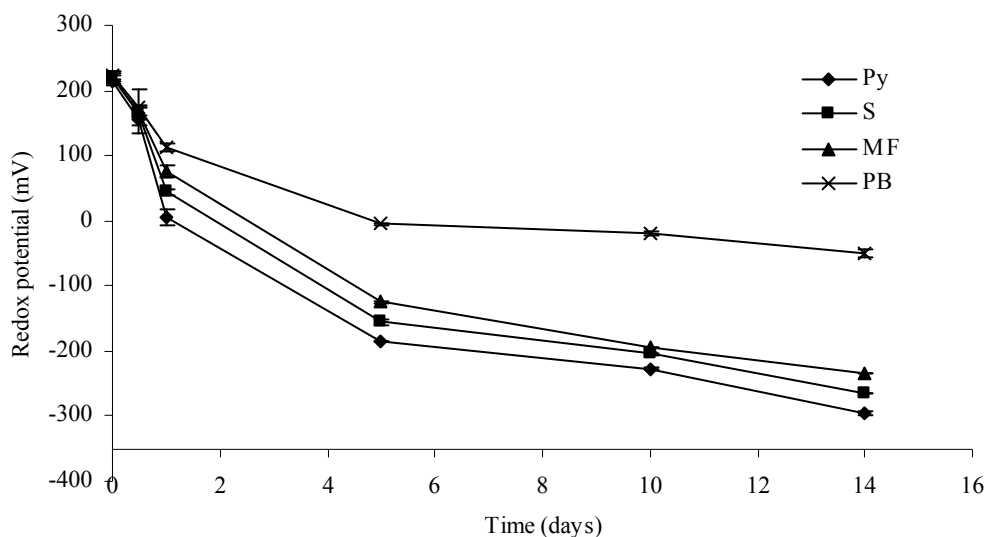
Figure 4.3 Changes in redox potential as a function of time (days) in flasks with polystyrene (Py), sand (S) and pine bark (PB) as support matrix and in a matrix-free (MF) system. Error bars represent standard deviation between 3 measurements. Statistically non-significant at P=0.05.

Sulphate Reduction and Sulphide Production

Figure 4.4 shows the level of sulphate reduction measured in the pine bark, sand and polystyrene immobilised flasks and in the matrix-free batch cultures. After 14 days, highest sulphate reduction (49%) had occurred in the polystyrene-containing flasks, whereas the lowest level (~7%) was observed when pine bark was the support matrix. In the free-living SRB culture, sulphate reduction amounted to about 42%, while with sand approximately 36% reduction occurred.
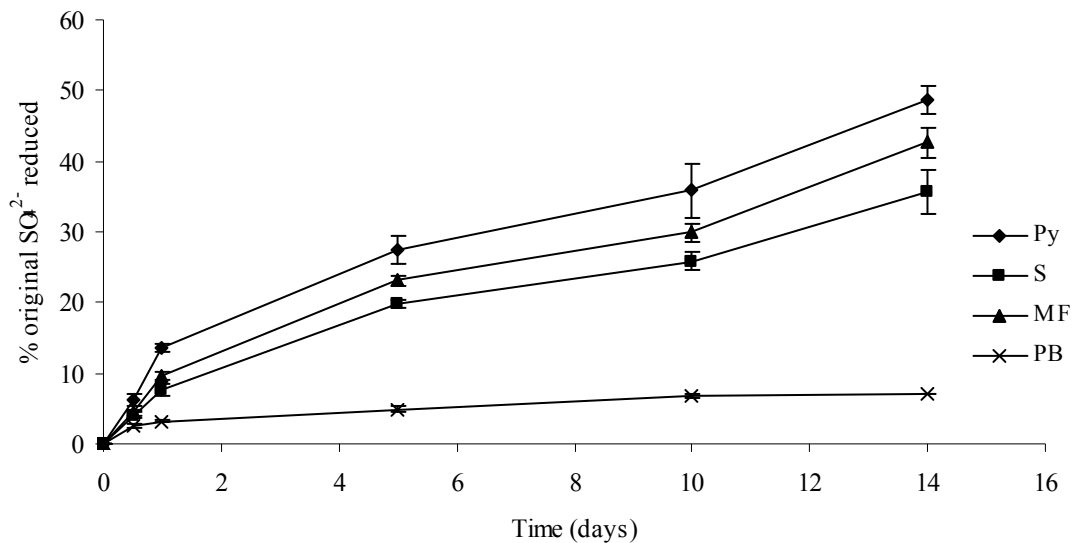
Figure 4.4 Percentage SO_4^{2-} reduction as a function of time (days) during growth of SRB on polystyrene (Py), sand (S), pine bark (PB) and in a matrix-free (MF) culture. Error bars represent standard deviation between 3 measurements. Statistically non-significant at P=0.05.

Sulphide production started on day 1 in the polystyrene and sand immobilised cultures and in the flasks with free-living SRB and reached about 10, 6.84 and 6.55 mg l^{-1} respectively at the end of the 14-day experiment. With pine bark as support matrix, only 4.5 mg l^{-1} sulphide was present at the end of the experiment (Figure 4.5).
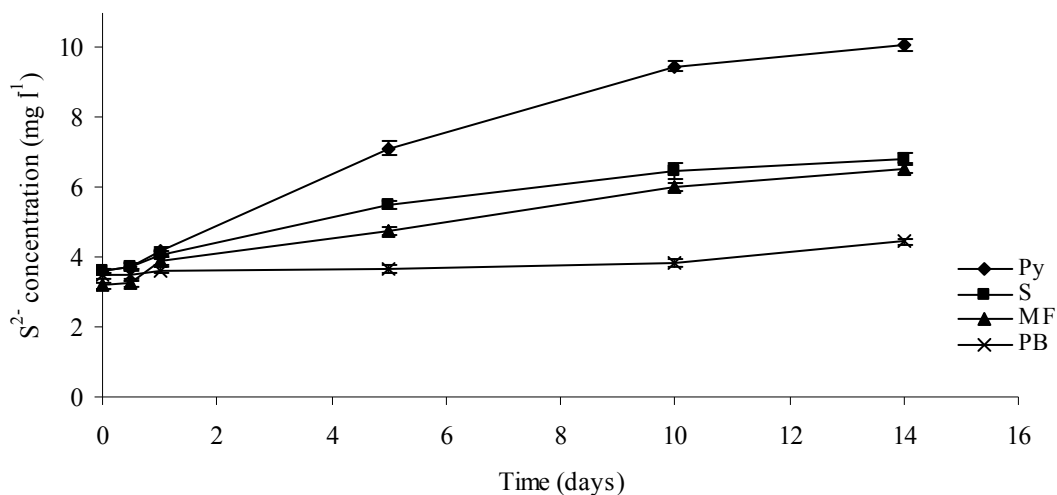
Figure 4.5 Changes in sulphide concentration as a function of time (days) during growth of SRB on polystyrene (Py), sand (S), pine bark (PB) and in a matrix-free (MF) system. Error bars represent standard deviation between 3 measurements. Statistically non-significant at P=0.05.

Table 4.2 shows the number of SRB cells per cm^2 detached from polystyrene, pine bark and sand at the end of 14 days of SRB growth when the support matrices were subjected to ultrasound.

Table 4.2   Cell number as a function of the surface area of the support material

Substrate	Number of cells per cm^2
Polystyrene	4.7×10^5
Pine bark	3.0×10^3
Sand	2.4×10^4

Growth of polystyrene-supported SRB in the presence of 20 mg l^{-1} As(III) and As(V) resulted in an increase in sulphide concentration from 3.2 mg l^{-1} to 6.6 and 7.2 mg l^{-1} respectively (Figure 4.6) over 14 days. The control (SRB growing on polystyrene without arsenic) showed a greater than 50% increase in sulphide concentration over that produced by a similar culture growing in the presence of 20 mg l^{-1} As(III).

111
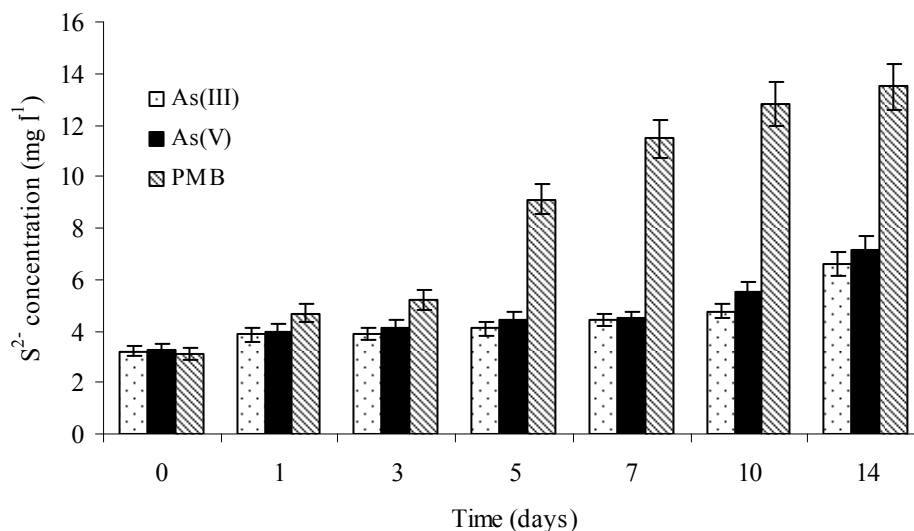
Figure 4.6 Changes in sulphide concentration during growth of polystyrene-supported SRB in Postgate medium B (PMB) and the same medium containing 20 mg l^{-1} As(III) or As(V). Error bars represent standard deviation between 3 measurements. Statistically significant at P=0.05.

The level of sulphate reduction in polystyrene immobilised SRB cultures occurring in the presence of 1, 5 and 20 mg l^{-1} of either arsenic species at the end of day 14 is depicted in Figure 4.7. The percentage sulphate reduction decreased as the concentration of either arsenic species was increased, with As(III) causing a larger decrease than As(V).
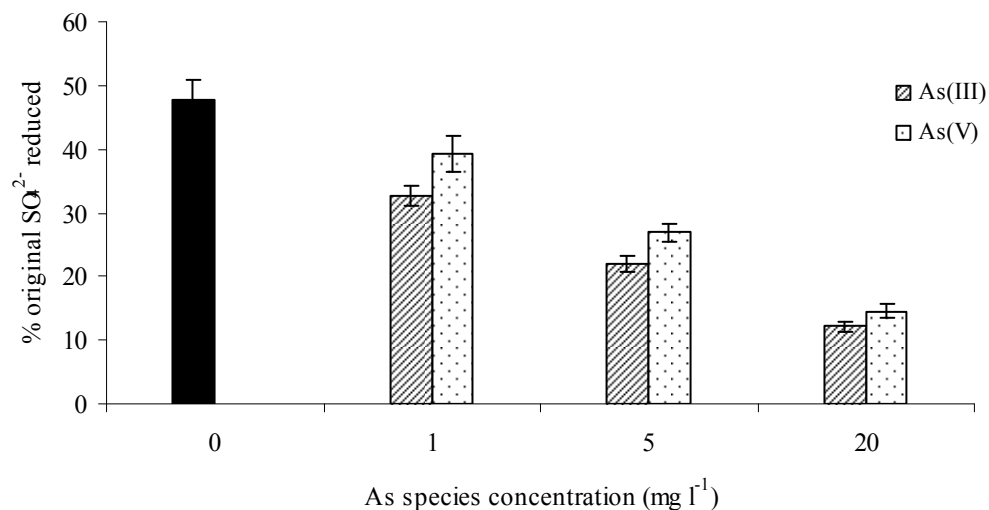
Figure 4.7 Percentage SO$_4^{2-}$ reduction by polystyrene-immobilised SRB as a function of arsenic species concentration. Error bars represent standard deviation between 3 measurements.

ESEM micrographs of the polystyrene immobilised SRB biomass after 14 days growth in Postgatge medium B in the presence [1 mg l^{-1} As(V)] and absence of arsenic are shown in Figure 4.8. At this concentration As(V) has little negative effect on the growth of the cells.
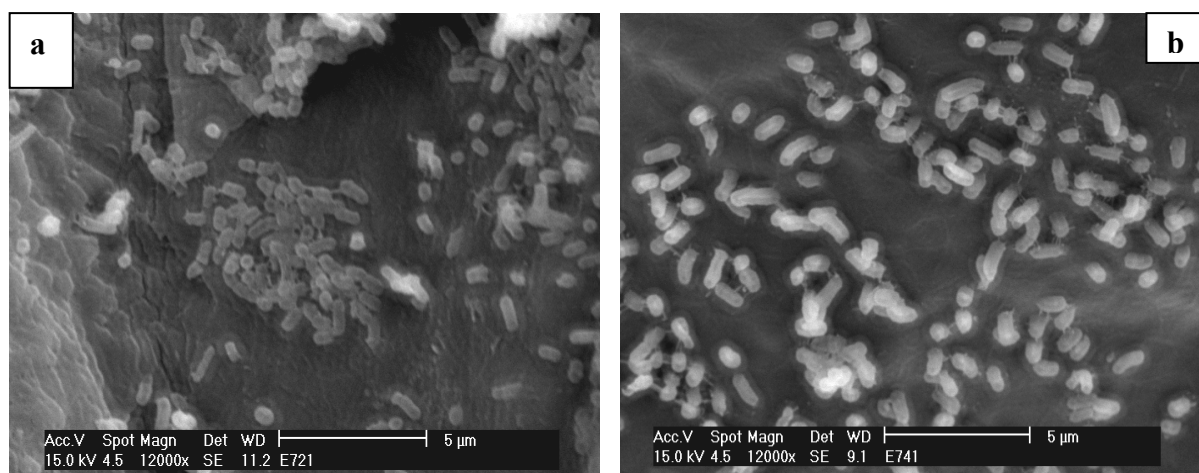
Figure 4.8 ESEM micrographs showing SRB growth on polystyrene with (a) arsenic amended [1 mg l^{-1} As(V)] and (b) arsenic-free Postgate medium B.

The pH of the pine bark extract and concentration of phenolic compounds therein following extraction times ranging from 15 min to 5-days and over an extended period (~ 2 months) are shown in Figure 4.9. The pH progressively decreased from ~ 5.9 after a 15 min extraction period to 4.0 after a 5 day extraction period.
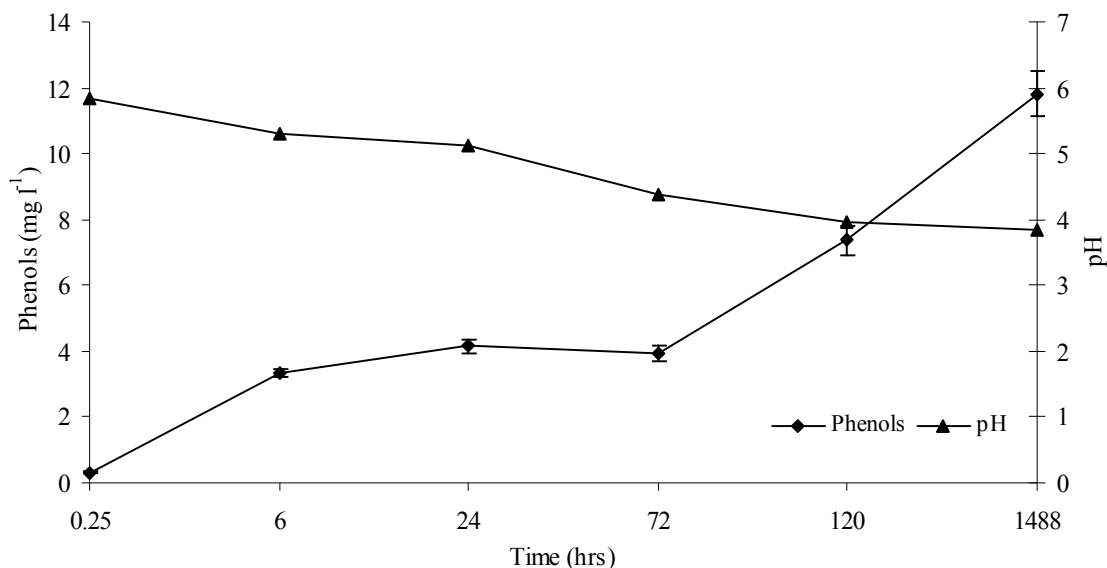
Figure 4.9 Phenolic content and pH of pine bark extract following extraction with distilled water for different periods of time. Error bars represent standard deviation between 3 measurements.

The extract from the 5-day treatment (in the proportions 16, 33 and 100%) were used *in lieu* of pure water to prepare modified Postgate medium B for culturing of SRB. Growth of SRB, expressed as number of cells ml^{-1}, in pine bark extract supplemented Postgate medium B and in standard Postgate medium B is shown in Figure 4.10. The elemental composition of digested pine bark is given in Table 4.3. The presence of 100% and 33% pine bark extract in the medium inhibited the growth of SRB, while a concentration of 16% caused an extended (96 h) lag period followed by a slow, small increase in cell population.
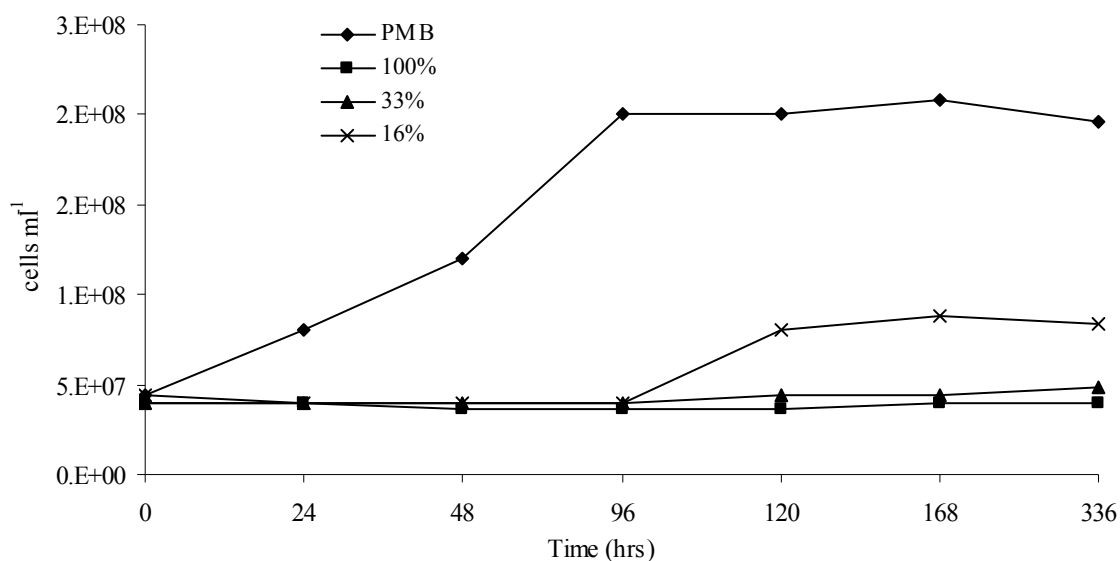
Figure 4.10 Growth of SRB in standard Postgate medium B (PMB) and in the same medium supplemented with pine bark extract at 100, 33 and 16% water replacement levels. Values are from a single measurement. Statistically highly significant at P=0.05.

Table 4.3   Elemental Composition of Pine Bark

Element	Concentration ($\mu g\ g^{-1}$)
As	ND[*]
Cd	ND
Cu	0.79
Fe	8.3
Mn	55
Ni	ND
Pb	ND
Zn	4.5

Key: *ND – not detected

4.4   Discussion

In batch experiments, the total surface area available for colonisation by the SRB can determine the extent of biofilm development. This process can also be influenced by both the electrical charge and pore size of the supporting matrices (Webb & Dervakos, 1996). A study by Silva *et al.* (2006) designed to evaluate support materials for the immobilisation of SRB and methanogenic archaea showed that SRB cells were predominant in the biofilms developing on vegetal carbon and lowest in those growing on alumina-based ceramics. No sulphate reduction occurred in the latter nor in systems with low-density polyethylene as a support matrix. Investigations by Silva *et al.* (2002), Vela *et al.* (2002) and Cadavid *et al.* (1999) showed that cells growing on polyurethane foam removed sulphate efficiently, indicating that this substance is a good support-matrix for SRB immobilisation.

The ESEM micrographs in Figure 4.1 show the diversity of morphotypes in the SRB-containing biofilms that developed on each of the three support matrices under investigation. Rod-shaped cells predominated and only a few cocci were evident. Extracellular polymeric material produced by the bacteria was observed in the biofilm that developed on the polystyrene support-matrix. The very slight increases in pH and decrease of redox potential observed in the sand, polystyrene, and matrix-free systems created an environment conducive to SRB growth. By contrast, these changes did not occur with pine bark as the support material, and SRB populations failed to establish.

Since it is important to assess bacterial abundance, biomass and community composition in biofilms (Buesing & Gessner, 2002), an effective cell detachment procedure is needed. There are different methodologies reported for the detachment of bacteria from substratum surfaces, but there is no agreement on which method gives the best results with which type of substratum (Buesing & Gessner, 2002). Gentle rinsing and dipping procedures have been used by many researches (Wirtanen *et al.*, 1996; Liu, 1995). Passing an air-liquid interface over the substratum has also been reported to detach bacteria adhering to solid surfaces (Gómez-Suárez *et al.*, 2001; Pitt *et al.* 1993). Buesing and Gessner, (2002) compared the efficiency of four detachment instruments (Ultrasonic probe, Ultrasonic bath, Ultra-Turax tissue homogeniser and a Stomacher 80 laboratory blender) to remove bacteria from leaf

litter and sediment and from epiphytic biofilms in a natural aquatic system. They indicated that the choice depends on the nature of the support-matrix under investigation and on whether the bacterial biomass is subsequently to be determined. After careful consideration, sonication in an ultrasound bath was adopted to detach the bacteria from the three support materials used in this study since it has been reported (Buesing & Gessner, 2002) that the maximum number of cells recovered by this method can be increased simply by increasing the treatment time. In this study, a 15-minute sonication time was found to be appropriate. Using a counting chamber (Neubauer) for enumeration, it was found that the number of bacterial cells detached from the polystyrene was considerably higher than the numbers removed from the other support-matrices. Image analysis revealed that the total surface areas potentially available for bacterial colonisation were similar for polystyrene and pine bark. Sand on the other hand had a much greater surface area and a considerably smaller void volume than did polystyrene and pine bark, but the former was not as good a support matrix as polystyrene, possibly because of unfavourable surface charge and hydrophobicity effects.

Sulphate reduction by the cells growing on polystyrene was superior to that by cells immobilised on the other materials tested, confirming the findings of other workers that the type of support-matrix used for the immobilisation of SRB does affect the efficiency of the process. As expected, sulphate reduction was lowest when pine bark was the support matrix, possibly due to the leaching of toxic chemicals, such as phenolics, that have shown to inhibit the growth of SRB. Bacterial adhesion to and desorption from solid surfaces can also be affected by their wettabilities. Hydrophobic microorganisms desorbed more readily from hydrophilic substrates than from hydrophobic substrates, and microorganisms with hydrophilic surface properties usually adher reversibly on hydrophobic surfaces (Meinders *et al.*, 1995). The pH of the medium plays a role in determining the electrophysical properties of the bacteria. Charge density and the electrophoretic mobility of SRB change according to pH changes in the medium (Ulanovskii *et al.*, 1980).

A study on anaerobic sulphate reduction by immobilised SRB in bioreactors containing various support matrices by Baskaran and Nemati (2006) showed that among these sand

outperformed biomass support particles (BSP) [BSP – a porous scouring pad sheet] and glass beads as a support-matrix, with a maximum sulphate reduction rate of 1.7 g l^{-1}. Conversely in the present study, sulphate reduction levels were higher in SRB cultures growing on polystyrene than on sand.

Decaying pine bark releases numerous phenolic compounds as the lignin within the bark contains hydroxylated benzene rings as a major component. Work by Uberoi and Bhattacharya (1997) on the effects of chlorophenols and nitrophenols on the kinetics of degradation of toxic substances by SRB showed that a concentration of about 12 mg l^{-1} of some of these phenolic compounds could be inhibitory. The activity of various bacterial enzymes may be inhibited by soluble polyphenols, phenolic acids and plant-derived tannins (Marschner & Kalbitz, 2003). These factors mitigate against pine bark as a suitable support material for immobilising SRB for the long-term treatment of arsenic contaminated water.

4.5   Conclusions

Our results show that the sulphate reduction kinetics of immobilised SRB cultures were affected by the support matrix used, with greater sulphate reduction occurring in cell populations attached to polystyrene than to pine bark or sand. When colonised polystyrene, sand and pine bark were subjected to ultrasound waves to detach the attached bacterial cells, polystyrene was found to have the highest number of cells attached to its surface.

In the presence of arsenite concentrations of 1, 5 and 20 mg l^{-1}, polystyrene-immobilised SRB cells caused sulphate reduction of 32.6, 21.9 and 12.2 mg l^{-1} respectively, whereas with the same concentrations of arsenate sulphate reduction levels of 39.4, 26.9 and 14.6 mg l^{-1} respectively were obtained after 14 days batch culturing.

Pine bark is not suitable as a support matrix since SRB populations immobilised on this material showed inferior sulphate reduction and sulphide production capacities to those immobilised on sand and polystyrene, and those cells growing in a matrix-free system. Phenolic compounds leached from the pine bark and concentrations of these substances in water extracts increased with extraction time, reaching about 12 mg l^{-1} on day 5. The pH

also decreased progressively over the extraction time. When these extracts were used to prepare media for culturing SRB, growth of the bacteria was completely inhibited. The high levels of Mn (55 mg kg^{-1}) and the presence of Fe (8.3 mg kg^{-1}) found in digested samples of pine bark might also make this an unsuitable material for immobilising SRB for use in the bioremediation of arsenic contaminated waters. By contrast, polystyrene was shown to be a good material for this purpose.

4.6   References

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Chapter 5

Effect of Nitrate, Sulphate and Iron on the Growth of Sulphate-Reducing Bacteria (SRB)

5.1   Introduction

Sulphate-reducing bacteria are one of the important groups of microorganisms that degrade organic matter and carry out sulphate reduction (Fauque, 1995; Postgate, 1979). As some SRB can grow in the absence of sulphate, this group of bacteria has become widely spread in different ecological environments (Moura *et al*., 1997). Electron acceptors other than sulphate have been recognised (Faugue & Ollivier, 2004; Fauque *et al*., 1991; Postgate, 1979), including inorganic compounds such as bisulphite, metabisulphite, dithionite, tetrathionate, thiosulphate, sulphur, nitrate and nitrite, and a wide range of organic compounds such as fumarate, malate, aspirate, cystine, oxidised glutathione and pyrvuate (Moura *et al*., 1997).

The utilisation of inorganic compounds and, in particular metals, by SRB as electron acceptor is less studied. There is geological evidence that microbial reduction of Fe(III) and other metals may have evolved before sulphate, nitrate and oxygen reduction (Lovley, 1993; Vargas *et al*., 1998). Several *Desulfovibrio* species that can oxidise H_2 with simultaneous reduction of Fe(III) are comparable to other Fe(III) reducers (Moura *et al*., 1997).

The reduction of sulphate in the presence of nitrate depends on the type of SRB population and the carbon source. Nitrate can efficiently provide energy to support growth of some SRB as shown in cell extracts or washed cell suspensions of *D. desulfuricans* (Liu & Peck, 1981), *Desulfovibrio* sp. (McCready *et al*., 1983) and *D. gigas* (Barton *et al*., 1983; Odom & Peck, 1981). The growth of *D. desulfuricans* on nitrate medium was better than when sulphate was the terminal electron acceptor (Fauque *et al*., 1991). Calculations of the free energy thermodynamics for the electron transfer confirmed this (Postgate, 1979):

$$NO_3^- + 4H_2 + 2H^+ = 3H_2O + NH_4^+ \qquad \Delta G = -149.2 \text{ kJ/H}_2 \qquad \textbf{(5.1)}$$

$$SO_4^{2-} + 4H_2 = S^{2-} + 4H_2O \qquad\qquad \Delta G = -172.0 \text{ kJ/H}_2 \qquad (5.2)$$

Even though the reduction of nitrate prevails energetically over sulphate reduction (Pietzsch & Babel, 2003; Thauer *et al.*, 1977), little attention has been given to it interms of ecological significance. This might be due to, in some cases, the preferential reduction of sulphate over nitrate when both are present in the culture medium (Pietzsch & Babel, 2003; Widdel & Pfennig, 1982), thereby suggesting that nitrate reduction was insignificant in the respiratory system; however, it has been proposed that some SRB use nitrate in preference to sulphate as electron acceptor (Seitz & Cypionka, 1986) reducing it to ammonium (Keith & Herbert, 1983; McCready *et al.*, 1983) or they may use both electron acceptors simultaneously (Keith & Herbert, 1983) making the real reduction of nitrate by SRB ambiguous in marine or terrestrial sediments (Lopez-Cortes *et al.*, 2006).

Among the SRB, members of the genera *Desulfovibrio* (McCready *et al.*, 1983; Liu & Peck, 1981), *Desulfobulbus* (e.g., *Desulfobulbus propionicus*; Widdel & Pfennig, 1982) *Desulforhopalus* (e.g., *Desulforhopalus singaporensis*; Lie *et al.*, 1999), *Desulfobacterium* (e.g., *Desulfobacterium catecholicum*; Szewzyk & Pfennig, 1987) and *Desulfomonas* (Widdel & Pfennig, 1984) have the ability to reduce nitrate. The dissimilatory reduction of nitrate by SRB is poorly studied (Moura *et al.*, 1997); however, in a review article these authors concluded that SRB possess two enzymes responsible for the stepwise reduction of nitrate through nitrite to ammonia.

It is recognised that nitrate as electron acceptor would avoid the odour caused by sulphate-reducing bacteria (SRB) in wastewater treatment (Jenneman *et al.*, 1986). The reason is that it can be reduced preferentially to sulphate under anaerobic conditions; but once the nitrate is metabolised, the remaining organic matter is used to reduce sulphate to sulphide (Jenneman *et al.*, 1986). The addition of sufficient nitrate to raise the redox potential above 300 mV and so control the production of sulphide (Poduska & Anderson, 1981).

The objective of this study was to examine the effect of sulphate, nitrate and ferrous iron amendments on the growth of a mixed SRB population.

5.2 Materials and Methods

5.2.1 Nutrient Medium and Source of Sulphate-Reducing Bacteria

The growth medium used was Postgate medium B (Postgate, 1979) as described in **section 2.2**.

The culture of sulphate-reducing bacteria (SRB) was isolated from anaerobic sediments from the Msunduzi River (Pietermaritzburg, South Africa) as described in **section**s **2.1** and **2.3**.

5.3 Batch Experiments

5.3.1 Effect of Nitrate and Sulphate on the Growth of SRB

The growth of SRB was investigated in batch cultures in media containing various sulphate and nitrate concentrations. Mixed cultures of SRB, pre-grown in standard Postgate medium B, were inoculated (20% v v^{-1}; ~2×10^7 cells ml^{-1}) into a series of autoclaved, sterilised 250 ml bottles containing a modified Postgate medium B (initially containing no NO$_3$ and SO$_4$) with the following combinations of sulphate and nitrate (Table 5.1) adjusted to the given concentrations using Na$_2$SO$_4$ and NaNO$_3$ respectively. The combinations were chosen on the basis of preliminary experimental results and the maximum level allowed in drinking water according to WHO.

Table 5.1   Combinations of different sulphate and nitrate concentrations

Combination	SO$_4^{2-}$ (mg l^{-1})	NO$_3^-$ (mg l^{-1})
A	150	5
B	50	5
C	50	30
D	150	30
E	0	5
F	0	30

SRB grown in standard Postgate medium B served as the control (G).

5.3.2 Effect of Nitrate and Ferrous Iron on the Growth of SRB

Additional batch cultures were set-up as described in **section 3.2.1** but with different concentrations of iron (II) (to make the cultures more reduced and so conducive to SRB growth) in the presence of 30 mg l^{-1} nitrate (Table 5.2).

Table 5.2   Concentrations of iron(II) in the presence of 30 mg l^{-1} nitrate

Combinations	Fe(II) (mg l^{-1})	NO$_3^-$ (mg l^{-1})
H	0	30
I	10	30
J	50	30
K	100	30

As a control (G) standard Postgate medium B was again used. The series of flasks (Table 5.2) were inoculated with the SRB culture (20% v v^{-1}; inoculum) and then filled with a modified Postgate medium B that did not contain ferrous ions.

5.3.3 Effect of Nitrate, Iron and Arsenic on the Growth of SRB

The effects of 20 mg l^{-1} either As(III) or As(V), 100 mg l^{-1} iron and 30 mg l^{-1} nitrate on the growth of SRB were tested as follows:

Appropriate solutions of nitrate (5 and 30 mg l^{-1}), sulphate (50 and 150 mg l^{-1}) and ferrous iron (10, 50, 100 mg l^{-1}) were prepared by diluting stock solutions of NaNO$_3$, Na$_2$SO$_4$ and FeCl$_2$ with deionised water. To these, arsenic [either As(III) or As(V)] was added to a final concentration of 20 mg l^{-1}. The experiments were conducted in duplicate and cultures were incubated statically at room temperature (25±2°C) in the dark for 14 days. Samples were collected at time 0 and then after 1, 3, 5, 7, 10 and 14 days with a syringe to determine pH, redox potential, sulphate, sulphide, nitrate, ammonia and ferrous iron concentrations and bacterial count.

5.4 Analytical Techniques

The pH and redox potential were determined using a Crison micropH 2000 meter. The turbidimetric method described in **section 2.20.2** was used to measure the sulphate concentration. Sulphide was determined colorimetrically (**section 2.21**) after preserving the samples with zinc acetate solution. Dissolved ferrous iron was determined according to the procedure given in **section 2.24**.

NH_4^+-N and NO_3^--N were analysed colorimetrically using a continuous flow TrAAcs auto analyser (**section 2.19**).

SRB cell counts were performed by direct counting using a Neubauer counting chamber and phase contrast microscopy (Zeiss).

5.5 Results

5.5.1 Effect of Nitrate and Sulphate Concentrations on the Growth of SRB

Various nitrate and sulphate concentrations were tested in batch cultures to determine their effect on the growth of the mixed SRB culture. After incubation for 14 days under static conditions, growth of the SRB was observed in all bottles (Figure 5.1). When the concentration of sulphate was increased from 50 to 150 mg l^{-1} with either concentration of nitrate (viz 5 or 30 mg l^{-1}), the SRB were higher. The presence of nitrate alone can support the growth of the mixed culture of SRB with 30 mg l^{-1} nitrate supporting better growth than 5 mg l^{-1} nitrate; but the growth was poorer than when either 50 or 150 mg l^{-1} sulphate was also present.
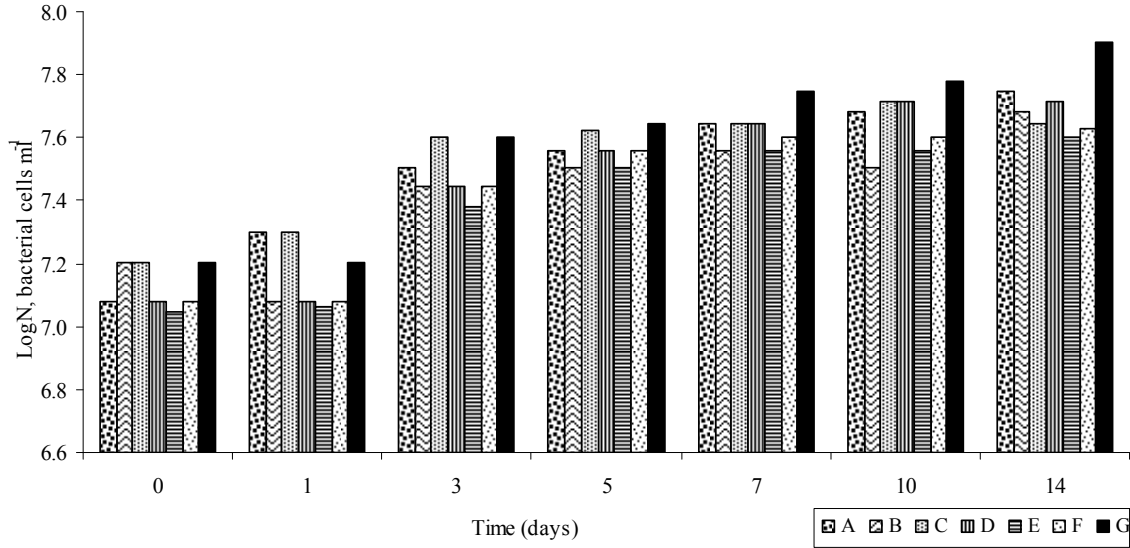
Figure 5.1 Cell number as a function of time during growth in sulphate and nitrate – supplemented PMB. Concentrations (mg l^{-1} of SO_4^{2-} + mg l^{-1} NO_3^-) are represented by: **A**, 150+5; **B**, 50+5; **C**, 50+30; **D**, 150+30; **E**, 0+5; **F**, 0+30 and **G**, Control (PMB, standard Postgate medium B). Values are from a single measurement. Statistically non-significant at P=0.05.

Table 5.3 shows the changes over time in the nitrate, ammonium, sulphate and sulphide concentrations, pH and redox potential in media with different initial concentrations and combinations of sulphate and nitrate following growth of SRB in batch cultures.

Table 5.3 Concentrations (average value±std. Dev.[1]; duplicate reading) of NO_3^-, NH_4^+, SO_4^{2-}, S^{2-}, logN and pH and redox potential after 14 days growth of a mixed culture of SRB in PMB medium with different combinations and initial concentrations of SO_4^{2-} and NO_3^-

	Time (days)	NO_3^--N (mg l^{-1})	NH_4^+-N (mg l^{-1})	SO_4^{2-} (mg l^{-1})	S^{2-} (mg l^{-1})	pH	logN	Redox (mV)
A*	0	10.64±0.91	26.31±2.35	285±18	1.94±0.08	6.8	7.08	207
	1	10.35±0.82	26.63±2.07	276±15	2.73±0.11	6.8	7.30	20
	3	10.09±0.65	26.62±2.20	257±17	4.24±0.21	6.9	7.51	-76
	5	9.86±0.83	27.21±2.33	219±17	7.31±0.49	7.0	7.56	-127
	7	9.77±0.69	27.78±2.27	211±16	7.82±0.39	7.2	7.64	-147
	10	9.44±0.74	28.36±2.18	180±14	11.65±0.80	7.3	7.68	-190
	14	9.36±0.80	28.94±2.18	168±8	11.91±0.74	7.5	7.75	-258
B	0	11.65±1.02	22.98±1.54	197±18	1.87±0.17	6.8	7.20	210
	1	11.29±0.69	23.47±2.22	189±12	2.91±0.18	6.8	7.08	20
	3	11.08±0.88	23.73±2.14	179±12	4.01±0.24	6.8	7.45	-72
	5	10.70±0.73	24.26±1.65	152±13	7.44±0.58	6.9	7.51	-125
	7	10.06±0.87	24.78±1.49	144±9	8.07±0.74	7.1	7.56	-137
	10	9.43±0.73	25.31±2.33	132±9	9.86±0.56	7.2	7.51	-181
	14	8.97±0.69	26.38±2.03	128±8	10.58±0.66	7.2	7.68	-235
C	0	20.81±2.22	24.37±2.01	201±11	1.91±0.13	6.9	7.20	206
	1	20.15±2.13	24.82±2.12	195±15	2.66±0.28	6.9	7.30	19
	3	19.72±2.13	26.43±2.47	187±15	3.60±0.17	7.1	7.60	-74
	5	19.11±1.61	27.46±2.02	163±14	6.41±0.35	7.1	7.62	-119
	7	17.86±1.82	28.22±2.72	155±8	7.41±0.70	7.2	7.64	-130
	10	16.35±1.53	29.35±2.32	151±13	7.84±0.82	7.1	7.72	-165
	14	15.18±1.39	30.46±3.11	143±7	8.51±0.79	7.2	7.64	-211
D	0	20.17±1.39	25.56±2.19	294±22	1.88±0.12	6.8	7.08	204
	1	19.54±1.73	25.93±2.15	279±27	2.71±0.19	6.9	7.08	20
	3	19.29±1.29	26.94±2.34	262±24	4.50±0.49	7.0	7.45	-75
	5	19.03±1.77	28.20±2.34	221±16	7.31±0.66	7.1	7.56	-127
	7	19.09±1.26	28.73±2.16	214±24	8.33±0.75	7.2	7.64	-137
	10	18.37±1.17	29.80±2.12	206±13	9.86±1.02	7.3	7.72	-182

	Time (days)	NO_3^--N (mg l^{-1})	NH_4^+-N (mg l^{-1})	SO_4^{2-} (mg l^{-1})	S^{2-} (mg l^{-1})	pH	logN	Redox (mV)
	14	17.50±1.66	30.78±2.54	188±11	10.89±0.87	7.3	7.72	-239
E	0	29.70±2.10	17.83±1.45	123±8	1.95±0.15	6.7	7.05	207
	1	28.51±1.88	18.33±1.31	123±11	2.63±0.15	6.7	7.06	20
	3	27.47±1.92	18.95±1.25	120±10	3.45±0.18	6.8	7.38	-71
	5	26.88±1.75	20.45±1.50	115±7	3.50±0.26	6.8	7.51	-114
	7	25.36±2.19	21.12±1.30	109±8	4.52±0.27	6.9	7.56	-125
	10	24.09±1.53	22.65±1.80	108±7	4.83±0.32	7.0	7.56	-160
	14	23.41±1.44	23.10±1.43	103±7	4.98±0.44	7.1	7.60	-205
F	0	31.65±3.05	18.95±1.65	127±12	1.92±0.15	6.8	7.08	206
	1	30.02±2.89	19.97±1.68	123±12	2.58±0.28	6.8	7.08	20
	3	29.39±2.85	19.70±1.78	118±12	3.65±0.20	6.8	7.45	-71
	5	27.49±3.28	21.70±1.89	116±10	3.93±0.31	6.9	7.56	-114
	7	25.60±2.42	22.22±1.96	109±11	5.01±0.44	7.0	7.60	-124
	10	23.70±2.32	24.01±2.11	110±10	5.26±0.58	7.1	7.60	-160
	14	23.11±2.54	24.58±2.19	108±10	6.49±0.74	7.1	7.63	-208
G	0	33.62±2.46	24.27±2.36	125±13	1.88±0.15	6.8	7.20	213
	1	32.93±1.93	24.23±2.94	113±11	2.86±0.34	6.9	7.20	21
	3	32.91±3.43	24.79±2.74	105±11	5.16±0.48	7.1	7.60	-82
	5	32.59±2.82	25.47±1.63	93±8	7.95±0.58	7.2	7.64	-137
	7	31.92±3.47	25.88±2.90	91±8	8.87±0.80	7.3	7.75	-152
	10	31.25±2.16	26.21±2.45	84±9	10.48±0.64	7.5	7.78	-201
	14	31.27±2.23	26.14±1.42	68±7	13.19±0.86	7.8	7.90	-275

*A-F According to section 5.3.1 (Table 5.1) and G – Control (PMB)
[1]Standard deviation of three readings

Redox potential became more reducing with time in all the cultures tested. For the nitrate and sulphate combinations 30 mg l^{-1}+ 150 mg l^{-1} respectively (Table 5.3 D) and 5 mg l^{-1}+ 150 mg l^{-1} (Table 5.3 A), the increase in pH was 0.5 and 0.7 units respectively while the redox potential decreased from 204 to -239 mV and 207 to -258 mV respectively over the 14-day period. The change in nitrate concentration was higher in the 30 mg l^{-1}+ 150 mg l^{-1} than in the 5 mg l^{-1} + 150 mg l^{-1} combination. With 50 mg l^{-1} sulphate and either 30 (Table

5.3 C) or 5 (Table 5.3 B) mg l^{-1} nitrate, the percentage decrease in concentration of original sulphate was 35% and 29% in the presence of 5 mg l^{-1} and 30 mg l^{-1} nitrate respectively. These results were reflected in the corresponding sulphide concentration, which increased from 1.88 to 10.89 and 1.94 to 11.91 mg l^{-1} respectively.

When no additional sulphate was added to the Postgate medium B, the nitrate originally available decreased by 21% while ammonium increased by 30% in the presence of 5 mg l^{-1} nitrate (Table 5.3 E); whereas when 30 mg l^{-1} nitrate were present (Table 5.3 F), the nitrate concentration was reduced by 27% and the increase in ammonium level was 30% at the end of the experiment. The decrease in nitrate concentration in the control (G) was 7%, the ammonium increase was 8% and the amount of sulphate present decreased by 46%.

5.5.2 Effect of Nitrate and Iron on the Growth of SRB

The effect of ferrous iron on the growth of the SRB culture in the presence of 30 mg l^{-1} nitrate was investigated. The initial and final (after 14-days incubation) pH and redox potential values are shown in Figures 5.2 and 5.3 respectively. The pH increased gradually throughout the experiment, in parallel with the increase of ferrous iron concentration with the exception of 50 and 100 mg l^{-1} iron, which gave very similar results. Likewise, the redox potential decreased to about -220, -250, -230, -240 mV in the presence of 0, 10, 50 and 100 mg l^{-1} iron, respectively.
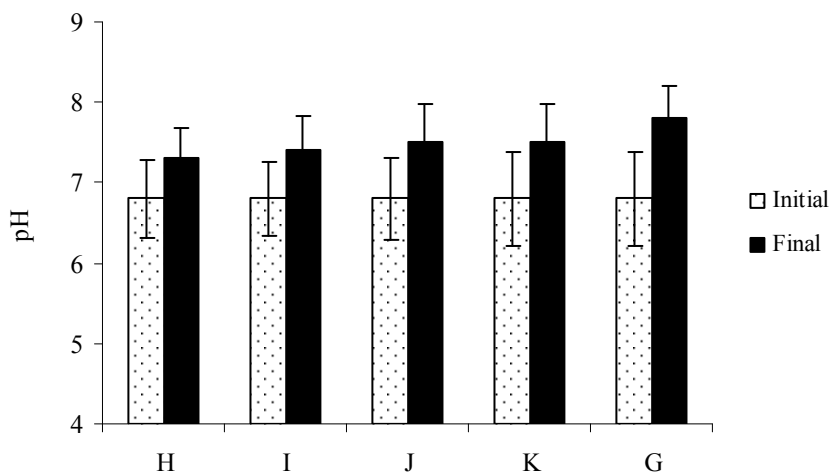
Figure 5.2 Initial and final pH values following growth of SRB in media containing: **H**, 0; **I**, 10; **J**, 50; **K**, 100 mg l^{-1} ferrous iron in the presence of 30 mg l^{-1} nitrate and **G**, control (standard PMB). Error bars represent standard deviation between 3 measurements.
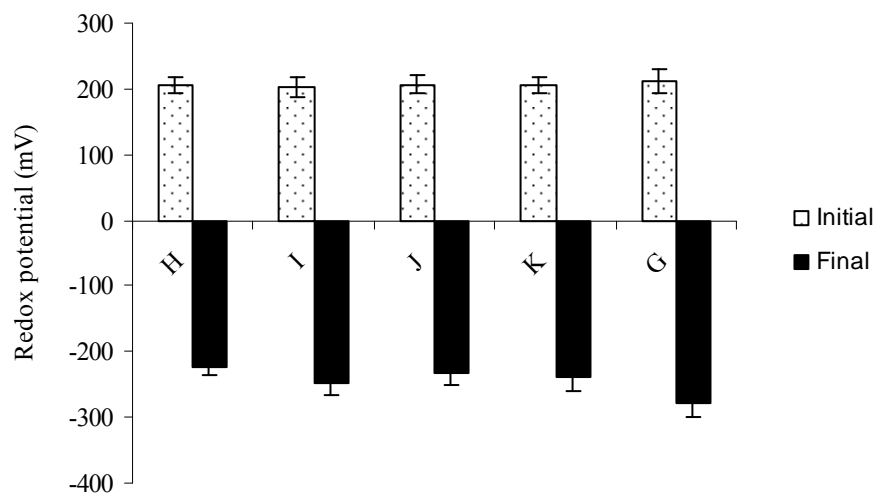
Figure 5.3 Initial and final redox potential values following growth of SRB in media containing: **H**, 0; **I**, 10; **J**, 50; **K**, 100 mg l^{-1} ferrous iron in the presence of 30 mg l^{-1} nitrate and **G**-control (standard PMB). Error bars represent standard deviation between 3 measurements.

The percentage sulphate reduction (Figure 5.4) and production of sulphide (Figure 5.5) increased steadily starting from day-2 of the batch culture experiment. The maximum percentage sulphate reduction of about 42% was observed in the presence of 100 mg l^{-1} iron

and 30 mg l^{-1} nitrate. Without addition of iron to the medium, the percentage sulphate reduction was ~ 34%. Concomitantly, 12.10, 11.98, 10.98 and 8.65 mg l^{-1} sulphide were produced with iron concentrations of 100, 50, 10 and 0 mg l^{-1}, respectively.
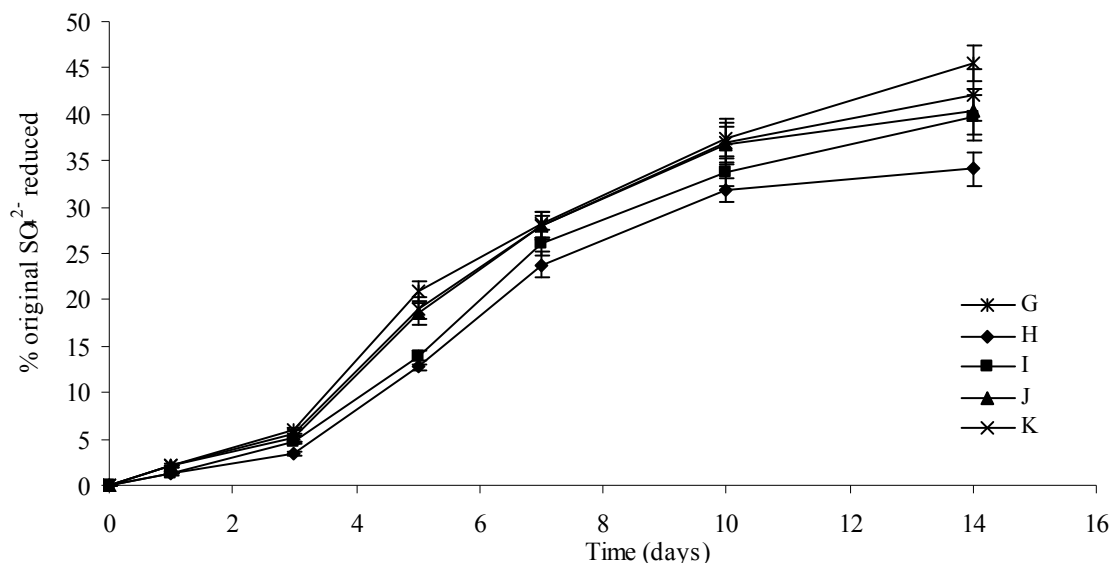
Figure 5.4 Percentage SO_4^{2-} reduction as a function of time (days) during growth of SRB on **H**, 0; **I**, 10; **J**, 50; **K**, 100 mg l^{-1} ferrous iron in the presence of 30 mg l^{-1} nitrate and **G**, control (standard PMB). Error bars represent standard deviation between 3 measurements. Statistically non-significant at P=0.05.
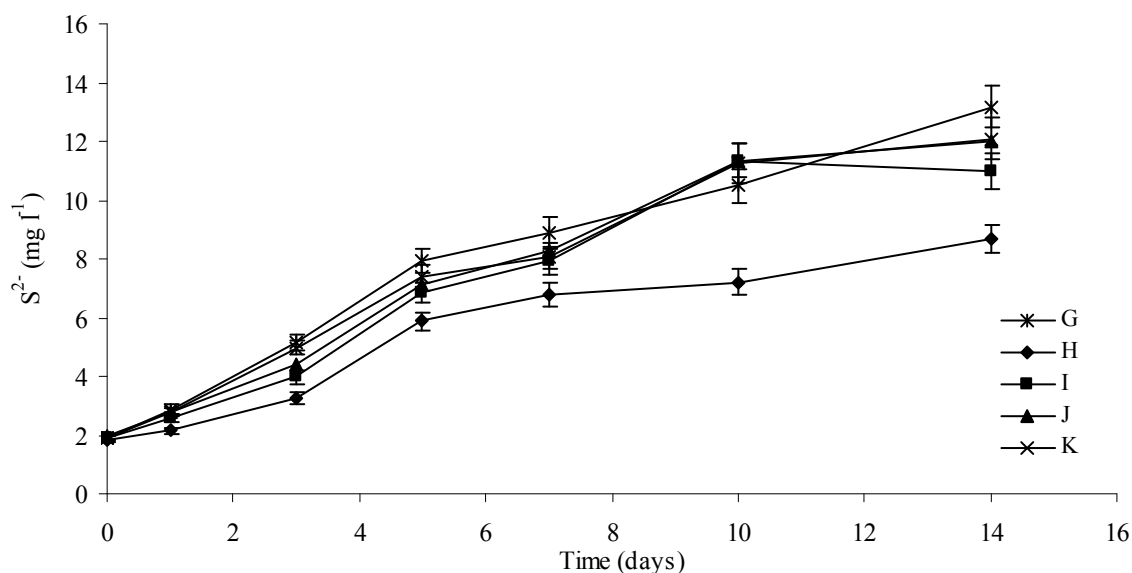
Figure 5.5 Changes in sulphide concentration as a function of time (days) during growth of SRB on **H**, 0; **I**, 10; **J**, 50; **K**, 100 mg l^{-1} ferrous iron in the presence of 30 mg l^{-1} nitrate and control (**G**; standard PMB). Error bars represent standard deviation between 3 measurements. Statistically non-significant at P=0.05.

The concentrations of ferrous iron, initially and at the end of the 14-day experiment, in media containing different amounts of ferrous iron together with 30 mg l^{-1} nitrate are shown in Table 5.4. Ferrous iron was below the detection limit of the method used when no iron was added to the growth medium (Table 5.2 H). The control G [Postgate medium B (PMB)], initially had the highest dissolved ferrous iron concentration and the lowest removal thereof. Almost all the dissolved ferrous iron present was removed when 10 mg l^{-1} was added initially. With 50 mg l^{-1} and 100 mg l^{-1} Fe(II) added initially, 38% and 25% was removed respectively.

Table 5.4 Initial and final ferrous iron concentrations (mg l^{-1}) in the different media tested for support of SRB culture growth

Time (days)	H[1]	I	J	K	G[2]
0	1.52	12.87	56.42	102.8	205.7
14	ND*	0.26	35.25	76.81	162.1

ND – Not Detected
[1]See Table 5.2 for details
[2]Control (standard PMB)

In combination H (no Fe added), a concentration of 1.52 mg l^{-1} Fe^{2+} was detected. This could have arisen from the 20% v v^{-1} Postgate medium B transferred to the experimental flasks with the inoculum. The composition of control (G, standard Postgate medium B) contained 0.5 gl-1 as FeSO$_4$·7H$_2$O that could explain the high concentration of Fe(II) at time 0 (Table 5.4).

5.5.3   Effect of Nitrate, Iron and Arsenic on the Growth of SRB

Table 5.5 shows the changes in pH and redox potential values after 14-days growth in batch cultures containing 30 mg l^{-1} nitrate, 100 mg l^{-1} iron and 20 mg l^{-1} of either arsenic species [**a** – As(III) and **b** – As(V)] and **c** - Postgate medium B without arsenic as the control. In Figure 5.6 the number of cells ml^{-1} under the same set of conditions is given. A standard inoculum of ~2×10^7 cell ml^{-1} was used in all cases. The results indicate that under the experimental conditions As(III) at 20 mg l^{-1} inhibited growth of the SRB much more strongly than did an equivalent amount of As(V).

Table 5.5 Changes pH and redox potential following growth SRB in media containing nitrate, iron and arsenic species

	pH		Redox potential (mV)	
	Initial	**Final**	**Initial**	**Final**
a	6.8	6.9	211	-67
b	6.8	7.2	209	-134
c	6.8	7.9	213	-282
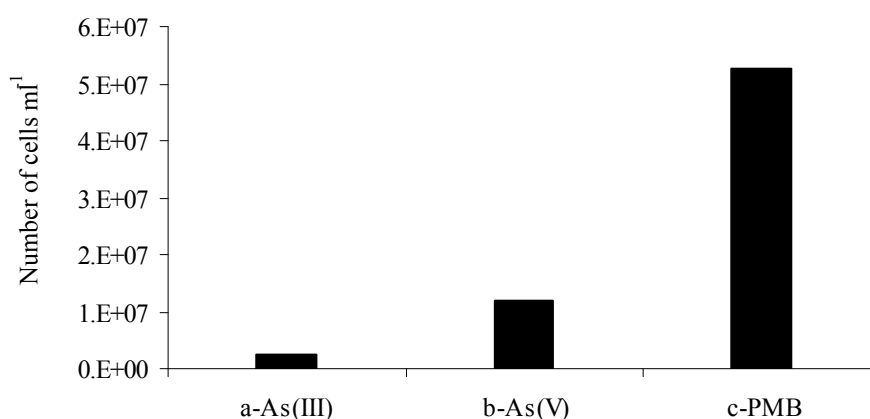
Figure 5.6 Cell number after 14 days growth of SRB in media containing 20 mg l^{-1} of **a**) As(III); **b**) As(V) and in a control medium (**c**) Postgate medium B – (PMB) lacking arsenic. Values are from a single measurement.

The results for percentage sulphate reduction (Figure 5.7) and production of sulphide (Figure 5.8) support the above conclusions that both arsenic species, but especially As(III) at a concentration of 20 mg l^{-1}, markedly inhibits the growth of SRB. At the end of the batch experiments, <10% of original sulphate reduction had occurred and only about 3 mg l^{-1} sulphide were produced in the presence of 20 mg l^{-1} As(III). In the presence of the same amount of As(V), sulphate reduction was slightly above 10% and about 5 mg l^{-1} sulphide was generated.
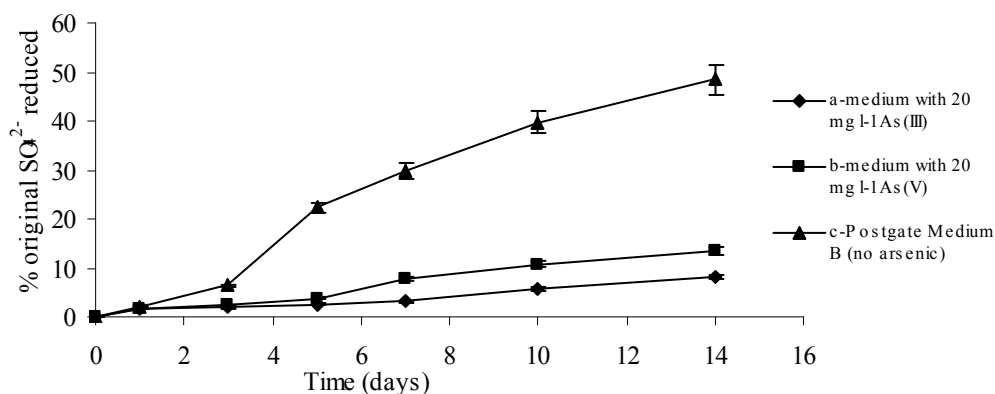
Figure 5.7 Percentage of original SO_4^{2-} reduction as a function of time (days) during growth of SRB on 30 mg l^{-1} nitrate and about 100 mg l^{-1} sulphate with **a**) 20 mg l^{-1} As(III); **b**) 20 mg l^{-1} As(V) and **c**) Control (Postgate medium B (PMB) lacking arsenic). Error bars represent standard deviation between 3 measurements. Statistically significant at P=0.05.
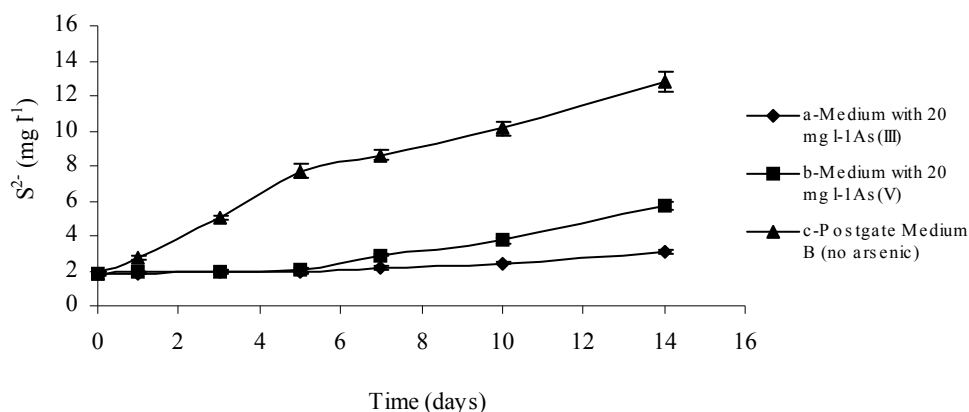
Figure 5.8 Changes in sulphide concentration as a function of time (days) during the growth of SRB on 30 mg l^{-1} nitrate about 100 mg l^{-1} sulphate with **a**) 20 mg l^{-1} As(III); **b**) 20 mg l^{-1} As(V) and **c**) Control (Postgate medium B (PMB) lacking arsenic). Error bars represent standard deviation between 3 measurements. Statistically significant at P=0.05.

5.6   Discussion

This study showed that nitrate at different concentrations, either in the presence of sulphate (50–150 mg l^{-1}) or in its absence, supported the growth of the mixed SRB population. Previous studies have shown the presence of nitrate-reduced sulphate reduction (Jenneman *et al*., 1986; Mancinelli & McKay, 1983). The presence of sulphide can affect the growth of

SRB when nitrate is used as electron acceptor (Dalsgaard & Bak, 1994). These authors reported that the concentration of sulphide that can inhibit the reduction of sulphate was 160 mg l^{-1} for *D. desulfuricans*. Reise *et al*. (1992) found that a concentration of 512 mg l^{-1} sulphide totally inhibited the growth of a mixed culture of SRB. Earlier studies on the reduction of nitrate by SRB were done in media containing sulphide as a reducing agent, often at a concentration of about 16 mg l^{-1} (Dalsgaard & Bak, 1994). Mitchell *et al*. (1986) examined 16 strains of SRB for their ability to grow with nitrate as electron acceptor in sulphate-containing medium reduced with 16 mg l^{-1} sulphide and found that only 1 strain could do so. In all experiments involving nitrate, sulphide concentration was always below 16 mg l^{-1}. During growth of SRB the concentration of sulphide in a medium can be further increased by the reduction of sulphate. In the present study, sulphide-free media were used to grow a consortium of SRB and this might explain the improved growth rates of the bacteria in the presence of nitrate. Throughout this investigation, thioglycolate was used as a reducing agent instead of sulphide to maintain the reducing environment conducive to the growth of SRB.

A study by McCready *et al*. (1983) showed that if a small amount of sulphate (96 mg l^{-1}) was present, the cells could reduce nitrate to ammonium using both electron acceptors simultaneously. But, if sulphate was absent or was present in high concentration (3 360 mg l^{-1}), nitrate was not reduced, i.e., the presence of large amounts of sulphate compared to nitrate will favour the reduction of sulphate to sulphide due to preferential use of sulphate as electron acceptor. The highest amount of sulphate used in the present study was 1 632 mg l^{-1}; the amount originating from the growth medium, i.e., Postgate medium B. In another study on a different strain of SRB, nitrate and sulphate were reduced concomitantly, and the strain was able to grow by reduction of nitrate in the absence of sulphate (Keith & Herbert, 1983). The study by Dalsgaad and Bak (1994) showed that simultaneous reduction of nitrate and sulphate was possible for a short period of time and at a very low concentration of sulphate. SRB reduce nitrate to ammonium in a true respiratory process coupled to electron transport associated phosphorylation (Seitz & Cypionka, 1986). There have been several studies on the reduction of nitrate to ammonia by different strains of SRB; some of these investigations were concerned mainly with the regulation of the

enzymes involved in this process (Mitchell *et al*., 1986; Seitz & Cypionka, 1986; Keith & Herbert, 1983; McCready *et al*., 1983).

It is known that the presence of iron (Fe^{2+}) in the nutrient medium is a relevant factor in the growth of SRB and is probably necessary for the biosynthesis of the iron-cytochromes in the respiratory chain (Postgate, 1979); hence, the presence of varying concentrations of iron may affect the growth of SRB cultures. A study by Marchal *et al*., (2001) on the environmental conditions controlling growth and metabolic activity of the sulphate-reducing bacterium *Desulfovibrio gabonensis* DSM 10636 showed that the maximum growth rate was markedly influenced by the ferrous ion concentration in the medium. Hence, the presence of ferrous iron could affect the growth of SRB and thereby the bioremediation of arsenic.

5.7   Conclusions

The investigation on the effect of nitrate and sulphate on the growth of a mixed SRB culture showed that in the presence of 30 mg l^{-1} nitrate, cell population was larger in 150 than in 50 mg l^{-1} sulphate. The presence of a large amount of nitrate stimulated the growth of SRB more than when a lower concentration of nitrate was used. The concentration of ferrous iron present also had an effect on the growth of the cells; when the level of iron in the medium was increased from 50 to 100 mg l^{-1}, the rate and degree of sulphate reduction was increased due to increased activity of the SRB. The presence of 20 mg l^{-1} of either As(III) or As(V) and 30 mg l^{-1} nitrate in the medium that contained about 100 mg l^{-1} sulphate inhibited the growth of the mixed culture of SRB with the inhibition being greater in the presence of As(III) than of As(V).

5.8   References

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Chapter 6

Study of the Bioremoval of Arsenic Species

6.1   Introduction

The presence of elevated concentrations of arsenic and other heavy metals in groundwater and surface waters is creating serious problems for humans as well as other living organisms (Jang *et al*., 2006). These contaminants may be removed through biological and/or chemical methods. The mechanisms of contaminant removed by microorganisms can include: i) extracellular accumulation/precipitation; ii) cell-surface adsorption or complexation; and iii) intracellular accumulation (Muraleadharan *et al*., 1991). The extracellular polymeric substances (EPSs) surrounding many microorganisms, especially bacteria, depending on the strain and culture conditions, comprise a mixture of polysaccharides, mucopolysaccharides and proteins (Zinkevich *et al*., 1996) that play a major role in the biosorption of heavy metals (Beveridge & Doyle, 1989). Cell-sorption occurs with dead or living microorganims whilst intracellular entrapment requires microbial activity (Igwe & Abia, 2006).

There are several conventional sorbents for arsenic species; some of the most widely studied are iron hydroxides and oxides such as amorphous hydrous ferric oxide, ferrihydrite and goethite (Appelo *et al*., 2002; Raven *et al*., 1998; Fendorf *et al*., 1997), activated alumina (Kuriakose *et al*., 2004; Singh & Pant, 2004), iron-modified activated carbon (Chen *et al*., 2007) and cellulose sponge (Munoz *et al*., 2002). The removal of arsenite and arsenate by hydrous ferric oxide (HFO) has been studied extensively due to HFOs high isoelectric point (IEP=8.1) (Dixit & Hering, 2003) and selectivity for arsenic species (Deliyanni *et al*., 2003). Chen *et al*. (2007) have shown that combining carbon and iron is effective in arsenic removal as the activated carbon supports the preloading of iron. Oxyanions (such as $H_2AsO_4^-$ and $HAsO_4^{2-}$) can react with iron species in ligand exchange reactions to form an inner sphere monodentate or bidentate surface complex (Grossl *et al*., 1997; Fuller *et al*., 1993).

Microbial sulphate reduction decreases the toxicity of several metals by precipitation of the dissolved metals as metal-sulphides; by incorporating them into sulphide minerals, or by adsorption onto mineral surfaces (O'Day *et al.*, 1998). Iron sulphide minerals can induce metal retention, mobility and bioavailability that are governed by reactions occurring at the surface of the iron sulphide moiety (Huerta-Diaz & Morse, 1992). Watson *et al.* (1995) investigated the adsorbent properties of iron sulphide produced by SRB. They found that the SRB-produced adsorbent had a considerably higher specific uptake capacity for different metal ions from solution than other adsorbents such as activated carbon. Moreover, the advantages of using biogenic sulphide to precipitate metals include lower sludge formation and formation low solubility products as compared to hydroxide precipitation (Hao, 2000). Increasing knowledge of biosorption during the past few decades has revealed the high adsorption capacities, low cost and regenerability of natural biosorption materials (Gavrilescu, 2004; Volesky, 2003).

Under anaerobic conditions, SRB can transform sulphate to hydrogen sulphide using simple organic substrates that can have importance in bioremediation of pollutants (Chang *et al.*, 2000). The resultant H_2S, in the absence (or limiting presence) of metal species, may dissociate according to the following equilibrium equations (Moosa & Harrison, 2006):

$$H_2S \leftrightarrow HS^- + H^+ \qquad \textbf{(6.1)}$$
$$HS^- \leftrightarrow S^{2-} + H^+ \qquad \textbf{(6.2)}$$
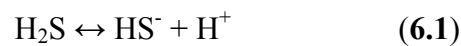
Total hydrogen sulphide concentration can be determined by the following relationship:
$$H_2S_{total} = H_2S_{aq} + HS^- \qquad \textbf{(6.3)}$$
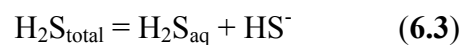
The following figure gives the relationship between species of H_2S and pH.
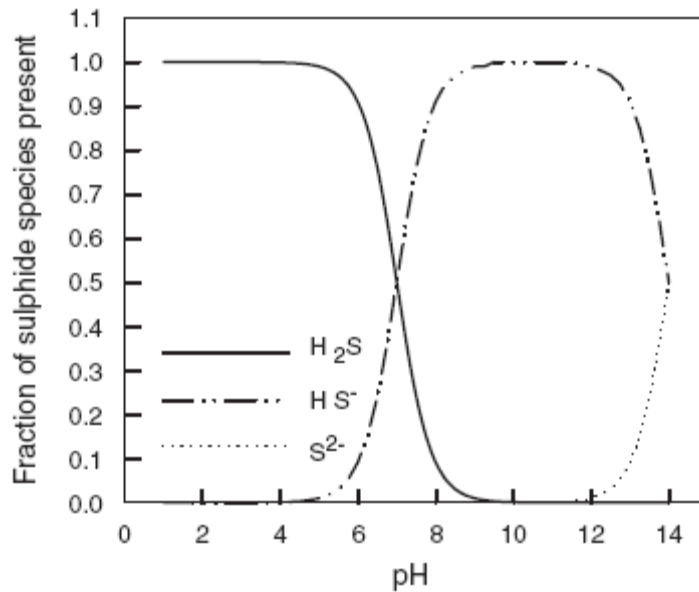
Figure 6.1 Relationship between hydrogen sulphide speciation and pH (taken from Moosa & Harrison, 2006).

It can be seen that in the pH range 6-8, hydrogen sulphide exists as a mixture of H_2S and HS^-. Below pH 6, the undissociated form (H_2S) dominates. The HS^- dissociates further to S^{2-} near pH 12. Even though SRB have the highest tolerance to sulphide of all anaerobic microorganisms, their activity is nonetheless inhibited in its presence (O'Flaherty *et al.*, 1997). Two of the hypotheses for the inhibition mechanisms are: (1) metal sulphides precipitate so that the SRB are deprived of the essential trace metals required as cofactors for their enzyme systems (Loka Bharathi *et al.*, 1990); (2) the sulphide is absorbed into the cells of the microorganisms and denatures proteins by acting as a cross-linking agent between the polypeptide chains (Postgate, 1979) thereby interfering with the metabolic coenzymes through sulphide bond formation. However, when present at concentrations below the inhibitory level, H_2S can react with dissolved metals to form insoluble precipitates that are non-toxic to microorganisms (Lyew & Sheppard, 2001). Metals may also be precipitated by bubbling H_2S through a metal-containing solution (Hao, 2000; Vogel, 1996). A study by Newman *et al.* (1997) showed that *Desulfotomaculum auripigmentum* precipitated arsenic trisulphide (As_2S_3) that resulted from the reduction of As(V) to As(III). The stability of As_2S_3 is highly dependent on pH and sulphide

concentrations. The following diagram shows the occurrence of arsenite and arsenate at different pHs which is significant as As(III) is more toxic than As(V).
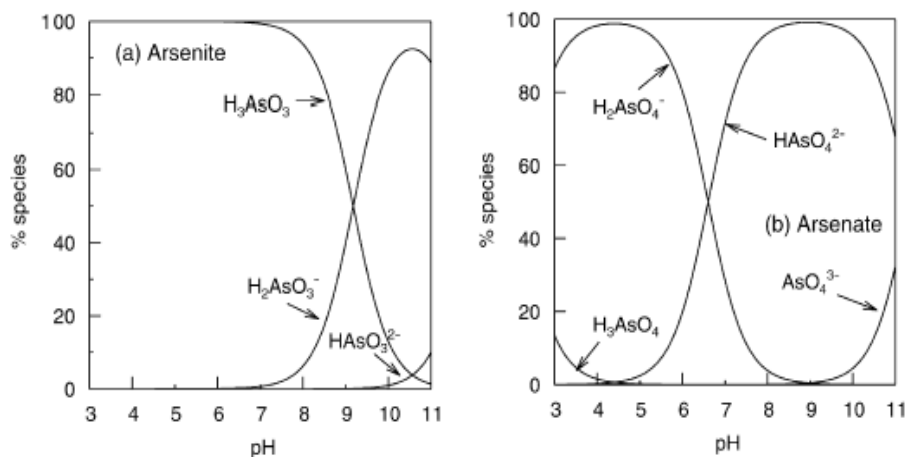
Figure 6.2 Occurrence of As(III) and As(V) with changing pH (taken from Sami & Druzynski, 2003).

The objectives of the study were: (1) to investigate the bioremoval of arsenic species [As(III) and/or As(V)] during the growth of a mixed culture of SRB; (2) to determine the sorption of the arsenic species on the surface components of the bacteria; and, (3) assess precipitation of the metalloid as a sulphide salt when it reacts with hydrogen sulphide.

6.2   Materials and Methods

6.2.1   Preparation of Arsenic Solutions

Stock solutions of As(III) and As(V) were prepared according to **section 2.4**.

6.2.2   Nutrient Medium and Source of Sulphate-Reducing Bacteria

The mixed culture of SRB used for the arsenic bioremoval studies was grown on Postgate medium B (PMB) (Postgate, 1979) as described in **section 2.2** and was originally isolated from anaerobic sediments from the Msunduzi River (Pietermaritzburg, South Africa) as described in **sections 2.1** and **2.3** and maintained by regular transfer to fresh PMB.

6.2.3   Bioremoval Studies

The cells present in 50 ml of an exponentially growing mixed SRB culture grown in the presence of either iron [Fe(III)] or arsenic species (either As(III) or As(V) at 0.1 mg l^{-1}) were collected by centrifuging late log phase cells at 10 000 rpm (12 096×g) (Avanti J-26 XPI high-performance centrifuge, Beckman Coulter) for 20 min for use in the experiments described below. All batch experiments were performed in duplicate using these cells with appropriate controls in each instance.

6.2.3.1 Precipitation of Arsenic Species as Sulphide

Cell pellets collected from SRB cultures adapted to grow in either 0.1 mg l^{-1} As(III) or As(V) as described in the previous section were used to study the bioremoval of arsenic as arsenic sulphide. The adapted SRB cultures were grown in PMB in which the $FeSO_4.7H_2O$ was replaced by either arsenic species at concentrations of 1 and 5 mg l^{-1}. As a control arsenic-free PMB were used.

6.2.3.2 Biosorption of Arsenic Species on SRB cells

Pellets containing SRB cells collected by centrifugation following growth in media containing iron were used to study the biosorption of arsenic from solutions that contained 1 and 5 mg l^{-1} each of the arsenic species [As(III) or As(V)]. Pellets which contained 0.16 g (wet weight) of cells per100 ml were inoculated into 250 ml flasks at pH 6.9 and temperature 25±2°C. Samples were taken at time 0 and then at intervals of 5, 15, 45, 90, 240, 600 and 1440 min. The samples were filtered (0.22 μm membrane filter, Sarotrius), and the filtrate analysed for residual arsenic. TEM-EDX (**section 2.25**) was used to characterise the absorbed arsenic species and other elements associated with the cells.

6.2.3.3 Adsorption of Arsenic Species on Biogenic Iron Sulphide

The iron sulphide precipitate generated during growth of the SRB was collected by centrifuging at 10 000 rpm (12 096×g) for 20 min and dried at 55°C. This material was used to investigate the adsorption of arsenic species from solutions containing 5 mg l^{-1} of either As(III) or As(V) at pH 6.9 and pH 8 at 25°C. The adsorption capacity of this biogenic iron sulphide was compared with that of synthetic iron sulphide (SIS) subjected to

the same adsorption procedure. The pellets of SIS were crushed using a pestle and mortar and sieved. Particles of <53 μm diameter were dried at 70°C for 8 h before being used as adsorbent according to the procedure described by Özverdi and Erdem (2006). The efficiencies of the SRB-produced precipitate (SRB-PP) and synthetic iron sulphide (SIS) to adsorb As(III) and As(V) were determined as follows: solutions containing 5 mg l^{-1} of either As(III) or As(V) were transferred to 250 ml flasks and either SRB-PP or SIS added to obtain final concentrations of 0.2 g l^{-1} and 2 g l^{-1}. The mixtures were incubated in a shaker (150 rpm) at 25°C. Approximately 20 ml aliquots were removed at time 0 and then after 5, 15, 45, 90, 240, 480, 960 and 1440 min. After filtering the samples the concentrations of arsenite and total arsenic in the supernatant were determined using the procedure given in **section 2.22.2.** From these values, the equilibrium times of the two adsorbents for As(III) and As(V) were determined. The pH was adjusted to the desired value using either HCl or NaOH.

6.2.3.3.1   Adsorption Isotherm Studies

Adsorption isotherm tests for SIS and SRB-PP were carried out at pH 6.9 and 25°C with a contact time of 24 h for both As(V) and As(III). The experimental data were fitted into Langmuir and Freundlich models. The Freundlich equation is given by:

$$q = KC_f^{\frac{1}{n}} \qquad\qquad \textbf{(6.4)}$$

To obtain *K* and 1/n, experimental data were fitted by logarithmic transfer of equation (6.4) to give:

$$\log(q) = \log(K) + \frac{1}{n}\log(C_f) \qquad \textbf{(6.5)}$$

where q is the amount of arsenic species sorbed per weight of adsorbent (dry weight in mg g^{-1}), C_f is the concentration of arsenic remaining at equilibrium (mg l^{-1}), *K* and 1/n are empirical constants and indicate the sorption capacity and sorption intensity, respectively (Weber, 1972).

The Langmuir model is given by:

$$q = \frac{q_{max}bC_f}{1+bC_f} \qquad (6.6)$$

where q_{max} represents the maximum metal uptake, b is a constant relating the affinity of the sorbent and sorbate.

The linearised form of equation (6.6) after rearrangement is given by:

$$\frac{C_f}{q} = \frac{1}{q_{max}b} + \frac{1}{q_{max}}C_f \qquad (6.7)$$

The experimental data was then fitted into the latter equation for linearisation by plotting C_f/q against C_f.

For each species the weight of arsenic adsorbed was calculated as the difference between the initial and final amounts of arsenic in solution divided by the weight of the adsorbent using the following equation:

$$q = \frac{V(C_i - C_f)}{m} \qquad (6.8)$$

where q is the metal uptake (mg g^{-1}), V is the volume of the liquid sample (ml), C_i and C_f are the initial and final concentrations of the arsenic respectively, and *m* the dry weight of adsorbent added (mg).

6.2.3.4 Precipitation of Arsenic by SRB-Produced Hydrogen Sulphide

SRB were grown anaerobically in 250 ml bottles in standard Postgate medium B leaving a small headspace. After 2 weeks incubation at 25±2°C in the dark, the gaseous hydrogen sulphide generated was removed by syringe and reacted within the syringe with 5 ml of a solution containing 0.1, 1 or 5 mg l^{-1} of either As(III) or As(V) for about 15 min. Samples were filtered through 0.22 μm cellulose acetate membrane filters and the filtrate analysed for arsenic species.

6.2.4 Adsorbent Characterisation

The surfaces of the adsorbents (SRB-PP and SIS) were investigated using environmental scanning electron microscopy (ESEM) (**section 2.9**) while energy dispersive x-ray (EDX) analysis (**section 2.10**) was employed to quantify (percentage wise) the elemental composition of these precipitates.

6.2.5. Mass Balance Experiment

The mixed culture of SRB was grown in glass or polystyrene cups containing 25 ml Postgate medium B supplemented with 5 mg l^{-1} of either As(III) or As(V). The cultures were incubated at 23 ± 2°C for 5 days. In every case, uninoculated Postgate medium B served as the control. The amounts of arsenic present in/on the bacterial cells, in the precipitate generated by the growing SRB, adsorbed onto the surfaces of the containers; and that remaining in aqueous solution (the supernatant) were quantified. A mass balance for the distribution of arsenic between these components was calculated. The cells (collected by filtration through a 0.22 µm cellulose acetate membrane, Sartorius) and the precipitate were digested with 0.5 M HCl for arsenic analysis. Energy dispersive x-ray (EDX) analysis coupled with both environmental scanning electron microscopy (ESEM, Philips, FEI XL 30) and transmission electron microscopy (TEM, Philips, CM 120, biotwin) (**section 2.25**) was used to study the mineralogy and morphology of the precipitates and the bacterial cells.

6.2.6 Analytical Methods

The pH of the samples was measured with a Crison micropH 2000 meter. Arsenic was analysed using hydride generation coupled to an ICP detection system according to the method described in **section 2.22.2**.

6.3   Results and Discussion

6.3.1   Precipitation of Arsenic Species as Sulphide

The change in As(III) and As(V) concentrations as a function of time during the growth of the mixed culture of SRB is shown in Figure 6.3. In the presence of 1 mg l^{-1} As(III), the concentration at the end of the 14-day batch experiment was about 0.3 ± 0.02 mg l^{-1} (70%

removal) while the corresponding value for As(V) was 0.13±0.01 mg l^{-1} (87% removal). When the concentration of each arsenic species was increased to 5 mg l^{-1}, the residual concentration of As(III) and As(V) at the end of the experiment was 1.95±0.10 (61% removal) and 0.95±0.05 mg l^{-1} (81% removal) respectively. Thus, when the concentration of arsenic species was increased from 1 to 5 mg l^{-1}, the percentage bioremoval efficiency decreased, especially for As(III). Previously, (**section 3.3.2**) it was shown that at higher arsenic concentrations [either As(III) or As(V)] the growth of SRB was inhibited. Bacterial growth characteristics can be changed by the presence of high levels of toxic metals, and consequently the ability for bioremoval (Rahdika *et al.*, 2006). The attachment of the SRB cells on the sulphide precipitate may inhibit the metabolic activity of the bacteria (Utgikar *et al.*, 2002) and the precipitate can also act as a barrier between the cells and the nutrients essential to their growth (Rahdika *et al*, 2006). The sulphide produced by the metabolic activity of the SRB may react with the dissolved arsenic species to form an arsenic sulphide precipitate that can lead to a decrease in the availability of dissolved arsenic and thus exposure to this toxic metalloid (Temple & LeRoux, 1964; Bååth, 1989; White & Gadd, 2000).
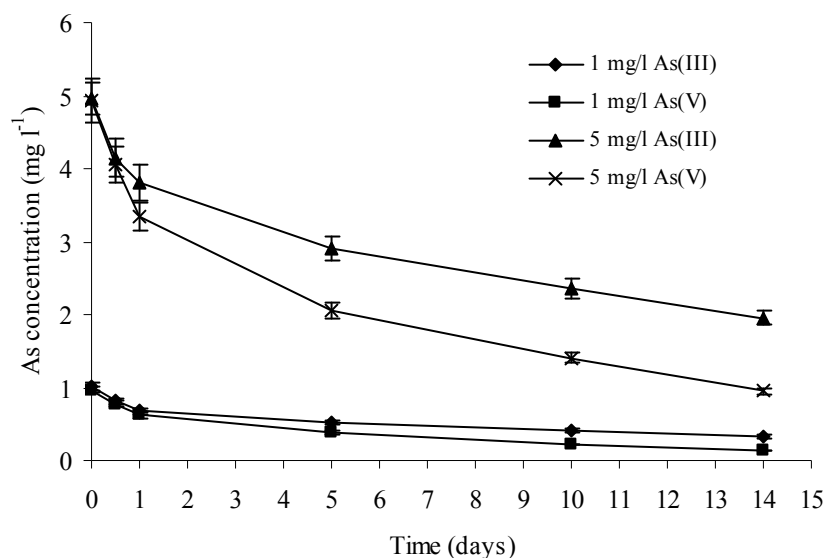
Figure 6.3 Changes in arsenic concentrations during growth of a mixed SRB culture. Error bars represent standard deviation between 3 measurements.

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6.3.2   Biosorption of Arsenic Species on SRB Cells

Cell pellets of SRB were examined for their capacity to sequester arsenic species from contaminated waters. The biosorption of As(III) and As(V), at initial concentrations of either 1 or 5 mg l^{-1}, on the surfaces of the SRB cells collected from 50 ml culture samples are shown in Figure 6.4. The cells removed about 6.6% of the As(III) and 10.5% of the As(V) when both species were at an initial concentration of 1 mg l^{-1}; whereas if the initial arsenic species concentrations were increased to 5 mg l^{-1}, the removal rate was 6.4% for As(III) and 10.0% for As(V) after 24 h contact. These biosorption results show that As(III) removal was lower than that of As(V) and generally removal of both arsenic species at the concentrations studied was very low compared to the bioremoval efficiencies exhibited by actively growing SRB. Both the nature of the surface charge on the SRB cells and the prevailing pH play a role in biosorption of arsenic species. The isoelectric point of most microorganisms is around pH 2 and their surfaces should be negatively charged at near-neutral pH (Seki *et al.* 2005). Hence, it is to be expected that anions like As(V) will not adsorb onto microorganisms at near-neutral pH. The dissociation of arsenic species at different pH values (see Figure 6.2) also plays a role in their biosorption.
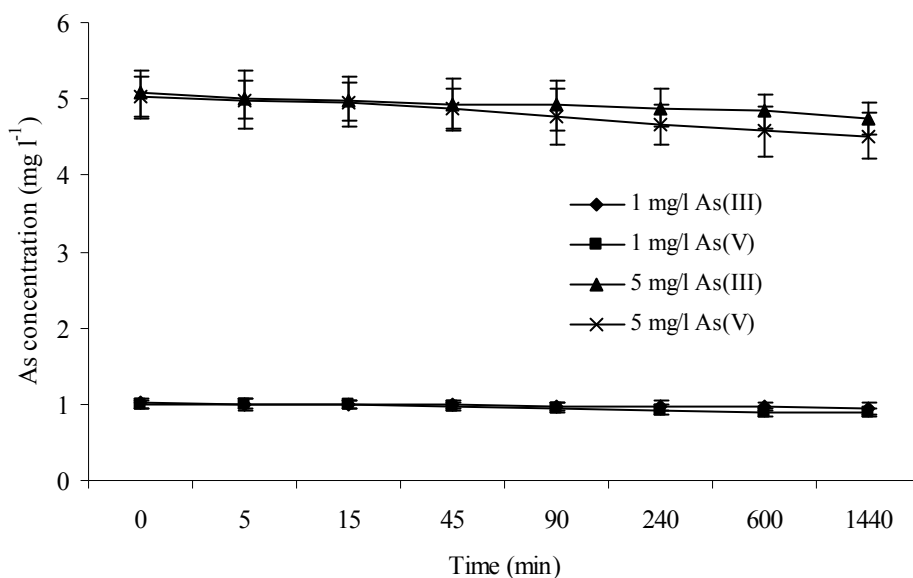
Figure 6.4 Changes in concentration of arsenic species as a function of time during the biosorption of the metalloid on SRB cells. Error bars represent standard deviation between 3 measurements.

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6.3.3 Precipitation of Arsenic by SRB-Produced Hydrogen Sulphide

The hydrogen sulphide generated by the mixed SRB culture was reacted with 0.1, 1 and 5 mg l^{-1} of either As(III) or As(V) at pH 6.9. Table 6.1 shows the initial and final (after 14 days growth) concentrations of the two arsenic species investigated. The data indicate that the removal of both As(III) and As(V) at all initial concentrations was low; with the maximum removal occurring for As(III) at an initial concentration of 0.1 mg l^{-1}. It can be seen from Figure 6.1 that the fraction of gaseous H_2S present at pH 6.9 is about 50% of the total sulphide concentration (the remaining dissociated sulphide exists in the liquid) and this can have an effect on the efficiency of arsenic removal. The removal of metals by H_2S can vary depending on the metal and the ratio of metal:sulphide (Bhagat *et al.*, 2004).

Table 6.1 Initial and final arsenic species concentrations during reaction with gaseous hydrogen sulphide (H_2S)

Arsenic concentration (mg l^{-1})					
Species	Initial	Final	Initial	Final	Initial	Final
As(III)	0.11±0.01	0.09±0.02	1.04±0.05	0.89±0.01	4.97±0.31	4.42±0.28
As(V)	0.12±0.01	0.10±0.01	1.02±0.04	0.93±0.01	5.01±0.27	4.71±0.30

6.3.4 Adsorption of Arsenic Species on Precipitate Produced by Sulphate-Reducing Bacteria (SRB-PP) and on Synthetic Iron Sulphide (SIS)

The sorption rates of biogenic sulphides (SRB-PP) produced during the growth of SRB and used as an adsorbent for the removal of arsenic species from artificial groundwater were investigated. The results were compared with those obtained with SIS (Figures 6.5 a, b, c and d).
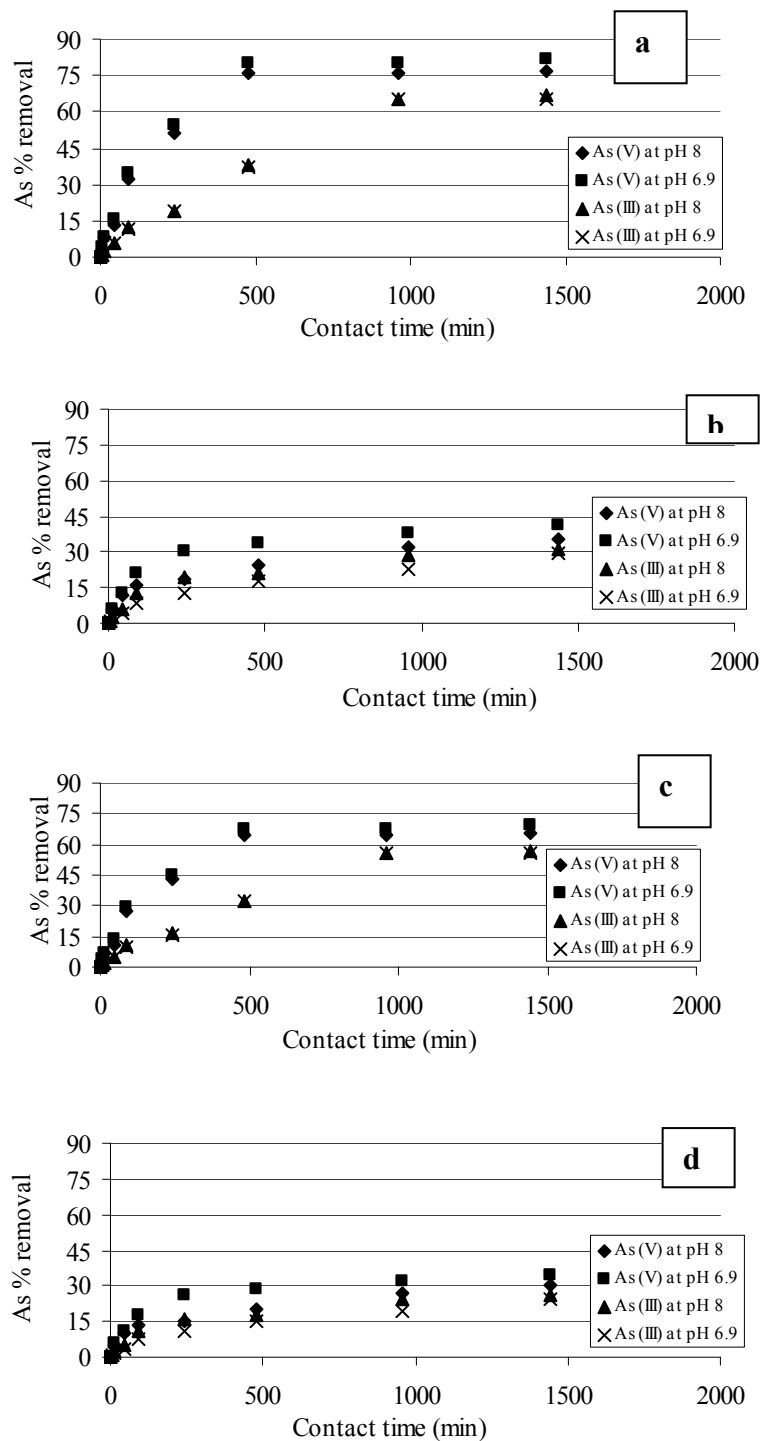
Figure 6.5 Adsorption kinetics of As(III) and As(V) at 25°C on (**a**) 2 g l^{-1} SIS; (**b**) 0.5 g l^{-1} SIS; (**c**) 2 g l^{-1} SRB-PP; (**d**) 0.5 g l^{-1} SRB-PP at an initial arsenic concentration of 5 mg l^{-1} at pH 6.9 and 8.

The sorption capacities of SIS and SRB-PP were investigated under the same environmental conditions (e.g., pH, temperature). When SRB-PP was used as adsorbent at 2 g l^{-1} (Figure 6.5c), the percentage removal was at equilibrium after about 8 h at both pH 8 and 6.9; however, when the amount of SRB-PP was decreased to 0.5 g l^{-1}, the rate of removal increased slowly with time beyond 8 h contact time (Figure 6.5d) for both arsenic species; the same trend was observed for SIS at the same level. The overall removal rates achieved with 2 g l^{-1} SIS were higher than those with the corresponding amounts of SRB-PP under the same experimental conditions. Both adsorbents adsorbed As(V) better than As(III). The removal of arsenic species was very fast initially, possibly due to the presence of active sites on the finely produced adsorbent providing the opportunity for the arsenic particles to diffuse into intraparticle sites where, according to Zhang *et al.* (2007), the adsorption rate is slow. The removal of As(III) and As(V) depends on the prevailing pH with largest percentage adsorption occurring in the lower pH range, particularly the with respect to As(V) which is also adsorbed considerably more rapidly than As(III). Arsenate in the range pH 4-9 exists as $H_2AsO_4^-$ and $HAsO_4^{2-}$ (Figure 6.2). A lower pH is ideal for protonation of sorbent surfaces (Zhang *et al.*, 2007). With increased protonation, the positive charge on the surface of the adsorbent will increase and, as a result attract the negatively charged arsenic species more strongly. At high pH, negatively charged sites are predominant and there would be repulsion between these sites and the arsenic species. Consequently, adsorption would decrease. The same trend was observed with As(III) but the effect was not as pronounced as with As(V), particularly at high pH values. Other studies have shown that as the pH increases the adsorption of As(V) on iron or iron-containing adsorbents decreases (Raven *et al.*, 1998; Jang *et al.*, 2006). A pH of 6.9 is suitable for the removal of arsenic species from drinking water supplies that generally have a pH in the range 6.5-8.5 (Gu *et al.*, 2005). Figure 6.6 compares the percentage removal of arsenic species at different initial concentrations when 2 g l^{-1} of either SRB-PP or SIS was used as adsorbent.
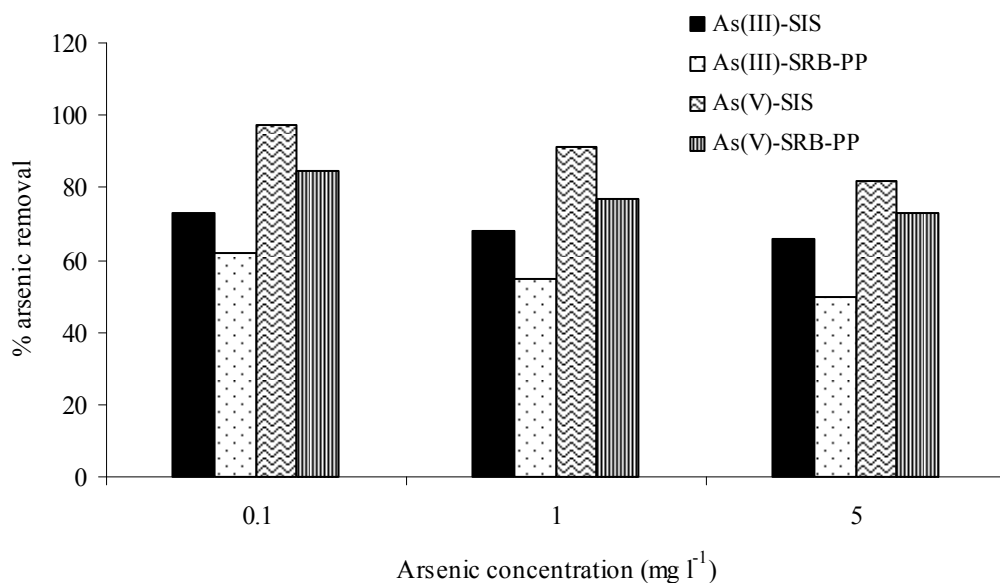
Figure 6.6 The effect of initial concentration on the percentage removal of arsenic by adsorption on SIS (2 g l^{-1}) and SRB-PP (2 g l^{-1}) at pH 6.9, T 25°C, and contact time 24 h.

6.3.5   Adsorption Isotherms

The Freundlich plots of As(III) adsorption on either SIS or SRB-PP did not fit well to the model and gave a poor correlation coefficient ($r^2 < 0.45$). Conversely the Langmuir isotherm was used satisfactorily to characterise the sorption of both As(III) and As(V) to either SIS or SRB-PP at pH 6.9. Langmuir isotherm behaviour of As(III) and As(V) with SIS and SRB-PP is shown in Figure 6.7 while the Freundlich isotherm for As(V) with either SIS or SRB-PP is given in Figure 6.8.
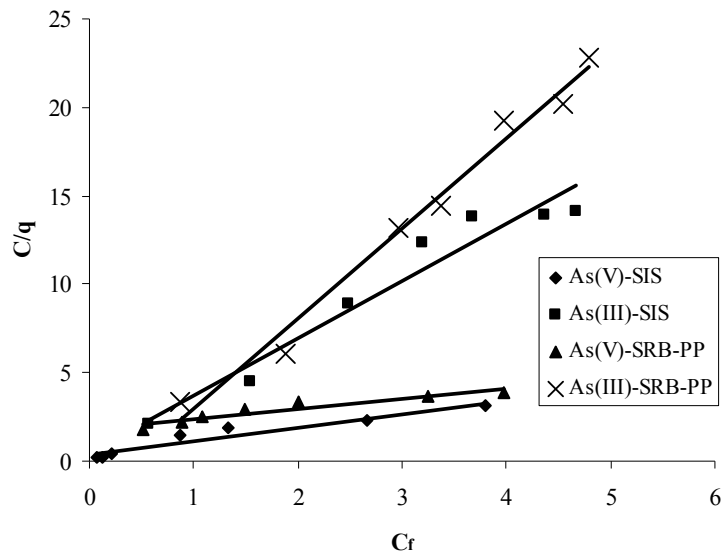
Figure 6.7 Langmuir isotherm for adsorption of As(III) and As(V) on SIS and SRB-PP.
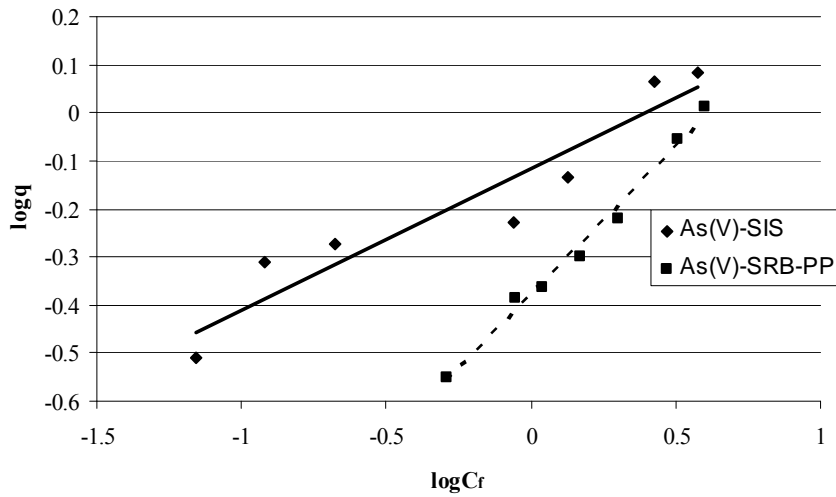
Figure 6.8 Freundlich isotherm for the adsorption of As(V) on SIS and SRB-PP.

Table 6.2 gives the constants for the Langmuir isotherms for SIS and SRB-PP adsorption of As(III) and As(V) and the Freundlich isotherms for adsorption of As(V) on SIS and SRB-PP.

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Table 6.2   Adsorption isotherm parameters from experimental data

Adsorbent	Species	Freundlich Parameters		
		K	1/n	R^2
SIS	As(V)	0.76489	0.2959	0.9005
SRB-PP	As(V)	0.4176	0.6181	0.9877
		Langmuir Parameters		
		b	q_{max}	R^2
SIS	As(III)	6.2739	0.31076	0.9438
SRB-PP	As(III)	2.4408	0.1966	0.9817
SIS	As(V)	1.9888	1.3118	0.9326
SRB-PP	As(V)	0.3095	1.7612	0.8885

The uptake of As(III) was higher on SIS than on SRB-PP or intact SRB cell surfaces, while the maximum adsorption capacity of SIS was lower than that of SRB-PP for As(V).

A wide variety of adsorbents have been investigated under different experimental conditions for their abilities to sorb arsenic species. Table 6.3 compares some of these with the adsorbents used in the present study. It must be kept in mind, however, when comparing the results that the studies were conducted under different conditions.

Table 6.3   Comparison of the adsorption capacities of different adsorbents

Adsorbent	pH	Temp ($^\circ$C)	Model used	Capacity (mg g^{-1}) As(III)	As(V)	Reference
SIS	6.5	25	Langmuir	0.31	1.31	This study
SRB-PP	6.5	25	Langmuir	0.20	1.76	This study
Iron coated sand	7.6	22±2	Langmuir	0.04	0.04	Thirunavukka-rasu *et al.* (2003)
TiO$_2$	4.0	22	Langmuir	3.45	4.65	Lenoble *et al.* (2002)
Manganese ore	6.3 for As(III); 6.5 for As(V)	-	Langmuir	0.53	15.38	Chakravarty *et al* (2002)
Tea fungal biomass	7.2	30	Freundlich	1.11	4.95	Murugesan *et al.* (2006)
Iron hydroxide coated alumina	6.6-6.7 for As(III); 7.1-7.2 for As(V)	25	Langmuir	7.64	36.64	Hlavay and Polyak (2005)

6.3.6   Adsorption Characterisation Studies

Growth of the mixed culture of sulphate-reducing bacteria resulted in the production a precipitate (SRB-PP), arising from the reaction of biogenic hydrogen sulphide with the arsenic present in the medium. The precipitate was collected by filtration and characterised with respect to surface morphology and elemental composition using ESEM combined with EDX. The image obtained for the adsorbent (SRB-PP) showed that it was comprised of many aggregated particles (Figure 6.9a). These have rough surfaces that can help increase the surface area available for adsorption of arsenic. The EDX analysis of the SRB-PP is shown in Figure 6.9b. Table 6.4 reveals that the chemical composition of this material differs considerably from that of SIS, which contains larger amounts of iron and especially sulphur than does SRB-PP. The differences might be due to: (1) the adherence of SRB cells to the sulphide precipitate; (2) reactions between components of the growth medium and the sulphide precipitate. The nature of the surface (Zhang *et al.*, 2007), availability of

functional groups, metal speciation (Niu & Volesky, 2003) and other characteristics of the adsorbent can have an influence on overall solute adsorption.
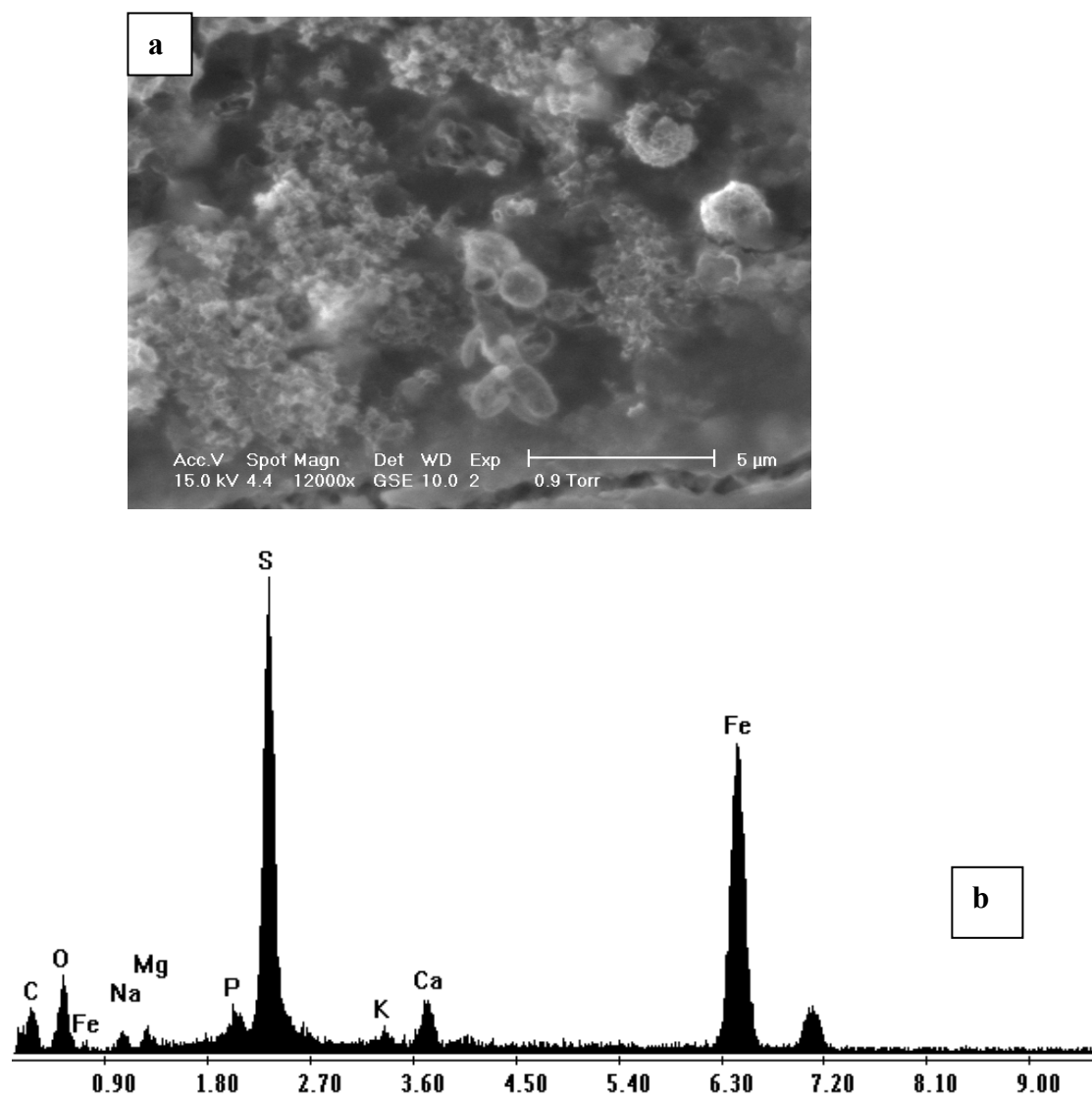
Figure 6.9   (**a**) ESEM micrograph of SRB-PP; (**b**) EDX spectrum of SRB-PP.

Table 6.4   Elemental composition of SRB-PP and SIS

Element	SRB-PP	SIS
Fe	30.77	31.23
S	51.21	68.77
Na	3.55	-
Mg	3.15	-
K	2.05	-
P	2.33	-
Ca	6.94	-
Total	**100**	**100**

The composition of SRB-PP as depicted in the EDX spectrum (Figure 6.9b) included substances such as Mg, Ca, K that may have originated from the growth medium. Results from the study of zinc bioremediation by Radhika *et al*. (2006) showed that the biogenic zinc sulphide produced during the growth of SRB had lower sulphur and zinc content than ZnS precipitated using bacterially produced hydrogen sulphide or those synthesised chemically.

6.4   Mass Balance and TEM-EDX Results

The results for the mass balance experiments are presented in Table 6.5.

Table 6.5 Mass balance results for 5 mg l^{-1} of As(III) and As(V) contained in either polystyrene or glass flasks inoculated with SRB

	Precipitate (mg l^{-1})	Cells (mg l^{-1})	Dissolved arsenic (mg l^{-1})	Total arsenic accounted for (mg l^{-1})	% Error
Polystyrene					
As(III)	1.12	0.02	3.32	4.46	10.8
As(V)	2.12	0.06	2.43	4.61	7.8
Glass					
As(III)	1.02	0.02	3.27	4.31	13.8
As(V)	1.87	0.04	2.65	4.56	8.8

The experiment showed that there was no significant adsorption of arsenic on the walls of either the glass or polystyrene (data not shown) and the volatilisation of arsenic (e.g., as arsine) was not taken into account as its contribution was assumed to be very minimal. The contribution of the SRB (both the cells themselves and the precipitate formed as a result of their metabolic activity) was very significant in the removal of arsenic from the culture media since in the control flasks, that did not contain SRBs, no removal of either of the arsenic species occurred (data not shown).

Figure 6.10 shows the EDX results of a sample of the precipitate obtained in the ESEM which was formed during the growth of SRB mixed cultures in the presence of 5 mg l^{-1} As(III). It contains amorphous and crystalline structures with relatively high percentages of phosphorus (Figure 6.10B) and sulphur (Figure 6.10C) respectively.
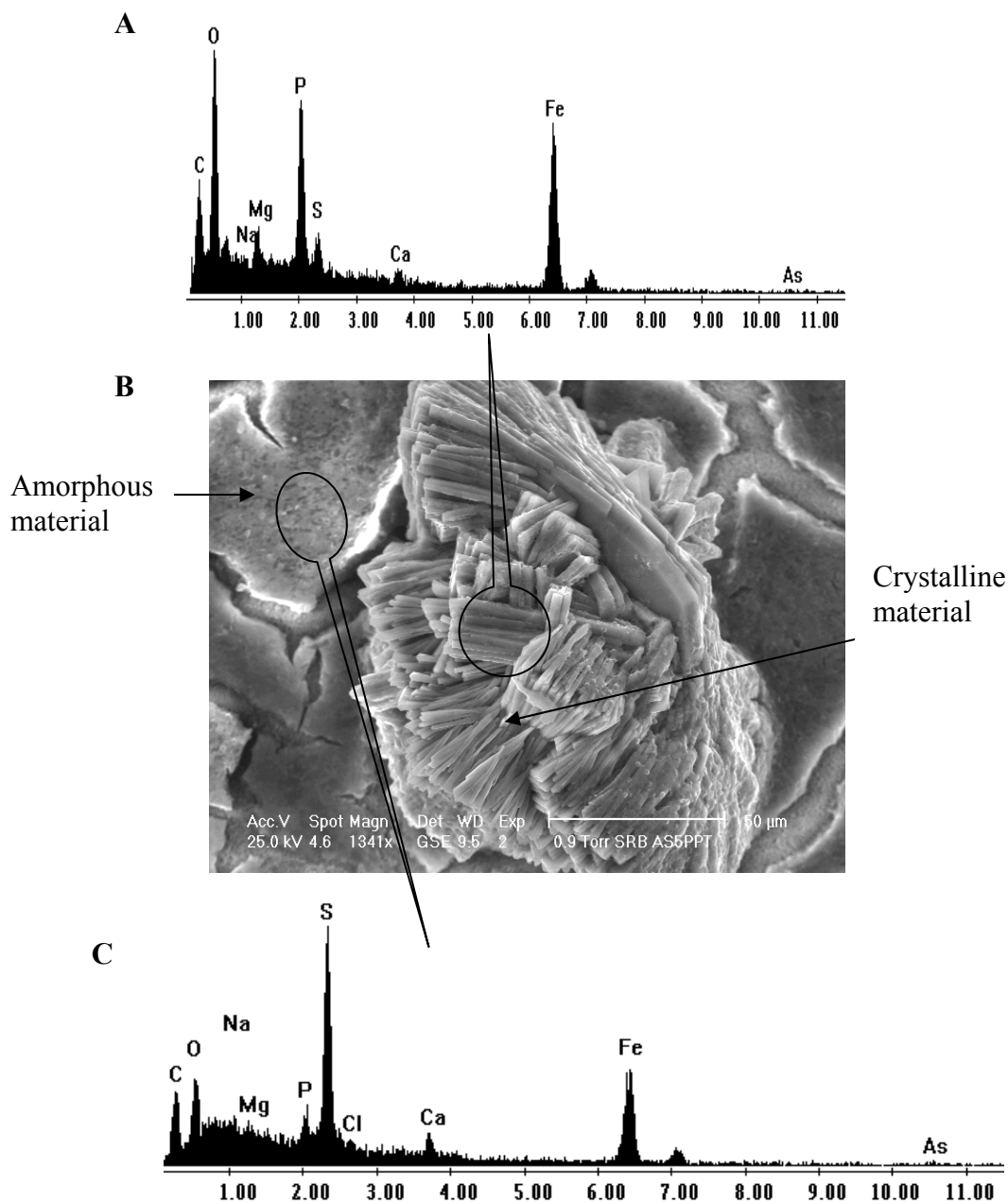
Figure 6.10   ESEM micrograph of the precipitate generated during the growth of SRB in the presence of 5 mg l^{-1} As(III). Note the presence of amorphous and crystalline material and the difference in elemental spectra of the two components.

The rod-shaped SRB (Figure 6.11A) were exposed to TEM-EDX analysis and the result is shown in Figure 6.11B.
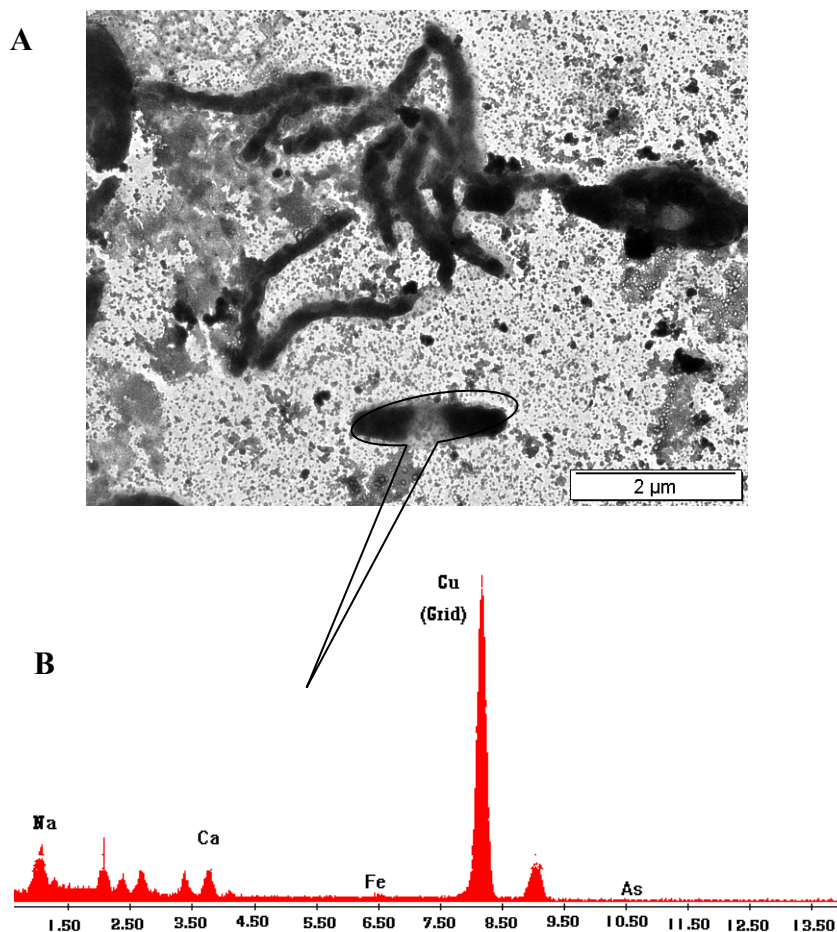
Figure 6.11 TEM-EDX spectrum of a single SRB cell.

Figure 6.11B indicates that the metals (iron and arsenic) were not actively adsorbed on the cell surface or absorbed into the cytoplasm. This could be due to the low concentration of the elements present in the samples and the moderately high detection limit of the TEM-EDX technique

6.5 Conclusions

This study has shown that arsenic species at concentrations of 5 mg l^{-1} or less can be removed by precipitating the metalloid out of solution as the metal sulphide by reacting

with the H_2S produced by sulphate-reducing bacteria (SRB). The efficiencies of removal by biosorption on the cells were 6.4% and 10% respectively when 5 mg l^{-1} of either As(III) or As(V) were present initially. When gaseous hydrogen sulphide was reacted with different initial concentrations of arsenic species to precipitate the metal sulphide, it was found that better removal was achieved at an initial concentration of 0.1 mg l^{-1} for both As(III) and As(V) than when the initial concentrations were 1 or 5 mg l^{-1} at pH 6.9. At 5 mg l^{-1}, As(V) removal by sorption on the precipitate produced by the sulphate-reducing bacteria (SRB-PP) was comparable to that of synthetic iron sulphide (SIS) at an adsorbent dosage of 0.5 g l^{-1}. However, when the amount of adsorbent was increased to 2 g l^{-1}, SIS was superior to SRB-PP for both As(V) and As(III) at pH 6.9 and pH 8. pH had an effect on the removal of arsenic species so that when the pH was increased to pH 8 the adsorption of As(V) on either SIS or SRB-PP was decreased. It was found that the Langmuir isotherm is more suitable to evaluate the sorption of As(III) or As(V) to either SIS or SRB-PP at pH 6.9 than is the Freundlich adsorption isotherm.

6.6 References

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Chapter 7

Removal of Arsenic Species in Purpose Designed Laboratory-Scale Constructed Bioreactors

7.1 Introduction

The use of bioreactors for the treatment of wastewater has been practiced for more than 100 years, and the basic designs have recently been adapted for groundwater treatment purposes (Peters & Alleman, 1983). Bioreactors for groundwater treatment of drinking water should take into consideration the low concentration of the effluent (i.e., $\mu g\ l^{-1}$ compared to mg l^{-1} level for wastewater) (Langwaldt & Puhakka, 2000). Bioreactor processes can be distinguished on the basis of biomass retention, i.e., the microorganisms grow as a carrier attached biomass or as a cell suspension, and the system can be operated under aerobic or anaerobic conditions (Langwaldt & Puhakka, 2000). Bioreactors must be constructed to a specific design to meet the technical, environmental and economic requirements. These include functioning at low pollutant concentrations and operating under varying conditions at low cost for long periods of time. Bioprocess design limitations in the cleanup of contaminated groundwater include low nutrient loads for the microorganisms to grow, resulting in slow biomass build-up.

Several strategies exist for the treatment of groundwater. The main categories are: ex-situ technologies such as "pump-and-treat" systems; and in-situ technologies such as "permeable reactive barriers" (PRBs) (Zouboulis & Katsoyiannis, 2005). Physical, chemical, and/or biological processes can be applied in both ex- and in-situ treatment strategies. Recently, the application of biological treatment has gained increasing support, as it does not require the use of chemical reagents; instead it uses microorganisms to oxidise, reduce or eliminate the contaminant(s). It can be used alone or in combination with physico-chemical processes (Zouboulis & Katsoyiannis, 2005).

Coagulation/filtration, ion exchange, lime softening, adsorption on iron oxides or activated alumina, and reverse osmosis have been used to treat groundwater, contaminated with arsenic, particularly As(V) (Zouboulis & Katsoyiannis, 2002; Jekel, 1994). For efficient removal of As(III), an oxidation step may be performed by the addition of chemical

reagents, such as potassium permanganate, chlorine, ozone, hydrogen peroxide, or manganese oxide prior to applying the above mentioned processes (Kim & Nriagu, 2000; Jekel, 1994).

Some of the important advantages of biological technologies in removing metals from solution are: a) lower costs; b) higher efficiency when metal ion concentrations are below 1 mg l^{-1} and c) selectivity (Brierley, 1990). There is also minimal generation of precipitate. By contrast, chemical treatment methods have high operational and maintenance costs and produce large amounts of sludge that require disposal (Zouboulis & Katsoyiannis, 2005).

Developing countries cannot afford many of the most commonly used physico-chemical treatment methods due to their high costs. But, by applying biotechnological approaches that make use of the natural capabilities of microorganisms, many of the drawbacks can be eliminated.

This study was undertaken to investigate the removal of arsenic species from groundwater using bioreactors containing SRB, growing on molasses as carbon source, sulphate as electron acceptor and polystyrene as bacterial support matrix in the presence of 20, 10, 5, 1 and 0.1 mg l^{-1} As(III) or As(V) alone or in the ratio As(III):As(V) 0.25:4. Growth of the bacteria on the surfaces of the polystyrene was investigated using environmental scanning electron microscopy (ESEM) and ESEM-EDX was used to identify metallic elements associated with the cells. Chemical oxidising agents were used in combination with the biological process in order to assess the efficiency of the removal of arsenic species, particularly As(III).

7.2   Materials and Methods

7.2.1   Preparation of Arsenic Solutions

Solutions of the arsenic species used in this study were prepared according to **section 2.4**.

The arsenic-contaminated synthetic groundwater used in this study was prepared by spiking tap water with As(III) and/or As(V). The concentrations used for both forms of arsenic were 20, 10, 5, 1 and 0.1 mg l^{-1}.

7.2.2   Nutrient Medium and Source of Sulphate-Reducing Bacteria

The mixed culture of SRB used in this study was grown on Postgate medium B (Postgate, 1979) as described in **section 2.2** and was isolated from anaerobic sediments in the Msunduzi River (Pietermaritzburg, South Africa) as indicated in **sections 2.1** and **2.3**.

7.2.3   Bioreactor Configuration and Experimental Set-up

A full description of the configuration of the bioreactors used in this study with detailed experimental procedures was given in **section 2.8**. The main characteristics of the synthetic groundwater used in the study and adapted from the literature are shown in Table 7.1.

Table 7.1   Characteristics of the synthetic groundwater

Parameter	Value
pH	6.9
Redox potential, mV	227±6
Temp., °C	25±3
SO_4^{2-}, mg l^{-1}	175±5
NO_3^-, mg l^{-1}	6.29±0.31
Ca, mg l^{-1}	112±6
Mg, mg l^{-1}	64.4±1.8
Na, mg l^{-1}	102±6
Fe (total), mg l^{-1}	3.2±0.09
As, µg l^{-1}	<2
Conductivity, µS cm^{-1} (25°C)	1120

7.2.4 Oxidation of Arsenite

Batch experiments were set-up to study the oxidation of arsenite to arsenate using pumped air, atmospheric air and MnO_2. Mixtures of As(III) and As(V) in: 80:20; 70:30; 60:40; 40:60; 30:70 and 20:80 ratios (total arsenic concentration, 100 μg l^{-1}) were exposed for 24 h to 0.1, 1 and 2 g l^{-1} MnO_2 at 25±2°C, pH 6.9. The air treatments were of similar duration.

7.2.5 Analytical Determinations

The parameters monitored over the experimental period were SRB populations (cells ml^{-1}); pH; redox potential; SO_4^{2-}; S^{2-} and arsenic species concentrations. All the pH and redox potential measurements were made using a Crison combination pH electrode and a platinum electrode paired with an Ag/AgCl reference electrode coupled to a Crison 2000 pH meter. Total arsenic and arsenite [As(III)] were analysed according to **section 2.22.2** and metals were analysed using ICP-OES (**section 2.22.1**). Sulphate and sulphide were analysed using the methods described in **sections 2.20.2** and **2.21** resepectively. Environmental scanning electron microscopy (ESEM) was used to study the biofilms and the surface characteristics of the polystyrene support matrix. Samples of polystyrene from the bioreactors were dry ashed using 20% $Mg(NO_3)_2.6H_2O$ (**section 2.18.2**) to determine its arsenic and other metal content using ICP-OES.

7.3   Results and Discussion

ESEM photomicrographs of the biofilms from the bioreactors showed that the SRB were successfully immobilised on the polystyrene support matrix (Figure 7.1) when grown with molasses as carbon source. The SRB count in the bioreactors with 0.1 mg l^{-1} of either As(III) or As(V) after sonication ranged from 3×10^6 to 5×10^7 cells ml^{-1}from an initial population of about 6×10^4 thus showing that even though growth rates depend on the availability of essential nutrients and absence of large amounts of toxic substances, biological process can be applied for the bioremediation of arsenic contaminated waters provided wash-out of the SRB can be avoided by immobilising the cells on a solid support. Similar results have been reported by Glombitza, 2001.
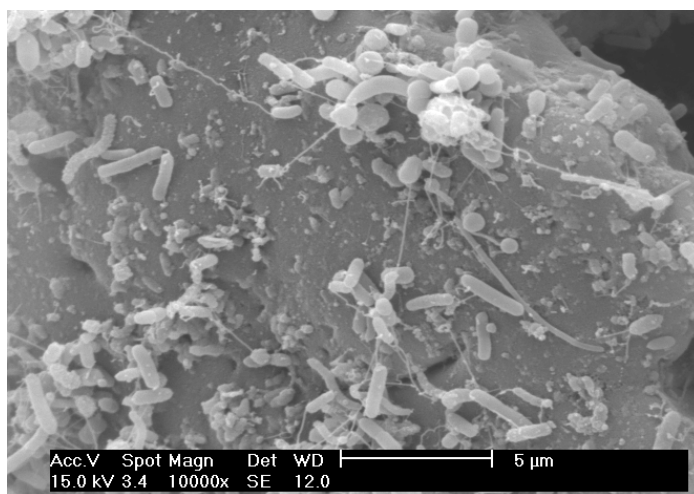
Figure 7.1   ESEM micrographs of SRB colonising the polystyrene surface.

Changes in redox potential and pH within the bioreactors in the presence of different of arsenic species were monitored over a period of 14 days. Initially, the pH was about 6.9 and the redox potential was around 215 mV in all the bioreactors. Figures 7.2, 7.3 and 7.4 give the final redox potential and pH in SRB cultures comprising either immobilised or free-living cells growing in the presence of different levels of As(III), As(V) and in various ratios of As(III):As(V) while keeping the total initial arsenic content at 0.1 mg l^{-1}. In an earlier flask study (**section 3.3.2**), it was found that 20 mg l^{-1} of either As(III) or As(V) inhibited the growth of SRB and the data given below support this finding.

Figure 7.2 Changes in (**a**) redox potential (Statistically significant at P=0.05) and (**b**) pH as a function of As(III) concentration in the presence of SRB and polystyrene as support matrix [SRB(+) Py(+)]; absence of SRB and polystyrene [SRB(-) Py(-)] and in the presence of SRB with no polystyrene [SRB(+) Py(-)].Error bars represent standard deviation between 3 measurements.
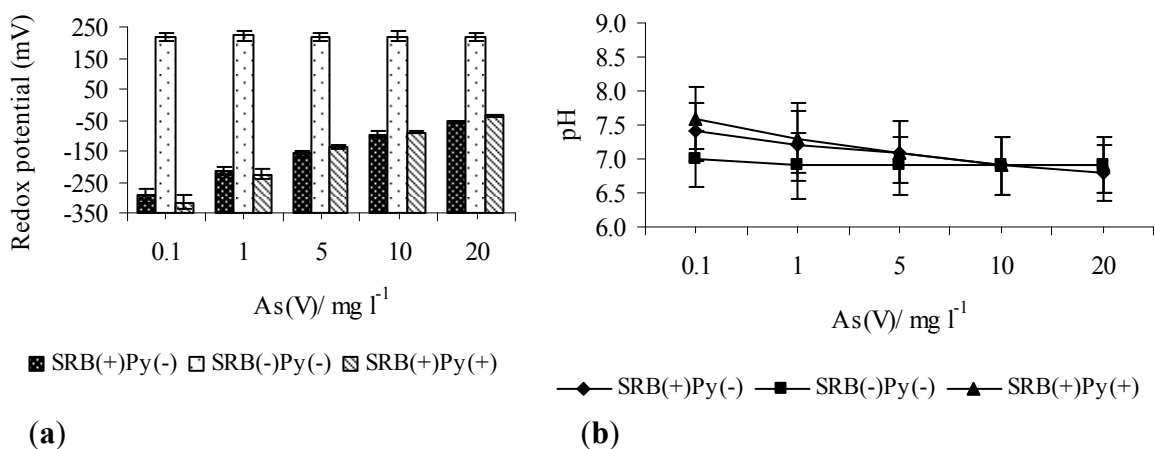
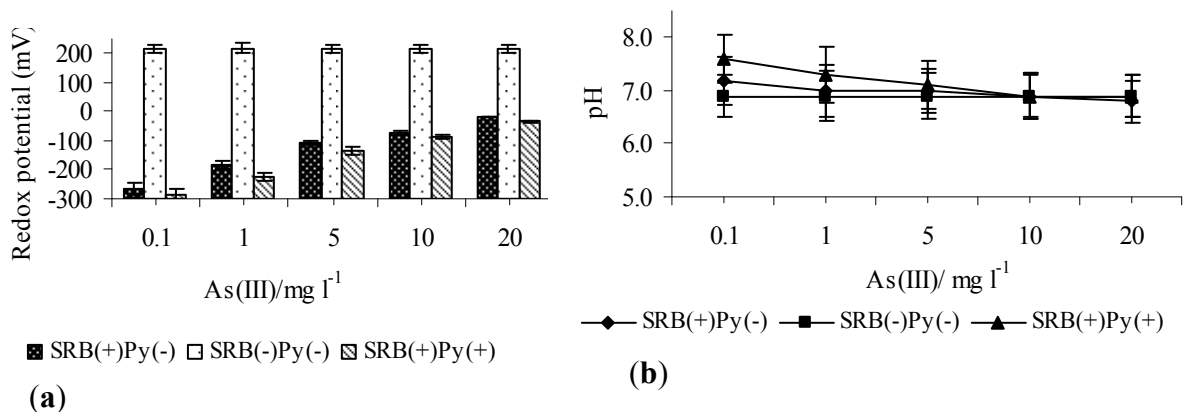**Figure 7.3** Changes in (**a**) redox potential (Statistically significant at P=0.05) and (**b**) pH as a function of As(V) concentration in the presence of SRB and polystyrene as support matrix [SRB(+) Py(+)]; absence of SRB and polystyrene [SRB(-) Py(-)] and in the presence of SRB with no polystyrene [SRB(+) Py(-)].Error bars represent standard deviation between 3 measurements.

Figure 7.4 Changes in (**a**) redox potential (Statistically significant at P=0.05) and (**b**) pH as a function of initial % As(III) in a total arsenic concentration of 0.1 mg l⁻¹ in the presence of SRB and polystyrene as support matrix [SRB(+) Py(+)]; absence of SRB and polystyrene [SRB(-) Py(-)] and in the presence of SRB with no polystyrene [SRB(+) Py(-)].Error bars represent standard deviation between 3 measurements.
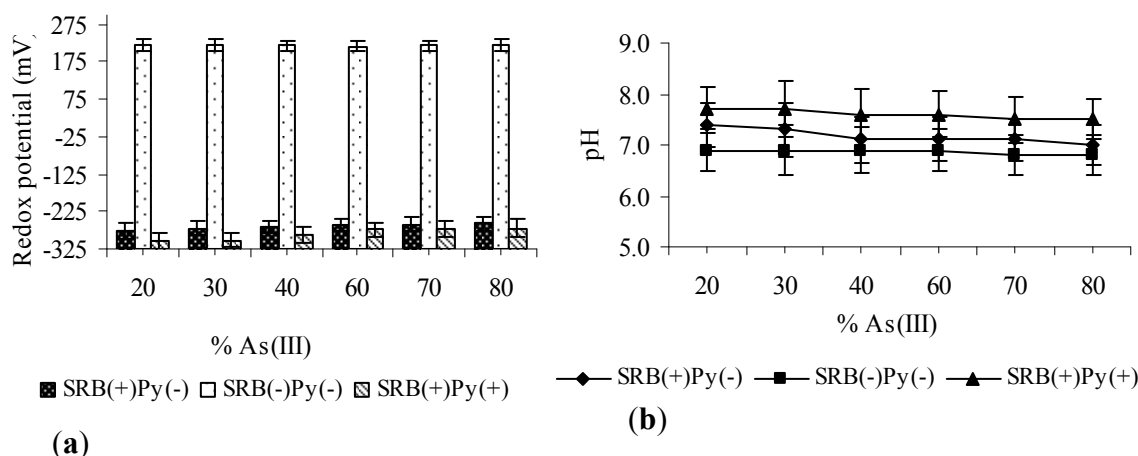
Figures 7.2, 7.3 and 7.4 provided evidence that in the presence of either As(III) or As(V), the redox potential became more reducing (more negative) as the arsenic concentration decreased from 20 to 0.1 mg l⁻¹. In every instance the pH also increased in those bioreactors inoculated with SRB and in which polystyrene was present as support matrix. In the positive control (inoculated with SRB in the absence of polystyrene), there was a decrease in redox potential and an increase of pH, but the changes were smaller compared to those in the bioreactors containing polystyrene as support matrix. In all bioreactors containing neither SRB nor polystyrene, there was no change in either redox potential or pH. With changing As(III):As(V) ratios, viz 20:80, 30:70, 40:60, 60:40, 70:30 and 80:20 in which the total initial arsenic concentration was always 0.1 mg l⁻¹, only small differences in pH and redox potential were observed with increasing As(III) concentration causing less reducing conditions and a smaller decrease in pH.

Polystyrene appeared to contribute more significantly to the lowering of the redox potential as the As(III) concentration increased (Figure 7.2a). This could be a reflection of the greater toxicity of As(III) than As(V). As the As(V) concentration increased, the polystyrene contribution to redox appeared less important, and the pH was little affected by the various

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treatments toward the end of the experiment. Care must be taken in interpreting these results since the initial pHs were somewhat different (Figure 7.3).

The activities of SRB within the bioreactors were assayed by their ability to reduce sulphate and generate sulphide. Figures 7.5-7.7 show that the levels of sulphate reduction and sulphide production increased during the experimental period in the presence of SRB with or without the polystyrene support matrix. Irrespective of the proportions of the two arsenic species, changes in sulphate and sulphide concentrations were insignificant in the uninoculated and polystyrene-free bioreactors (data not shown).

Figure 7.5 (a) Percentage SO_4^{2-} reduction as a function of As(III) concentration in the presence of SRB and polystyrene as support matrix [SRB(+) Py(+)] and in the presence of SRB with no polystyrene [SRB(+) Py(-)]; **(b)** Changes in S^{2-} concentration as a function of time in the presence of polystyrene-immobilised SRB [SRB(+) Py(+)] growing in the presence of different As(III) levels. Error bars represent standard deviation between 3 measurements. Statistically non-significant at P=0.05.

Sulphate reduction in bioreactors with immobilised microorganisms was higher than in those containing suspended SRB (Figures 7.5a, 7.6a and 7.7a). The change in sulphate reduction levels in the presence of different proportions of arsenic species (total initial concentration, 0.1 mg l^{-1}) was small, and the level of sulphate reduction increased with increase in proportional As(V) concentration. Due to the complexity of the reactions involved in sulphate reduction by anaerobic bacteria, different parameters will affect this reduction process including: availability and type of electron donor, pH, temperature,

sulphate concentration as well as inhibitory effects of any heavy metals present and sulphide (Elferink *et al*., 1994; Rintala & Lettinga, 1992; Zehnder, 1988; Postgate, 1979). Temperature has an effect on the magnitude of sulphate reduction with increases in temperature resulting in increased reduction levels (Barnes *et al*., 1992; Middleton & Lawrence, 1977). The concentration of sulphate has been shown to affect the activity of SRB (Dries *et al*., 1998; White & Gadd, 1996). Moosa *et al*. (2002) have studied the effect of sulphate concentration and its volumetric loading on the kinetics of bacterial growth and bioreduction of sulphate. They found that an increase in sulphate concentration results in an enhanced reaction rate. For a given initial concentration of sulphate and for dilution rates below the wash out value, an increase in volumetric loading of sulphate led to a linear increase in its volumetric reduction rate.
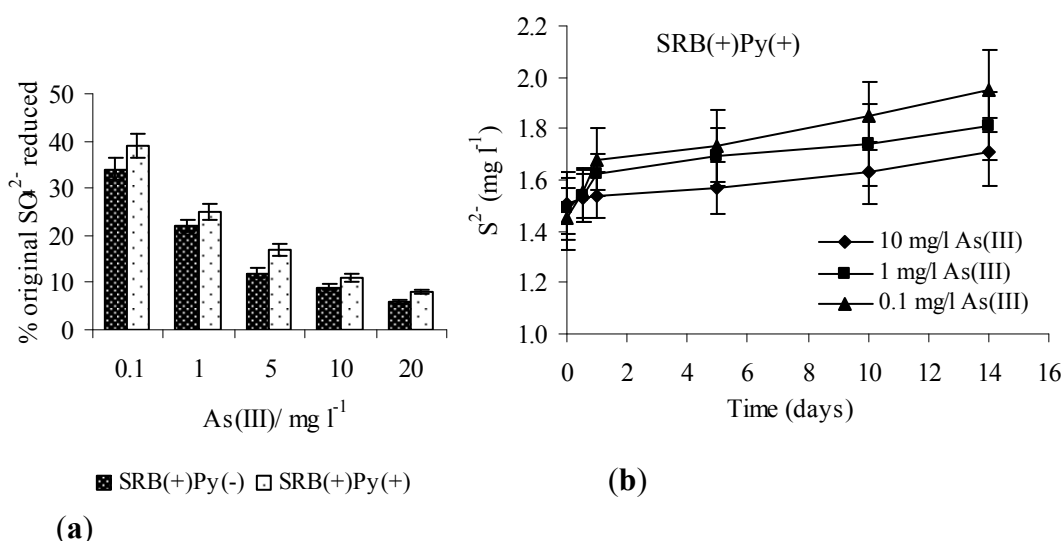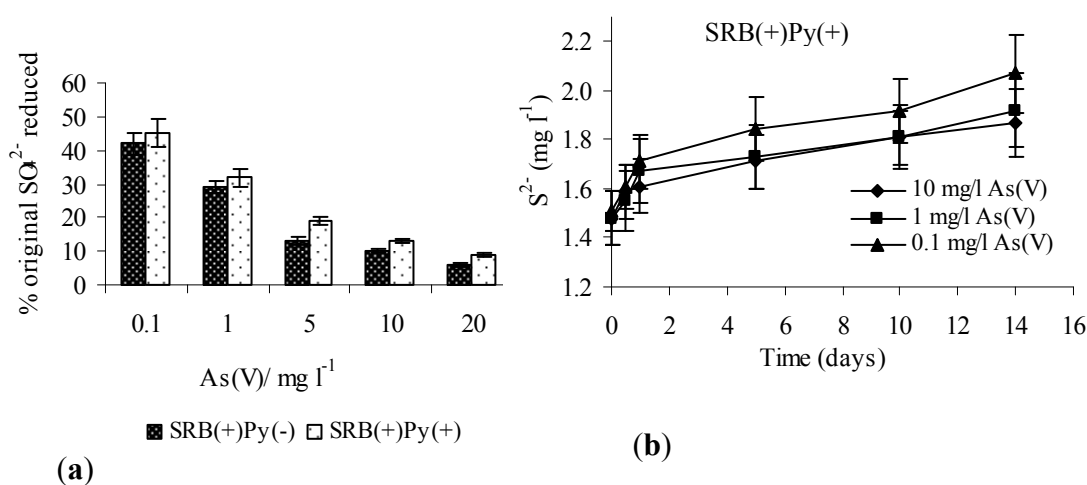

Figure 7.6 (a) Percentage SO_4^{2-} reduction as a function of As(V) concentration in the presence of SRB and polystyrene as support matrix [SRB(+) Py(+)] and in the presence of SRB with no polystyrene [SRB(+) Py(-)]. **(b)** Changes in S^{2-} concentration as a function of time in the presence of polystyrene-immobilised SRB [SRB(+) Py(+)] growing in the presence of different As(V) levels. Error bars represent standard deviation between 3 measurements. Statistically non-significant at P=0.05.
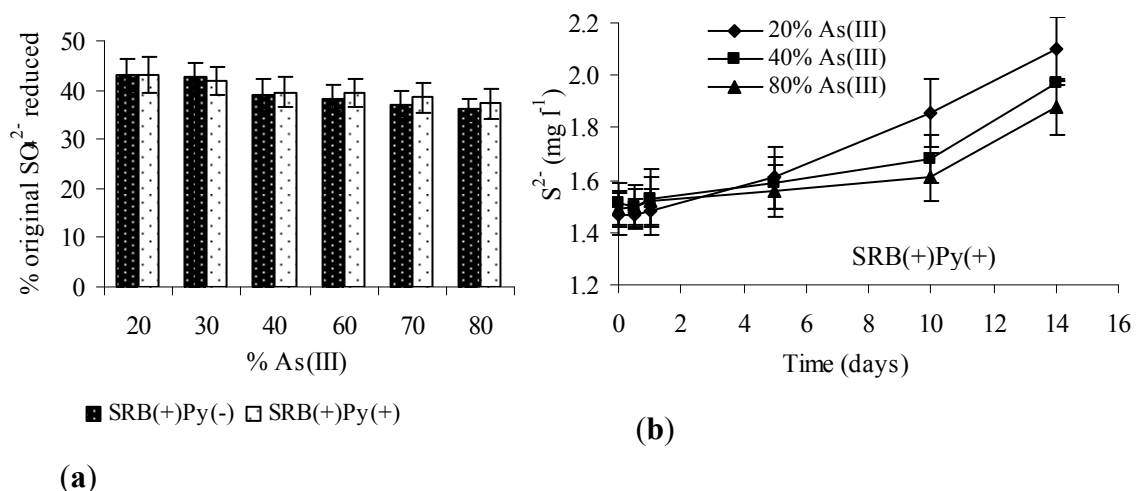
Figure 7.7 (a) Percentage SO_4^{2-} reduction as a function of the % As(III) in a mixture of As(III) and As(V) (total arsenic = 0.1 mg l^{-1}) in the presence of SRB and polystyrene as support matrix [SRB(+) Py(+)] and in the presence of SRB with no polystyrene [SRB(+) Py(-)]. **(b)** Changes in S^{2-} concentration as a function of time in the presence of polystyrene-immobilised SRB [SRB(+) Py(+)] growing in the presence of different As(III) and As(V) concentrations. Error bars represent standard deviation between 3 measurements. Statistically non-significant at P=0.05.

The biomass hold-up in an immobilised cell bioreactor and any freely suspended cells present in a system are important in influencing the rate of sulphate reduction (Webb & Dervakos, 1996). The contribution by freely suspended cells is significant at low volumetric loading rates but not at high volumetric loading because wash out of the cells can occur (Baskaran & Nemati, 2006). Immobilised cells usually show more tolerance to environmental stresses such as high levels of toxic substances (Costerton *et al*., 1994) and positively influence the sorption, transportation and decomposition of pollutants (White & Gadd, 1998; Schorer & Eisele, 1997). Notwithstanding this generation of a high concentration of hydrogen sulphide can negatively affect SRB activity due to its increase toxicity and through precipitation of key essential metals (Patidar & Tare, 2005).

The efficiencies of arsenic species removal within the bioreactors during the growth of polystyrene-immobilised and free-living SRB were studied. Figures 7.8-7.10 show the changes in concentration of As(III), As(V) and in mixtures of As(III) and As(V), respectively. Both As(III) and As(V) were removed by the mixed culture of SRB either in the presence or absence of the support matrix. Irrespective of the initial concentration, the

removal efficiency of As(III) was always inferior to that of As(V). Also, immobilised SRB were superior to freely suspended SRB in removing arsenic species. Percentage removal of As(III) improved from about 10% to 47% when the concentration was reduced from 20 to 1 mg l^{-1} (Figure 7.8) whereas the corresponding improvement for As(V) was from 39% to 92% removal (Figure 7.9) during the 14-day experiment in the immobilised system. In the free-living cell systems, the percentage removals at the end of the 14 day experiment was 43, 33, 12 and 12% for initial As(III) concentrations of 1, 5, 10 and 20 mg l^{-1} respectively whilst for As(V) the corresponding removal values were 88, 76, 69 and 34%.

Figure 7.8 Changes in As(III) concentration as a function of time in the presence of: **(a)** SRB with polystyrene as support matrix [SRB(+) Py(+)] and **(b)** in the presence of SRB with no polystyrene [SRB(+) Py(-)].
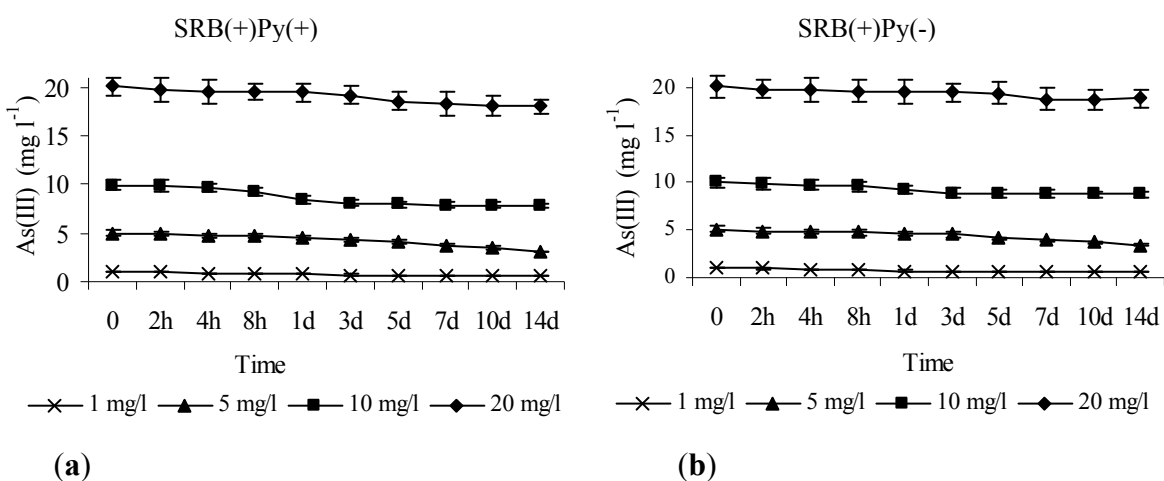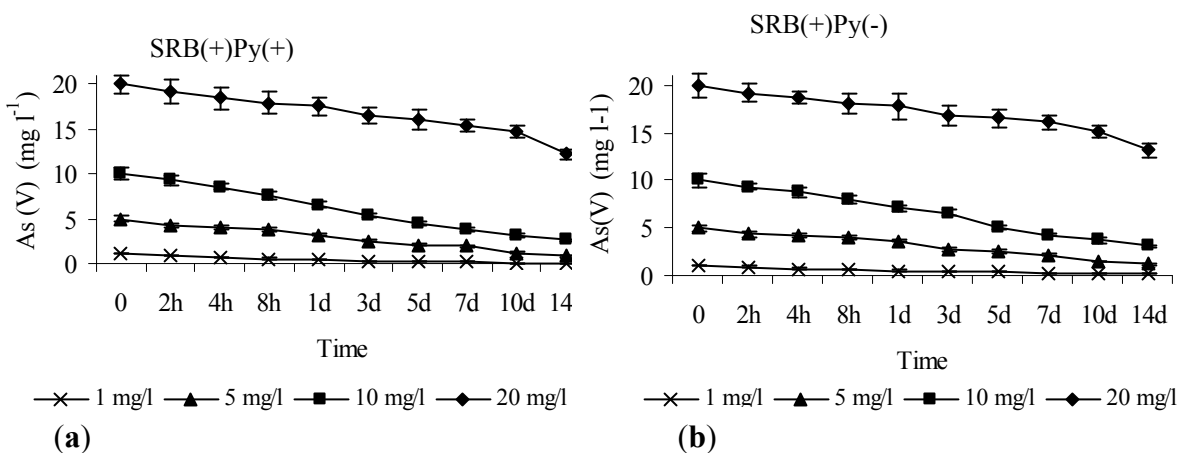

Figure 7.9 Changes in As(V) concentration as a function of time in the presence of: **(a)** SRB with polystyrene as support matrix [SRB(+) Py(+)] and **(b)** in the presence of SRB with no polystyrene [SRB(+) Py(-)].

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When the total arsenic concentration (i.e., As(III) + As(V) in different proportions) was 100 μg l^{-1}, the removal efficiencies were improved for both As(III) and As(V) and the percentage removal was 52%, 73% and 96% at the end of the 14 day experiment when As(III) comprised 100%, 60% and 0% of the total arsenic respectively (Figure 7.10). When the residence time was increased to 21 days, the solutions containing 40% As(III) or less (i.e., 40 μg l^{-1} As(III) or less in a total arsenic concentration 100 μg l^{-1}) were efficiently bioremediated to below the WHO acceptance limit of 10 μg l^{-1} (Figure 7.10). Retaining water in the bioreactors for longer than 21-36 days is not advisable since the quality of the water could deteriorate due to the growth of harmful microorganisms.

Figure 7.10 Changes in total arsenic concentration in solutions with different ratios of As(III):As(V) as a function of time in the presence of polystyrene-immobilised SRB.

Polystyrene samples were taken from bioreactors inoculated with SRB [SRB(+) Py(+)] and from control (uninoculated) bioreactors [SRB(-) Py(+)] and dry-ashed to quantify arsenic and iron content. The results are given in Table 7.2.

Table 7.2 Arsenic and iron content of dry ashed polystyrene samples from SRB-inoculated [SRB(+) Py(+)] and uninoculated [SRB(-) Py(+)] bioreactors

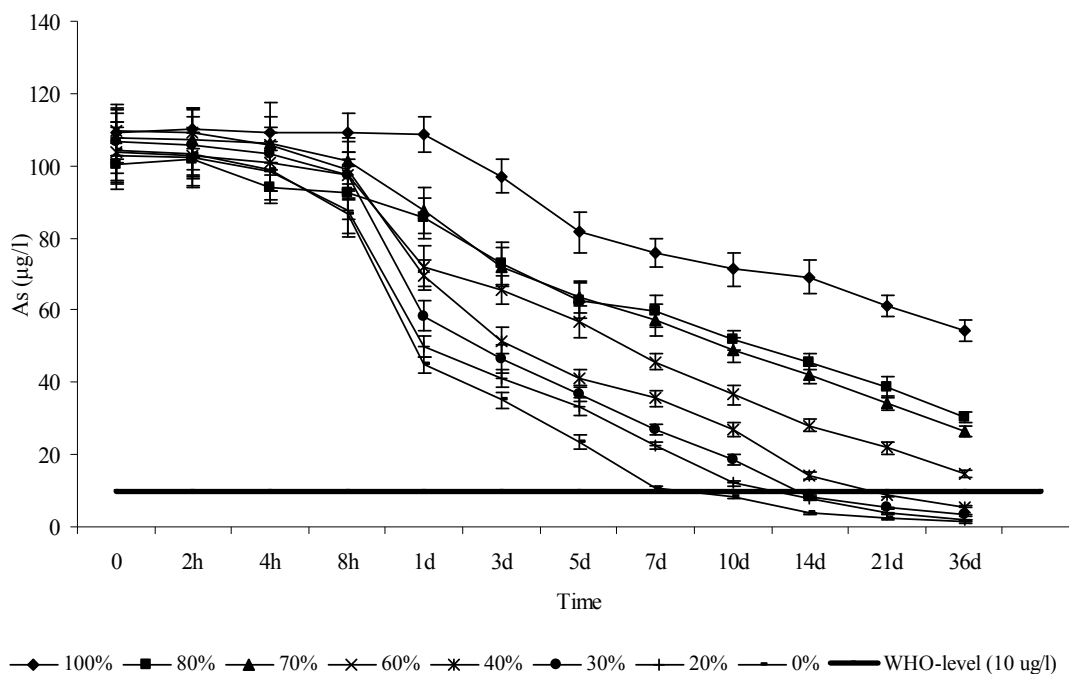| Samples	As concentration $(mg\ g^{-1})$	Fe concentration $(mg\ g^{-1})$
As(III)		
SRB(+) Py(+)	1.79±0.03	2.52±0.01
SRB(-) Py(+)	0.23±0.01	1.86±0.01
As(V)		
SRB(+) Py(+)	2.43±0.03	3.01±0.04
SRB(-) Py(+)	2.07±0.02	2.94±0.03

The concentration of arsenic [either As(III) or As(V)] associated with the SRB-colonised polystyrene samples was higher than that associated with this material in the bioreactors lacking biofilms. Digestion of the former showed that the concentration of As(V) was higher than that of As(III). This might be due to the charged nature of As(V) in the pH range used in this study, whereas As(III) would exist mainly as a neutral compound under these conditions. The concentration of arsenic species correlates positively with the concentration of iron in the polystyrene samples. Previously, it had been shown that the surface of microorganisms covered by iron oxides could provide a favourable environment for arsenic to be adsorbed and thus removed from aqueous streams (Katsoyiannis & Zouboulis, 2004). The iron in the influent water could be the source for the formation of adsorbents that subsequently remove arsenic species.

It is evident that As(V) was removed more efficiently than was As(III). Hence, a pre-oxidation of As(III) to As(V) using air (atmospheric and pumped) and MnO_2 was investigated. MnO_2 was the oxidising agent preferred by Ghurye and Clifford, (2001) for the treatment of drinking water prior to the removal of arsenic. Synthetic groundwater still containing about 69 $\mu g\ l^{-1}$ As(III) on day-14 (Figure 7.10) was removed from the appropriate bioreactors and exposed to atmospheric air, pumped air and MnO_2 (0.1, 1, and 2 g l^{-1}) for 24 h at pH ~ 7.0. Atmospheric and pumped air did not cause significant oxidation of As(III), whereas MnO_2 did oxidise As(III) with the oxidation rate increasing

with increasing concentration of MnO_2. This oxidising agent was further tested at a total arsenic concentration of 0.1 mg l^{-1} comprising 80, 70, 60, 40, 30 and 20% As(III). The results are given in Figure 7.11.

As(III) concentrations: ■ Initial; after treatment with ⬚ 0.1g ⊠ 1g and ▤ 2g $MnO2$ /l
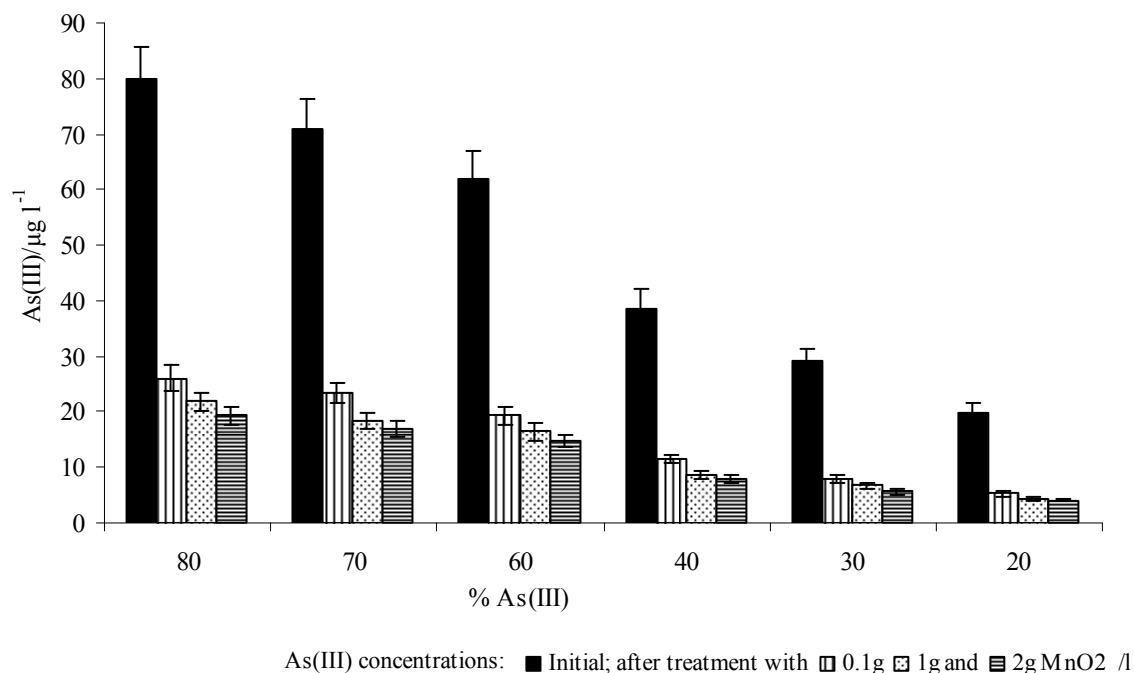
Figure 7.11 Changes in As(III) concentration when solutions containing various initial concentrations of this arsenic species were contacted with 0.1, 1 and 2 g l^{-1} MnO_2 for 24h at pH 6.9. Error bars represent standard deviation between 3 measurements.

The differences in percentage oxidation of the initial amounts of As(III) to As(V) by 0.1, 1 and 2 g l^{-1} MnO_2 were not significant (Figure 7.11); however, choosing the amount of the oxidising agent to use will depend on the initial As(III) concentration. Thus, in the case of 80% As(III), it would be appropriate to use 2 g l^{-1} MnO_2, whereas for 20% As(III) 0.1 g l^{-1} MnO_2 would suffice. It would be easier to remove arsenic species from groundwaters using bioreactors inoculated with SRB if such water contained much lower concentrations of As(III) than As(V).

The total dissolved arsenic concentrations remained fairly constant (data not shown) indicating that the decrease in As(III) was simply the result of its oxidation to As(V) and

there was very little adsorption of either As(III) or As(V). Similar results were reported by Scott and Morgan (1995). The adsorption of As(V) onto MnO_2 minerals has been previously reported (Chiu & Hering, 2000; Manning *et al*., 2002; Ouvrard *et al*., 2002). In the present investigation, very low MnO_2 concentrations were used and as a result few surface sites were available for arsenic sorption. A study by Radu *et al*. (2008) using MnO_2 as adsorbent for arsenic found that the adsorption kinetics were very fast, with the concentration of sorbed arsenic remaining constant after about 2 min., whereas As(III) continued to be oxidised for a long time (Tournassat *et al*., 2002; Driehaus *et al*., 1995) and its sorption on MnO_2 has not been observed (Amirbahman *et al*., 2006). Radu *et al* (2008) hypothesised that MnO_2 consists of oxidative sites and non-oxidative sorption sites. The oxidative sites are renewable, and they rapidly oxidise As(III) and release As(V) to the solution through the mechanism postulated by Scott and Morgan (1995). The mechanism involves a multi-step reaction model, where the first step is the formation of an inner spherical surface complex where As(III) diffuses into oxidative sites and displaces surface-bound OH^- and H_2O via ligand substitution and binds to the oxide metal ion. The second step is the transfer of two electrons from As(III) to the surface. In the third and fourth steps, the surface-bound oxidised As(V) and the reduced metal Mn(II) are released into the solution. In the above process, the total number of reactive surface sites will remain constant as the result of the formation of a new site when the reduced Mn(II) is released and the near-surface Mn–O group is protonated (Scott & Morgan, 1995).

Different mechanisms can be suggested for lowering concentration of arsenic species in the bioreactors. In Chapter 6, bioremoval mechanisms such as bioprecipitation of arsenic as sulphides and adsorption on biogenic sulphide precipitates were discussed. In addition to these microbiologically induced mechanisms, adsorption of As(III) or As(V) on the walls of the bioreactors and on polystyrene was investigated and found to be negligible.

The design of the bioreactor and construction material used in its manufacture can influence the removal of arsenic species. The design should include a sludge (precipitate) trap that can be easily removed so as to avoid the re-solublisation of arsenic in the water. Alvarez *et al*. (2007) noted the major problem associated with the use of SRB for

precipitating metal sulphides is the build-up of the metal-containing precipitate in the same reactor where the bacteria are reducing sulphate, as the viability of the SRB culture can be adversely affected by metal toxicity (Chen *et al*., 2000; Poulson *et al*., 1997). Extended use of bioreactors can be achieved by optimising matrix size and having a system with the ability to flush out the precipitates (Tsukamoto *et al*., 2004) since large size will allow easy maintenance of hydraulic conductivity but decrease available surface area for biomass formation (Lyew & Sheppard, 1999).

7.4   Conclusions

The bioreactors inoculated with SRB and polystyrene as a support matrix showed a decrease in redox potential and an increase in pH during the removal of both As(III) and As(V) at initial concentrations of 20, 10, 5, 1 and 0.1 mg l^{-1}; howerver, the change were markedly greater in solutions containing the lower concentrations of the metalloid. Similarily, sulphate reduction and generation of sulphide were observed throughout the duration of the study. Arsenite removal from bioreactors supporting a culture of SRB immobilised on polystyrene was only about 10% when the initial concentration was 20 mg l^{-1}; the result for the same initial concentration of As(V) was 39%. Planktonic SRB cultures removed less As(III) and As(V) than their immobilised counterparts. When the total arsenic concentration of 0.1 mg l^{-1} was solely As(V) or when the percentage As(III) in the total weight of arsenic was 20% and 30% of the total arsenic concentration, the metalloid was reduced to below the WHO's permissible level (10 µg l^{-1}) was achieved after 14 days. When the residence time was extended to 21 days, the solution containing 40% As(III) in a total arsenic concentration of 0.1 mg l^{-1} was also bioremediated to below this level. Planktonic SRB removed both arsenic species with lower efficiency than their immobilised counterparts. The presence of SRB markedly improved the arsenic removal capacity of the system. The efficiency of As(III) removal was enhanced by oxidising it to the less toxic As(V) using MnO_2.

7.5   References

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Chapter 8

General Summary

The research results with respect to the specific objectives cited at the beginning of the dissertation are summarised in the following points:

- Investigation of the bioremoval of arsenic species [As(III) or As(V)] during the growth of a mixed culture of SRB, sorption of the species on precipitates resulting from sulphate-reducing bacterial activity and precipitation as sulphide when the species react with gaseous hydrogen sulphide.

  - Arsenic species at concentrations of 5 mg l^{-1} or less can be removed by precipitating the metalloid out of solution as the metal sulphide by reacting with the H$_2$S produced by sulphate-reducing bacteria (SRB). When gaseous hydrogen sulphide was reacted with different initial concentrations of arsenic species to precipitate the metal sulphide, it was found that better removal was achieved at an initial concentration of 0.1 mg l^{-1} for both As(III) and As(V). Arsenate removal by sorption on the precipitate produced by the sulphate-reducing bacteria (SRB-PP) was comparable to that of synthetic iron sulphide (SIS) at an adsorbent dosage of 0.5 g l^{-1} with initial concentration of 5 mg l^{-1}; however, when the amount of adsorbent was increased to 2 g l^{-1}, SIS was superior to SRB-PP for both As(V) and As(III) at pH 6.9 and pH 8. Langmuir isotherm is more suitable to evaluate the sorption of As(III) or As(V) to either SIS or SRB-PP at pH 6.9 than is the Freundlich adsorption isotherm.

- Assessment of molasses's suitability as carbon/energy source to sustain SRB activity and investigation of the effect of arsenic species [As(III) and As(V)] on the growth of a mixed culture of SRB

  - Molasses provided as the source of carbon at concentrations of 1, 2.5 and 5 g l^{-1} supports the growth of SRB. The heavy metals occurring in molasses are not present in high enough concentrations to inhibit the growth of SRB.

- At 20 mg l^{-1}, both arsenic species, but particularly As(III), were shown to reduce the growth of SRB. At much lower concentration of both arsenic species, the growth of SRB is considerably better but a prolonged lag phase is evident.

- Evaluation of the adhesion capability of a mixed culture containing SRB to pine bark, polystyrene and sand, and comparison of the arsenic removal capacity of this immobilised biomass with that of planktonic SRB populations

  - Sulphate reduction kinetics of immobilised SRB cultures were affected by the support matrix used, with greater sulphate reduction occurring in cell populations attached to polystyrene than to pine bark or sand

  - In the presence of As(III) concentrations ranging 1-20 mg l^{-1}, polystyrene-immobilised SRB cells caused less original sulphate reduction as compared to As(V) of the same concentration level

  - Pine bark is not suitable as a support matrix since SRB populations immobilised on this material showed inferior sulphate reduction and sulphide production capacities to those immobilised on sand and polystyrene, and those cells growing in a matrix-free system. Growth of SRB was completely inhibited in media prepared using pine bark extracts

- Examination of the effect of sulphate, nitrate and ferrous iron amendments on the growth of mixed SRB.

  - In the presence of 30 mg l^{-1} nitrate, SRB grew better in 150 than in 50 mg l^{-1} sulphate. The presence of a large amount of nitrate stimulated the growth of SRB more than when a lower concentration of nitrate was used. The concentration of ferrous iron present also had an effect on the growth of the cells; when the level of iron in the medium was increased from 50 to 100 mg l^{-1}

- Investigation of the removal of arsenic species in bioreactors inoculated with SRB, with molasses as carbon source, sulphate as electron acceptor and polystyrene as support matrix in the presence of various concentrations of either As(III) or As(V) and in the ratio of the species As(III):As(V) 0.25 to 4; assessment of chemical oxidising reagents in combination with the biological process during of the removal of arsenic species, particularly As(III)

  ➢ Bioreactors inoculated with SRB and polystyrene as a support matrix showed a decrease in redox potential, particularly in solutions containing the lower concentrations of the metalloid. Planktonic SRB cultures removed less As(III) and As(V) than their immobilised counterparts. When the total arsenic concentration of 0.1 mg l^{-1} was solely As(V) or when the percentage As(III) in the total weight of arsenic was 20% and 30% of the total arsenic concentration, the metalloid was reduced to below the WHO's permissible level (10 µg l^{-1}) was achieved after 14 days. The presence of SRB markedly improved the arsenic removal capacity of the bioreactor. Arsenite removal was enhanced by oxidising it to the less toxic As(V) using MnO_2.

Finally, the following further research studies would assist future research in this field:

- ❖ Investigation of alternative systems for oxidising arsenite such as photocatalytic, biological oxidation to couple with the bioreactors

- ❖ Study of the mixed culture of SRB to identify the individual species present using an array of molecular techniques.

Appendix

A.1   Reagents for Nitrate Analysis using TrAAcs

The dilution water and system wash solution were prepared by adding 2 ml Brij-35 (polyoxyethyleneglycol dodecyl ether) to 1000 ml distilled water and mixing thoroughly.

a) Colour Developing Reagent

Ten grams of sulphanilamide were dissolved in about 600 ml of distilled water. After complete dissolution, 0.5 g of NEDD was added and the solution was mixed thoroughly. One hundred ml of phosphoric acid were added to the mixture and diluted to one litre with distilled water. The final solution was stored in a dark bottle. Fresh colour-developing reagent was prepared weekly.

b) Copper Sulphate Stock Solution

One gram of cupric sulphate ($CuSO_4.5H_2O$) were dissolved in about 600 ml distilled water. The solution was diluted to one litre with distilled water and mixed thoroughly to give a final concentration of 1000 mg l^{-1} cupric sulphate.

c) Zinc Sulphate Stock Solution

Ten grams of zinc sulphate were dissolved in about 600 ml distilled water. The solution was diluted to one litre with distilled water and mixed thoroughly to give a final concentration of 10 000 mg l^{-1} zinc sulphate.

d) Sodium Hydroxide Stock Solution

Ten grams of sodium hydroxide (NaOH) were dissolved in about 600 ml distilled water. Three millilitres of ortho-phosphoric acid were added cautiously and mixed thoroughly. The solution was diluted to one litre with distilled water and 1 ml of Brij-35 solution was added.

e) Hydrazine Sulphate Stock Solution

A stock solution of hydrazine sulphate was prepared by dissolving 10 g of hydrazine sulphate in about 600 ml distilled water. The solution was then diluted to one litre with distilled water and mixed thoroughly.

f) Working Hydrazine Sulphate Solution

To about 600 ml distilled water 10 ml stock copper sulphate solution, 10 ml zinc sulphate solution and 200 ml stock hydrazine sulphate were added.

g) Standard Nitrate Solution

A stock nitrate standard solution, 1000 mg N l^{-1} was prepared by dissolving 7.218 g potassium nitrate in about 600 ml distilled water and making the volume up to one litre with distilled water.

The working standard solutions for linear calibration were prepared by diluting the stock solution to a concentration sequence of 1, 2.5, 5, 10 and 15 mg l^{-1}.

A.2   Reagents for Ammonia Analysis using TrAAcs

The same system wash solution as used for nitrate determination was employed for ammonia concentration.

a) Tri-sodium Citrate ($C_6H_5Na_3O_7.2H_2O$)

Forty grams of tri-sodium citrate were dissolved in about 600 ml of distilled water. The solution was diluted to 1 litre with distilled water, thoroughly mixed and 2 ml of Brij-35 solution added. The final solution was stable for 1 week.

b) Sodium Salicylate ($NaC_7H_5O_3$)

Forty grams of sodium salicylate were dissolved in about 600 ml of distilled water and 1 g of sodium nitroprusside ($Na_2[Fe(CN)_5NO].2H_2O$) was added. The mixture was diluted to 1 litre with distilled water and mixed thoroughly. The final solution was stable for 1 week.

c) Dichloro isocyanuric Acid

Twenty grams of sodium hydroxide and 3 g of dichloro isocyanuric acid sodium salt dihydrate ($C_3Cl_2N_3NaO_3.2H_2O$) were dissolved in about 600 ml of distilled water. After mixing thoroughly, the solution was diluted to 1 litre with distilled water.

d) Stock Ammonia Standard

A 1000 mg l^{-1} (as N) ammonia standard stock solution was prepared by dissolving 4.717 g of ammonium sulphate (($NH_4)_2SO_4$) in about 600 ml of distilled water. The solution was diluted to one litre with distilled water. The working standard solutions for linear calibration were prepared by diluting the stock solution to a concentration sequence of 1, 2.5, 5, 10 and 15 mg l^{-1}.

**B.1.   Graph of calibration curve for As(III) generated using HG-ICP-OES**
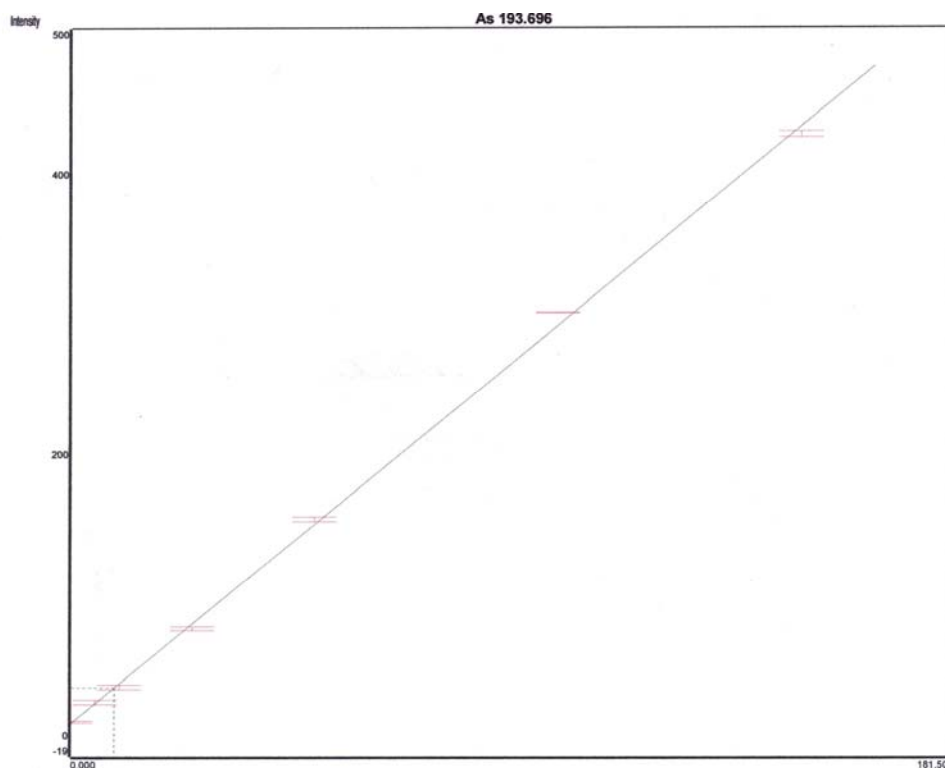
As 193.696 Calibration (ug/L) on Sep 2 2007, 09:13:45 am

Label	Flags	Int. (c/s)	Std Conc.	Calc Conc.	Error	%Error
Blank	---	5.6	0.000	0.282	0.282	
Standard 1	---	19.5	5.00	5.19	0.186	3.715
Standard 2	---	30.0	10.0	8.90	-1.102	-11.020
Standard 3	---	71.6	25.0	23.5	-1.459	-5.836
Standard 4	---	149.8	50.0	51.1	1.137	2.273
Standard 5	---	297.2	100	103	3.076	3.076
Standard 6	---	424.3	150	148	-2.119	-1.413

Correlation Coefficient	0.999514
Status	Calibrated.
Curve Type	Linear
Curve Coefficient 1:	4.787
Curve Coefficient 2:	2.837
Blank Offset	4.8

B.2.   Graph of calibration curve for total arsenic generated using HG-ICP-OES
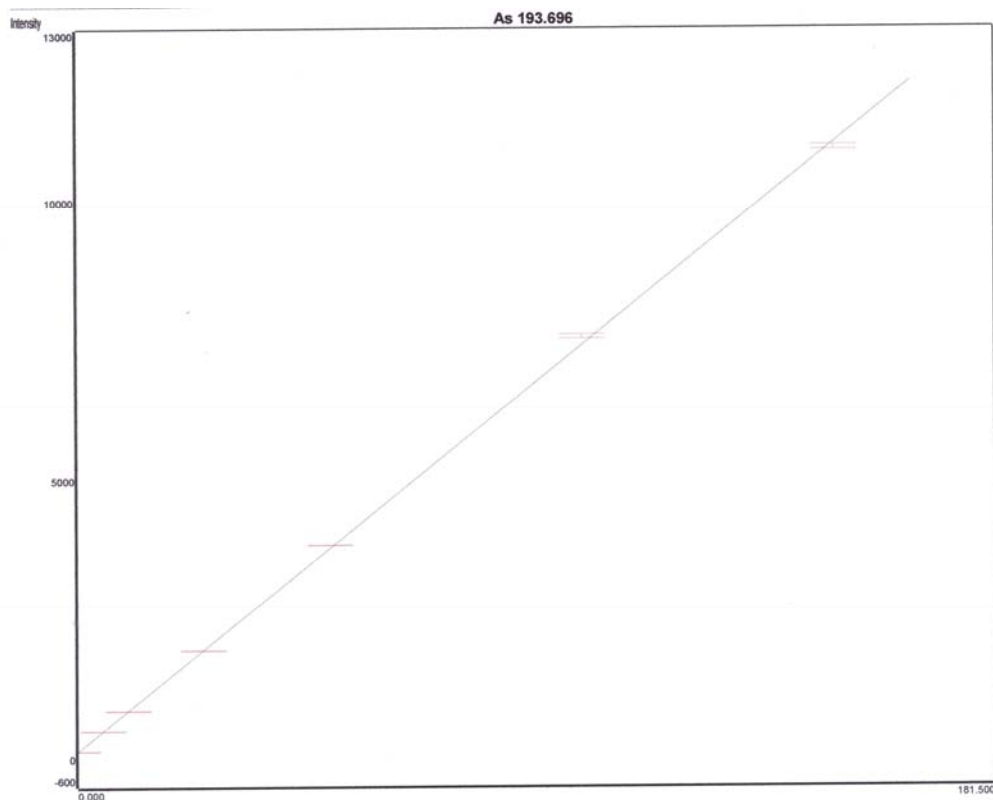
As 193.696 Calibration (ug/L) on Aug 18 2007, 12:23:00 pm

Label	Flags	Int. (c/s)	Std Conc.	Calc Conc.	Error	%Error
Blank	---	36.4	0.000	-0.205	-0.205	
Standard 1	---	398.6	5.00	4.78	-0.223	-4.458
Standard 2	---	757.3	10.0	9.71	-0.287	-2.868
Standard 3	---	1838.1	25.0	24.6	-0.417	-1.670
Standard 4	---	3725.5	50.0	50.5	0.550	1.100
Standard 5	---	7465.7	100	102	2.009	2.009
Standard 6	---	10850.1	150	149	-1.427	-0.951

Correlation Coefficient	0.999825
Status	Calibrated.
Curve Type	Linear
Curve Coefficient 1:	51.347
Curve Coefficient 2:	72.683
Blank Offset	51.3