Environmental Chemistry and Biological Degradation of Metallocyanide Complexes in Gold Mines

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'Whatever befalls the earth befalls the sons of the earth. Man did not weave the web of life; he is merely a strand in it. Whatever he does to the web, he does to himself.'

(Quote from a speech attributed to Chief Seattle, Chief of the Suquamish, in 1854)

SUMMARY

Gold from ancient times to the present, has been prized. The inert quality, bright yellow colour, lustre and scarcity of gold has ensured its lasting value. Few chemicals react with this metal, but with sodium cyanide, auro complexes are formed.

Mining gold results in some toxic cyanide wastes which are usually stored in impoundment areas. This can be problematic since huge volumes of tailings need to be held in storage for many years and monitored to eliminate spillage and leakage. Environmental pollution by toxic compounds has occurred since the development of many modern industrial processes. One such process being the inclusion of sodium cyanide as a lixiviant for gold in the mining industry.

A possible alternative to long-term tailings storage is biodegradation of the cyanide compounds and this has often been a subject for research. Investigative research into the possible utilization of the cyanide (CN^-) as a food source for microorganism has been carried out for many years, usually concentrating on *Pseudomonas* species, with a few studies favouring the *Bacillus* group. Also investigations have centred around the simpler cyanides e.g. hydrogen cyanide and sodium or potassium cyanides.

The subject of this thesis is the comparison of three systems for the degradation of three heavy metal cyanide compounds, copper(I) cyanide, sodium tetracyanonickelate and potassium hexacyanoferrate(III). It was concluded that the three heavy metal cyanides could be degraded by microorganisms, with

varying degrees of efficiency in the following three systems; in shake flask cultures, bioreactors and using individual bacterial species in test tube cultures. The advantages of biodegradation include self generation and perpetuation, simple designs for equipment and the use of innocuous chemicals.

Copper(I) cyanide degradation research

Copper(I) cyanide is generally regarded as a simple cyanide compound with a relative low solubility in water. However, over a period of 7 days, the solubility was found to increase from 2.26 to 41.98 mg l^{-1}, measured by using Reverse Phase Ion-interaction High Performance Liquid Chromatography. The cyanide ligands can be removed from the copper(I) cyanide complex by treatment with weak acids. Thermodynamic and kinetic reasons cause the cyanide ligands to be labile in this complex. As the cyanide anion concentration increased in solution, the bacteria were able to use the carbon and nitrogen for metabolic processes thereby, removing the cyanide from the system.

In the shake flask cultures, the consortium of bacteria utilized the cyanide more efficiently when provided with additional organic material e.g. peptone. A build-up of the by-product cyanate was measured at pH 8 but no such accumulation was found in the larger bioreactor system, where the cyanate was in turn hydrolyzed to ammonia and carbon dioxide. Moreover, an acceleration of the degradation process after a pretreatment with peptone was confirmed in the bioreactor experiments.

The main bacterial species identified both in the shake flask cultures and the bioreactors were *Pseudomonas stutzeri* and *Bacillus pumilus*. Although, when two

strains of *P. stutzeri* and three strains of *B. pumilus* were inoculated as pure cultures into the copper(I) cyanide mineral salts medium, either very low or no degradation was noted. The best species for degradation was found to be *Bacillus sphaericus* followed by *Sphingomonas paucimobilis*.

The *B. sphaericus* and *S. paucimobilis* strains appeared to be neither efficient cyanate producers nor degraders, which inferred that the enzymes cyanide monoxygenase and cyanase were inactive during the pure culture tests. This was contrary to what was seen with the bacterial consortium active in the bioreactors. Therefore, the biomass in the bioreactors contained some bacterial species that degraded the cyanide to cyanate which was further hydrolysed to ammonium-nitrogen and carbon dioxide by other strains. Some bacteria were able to convert the cyanide directly to ammonium-nitrogen through the action of the two enzymes cyanide dioxygenase and cyanidase.

The consortium of bacteria active in the biomass of the shake flask cultures and bioreactors was more effective than when using the pure cultures.

Sodium tetracyanonickelate degradation research

Sodium tetracyanonickelate is a moderately stable complex which is soluble in water. Utilization of the cyanide anion occurred at pH 8 and pH 10 in the shake flasks when an organic supplement, peptone, was added. Furthermore, degradation also proceeded in the absence of peptone but only at pH 8. The independence of the degradation process in regards to peptone was more clearly apparent in the bioreactors.

The by-product cyanate accumulated at pH 8 and reached a higher level when

peptone was added. This was confirmed in the bioreactor experiments where cyanate was detected for a longer period in the bioreactor which had the peptone pretreatment.

A faster conversion of cyanate to ammonium-nitrogen occurred in the bioreactor where no pretreatment with peptone was carried out. This indicated that the sodium tetracyanonickelate was degraded by the enzyme cyanide monoxygenase, followed by the action of cyanase.

Bacterial isolations were only successful from the peptone treatments with *Pseudomonas stutzeri*, *Bacillus firmus*, *B. sphaericus, B. filicolonicus* and *Sphingomonas paucimobilis* being identified from the shake flask cultures. Some of these species were also isolated from the bioreactor experiments, in addition to *B. cereus,* and *B. pumilus.*

In the pure cultures trials, two unnamed strains nos. 101 and 94 performed the best, followed by *S. paucimobilis, Bacillus globisporus* and the unnamed strain no.157. Cyanate was not detected in 4 of the 5 cultures with only *B. globisporus* producing a small amount.

The bacterial strains, no.101 and no. 94 were able to use sodium cyanate showing that the enzyme cyanase was induced. Other bacterial species isolated during the degradation experiments in the shake flasks and bioreactors were *P. stutzeri*, *B. filicolonicus* and *B. firmus* which also utilized cyanate as a substrate to form ammonium-nitrogen.

The formation of ammonium-nitrogen by the *S. paucimobilis* culture may have been due to the enzymes cyanide dioxygenase and/or cyanidase since no cyanate was produced. Bacterial species isolated from the shake flask and bioreactor

experiments, were predominantly different to those that best degraded sodium tetracyanonickelate in the pure cultures. Only *S. paucimobilis* was regularly isolated from the biomass in both the shake flask cultures and the bioreactors and also performed well as a pure culture.

Potassium hexacyanoferrate(III) degradation research

The potassium hexacyanoferrate(III) complex is very soluble in water. This complex has been regarded as a stable metallocyanide where decomposition required extreme conditions and UV irradiation. However, in the research, potassium hexacyanoferrate was found to be very reactive.

The better conditions for degradation in the shake flask cultures were at pH 8 and in the presence of peptone, but a high level of cyanate accumulated under these conditions. In the bioreactors, however, degradation proceeded efficiently regardless of any pretreatment with peptone and with no build-up of cyanate.

From the shake flask cultures many bacterial species were isolated including, *Cellulomonas cellulans*, *Bacillus cereus*, *B. filicolonicus*, *B. pumilus*, *B. sphaericus* and *Pseudomonas stutzeri*. Similar species were present in the bioreactors, including *Sphingomonas paucimobilis, B. firmus* and *B. thuringiensis*.

As pure cultures, 30 out of the 31 species tested were effective in the degradation of potassium hexacyanoferrate(III) with *B. sphaericus* being the best. Some species formed cyanate during the degradation process with *S. paucimobilis* producing the greatest amount. This signified that the enzyme cyanide monoxygenase was activated in these species.

In addition, high levels of ammonium-nitrogen but no cyanate was detected during the pure bacterial species tests, indicating that the degradation of potassium hexacyanoferrate was facilitated by the enzymes cyanide dioxygenase and/or cyanidase.

Ammonium-nitrogen values were low when peptone was absent in the shake flasks. This confirmed that the enzyme cyanase was present at a low level.

The degradation of potassium hexacyanoferrate was most efficient when the bacteria were used as pure cultures, followed by degradation involving the biomass in the bioreactor and shake flask systems.

Sodium cyanate utilization

When sodium cyanate was introduced as the substrate, it was best utilized by *P. stutzeri*, with only 9 out of the 31 species being able to hydrolyse the sodium cyanate. The production of ammonium-nitrogen in the 20 to 38 mg l^{-1} range, indicating that the enzyme cyanase was present but only in a few of the bacterial species tested.

The nine bacterial strains included three *Bacillus* spp., three *Pseudomonas* spp. and three of the four unnamed strains. Furthermore, each time when bacterial isolations were carried out during the shake flask culture and bioreactor experiments, some of the sodium cyanide utilizing strains were found to be present.

This therefore explained the disappearance of cyanate that was measured during the degradation experiments of the three metal cyanides in the shake flask cultures and the bioreactors.

Declaration of Candidate

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference has been made in the text.

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Chapter 1.0 Literature Review

1.1 *Introduction*

Gold is one of the most precious of metals and in high demand especially for the manufacture of exquisite jewellery. It is estimated that some 3 billion kg of cyanide are used yearly worldwide in various industries including the gold mining industry (Basheer *et al.*, 1992). After the extraction and gold recovery processes, the waste (tailings) is pumped into impoundment areas. Toxic compounds present in these tailings which are stored at the mines, can be reduced in toxicity by both chemical and biological degradation processes. Although expensive, chemical removal is generally preferred, while biodegradation, an inexpensive and self sustaining technique is rarely used (Hoecker and Muir, 1987; Dubey and Holmes, 1995).

Over 40 years of research into biodegradation of cyanide has provided information on the successful use of this method. Details relating to types of organisms tested, conditions most favoured by these organisms, end products and the identity of activated enzymes are available (Dubey and Holmes, 1995). Although there have been a number of genera and species tested for the transformation of simple cyanides, HCN, KCN and NaCN, overall there are only about half a dozen microorganisms regularly studied in relation to this process (Harris and Knowles, 1983b; Kunz *et al.*, 1992; Chapatwala *et al.*, 1993).

Encouraging results with bacterial and fungal isolates, led to the identification of some enzymes responsible for the biodegradation of cyanide compounds with three

enzymes, cyanidase, rhodanese and cyanide hydratase, being particularly active. With molecular genetic research becoming more common, the genes regulating these enzymes are now being discovered and mapped (Watanabe *et al.*, 1998). Already cell-free enzymes have been tested and shown to degrade toxic cyanide compounds in wastewaters from the food industry (Basheer *et al.*, 1993) and when production costs decrease, toxic wastes from gold mines may be treated and rendered harmless by these special proteins.

Heavy metal cyanide complexes have been less frequently studied but it appears that some of the above enzymes are also active in the degradation processes for these compounds (Rollinson *et al.*, 1987). However, the huge volumes of wastes produced at gold mines make the use of cell-free enzymes impractical at this stage, therefore the use of whole bacterial cells is still considered to be the best option. The cost of obtaining cell-free enzymes and immobilizing these proteins in some inert substance, is much greater than growing a large biomass of bacterial cells. Tailings contain a pool of both known and unknown compounds because of the many interactions between metals, cyanide, water and intermediary products. Due to their adaptability, it is expected that the bacterial cells would cope better in this harsh environment.

A review of the literature, covering the extraction of gold from the ore, the compounds formed during the process and resultant waste material stored in the tailing dams, shows the complexity of the whole procedure. Although some of the physical and chemical methods used to destroy residual cyanide compounds are

very efficient, biological treatments can also be effective, as shown at the Homestake Mine.

The research programme for this thesis, included investigating whether bacterial species residing on the gold ore particles could break down the cyanide anion in three metal cyanides. These compounds are some of the heavy metal complexes manufactured during the gold extraction process and form part of the toxic waste material.

Lessening the toxicity of the tailings through biodegradation could reduce the volumes of dangerous wastes which have to be keep in storage.

1.2 *Aims of the project*

The objectives of this research project were:

- To examine the ability of some common mine-soil bacteria to degrade three metal cyanides and to determine the degradation rates under three growth systems;

- To study the influence of two pH levels and an organic supplement on the degradation of the three metal cyanides;

- To estimate the levels of two by-products, ammonium-nitrogen and cyanate, formed during the degradation of the three metal cyanides;

- To isolate and identify the bacterial species involved in the degradation processes and to determine the degradation potential of each species.

1.3 *Background*

1.3.1 *Gold ore types, extraction and recovery processes*

There are three main economic sources of gold, native gold, gold tellurides and gold bearing sulfides (Osseo-Asare *et al.*, 1984). In particular, gold is found to be associated with the pyrite-pyrrhotite group of sulfide minerals (Menne and Muhtadi, 1988). Extraction of the gold from the ores is very difficult until it is exposed to a solution of the complexing agent, sodium cyanide, with the resulting formation of soluble sodium aurocyanide (Scott, 1984). Mineral contents of ores ultimately govern the levels and types of metallocyanides formed and subsequently, the final breakdown products. In particular iron sulfide in the pyrite ore is a source of ferrous and ferric ions which readily react with cyanide to form ferro and ferri hexacyanide complexes of varying stabilities.

A significant by-product from the degradation of these metal cyanide complexes, is cyanate (CNO^-) which further hydrolyses to carbonate (CO_3^{2-}) and ammonium-nitrogen (NH_4^+), in conditions favouring biological activity (Hoecker and Muir, 1987). If the ore is sulfidic, thiocyanate (SCN^-) is formed as a by-product which under alkaline conditions, degrades slowly to carbon dioxide, ammonia and hydrogen sulfide (H_2S).

For over 100 years (Longe and Devries, 1988; Jones and Staunton, 1991) gold has been leached with alkaline sodium cyanide in the Merrill-Crowe extraction process (Muir, 1982; Scott, 1984). In this method, gold is recovered from the aurocyanide solution by precipitation initiated by the addition of zinc dust and followed by some purification steps (Ritcey, 1989).

Other processes have evolved over the past 20 years for the recovery of gold from the soluble aurocyanide complex (Fleming, 1998). There are three main processes used at present, 1) carbon-in-pulp (CIP), 2) carbon-in-leach (CIL) and 3) carbon-in-column (CIC). For carbon-in-pulp, the collection of gold is accomplished by the adsorption onto activated carbon granules. This is carried out in specially designed tanks which receive the aurocyanide pulp from the leaching tanks. Thus the leaching is completed before the recovery of gold commences. Whilst in the carbon-in-leach process, the carbon granules are added to the leach tanks where adsorption of the gold from the aurocyanide occurs at the same time as the leaching (Ritcey, 1989; Fleming, 1998). In the last process, carbon-in-column, the aurocyanide solution is pumped upwards through a series of columns packed with activated carbon granules (Fleming, 1998).

Successful liberation of gold from the ores is also governed by the crushing process and the final grain size has to be optimised for each method of extraction used. The best size for extraction in tanks is <20 μm but when heap leach pads are used the ore is left in a coarser form. In the final step, recovery of the gold from the loaded carbon granules is usually done by stripping with hot caustic sodium cyanide or hot sodium hydroxide solution and by electrowinning from the strip solution (Ritcey, 1989).

The aurocyanide forming reaction between gold (Au) and sodium cyanide (NaCN), is dependent on the amount of oxygen available, as is the efficiency of extraction from the ore when using heap leach pads (Menne and Muhtadi, 1988). In these pads, ore is left coarse (approx. 30 mm average diameter) to allow the aerated

sodium cyanide solution to flow easily through the ore material dissolving gold particles during the percolation. After leaching the heaped ore, the leachate (called the pregnant solution) flows through activated carbon packed in towers where the gold is adsorbed. The residual liquid (called the barren solution) is topped up with sodium cyanide so that the concentration is kept high, and recycled back to the heap leach pads (Ritcey, 1989). The residue after the adsorption of gold onto the carbon, is waste material. Only comparatively small volumes of aqueous tailings are produced from the heap leach pad system since the ore remains in the heaps but very large volumes of tailings result from all of the other methods of gold mining.

1.3.2 *Tailing dams contents, toxicities and case studies*

1.3.2.1 *Construction of tailings dams and contents*

Collection and containment of the wastes produced (called tailings) is labour intensive. The containment areas (tailing dams), being large earth structures, require constant monitoring for damage such as cracking (Ritcey, 1989), as well as regular deepening for greater storage. The cost of maintaining these dams which contain a complex and toxic mixture of heavy metals originally present in the ore, including many cyanide complexes and some free cyanide, is high. The tailings are deposited and stored in these specially constructed areas (tailing dams) which sometimes have dimensions of 10 m to 30 m high walls and length and breadth of up to 1 km (Photo 1.1.).

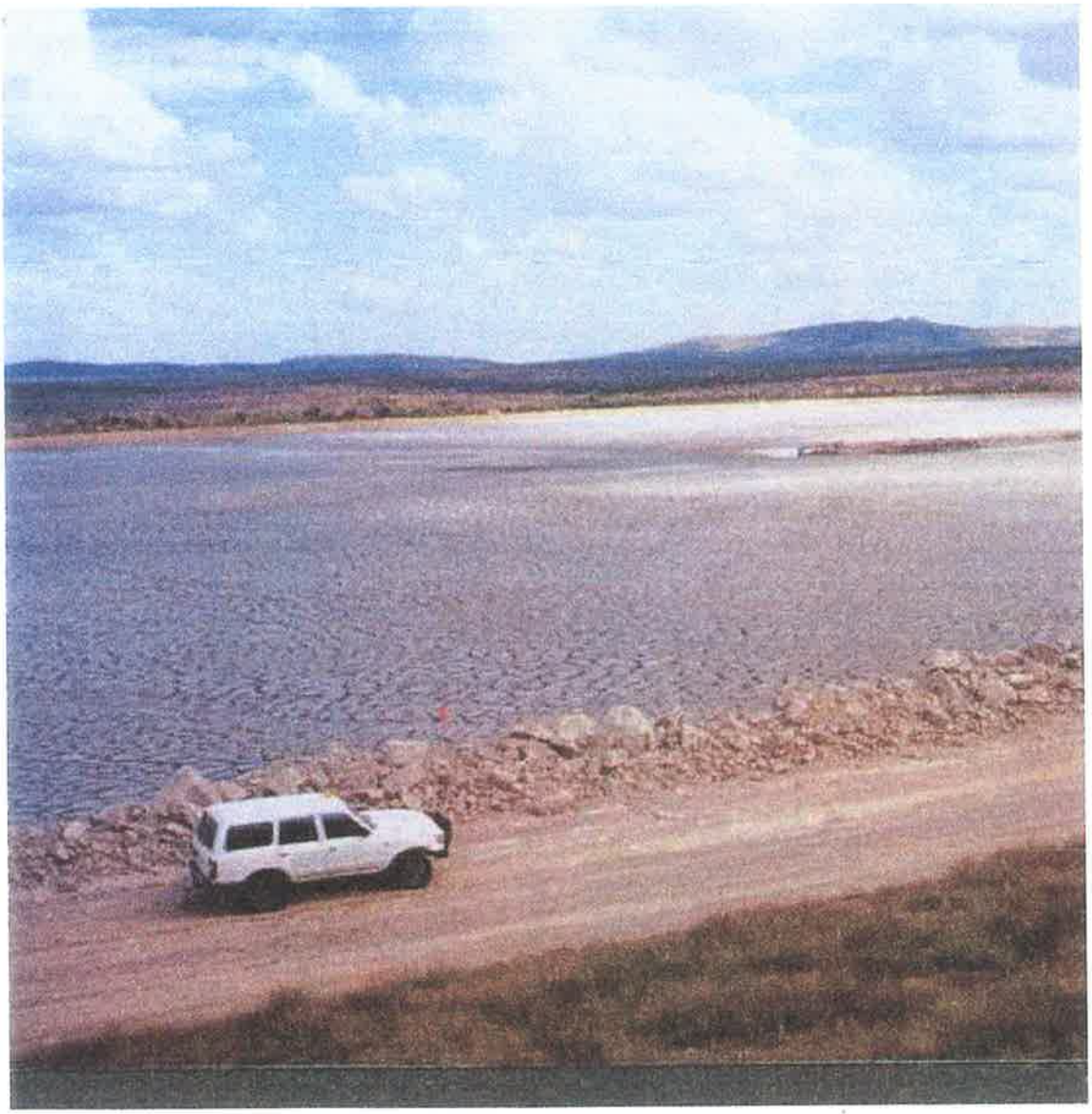

Photo 1.1. A Tailings Dam

Amongst the main concerns for gold mine managers are included the following:

1) dewatering of waste material,

2) safety and stability of the tailings dams,

3) destruction of residual cyanide and

4) maintaining low volumes of acid mine drainage (Dorey, 1988).

Of the four points, destruction of residual cyanide and maintaining low volumes of acid mine drainage (AMD) are the hardest to control. Precautions with construction are required since it may be necessary to store this material for hundreds of years. In just one year (1991) 80 million kg of sodium cyanide was used in Australia in the gold mining industry and yearly large volumes of toxic gold tailings are permanently sited at most gold mines (Jones and Staunton, 1991). If leakages and spills occur the toxic substances from the dams are released into the environs.

Tailings contain a mixture of free and simple cyanides, weak acid dissociable metal cyanides (WAD-CN) and a wide range of moderately and strongly complexed metal cyanides. The stability of each complex in the tailings varies, depending on pH, temperature, metal concentration, radiation energy (sunlight) and the redox potential (Hoecker and Muir, 1987).

Some of these compounds are easily degraded while others are considered stable and settle out as a precipitate. The levels of total cyanide (Total-CN) present in the impoundment waters after gold extraction are below 50 mg l^{-1}, for example, at the Boddington Gold Mine in Western Australia, the range is 5-38 mg l^{-1} (Miller *et al.*, 1991).

The reason for the extensive use of cyanide in the gold industry is the high reactivity of the cyanide anion (CN^-) which can complex with some 28 metals resulting in 72 metallic complexes (Chatwin and Hendrix, 1988). This ability of the CN^- has the advantage of solubilising precious metals that are economically important but has the disadvantage in extracting metals that are not targeted and are superfluous. Cyanide can be present in solution as hydrogen cyanide (HCN) gas or in solution as CN^- (Windholz, 1983) and the state of the cyanide or the proportion of each component, depends on parameters such as ionic strength, pH and temperature.

Ionic cyanide compounds are formed between HCN and the alkali metals hydroxides (sodium or potassium hydroxides), which have high solubility but low stability that is, are easily dissociated. The complexes of copper, nickel and zinc can be dissociated by weak acids and are considered unstable but are soluble in water (Kurnia *et al.*, 1996). The compounds mentioned above are the most common found in the tailings and are categorized as weak or moderately strong cyanide complexes. Strongly complexed cobalt, mercury, gold and iron cyanides are very stable and soluble in water. The stability of the labile cyanide complexes is dependent on the availability of cyanide and will increase if free cyanide is present in the water (Huiatt, 1984).

There are three main groups of compounds found in tailings dams (Fuller, 1984; Zhang, 1991):

1 Free cyanides with the general formula of CN^- and HCN, where the equilibrium is described by:

$$CN^- + H_2O \leftrightarrow HCN + OH^-$$

high pH dependence on position of equilibrium

2 Simple cyanides (ionic salts) with the general formula of $A(CN)_x$, where A is a cation of an alkali metal and x is the valence of A which indicates the number of cyano groups present in the salt.

$$A^{x+} + x(CN^-) \leftrightarrow A(CN)_x$$

3 Complex cyanides with the general formula of $A_yM(CN)_x$, where A is the alkali metal, y is the number of alkali species (Na^+, K^+, NH_4^+), M is the heavy metal (Fe^{+++}, Fe^{++}, Cu^{++}, Au^{+++} and Ni^{++}) and x is the number of cyano groups.

$$yA^+ + M + x(CN^-) \leftrightarrow A_yM(CN)_x$$

these soluble cyanide compounds dissociate giving complex ions

Compounds other than those represented by the general formulae above, are formed in the tailings by the process of degradation of the cyanide complexes, including oxidation of the cyanide to cyanate or hydrolysis to carbon dioxide and ammonia. In addition, thiocyanate can be produced as a by-product of the cyanidation of sulfidic gold ores. Thiocyanate is regarded as a stable compound (Williams, 1948). In the tailings with respect to the stability, dissociability and solubility constants, the important parameters are pH, metal involved in the complex, level of free cyanide, temperature, pressure, oxygen levels and light (Garrels, 1960).

The main concern about cyanide in dams is that in the form of hydrogen cyanide gas it is very soluble in water and volatilizes below pH 9.2 especially in an open, well aerated area. At pH 9.2, 50% of the cyanide is in the HCN form and 50% as

CN^-, with 100% CN^- present at pH 11 and 100% HCN at pH 7 (Smith and Mudder, 1991). The pH of the new tailings is in the 9-10 range but the pH tends to slowly drift towards neutrality thereby increasing in the HCN level. The toxicology of simple cyanide compounds has been extensively investigated and data on safety levels is readily available (Windholz, 1983), less is known about the toxicity of metallic cyanocomplexes.

At lower pH values, changes are known to occur in copper and iron cyanide complexes with less cyanide ligands being present in the complexes. Also, the stability of some complexes is dependent on the amount of free cyanide present in the water (Huiatt, 1984). All ferri and ferrocyanide complexes $Fe(CN)_6$(total) have stabilities dependent on pH, redox potential, soil composition and the equilibrium with Prussian blue ($Fe_4[Fe(CN)_6]_3$) in solution. As the pH of a soil increases (more alkaline) the solubility and mobility of Prussian blue was also found to increase (Meeussen *et al.*, 1990). There was a misconception until recently that strongly bound iron cyanide complexes would precipitate and remain immobile for hundreds of years. This misconception was corrected when Meeussen *et al.* (1992) showed that reduced forms of the iron cyanide complexes are stable in anaerobic but not in aerobic conditions. Therefore, as the tailings material dries out, cracks will develop allowing oxygen to penetrate deeper into the tailings (Photo 1.2.). This causes the tailings to become aerobic and as a consequence the iron cyanides become less stable.

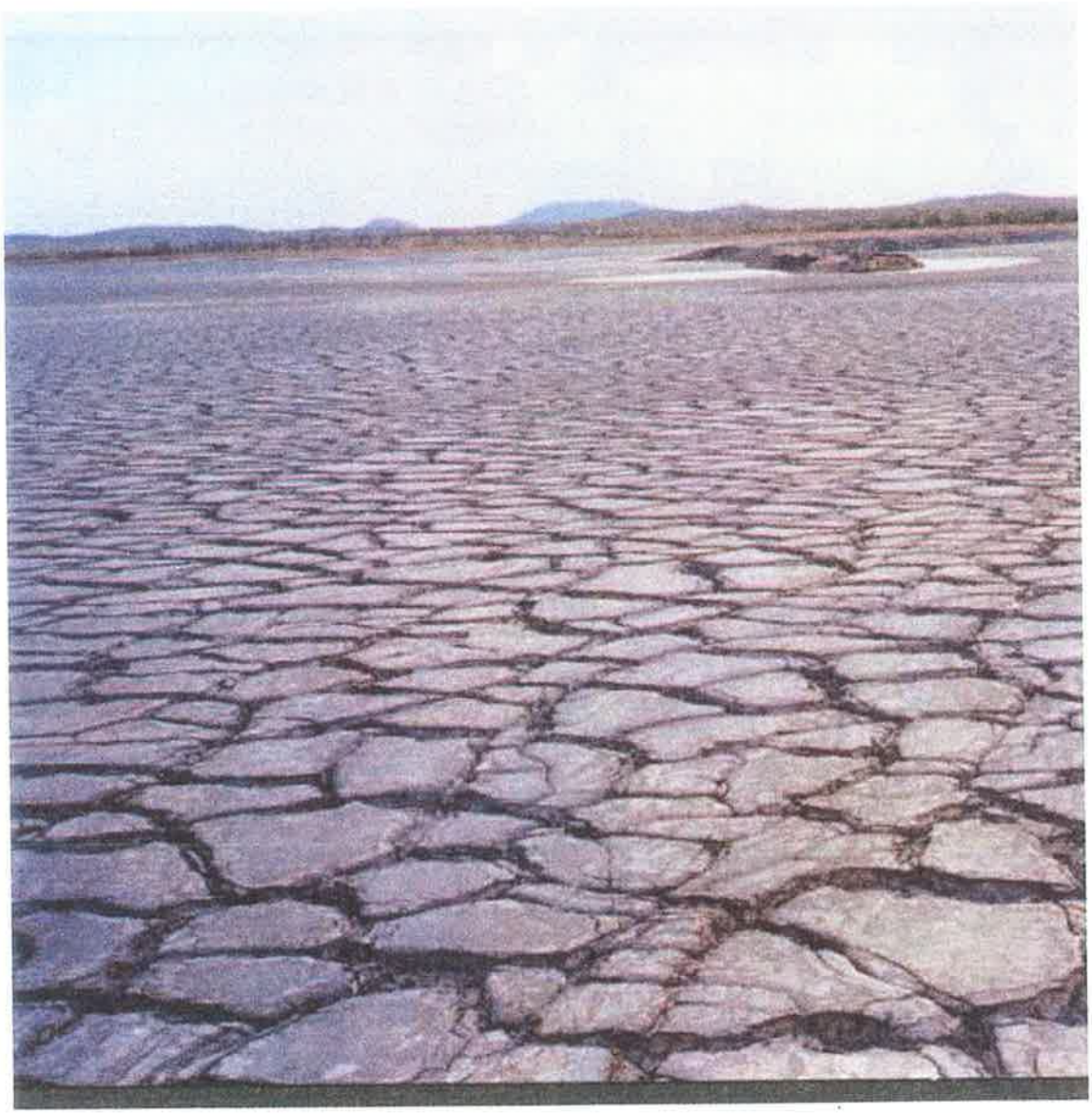

Photo 1.2. Surface cracks in the tailings material

1.3.2.2 *Toxicity of cyanide and cyanide compounds*

During the lifetime of impoundment areas, toxic sodium cyanide may be brought into contact with humans through leakages from these dams. For humans, death will result at rates of 0.5-3.5 mg cyanide per kilo of body weight (Raybuck, 1992). The concerns about the highly toxic nature of cyanide is reflected by the Environmental Protection Agencies (EPA) setting standards in many countries. In the USA, the level set by the EPA is 0.02 ug ml^{-1} (0.02 mg l^{-1}) of free cyanide for drinking water (Ritcey, 1989) while the National Health and Medical Research Council (NHMRC) in Australia has set the standard at 0.1 ug ml^{-1} (0.1 mg l^{-1}) of total cyanide.

In many bacterial species, the cyanide ion is an inhibitor of growth and cellular metabolism (Dubey and Holmes, 1995). In some microorganisms, cyanide inhibits the cytochrome oxidases which are necessary for the process of aerobic respiration (Davis *et al.*, 1990). Apart from the inhibition of mitochondrial cytochrome oxidase, cellular catalase, peroxidase, tyrosinase, ascorbic acid oxidase and phosphatase can also be affected (Dubey and Holmes, 1995).

As conditions change in the tailings, the chemistry and mobility of the complexes also changes. Metals, when associated with cyanide, increase their solubility and mobility thereby causing a potential hazard due to both heavy metal and cyanide toxicity. For aquatic life zinc at levels of 0.05 ug ml^{-1}, copper 0.005 ug ml^{-1}, iron 1.0 ug ml^{-1}, and lead at 0.005 ug ml^{-1} are permitted (ANZECC, 1992). The levels of cyanide and metals allowed in our reservoirs and other bodies of water are extremely low for the protection of aquatic life. Also the recommended pH range for aquatic life is between 6.5 and 9.0 since lower pH values increase both the

solubility (Garrels, 1960) and toxicity (ANZECC, 1992) of heavy metals. The weak acid dissociable cyanide complexes, $Cu(CN)_2$ and $Ni(CN)_2$ are toxic due to the easily available metal and the available cyanide. Progressively focussing on the stronger complexes the situation becomes more complicated since both the stability and solubility are pH and temperature dependent. Some researchers (Sharpe, 1976; Dreisbach and Robertson, 1974) considered these complexes to be non mobile and non toxic while others have shown that even the most stable cyanide complex, ferri-ferro hexacyanide or Prussian blue has some solubility and mobility at alkaline pH and in soils of high redox potential (Meeussen *et al.*, 1992).

End products such as cyanate and thiocyanate are also toxic, however, cyanide is the most toxic component with an LC_{50} for trout of 0.05-0.18 mg l^{-1} whereas cyanate has an LC_{50} of 30-40 mg l^{-1} with thiocyanate having the lowest toxicity of LC_{50} equal to 50-500 mg l^{-1} (Ritcey, 1989).

1.3.2.3 *Case studies*

1) The Romanian River Tisza was contaminated when an overflow from a gold mine dam caused a cyanide spill which travelled down the Tisza River through Hungary to Yugoslavia and into the River Danube. A conservation director for The Worldwide Fund for Nature in Hungary concluded that the spill had eradicated all life in a 400 km region of the Tisza River (Middap, 2000).

2) In Papua New Guinea, for almost 10 years 80,000 tonnes day^{-1} of polluting waste was released into the Ok Tedi and Fly Rivers by the Ok Tedi Gold-

Copper Mine (Henry, 2002). This pollution was the subject of litigation and a settlement was reached in 1994, in favour of the people living in the area downstream from the mine, with a payout from BHP of $150 million (Konkes, 2000).

3) In the Free State goldfields, South Africa in 1994, the Merriespruit tailings dam failed after heavy rains and a massive slide of 600,000 m^3 of tailings flowed into a village with death of 17 people and widespread devastation and environmental damage (Wagener *et al.*, 1998). In this case the tailings mass caused the immediate problem. A 31 m high tailings dam failed, only a few hours after approximately 50 mm of rain fell in 30 minutes during a thunderstorm. No indication of the damage done due to the heavy metals and cyanide compounds present in the tailings was reported.

4) Similar tailings dam failures have occurred at a coal mine in Aberfan (Wales), Bafokeng (South Africa) and Stava (Italy). In all cases poor engineering practices and mine tailings management contributed to the failures (Wagener *et al.*, 1998)

1.3.3 *Overview*

The situation in gold mines is such that although as much sodium cyanide as possible is recycled through the plant nevertheless some is discharged with the solids into the tailings dam. The slurry can contain 40 to 65% of solids consisting of crushed ore, metals and cyanide. This waste oozes through pipes and onto the existing solids in the dam forming a new surface. However, settlement of the newly

deposited solids is slow due to the fineness (<20 μm) of the suspension. The supernatant water then either collects forming a lake or evaporates quickly depending on the deposition rates of the slurry and on daily temperatures. In this way, the depth of the tailings material increases in the dam and can reach 10 to 30 m over an area of 1 km^2. At the Osborne copper-gold mine, a member of the Placer Dome Group, and located near Mt Isa, Queensland (Australia), a tailings dam was designed for the impoundment of 11 million tonnes of tailings (Hallman, 1998). This company alone has 19 mines in the group. Another company, WMC which is a major producer of gold and base metals has constructed a 250 million tonne tailings storage facility. This tailings impoundment area has a average diameter of 4.6 km and covers approximately 1,700 ha (Bentel, 1998). Volumes of tailings stored from gold and other minerals production are very large and scattered throughout Australia and the World.

Attenuation of the cyanide by the solids which may contain clay minerals and organics, occurs through a) chemical reaction, b) adsorption onto the mineral particles and c) reaction with organic matter although the latter is usually low (Longe and Devries, 1988), with adsorption of metals onto soil minerals being governed by the redox potential and the pH (Garrels, 1960) of the tailings. The physical and chemical processes involving cyanide at a tailing site may be divided into processes occurring in the atmosphere (air just above the tailings), in the tailings pond and in the tailings solids (Smith and Mudder, 1991).

All forms of cyanide, including free and WAD, can be introduced into the surrounding environment via water overflowing from the dam and water seepage

either through the dam wall or permeable dam foundation (Mehling and Broughton, 1989). In most dams, when based on proper engineering designs, the water evaporation exceeds the inflow from different sources. In addition, there are overflow dams for the rare cases where there is an excess of water. If a permeable foundation underlies the dam floor, cyanide present in pore spaces can be pushed through the tailing material by water moving downwards and this cyanide may enter the underlying soil and eventually the groundwater. If the water table rises, cyanide in solution can be forced upwards into the surface water where wildlife could have access to toxic levels of cyanide (Mehling and Broughton, 1989). Safeguard systems are in place during the lifetime of mines, however, when decommissioning tailings dams the residual cyanide in the pore spaces needs to be considered. If the volume of tailings is decreased by the practice of spreading the material over unused areas of the mining lease, a knowledge of the local soil minerals becomes important since minerals including kaolin, K-feldspar and hematite will adsorb cyanide. Under aerobic conditions, this cyanide may be biologically converted to cyanate which will become soluble in any surface water and find its way to nearby rivers or creeks. Since cyanate is also mildly toxic this practice should not be followed (Chatwin and Hendrix, 1988; Jones and Staunton, 1991).

Table 1.1 shows that Total cyanide and thiocyanate (SCN^-) in the Canadian mine (Byerley *et al.*, 1988) are quite high compared to the Homestake Mine in the USA (Scott, 1984). Pine Creek in Australia, had the highest sulfate level and may have problems with AMD in the future. All of the metals listed can form metallocyanide complexes thereby maintaining the cyanide locked in the tailings material.

The range of potential environmental pollutants that are stored in tailings dams is wide. Not only is the incoming waste toxic but complicated ecosystem develops inside the tailings which can contribute to the overall toxicity.

Table 1.1. Effluent composition (ug ml^{-1}) for three mines

Component	Canadian mine[a]	Pine Creek[b]	Homestake Mine[c]
CN^- total	360-1200	NA	7 - 30
CN^- free	200-700	NA	NA
SCN^-	50-1400	90	110 - 240
SO_4^{2-}	up to 250	2420-3200	NA
Cu	25-700	1-19	1.4 - 6
Fe	0.5-15	< 0.1	1.5 - 6
Zn	2-60	0.5-7	0.01 – 1.5
Co + Ni	0-160	2.9-3.2	0.2 – 0.4 (Ni only)
Ca	6-800	613-634	NA
pH	10.4-11.6	7.5-8.3	7 – 9.5

* NA indicates that the data is not available
references for a = Byerley *et al.*, 1988, b = personal communications, c = Scott, 1984

Added to the ongoing chemical reactions, are the reactions initiated or accelerated by the tailings microflora and end products from the biological activity include ammonia, carbon dioxide, cyanate, formate and thiocyanate (Mudder, 1984; Dubey and Holmes, 1995). Tailing material has not been extensively studied and more intermediary compounds may be discovered when more research is carried out.

Analyses of tailings have indicated that there still remain many valuable metals in

these wastes. Present trends worldwide are to recover these metals by improved technology now available. At the New Celebration Mine retreatment plant in Western Australia 49,824 tonnes of cyanide gold tailings have already been reworked (Martins *et al.*, 1993).

Old gold tailings at Kalgoorlie were reworked to recovery 9,125 kg of fine gold and 25,011 kg of fine silver. In five years the Kaltails Project reprocessed over 28 million tonnes of tailings (Brett and Kadushkin, 1996).

A biological pilot plant using mushroom compost for sulfate reduction of metal-rich mine waters is already operating and concentrations of iron, zinc, manganese, nickel and cadmium in the waters are reduced by 95%. These heavy metals are being precipitated as insoluble metal sulfides by the bacteriologically produced hydrogen sulfide (Dvorak *et al.*, 1991).

In the future, recycling wastes from all sources including gold tailings, will increase profits by reducing costs of maintaining huge containment areas and from the sale of the recovered metals. Most importantly these new techniques will decrease the levels of pollutants stored in the environment (Brett and Kadushkin, 1996).

1.4 *Physical and chemical processes used to destroy residual cyanide in gold mine tailings*

1.4.1 *Physical processes*

Simple cyanides

Cyanide can be destroyed by physical, chemical and biological processes (Scott, 1984; Fleming, 1998). Cyanide destruction by natural processes are mainly due to

volatilisation where cyanide (CN^-) in solution is converted to hydrocyanic acid (HCN) and this diffuses out of the solution as the pH of the tailings decreases (Scott and Ingles, 1981; Miller *et al.*, 1991). For this process to be most effective, the tailings are spread out in the open on the surface of the specially constructed tailings dam. Here the UV radiation in sunlight will degrade the cyanide and the concentration will slowly decrease. Photodecomposition occurs mainly on the surface tailings but penetration into the pond water up to 1 m is known. Simultaneously solar energy increases the temperature of the pond water thereby encouraging further degradation and volatilisation (Hoecker and Muir, 1987).

The mining industry prefers this minimalist approach but degradation by this method is very slow with storage of tailings required for many decades. Cyanate and thiocyanate are formed as by-products during the impoundment period but are subsequently slowly degraded as total cyanide decreases (Smith and Mudder, 1991).

Metal cyanide degradation

Complexation of cyanide with metals results in decreasing the toxicity of cyanide (Meeussen *et al.*, 1992; Wild *et al.*, 1994). Copper cyanide complexes are less toxic than the CN^- or HCN in solution. While strongly complexed cyanides of iron, potassium ferricyanide [$K_3Fe(CN)_6$] and potassium ferrocyanide [$K_4Fe(CN)_6$] are considered non toxic (Blaha, 1976b; Chapman, 1992), weak complexes of zinc, lead and cadmium are very toxic.

Removal of complexed cyanide from wastewater may occur by associations with

activated sludge floc in some sewage treatment systems. This organic material will bind the metals in precipitates or dissolved forms (Stoveland *et al.*, 1979; Stoveland and Lester, 1980). Mechanisms for this removal were not specified, however, efficiency was found to decrease at low temperatures and biodegradation was most suitable for the simple cyanide species (Wild *et al.*, 1994). In the sewage treatment works, 64-87% of the cyanide present was complexed and treated wastewaters were richer in complexed compounds compared to the untreated. The authors (Wild *et al.*, 1994) suggest this is an indication that biodegradation occurs primarily on the simple compounds and that degradation to CO_2, H_2O and N_2 is generally believed to be the main cyanide removal process. After constant exposure to cyanide, microorganisms have a lower rate of reproduction but the bacterial cells are not killed (Wild *et al.*, 1994).

In gold mines, wastewater after the cyanidation process consists of free cyanide, together with the metallocyanide complexes of copper, nickel, iron and zinc plus reaction end products, which include cyanate, ammonia and sometimes thiocyanate (Scott and Ingles, 1987; Miller *et al.*, 1991).
In the tailings, levels of Cu, Fe and CN^- down a sediment profile suggests that many process are occurring within the residues:

1 In the top 3-4 m, where copper cyanide (CuCN) and copper oxyhydroxides may be precipitated and volatilisation of free cyanide can more easily occur, there is a rapid decrease in concentration of Cu and CN^-. The change in CN^- concentration in the retained liquor incurs a marked change on the

chemical equilibrium of the metal cyanide complexes and induces the precipitation of CuCN (Miller *et al.*, 1991).

2 Between 4-8 m, chemical reactions and transportation processes dominate. A steady increase in Fe and a reduction of Cu indicates that Fe is replacing Cu in solution and possibly other metals in the metallocyanide complexes. This is favoured because of the large formation constant for iron cyanide over Cu, Ni and Zn cyanide complexes (Broderius, 1973).

3 Below 8 m, there is an increase in sodium hydroxide extractable Fe, indicating that soluble iron cyanide complexes have changed to insoluble iron cyanide complexes either by adsorption or precipitation onto residue solids. These changes which are dictated by depth and ageing (Miller *et al.*, 1991), will have the effect of prolonging the retention of the complexed cyanide in the tailings by reducing the mobility of the metal cyanides.

1.4.2 *Chemical degradation of cyanide*

1.4.2.1 *Alkaline chlorination*

Simple cyanides

Chemical destruction of cyanide by oxidation is one of the techniques commonly used (Scott and Ingles, 1981; Wild *et al.*, 1994; Smith and Mudder, 1995). In the alkaline chlorination method, sodium hypochlorite (NaOCl), destroys the cyanide ion (CN^-) by oxidation to cyanate (CNO^-), (Slavnic, 1994).

$$H_2O + CN^- + OCl^- \leftrightharpoons CNCl + 2OH^- \qquad (1)$$

$$CNCl + 2OH^- \rightleftharpoons CNO^- + Cl^- + H_2O \qquad (2)$$

$$2CNO^- + 3OCl^- + 2H^+ \rightarrow 2CO_2 + N_2 + H_2O + 3Cl^- \qquad (3)$$

During this method cyanogen chloride (CNCl), an extremely toxic gas may be produced if the hypochlorite levels drop (reaction 1). In the presence of the hydroxide anion, the end product cyanate (reaction 2) which is far less toxic than cyanide, is formed. Cyanate will further break down in excess hypochlorite to produce nitrogen and carbon dioxide (reaction 3). Alkaline chlorination is a popularly used method in the mining industry, although cyanogen chloride gas and trihalomethanes which are carcinogenic compounds, can be produced if insufficient hypochlorite is added (Slavnic, 1994).

In tailings, many heavy metals are present which are known to interfere with cyanide destruction by chlorination (Slavnic, 1994), with the presence of copper and nickel decreasing the overall efficiency of the alkaline chlorination. The advantage of this method is that the process equipment and controls are reliable, the heavy metals precipitate as hydroxides and thiocyanate, if present, is oxidized and removed (Smith and Mudder, 1991).

Metal complexed cyanides

The leading treatment for the destruction of all forms of free cyanide and the WAD-CN complexes of Cu, Ni, Zn and Cd is alkaline chlorination. Many cyanide compounds are removed during the process but not the very stable iron and cobalt cyanides (Scott and Ingles, 1981). Residual chlorine or chloramines which form by reacting with ammonia, are also very toxic to aquatic life and must be removed

before waters are discharged. Government guidelines require a level of 0.02 mg l^{-1} chlorine before release of waters into streams is approved (Smith and Mudder, 1995).

1.4.2.2 *INCO process*

Simple cyanides

A destruction method called the INCO process (reaction 4) is the best available technology at present. This method uses sulfur to break down cyanide at pH 9-10 and soluble copper sulfate ($CuSO_4$) as a catalyst (Scott, 1984; Hoecker and Muir, 1987; Ritcey, 1989).

$$CN^- + SO_2 + O_2 + H_2O \leftrightharpoons CNO^- + H_2SO_4 \qquad (4)$$

This process is suitable for all levels of cyanide from 1 mg l^{-1} to 1000's mg l^{-1} where metals are precipitated as hydroxides.

Metal complexed cyanides

The INCO method has been licensed in 45 sites world-wide. In this method, metals such as Cu, Zn and Ni which were dissociated during the oxidation reaction, precipitate the iron cyanide. This precipitate is an insoluble salt, $Me_2Fe(CN)_6$ where Me is one of the metals mentioned above and it is stable in a wide range of pH. Other metals excess in solution, including the copper catalyst, are precipitated as hydroxides. For every gram of WAD-CN, 2.46 g of SO_2 is required. Costs can vary from $0.1 to $1.75 Canadian, per tonne of ore with the higher prices required for slurry treatment using sodium metabisulfite. Sources of SO_2 with increasing

costs are; burning elemental sulfur, liquid SO_2, ammonium bisulfite, sodium sulfite or sodium metabisulfite (Robbins and Devuyst, 1995).

1.4.2.3 *Hydrogen peroxide process (Degussa's peroxide process)*

Simple cyanides

This latest method which is fast and does not form toxic intermediaries (reaction 5), is becoming popular in the gold mining sector because no further pollutant is added by its use, with the excess hydrogen peroxide degrading to water and oxygen (Norcross and Steiner, 1995). The strong oxidizing agent hydrogen peroxide (H_2O_2) is used to treat low cyanide levels in mine wastes (Scott and Ingles, 1981; Castrantas *et al.*, 1988). Cyanate, which hydrolyses to carbonate and ammonia (reaction 6), is formed during the treatment carried out at pH 10-11 and in the presence of copper sulfate, nickel or aluminium salts (Norcross and Steiner, 1995). This process which is second only to alkaline chlorination in popularity, was used at the Ok Tedi Mine in Papua New Guinea where the tailings were discharged down the Ok Tedi and Fly Rivers (Scott, 1984; Knorre and Griffith, 1984).

$$H_2O_2 + CN^- \rightleftharpoons CNO^- + H_2O \qquad (5)$$

$$CNO^- + 2H_2O \rightleftharpoons NH_4^+ + CO_3^{2-} \qquad (6)$$

Metal complexed cyanides

Moderately strong complexes forming WAD cyanides and strong complexes like the ferro/ferricyanides are degraded by the hydrogen peroxide. While heavy metals

like Cu, Zn and Cd are simultaneously precipitated as hydroxides with the formation of cyanate (reaction 7).

$$Cu(CN)_3^{2-} + 7H_2O_2 + 2OH^- \leftrightharpoons 6CNO^- + 2Cu(OH)_2\downarrow + 6H_2O \quad (7)$$

Iron cyanide complexes can also be removed with an additional treatment step (reaction 8). Direct addition of the H_2O_2 into tailings ponded water, is regarded as safe but suitable mainly for emergencies since sunlight can easily denature H_2O_2, its effect can only be short term when used in this way but cyanide levels of 0.2 mg l^{-1} can be reached.

$$2Cu_2+ + Fe(CN)_6^{4-} \leftrightharpoons Cu_2Fe(CN)_6\downarrow \quad (8)$$

Some non metal inorganic compounds are found to accelerate the detoxification process. The best is peroxymonosulfuric acid (Caro's acid, H_2SO_5), which is found to be the most cost effective and formed by the hydrogen peroxide reaction with sulfuric acid (reaction 9). As a stronger oxidizing agent than hydrogen peroxide, Caro's acid reacts quickly with free cyanide and WAD cyanides (reaction 10) thereby reducing the toxicity of the wastes prior to discharge into the tailings pond (Norcross and Steiner, 1995).

$$H_2SO_4 + H_2O_2 \leftrightharpoons H_2SO_5 + H_2O \quad (9)$$

$$H_2SO_5 + CN^- \leftrightharpoons CNO^- + H_2SO_4 \quad (10)$$

1.5 *Biological degradation of cyanide and metal cyanides*

Apart from the chemical breakdown of cyanide and metallocyanide complexes, biological degradation has been documented in the literature for over 40 years (Pettet and Mills, 1954). Microorganisms from various sources have played a part in this process e.g. from sewage sludge, minewaters, contaminated soil sites and from type cultures. It appears that the bacteria or fungi involved in these degradations, have no strict habitat preferences. The ubiquitous nature of these organisms has encouraged researchers to suggest that bioremediation may be an effective way of eliminating pollutants from the environment.

1.5.1 *Types of microorganisms*

Microflora from sludge

The waste material, sewage, has high levels of nutrients which are readily available to microorganisms, making this waste product a good source of many species of bacteria. Cyanide at 100 mg l^{-1} was utilized and 75% of copper and nickel complexes at 100 mg l^{-1} CN^- concentrations were degraded over the period of a few months when treated with sewage in a percolating filter (Pettet and Mills, 1954). This degradation at a rate of approximately 0.5 mg l^{-1} day^{-1}, was also found to take place under natural conditions without the assistance of sewage and formation of ammonia and nitrate was detected as end products.

Complex metal cyanides of cadmium $[Cd(CN)_4]^{2-}$ and zinc $[Zn(CN)_4]^{2-}$ were also destroyed by the sewage bacteria. The authors concluded that dilute wastewaters containing metal cyanides could be treated by biological oxidation.

Other than the organic matter in the sewage sludge, no additional organic

supplement was introduced by Pettet and Mills (1954), whereas in a study by Shivaraman and Parhad (1984) peptone, glucose or acetate was included. Organic matter increases the level of heterotrophic bacteria since these genera require the presence of organic carbon to enable the metabolism of proteins when organic carbon is absent autotrophic bacteria dominate. Most of the organic supplements assisted the bacteria to effectively utilize cyanide with the formation ammonia (Shivaraman and Parhad, 1984).

Rapid degradation of >100 mg l^{-1} cyanide in the presence of glucose, in 8 hr was also reported for a mixed bacterial culture isolated from activated sludge of coal tar wastewaters. Mixed cultures were found to be more efficient than single species cultures (Kang and Kim, 1993).

An activated sludge plus dextrose treatment was efficient in the aerobic removal of cyanide, cyanate and thiocyanate with end products of carbon dioxide, nitrite, nitrate and sulfate (Ludzack and Schaffer, 1962). Cyanate was harder to degrade than thiocyanate but eventually was converted to ammonia, nitrite, nitrate and carbon dioxide. In the removal of cyanide and metal cyanides, a consortium of organisms was more successful (Kang and Kim, 1993) with the accumulation of ammonia as an intermediary followed by nitrification to nitrite, nitrate and carbon dioxide (Pettet and Mills, 1954). Most researchers reported that adding an organic source accelerated the degradation process (Goncalves *et al.*, 1998).

Anaerobic investigations for the degradation of cyanide are scarce. But Fallon (1992) concluded that the products of the cyanide transformation under anaerobic

conditions were formate and ammonia, with formamide as an intermediary. The anaerobic consortium rapidly hydrolysed the formamide to formate which was further converted to bicarbonate.

Facultative anaerobic bacteria were investigated for biodegradation of cyanide by Noel (1991). This group utilized 300 mg l^{-1} sodium cyanide at pH 10.5 thereby reducing the cyanide to almost zero in 50 days. The nutrients, ferrous sulfate ($FeSO_4$), magnesium sulfate ($MgSO_4$) and potassium phosphate (K_2HPO_4) were required in the medium for bacterial growth.

Degradation by Fungi

It was discovered that although some plants were cyanogenic, certain fungi could still cause disease. The pathogen *Stemphylium loti* invades tissue of *Lotus corniculatus* by means of converting cyanide to formamide (reaction 1) through activation of the enzyme formamide hydrolyase or cyanide hydratase, EC 4.2.1.66, (Fry and Millar, 1972). Other phytopathogenic fungi able to induce the formation of this enzyme are *Gloeocercospora serghi*, *Fusarium moniliforme* and *Mycoleptodiscus terrestris* (Nazly *et al.*, 1983).

$$HCN + H_2O \leftrightharpoons HCONH_2 \qquad (1)$$

Two other *Fusarium* species, *F. solani* and *F. oxysporum*, can convert cyanide to formamide by formamide hydrolyase. When additional cyanide and *F. solani* were present, a further conversion to formate occurred but no transformation to formate if *F. oxysporum* was used (Pereira *et al.*, 1996).

Biotransformation of potassium tetracyanonickelate ($K_2Ni(CN)_4$) and potassium ferrocyanide ($K_4Fe(CN)_6$) in neutral and acidic pH conditions, to ammonia was achieved by formamide hydrolyase of *Fusarium solani* (Barclay *et al.*, 1998). Interestingly thermodynamic diagrams indicate that the lower pH limit for $K_4Fe(CN)_6$ stability, is pH 6 (Osseo-Asare *et al.*, 1984). However, Barclay *et al.* (1998) showed degradation of $K_4Fe(CN)_6$ could proceed at pH 4, by employing a mixed culture containing *Fusarium solani*, *Fusarium oxysporum*, *Scytalidium thermophilum* and *Penicillium miczynski*. Alternately, $K_2Ni(CN)_4$ was removed at pH 7 by a mixed culture of *Fusarium solani* and *Trichoderma polysporum*. The authors followed the degradation pathways using C^{14} labelling with 50% being traced to carbon dioxide (Barclay *et al.*, 1998).

In the degradation of either cyanide or metal cyanide complexes, the main enzyme involved is formamide hydrolyase which can be induced in many fungal genera although *Fusarium* species are often the chosen microorganisms. The enzyme cyanide hydratase or formamide hydrolyase which is present in fungi, hydrates cyanide to formamide (Fry and Millar, 1972).

Degradation by bacteria

Overwhelmingly, the majority of microorganisms being studied in relation to aerobic biodegradation of cyanide and metal cyanide complexes are the bacteria, and in particular the genus *Pseudomonas*. At times, the pseudomonad isolate originated in wastes contaminated with cyanide but equally often type cultures have been used in the studies.

A type culture that frequently has appeared in reports in the last 19 years, is *Pseudomonas fluorescens* NCIMB 11764 (Harris and Knowles, 1983a; Kunz *et al.*, 1992; Suh *et al.*, 1994). This bacterium was investigated for pathways defining cyanide assimilation, evaluation of its survival ability and performance while the cells were immobilized in zeolite (Suh *et al.*, 1994), and for the consistent formation of ammonia as an end product (Harris and Knowles, 1983a). Others have used *Pseudomonas putida* cells encapsulated in calcium alginate with the formation of CO_2 and NH_3 (Babu *et al.*, 1994).

Work by Silva-Avalos *et al.* (1990) has shown that bacteria readily utilize the nitrogen in K_2NiCN_4. In particular, *Pseudomonas putida* strain BCN 3 and a *Klebsiella* sp. isolated from enriched cultures converted the K_2NiCN_4 complex to $NiCN_2$.

Some *Bacillus* species have indicated changes of cyanide to formate and NH_3 (Meyers *et al.*, 1991; Meyers *et al.*, 1993) when an organic compound was added (Skowronski and Strobel, 1969). If a sulfur compound is available, an enzyme called rhodanese can be activated in some *Bacillus* species with the formation of thiocyanate (SCN^-) and sulfide (S^-) (Atkinson, 1975).

1.5.2 *Bacterial enzymes*

Bacterial cells contain enzymes which can be induced or "switched-on" when favourable conditions arise. Enzymes can be present all the time (constitutive) or are activated (induced) when an appropriate substrate is available (Abercrombie *et al.*, 1990). Intracellular and extracellular enzymes are proteins which act as catalysts for specific biochemical reactions and their activity is dependent on pH, temperature and substrate availability.

Cyanide resistant enzymes (Knowles, 1976) allow some microorganism to continue living in cyanide toxic environments even though cyanide inhibits respiration due to it binding to cytochrome c oxidase (Solomonson, 1981).

Alternative respiration pathways are activated when cyanide is present, and although the bacteria may not flourish under these conditions, the cells survive. Babu *et al.* (1996) showed that enzymes from *Pseudomonas putida* tested on cyanides, including iron cyanides, thiocyanates, cyanates, formamide, acetonitrile and acetamide gave the highest activity measurement for acetonitrile and acetamide at pH 7.5. Whilst at pH 9.5, formamide was found to be the best substrate. Low enzyme activity, measured in μmol NH_3, was noted for potassium ferricyanide at either pH but for potassium ferrocyanide a moderate level was observed at pH 9.5. Both the sodium and potassium cyanates were converted to ammonia at either pH but for the thiocyanates there was only low levels of conversion.

The enzymes present in *P. putida* are able to convert both the cyanide (CN^-) and the amide (NH_2^-) groups to ammonia (Babu *et al.*, 1996).

A short study with minewaters also showed that the same enzymes converted cyanide to ammonia, however, the enzymes were not named (Babu *et al.*, 1996).

The cyanase enzyme which was able to break down potassium cyanate (KCNO), was demonstrated in *Escherichia coli* (Taussig, 1960). A gene responsible for the enzyme cyanase (cyanate hydrolase, EC 3.5.5.3) has been isolated. This gene cynS, was mapped and cloned into *Escherichia coli* K-12 where cyanase production increased by 5 times (Sung *et al.*, 1987). The cyanase activity was restricted to conversion of cyanate to carbon dioxide and ammonia. Another enzyme that transforms cyanide to formate and ammonia was found in *Bacillus pumilus* CI

(Meyers *et al.*, 1993) and named cyanide dihydratase, however, no hydrolysis of cyanate and thiocyanate to ammonia occurred. Many physio-chemical similarities with cyanidase were reported.

Enzymatic reaction types for cyanide degradation:

1) In substitution/addition reactions by rhodanese or cyanide sulfurtransferase, EC 2.8.1.1, (Westley, 1988), cyanide is converted to thiocyanate (reaction 2).

$$CN^- + S_2O_3^{2-} \leftrightharpoons SCN^- + SO_3^{2-} \qquad (2)$$

2) Hydrolytic pathways of hydration and hydrolysis are catalysed by cyanide hydratase, EC 4.2.1.66, (fungal enzyme) and cyanidase (cyanide hydrolase), with the enzyme cyanidase hydrolysing cyanide (reaction 3) to formic acid and ammonia (White *et al.*, 1988).

$$HCN + 2H_2O \leftrightharpoons HCOOH + NH_3 \qquad (3)$$

3) Oxidation by cyanase, EC 3.5.5.3 (reaction 4), with conversion of cyanate to carbon dioxide and ammonia (Taussig, 1960).

$$CN^- + \tfrac{1}{2}O_2 \leftrightharpoons CNO^- \leftrightharpoons CO_2 + NH_3 \qquad (4)$$

4) Reductive pathways by nitrogenase under anaerobic conditions (Hardy and Knight, 1967) with formation of methane and ammonia (reaction 5).

$$HCN + H_2O \leftrightharpoons CH_2NH + H_2O \leftrightharpoons CH_3NH_2 + H_2O \leftrightharpoons CH_4 + NH_3 \quad (5)$$

Seven enzymes in bacterial and fungal (Table 1.2) cells are believed to be responsible for cyanide degradation (Dubey and Holmes, 1995).

Table 1.2. Known cyanide degrading enzymes

Name of Enzyme	Host microorganism	Substrate	End products
cyanase [1]	*Escherichia coli*	cyanate	carbon dioxide (CO_2), ammonia (NH_3)
cyanide monoxygenase [2]	*Pseudomonas* sp.	hydrogen cyanide (HCN)	cyanate (CNO)
cyanide dioxygenase [3]	*Pseudomonas fluorescens*	HCN	CO_2, NH_3
cyanidase [4]	*Alcaligenes xylosoxidans* subsp. *denitrificans*	HCN	formate $(COOH)^-$, NH_3
rhodanese [5]	*Bacillus subtilis*	HCN, $S_2O_3^=$	Thiocyanate (SCN), sulfite (SO_3)
cyanoalanine synthase [6]	*Bacillus megaterium*	HCN	β-cyanoalanine
cyanide hydratase [7]	snow moulds and common pathogenic fungi	HCN	formamide ($HCONH_2$)

[1]Taussig,1960; [2] Harris and Knowles, 1983b; [3] Harris and Knowles, 1983a; [4] Ingvorsen *et al.*, 1991; [5] Atkinson, 1975; [6] Dunnill and Fowden, 1965; [7] Fry and Myers, 1981.

The most promising enzyme has been cyanidase and Novo Industries reported its presence in and extraction from *Alcaligenes xylosoxidans* subsp. *denitrificans*. This work has progressed to the patenting of the enzyme as a product called CYANIDASE® (Ingvorsen *et al.*, 1991). Both the Danish (Ingvorsen *et al.*, 1991) and Swiss (Basheer *et al.*, 1992) groups have done extensive studies trying to establish optimal operational enzymic parameters. A concentrated suspension of resting (not actively growing) and acclimatized to cyanide *Alcaligenes* cells was found to reduce 25,220 mg l^{-1} cyanide to 0.002 mg l^{-1} in 55 h. Storing the cell extracts for two weeks at 4°C did not cause a loss of enzyme activity but cyanide concentrations greater than 100 mM (5200 mg l^{-1}) were inhibitory to the cell free enzyme although intact cells could withstand more than 25,000 mg l^{-1}.

Dubey and Holmes (1995) state in their paper that "among the enzymes involved in cyanide degradation, only cyanidase has been used to detoxify wastes containing metal cyanocomplexes under experimental field conditions". Cyanidase was tested on waste from the food industry but not the mining industry by Basheer *et al.* (1992). These wastes contained cyanide which originated from cyanogenic glycosides found in various crop materials but not toxic metals cyanocomplexes such as those of copper, arsenic and mercury.

1.5.3 *Biological degradation processes*

Simple cyanides

Degradation of cyanide by microorganisms is a feasible alternative to conventional chemical processes (Scott and Ingles, 1981; Pandey *et al.*, 1987; Haden *et al.*, 1993). Aerobic microorganisms present in sludge can oxidize cyanide, cyanate and

thiocyanate to carbon dioxide, ammonia and sulfate. However, cyanide complexes and high levels of cyanide (>10 mg l^{-1}) cannot be degraded by conventional biological systems according to Slavnic (1994). In addition, the production of nitrate, ammonia and heavy metals as end products are deleterious to biological systems (Slavnic, 1994) and can slow the whole system down (Rouse and Gochnour, 1992). If more oxygen is introduced into the system higher concentrations of cyanide complexes are found to degrade. End products from the degradation of cyanide and associated metal compounds by biological systems, are less toxic than from chemical processes (Hoecker and Muir, 1987; Ritcey, 1989; Mehling and Broughton, 1989).

Metal complexed cyanides

After preliminary work including a pilot trial to test various systems, a biological method in 1981 was deemed the most suitable by the Homestake Mining Co. The process had two stages; first stage was the oxidative breakdown of cyanides, thiocyanate and adsorption or precipitation of free metals on the biofilm in the bioreactors using cultured preparations of various *Pseudomonas* spp. isolated from the Homestake Mine waste streams. The second stage involved the conversion of ammonia to nitrate through conventional nitrification. Sewage sludge was used to seed various biological pilot plants and as a source of the nitrifying bacteria (Mudder *et al.*, 1984: Waterland, 1995).

Activated sludge was used as a bacterial inoculum by Boucabeille *et al.* (1994) to degrade both WAD-CN and thiocyanate, 82 mg l^{-1} and 95 mg l^{-1} respectively, in 7 and 15 days. A study by Shpak *et al.* (1995) used *Pseudomonas fluorescens* in

degradation studies of Zn, Cu, Ag and Fe cyanocomplexes which were degraded in the order Zn>Cu>Ag>Fe. The stability constants increased in the same order as the ability of the pseudomonad to degrade the complexes decreased.

In work by Rollinson *et al.* (1987), a *Pseudomonas fluorescens* strain was tested in a $Ni(CN)_4^{2-}$ solution and it was found that at Ni^{2+} values of 0.1-0.3% w/v, the $Ni(CN)_4^{2-}$ was bound to the cell wall polymers of the bacteria. The nickel cyanide complex was used as a nitrogen source for cellular growth.

An *Acinetobacter* sp. was identified by Finnegan *et al.* (1991) and tested against 13 simple and complex cyanides. The range of tolerance was 0.25 mg l^{-1} for $K_2Au(CN)_2$ to 100 mg l^{-1} for $K_3Fe(CN)_6$. The chemicals tested included $K_2Co(CN)_4$, KCN, $Ca(CN)_2$ as well as nine organonitrile compounds. No isolation of the enzyme was carried out but the researchers believed it to be extracellular and constitutive.

1.5.4 *Biological processes in tailings dams*

Chemical reactions subject to biological activity occur both in the tailings pond and in the tailings solids. Free hydrogen cyanide present undergoes hydrolysis and biological oxidation to give ammonia and formate. Ammonia is then nitrified to form nitrate (reaction 6). The cyanide ion is extremely reactive and combines with many metals especially nickel, copper, zinc and iron to form complexes (reaction 7), which are subject to biological oxidation (Mudder, 1984). Only partial biological oxidation occurs with iron complexes other than Prussian blue for which

no biological degradation is as yet known. In the tailings solids found in the lower part of the tailings pond, anaerobic conditions are in progress. Some processes are chemical and others bacteriological with end products of ammonia, hydrogen sulfide (if sulfides, sulfur or sulfates are present), methane and carbon dioxide (Mudder, 1984).

$$\overset{\text{Hydrolysis}}{HCN \leftrightharpoons HCOO^- + NH_4^+} \overset{\text{bio. oxid}}{\leftrightharpoons NO_2^-} \overset{\text{bio. oxid.}}{\leftrightharpoons NO_3^-} \quad (6)$$

$$CN^- + Ni^{2+} \overset{\text{bio. oxid.}}{\leftrightharpoons} Ni(CN)_4^{2-} \leftrightharpoons Ni + HCN \quad (7)$$

(Also for Cu^+ and Zn^{2+})

1.5.5 *Bioremediation of contaminated sites*

Case study 1

The Homestake Mine in South Dakota has the only commercial scale cyanide biodegradation plant reported (Dubey and Holmes, 1995). This plant, set up in 1984, needs flow equalization, 48 low and high density rotating biological contactors (RBC), residual solids/metals removal, secondary clarification, supplemental chemical addition for bacterial growth and multi-media pressure sand filtration. The operating costs for the plant including personnel, power and chemicals was estimated to be $US 700 per day (Mudder *et al.*, 1984).

The level of contaminants at the mine is relatively low, since the combined influent wastewater (process plus mine water) has values for Total-CN at 0.5-10.0 mg l^{-1}, WAD-CN at 0.5-5.0 mg l^{-1}, SCN^- at 45.0-75.0 mg l^{-1} and a pH of 7.5-8.5 (Mudder *et al.*, 1984).

Initially plate cultures from process solutions were prepared and from these cultures, several bacteria were found that could degrade both metal complexed cyanides and thiocyanate. The pilot study identified that the bioremedial process involved two stages:

1) the oxidation of metal cyanides and thiocyanate by a *Pseudomonas* species, with the formation of ammonia and sulfate and adsorption/precipitation of the free metal into the biofilm and
2) the conversion of the ammonia to nitrate through nitrification.

Originally it was thought that the *Pseudomonas* species was responsible for the complete polishing of the wastewater including oxidation of the ammonia, however, elevated ammonia and nitrate concentrations were found in pilot plant effluents and this indicated incomplete nitrification. Nitrifying bacteria from the local sewage treatment plant were obtained, added to the system and in two weeks the ammonia and nitrite effluent concentrations decreased.

During the pilot plant test three other factors were noted, a) the RBC was the best nitrification system, b) insufficient alkalinity for complete nitrification was present therefore soda ash (sodium carbonate) was needed and c) phosphorus was required for bacterial growth.

Additional information obtained from the early tests indicated that the oxidation of metal cyanides and thiocyanate proceeded almost five times faster than the oxidation of ammonia and that residual cyanide and metals inhibited the nitrification process which became the rate limiting step in the overall process.

The bacterium which made the whole system possible was identified as *Pseudomonas paucimobilis*, this system is still an acceptable alternative when compared to other detoxification (chemical) techniques.

Case study 2

Another company, located in the UK called Biotreatment Ltd. of Cardiff attempted to decontaminate a site polluted with polycyclic aromatic hydrocarbons (PAH) and cyanide complexes using microorganisms. Reductions in the PAH are given in the paper but no values for cyanide wastes which they included in non biodegradable materials. These wastes were encapsulated in clay on the site. The overall cleanup price was reported to be $US 70,000 cheaper than by other methods (Bewley and Thiele, 1988).

Case study 3

In another example, a gold mine called USMX Green Springs Gold, carried out field tests using *Pseudomonas pseudoalcaligenes* originally isolated from their site and this was used on wastewater with 20 mg l^{-1} WAD-CN mainly copper and nickel cyanides. The cyanide level was reduced to 8.5 mg l^{-1} by the end of the 15 week field trail (Lien and Altringer, 1993; Dubey and Holmes, 1995).

1.5.6 *Overview*

Diligent management is essential because of the dangers associated with the contents of the tailings dams. One way of lessening the danger is by reducing the toxicity of the waste material prior to impoundment and this can be done through biodegradation which can utilize the cyanide (a carbon-nitrogen anion) compounds

present in the tailings. Carbon (C) and nitrogen (N) being two of the main requirements for bacterial growth and for the formation of new bacterial cells.

Degradation of cyanide compounds by bacteria has been studied for nearly 40 years and biodegradation is generally regarded as cheaper but more temperature dependent than chemical treatments (Fleming, 1998). A biological method has been used in treatment of low concentration cyanide wastes, at the Homestake Mine for over a decade (Hoecker and Muir, 1987). In another case, indigenous bacteria present in the site soil of a Canadian mine were used in conjunction with hydrogen peroxide (H_2O_2) to degrade cyanide contaminated water from 80 mg l^{-1} WAD-CN level to <0.01 mg l^{-1} within a month (Rouse and Gochnour, 1992). Copper, gold, silver and zinc were present as metallocyanide complexes but became insoluble as the WAD-CN levels decreased.

Biodegradation of cyanide and metallocyanides has been carried out in two different ways, in past studies. One mode of research has been to use indigenous soil microflora whereby the cyanide containing waste material is either sprayed onto the soil or where a percentage of soil (as an inoculum) was added to watery tailings. Other researchers have immobilised fungal mycelia of *Stemphylium loti*, *Gloeocercospora sorghi* and *Fusarium moniliforme* in polyelectroyte flocculating agents (Nazly *et al.*, 1983) or mycelium of the fungal species, *Fusarium lateritium* (Gould *et al.*, 1987; Smith and Mudder, 1991) have been used for cyanide detoxification.

The main bacterial genera referred to in the literature as cyanide degraders are *Pseudomonas*, *Bacillus* and *Alcaligenes*. However, few common features are found between the cyanide tolerant species.

Seven different enzymes appear to operate during the degradation process (Dubey and Holmes, 1995). These enzymes are induced or switched-on when favourable conditions arise such as an appropriate substrate becoming available (Abercrombie *et al.*, 1990) but are dependent on pH and temperature. Consequently when sodium cyanide solution is added to a soil or vice versa, bacteria are able to activate the appropriate enzymes and degradation begins. To date, researchers have identified cyanidase as the most promising degrading enzyme (Ingvorsen *et al.*, 1991). But because enzyme extraction is long, laborious and costly, at present, it is not commercially viable. Thus the best application for cyanidase would be cyanide degradation in food wastes (Basheer *et al.*, 1992) where the volumes of wastes are smaller than in the mining sector. Many foods such as cassava, almonds and seeds from apples, pears, cherries, apricots and peaches contain cyanide compounds and reducing the toxicity in these wastes is important.

Bioreactors, seeded with storage basin soil, were used by Boucabeille *et al.* (1994) to test three systems, batch, fed-batch and a continuous feed, for the degradation of metal complexes and thiocyanate from mine wastewaters. The main bacteria identified in the soil were *Pseudomonas* spp., although species from the families Vibrioniaceae and Enterobacteriaceae were also present in the 42 isolates. Of the group of isolates, ten were able to adapt to 39 mg l^{-1} of cyanide but no further work on individual strains was carried out.

Researchers, Mudder *et al.* (1984) have successfully used 48 contactors (bioreactors) at the Homestake Mine to polish minewaters containing 0.5-10.0 mg l^{-1} Total cyanides. The volumes of cyanide wastes resulting from gold mining are huge and the utilization of whole bacterial cells rather than cell-free enzymes, for the degradation treatment, is more suitable. Cell walls and membranes help to protect the intracellular enzymes from external toxic substances and degradation processes therefore can continue for longer periods. Bioreactors can be used to degrade many cyanide compounds, simple and complexed (Boucabeille *et al.*, 1994) and destroying these toxic compounds will reduce the dangers of the wastes impounded in the tailings dams.

1.6 *Chemistry of metallocyanide complexes*

Reactions between cyanide and metals occur readily but are dependent on concentration of metal, level of cyanide, pH, redox potential and radiation energy (light). The cyanide ion CN^- is a ligand which readily complexes with metals to form cyanocomplexes with different coordination numbers (Dickerson *et al.*, 1984).

1.6.1 *Cuprous cyanide,* (CuCN)

Cuprous cyanide is formed by the addition of potassium cyanide to a solution of Cu^{2+} ions resulting in the precipitation of cupric cyanide $CuCN_2$, which easily decomposes to CuCN and cyanogen (C_2N_2). The white crystals of CuCN redissolve in excess KCN to form other cyanide complexes (Ford-Smith, 1964; Sharpe, 1976). Alternatively an alkali cyanide added to cuprous chloride dissolved

in sodium chloride results in a white precipitate of cuprous cyanide (Williams, 1948). There are many other methods for formation of CuCN mentioned in the literature (Cotton and Wilkinson, 1988).

$$2Cu^{2+}(aq) + 4CN^{-}(aq) \rightleftharpoons 2CuCN(s) + C_2N_2 \qquad (1)$$

Copper cyanide (CuCN) is insoluble in water and is considered very stable (reaction 1). The Cu(I)/Cu(II) equilibrium can easily be changed depending on the anions present in the aqueous solution (reaction 2). In the presence of cyanide, the anions (CN^-) and iodide (I^-) react with Cu(II) to give Cu(I) compounds due to redox reactions. Anions like perchlorate (ClO_4^-) and sulfate (SO_4^{2-}) will favour Cu(II) compounds (Cotton and Wilkinson, 1988).

$$2Cu(I) \rightleftharpoons Cu + Cu(II) \qquad (2)$$

The solubility of cuprous cyanide increases when dilute ammonia or ammonium salts are present. However, it is only very slightly soluble in dilute acids but very soluble in alkali cyanide solutions with the formation of cuprocyanides. There are three classes of cuprocyanides, which can be represented by the three formulae: $KCu_2(CN)_3.H_2O$, $K_2Cu_2(CN)_4$ and $K_6Cu_2(CN)_8$ according to Williams (1948). Since these compounds require temperatures around 100°C, they should not be present in tailings. Sharpe (1976) reported that complexes $K[Cu_2(CN)_3]$, $K[Cu(CN)_2]$ and $K_3[Cu(CN)_4]$ are formed between copper(1) cyanide and alkali metal cyanides. When copper cyanide compounds in water are discussed, it is

important to consider the pH and Eh of the system because the compounds present will depend on these two parameters.

Copper cyanide (CuCN) is soluble in excess cyanide (reaction 3) to give the ion $Cu(CN)_2^-$, with the progressive formation of $Cu(CN)_3^{2-}$ (reaction 4) and $Cu(CN)_4^{3-}$ (reaction 5), in the presence of additional cyanide (Kappenstein and Hugel, 1974; Cotton and Wilkinson, 1988) and in low pH solutions (Finkelstein, 1972). It is found that at pH 8 to 14 and Eh of +0.5 to -0.5 volts the main copper ion in solution is $Cu(CN)_3^{2-}$ (Osseo-Asare *et al.*, 1984).

$$CuCN + CN^- \rightleftharpoons (Cu[CN]_2)^- \qquad (3)$$

$$Cu(CN)_2^- + CN^- \rightleftharpoons (Cu(CN)_3)^{2-} \qquad (4)$$

$$Cu(CN)_3^{2-} + CN^- \rightleftharpoons (Cu(CN)_4)^{3-} \qquad (5)$$

All of the complexes formed between alkali metals such as sodium and potassium, copper(I) and cyanide, dependent on the pH, Eh and cyanide ion concentration. When end products such as ammonia or thiocyanate (amongst others) are formed, additional reactions with the various complexes were detected (Woodward and Owen, 1959; Jones, 1963). The chemical reactions in the tailings are not fully understood nor have they been fully studied.

1.6.2 *Sodium tetracyanonickelate*, $Na_2Ni(CN)_4$

The addition in stoichiometric quantities, of sodium or potassium cyanide to a solution of a nickel(II) salt (reaction 6), will result in the yellow cyanide complex,

sodium tetracyanonickelate (Ford-Smith, 1964; Sharpe, 1976).

$$NaCN + NiSO_4 \rightleftharpoons Na_2Ni(CN)_4 + SO_4^{2-} \qquad (6)$$

This complex ($Ni(CN)_4^{2-}$), can be destroyed by strong mineral acids to produce $Ni(CN)_2$. While in a solution of excess cyanide there may be the formation of the deep red $Ni(CN)_5^{3-}$ anion.

The acid form of $Ni(CN)_4^{2-}$ has not been purified but there is some evidence that it is formed and is a strong dibasic acid (Brigando, 1957).

In some papers, hydrates containing 7,4,3 and 2 water molecules for $Ni(CN)_2$ are reported while other authors only report 2 and 1.5 water molecule hydrates (Sharpe, 1976)

The tetracyanonickelate anion ($Ni(CN)_4^{2-}$) is one of the most stable complexes of nickel but can be decomposed by acids with the formation of hydrated $NiCN_2$.

Decomposition of $Ni(CN)_4^{2-}$ will also occur in the presence of hypochlorite in alkaline solution with the formation of hydrated Ni_2O_3.

If sulfide is present in the aqueous solution, a cyanide elimination process occurs with the formation of the yellow salt $K_3NiS(CN)_3.H_2O$ (Sharpe, 1976). No exchange of nickel occurs between a Ni^{2+} (aq) salt and $Ni(CN)_4^{2-}$ at pH 4-8, but a rapid exchange is noticed with $Ni(NH_3)_6^{2+}$ in alkaline solution (Sharpe, 1976). The rate of formation is first order in Ni^{2+} and fourth order in CN^- (HCN is a reactant as well as CN^-) when in the pH range 5.5-7.5, the rate is proportional to the concentration of $(Ni^{2+})(CN^-)^2(HCN)^2$. In more alkaline solutions the order with respect to CN^- increases and for HCN decreases but the rate of formation for total

cyanide remains fourth order. In acid solutions the decomposition of $Ni(CN)_4^{2-}$ results in the protonated species $HNi(CN)_4^-$, $H_2Ni(CN)_4$ and $H_3Ni(CN)_4^+$ (Kolski and Margerum, 1968).

A cyano/thiocyano complex, $K_2Ni(CN)_2(CNS)_2$ is obtained from nickel thiocyanate and potassium cyanide or with the sodium cation if that is present, where the nitrogen atom of the thiocyanate acts as a ligand (Sharpe, 1976).

For both heavy metals nickel and copper, reactions with the cyanide ligand are numerous, producing heavy metal cyanides, alkali metal/heavy metal cyanides and heavy metal/heavy metal cyanides. In addition, complexes which include ammonia or sulfur are formed. It is also possible that compounds like cyanate and other by-products present in the tailings material interact with nickel cyanide, but as yet these have not been studied.

1.6.3 *Potassium hexacyanoferrate*(III), $K_3Fe(CN)_6$

Formation of potassium ferricyanide is easily obtained using oxidizing reagents. Anhydrous, ruby-red crystals of potassium ferricyanide are prepared by passing chlorine through a solution of potassium ferrocyanide (reaction 7) without any heat being applied (Williams, 1948). Another common method for preparation of the hexacyanoferrate(III) involves oxidation of a hexacyanoferrate(II) acid solution with permanganate or other oxidising agents (Ford-Smith, 1964).

$$2K_4Fe(CN)_6 + Cl_2 \rightleftharpoons KCl + 2K_3Fe(CN)_6 \qquad (7)$$

The reaction between iron(III) and cyanide ions in aqueous solution results in the formation of some iron(III) hydroxide but mainly iron(III) hexacyanide, $Fe(CN)_6^{3-}$

(Sharpe, 1976). If excess cyanide is present this will not drive the reaction to completion but instead will reduce both $Fe(OH)_3$ and $Fe(CN)_6^{3-}$ with the formation of $Fe(CN)_6^{4-}$ and cyanate (CNO^-). The ferricyanide ion is more reactive than the ferrocyanide ion even though it is more thermodynamically stable and it is found to be more toxic (Cotton and Wilkinson, 1988).

Hydration of ferricyanide takes place more readily than for ferrocyanide with the formation of cyanide which reacts with any unchanged ferricyanide to form ferrocyanide plus cyanogen or cyanate (Sharpe, 1976). At times, this newly released cyanide may be in the tailings waters thereby increasing the total cyanide present.

The photochemistry of hexacyanoferrate in water leads to many products: $Fe(OH)_3$, $(Fe(CN)_5H_2O)^{2-}$, $(Fe(CN)_5H_2O)^{3-}$, $Fe(CN)_6^{4-}$, HCN, C_2N_2, along with Prussian blue (Balzani and Carassiti, 1970) under special conditions, being formed. In aqueous solution, $H_3Fe(CN)_6$ is formed by ion exchange and titration with alkali shows it is a strong acid as far as the dissociation of all three hydrogen ions (Qureshi, 1969). The hexacyanoferrate(III) for kinetic reasons dissociates and reacts rapidly (Ford-Smith, 1964; Cotton and Wilkinson, 1988). Both the $Fe(CN)_6^{4-}$ and $Fe(CN)_6^{3-}$ are kinetically inert species but the electron transfer between them (reaction 8) is very fast (Williams, 1948).

$$Fe^{3+} + Fe(CN)_6^{4-} \leftrightharpoons Fe^{2+} + Fe(CN)_6^{3-} \qquad (8)$$

The formation of either sodium or potassium hexacyanoferrate(III) during gold extraction is common and this complex is stored with all the other metal cyanides

in tailings dams. The reactivity of this complex has been documented for more than 50 years and is described as relatively harmless (Meeussen *et al.*, 1994). Furthermore because ferric thiocyanate can easily be produced and is very soluble in water, it is also likely to be present and at a high level in the tailings.

1.7 *Chapter overview*

The freedom enjoyed by mining companies for over 100 years has resulted in a great accumulation of toxic tailings at gold mines. Until recently, Australia had few laws that forced the rehabilitation of tailings dams and mine sites therefore little remedial work was done. Natural degradation by volatilization of the toxic compounds in the tailings is the most popular method for dealing with wastes, however, this method is very slow. Now that legislation has been enacted which ensures that mining companies rehabilitate their sites, other methods of cyanide degradation should be implemented.

Already new methods for gold extraction and recovery are being used and now faster degradation methods for the impounded cyanide compounds are necessary both for more efficient use of the site and to lower costs incurred from site monitoring.

Three main methods, alkaline chlorination, the INCO process and the Degussa's process, are extensively practised for removal of cyanide complexes by chemical means. In each, strong chemicals are required to transform toxic compounds, simple cyanides and metal cyanide complexes, to more environmentally friendly products. In the alkaline chlorination method, the chemical used is sodium hypochlorite where a deadly gas, cyanogen chloride (CNCl), can be formed as an

intermediary. In the INCO process sulfur dioxide is used where sulfuric acid is formed and in the Degussa process, hydrogen peroxide is the strong oxidizing agent.

Bacterial degradation is far milder and no harsh conditions are needed. The cyanide anion (CN^-) provides growth materials carbon and nitrogen, and an organic supplement can be introduced for speedier degradation rates but it is not essential. Moderate temperatures and water complete the requirements.

Degradation products and some of the problems that are encountered when using the various treatments for cyanide degradation (Hoecker and Muir, 1987) are listed in Table 1.3.

Table 1.3. Comparison of methods for the degradation of cyanide

Method	Suitable for low CN^-	Suitable for high CN^-	Degradation products	Problems
Passive breakdown	Yes	No	Cyanate, thiocyanate, insoluble iron cyanide complexes	Temperature, climate dependent, very slow
Oxidation via alkaline Cl_2/NaClO	Yes	Yes	Cyanate, cyanogen chloride (CNCl), N_2, CO_2, NH_3, metal hydroxides, trihalomethanes, residual chlorine, chloramines	Toxic CNCl, organochlorides, iron cyanides not degraded
Oxidation via SO_2	Yes	No	Metal hydroxides, cyanate, calcium sulfate	Needs Cu^{2+}, $Cu_2Fe(CN)_6$ ppts
Oxidation via H_2O_2	No	Yes	Cyanate, carbonate, NH_3, metal hydroxides	Reagent cost, elevated temperature
Bacteriological	Yes	No	Cyanate, CO_2, NH_3, nitrate, thiocyanate, sulfite, metal hydroxides	Nutrients needed, poor adaptation to surges

The most comprehensive method available for cyanide destruction is oxidation of the cyanide by alkaline Cl_2 and NaClO. This process will degrade low and high levels of cyanide as well as thiocyanate. All of the methods have some advantages and some disadvantages.

The two mildest methods are passive breakdown and biodegradation. Both involve low cost maintenance but the bacteriological degradation proceeds faster making this the better method. In the physical degradation method there is a biological component, where the naturally occurring bacteria in the tailings dam will slowly break down the cyanide. However, because of the low bacterial cell numbers present, the process can take much longer to be completed.

It is already known that many biological processes operate in tailings dams. More are doubtlessly occurring through aerobic or anaerobic bacterial processes and intra or extracellular enzymatic reactions on the original waste material or the by-products.

Therefore, because of the adaptability and diversity of bacteria, biological detoxification is seen as the best suited system for this complex environment.

Chapter 2.0 General materials and methods

2.1 *Introduction*

Papers in some chromatography journals during the last two decades, have reported that a quick and accurate way of separating the metallo-cyanide complexes (Hilton and Haddad, 1986) was achieved by Reverse-Phase Ion-Interaction High-Performance Liquid Chromatography (RPII-HPLC). Techniques such as atomic absorption spectroscopy (AAS) and inductively coupled plasma atomic emission spectroscopy (ICPAES) are normally used to analyse the precious and other metals. However, these two methods cannot differentiate between oxidation states of the same element.

The chromatographic method where cyanide is used as the ligand to form stable cyano complexes, is quick, convenient and accurate (Hilton and Haddad, 1986). Typically a C_{18} silica (ODS) column was employed with an aqueous eluent containing an organic modifier (such as acetonitrile) and an ion interaction reagent (IIR), such as a salt of tetrabutyl ammonium hydroxide. Cyano complexes of copper(I), silver(I), iron(II), iron(III), gold(I) and others were resolved on a C_{18} column using acetonitrile and water (v/v) as the eluent, with UV detection at 214 nm (Hilton and Haddad, 1986). Other authors (Haddad and Kalambaheti, 1991) used this technique to detect chromium(III) and nickel(II) cyano complexes. At present, the recommended standard method in Australia, is distillation but this technique is capable of only broad speciation between free cyanide, WAD cyanide and total cyanide (Huang *et al.*, 1996). Ten or more metal cyanide complexes can

be detected by liquid chromatographic separation, at mg l^{-1} levels in under 35 min (Huang *et al.*, 1996).

In the tailings water after gold extraction, many metal cyanide complexes are present along with by-products thiocyanate and cyanate. This water was also successfully analysed by reverse-phase ion-interaction liquid chromatography (Haddad and Rochester, 1988). Adaptations of the ion chromatographic method by Fagan and Haddad (1991) successfully measured the SCN^- ion in leach liquors and the procedure has being extended to measure cyanate by Fagan and Haddad (1997).

The diversity, accuracy and speed that was reported for the Reverse-Phase Ion-Interaction High-Performance Liquid Chromatography technique (RPII-HPLC), made it suitable for adaptation to biological degradation experiments of metallo-cyanide complexes. After ascertaining the interferences between the organic matter in the bacterial cultures and the metal cyanides, the modified technique was considered reliable and vital for monitoring the residual metal cyanides. This was essential to the research programme, in order to evaluate the effectiveness of the biomass in degrading the metal cyanide compounds.

2.2 *Materials and methods*

Many preliminary tests were carried out to establish the best conditions for this adapted RPII-HPLC method with all of the standards used in the experiments being prepared in the CSIRO laboratory. Bacterial cells were removed and the filtrates were injected onto the C_{18} column. Overall, four concentrations of

acetonitrile (BDH HiPerSolv, HPLC grade), 18%, 22%, 25% and 30%, were tried as the column eluents. Analyte elution was monitored by using UV and visible wavelengths with the resultant chromatograms showing peaks due to changes in detector response. The ion interaction reagent added to the eluent was tetrabutylammonium sulfate (Aldrich) at a concentration of 0.005 M.

Experiments using shake flask cultures (Chapters 3, 5 and 7) and bioreactors (Chapters 4, 6 and 8) were carried out for three cyanide compounds (CuCN, Na_2NiCN_4 and $K_3Fe(CN)_6$), in order to follow degradation processes under two very different conditions. In the shake flask cultures, sterility of the controls was of prime importance. While in the treatments, the only non sterile component was the gold ore since this was the source of bacteria required as the inoculum. At each sampling time, subsamples of 8 ml were taken aseptically. In contrast, all of the components in the bioreactors were non sterile.

The gold ore used throughout all the experiments was from heap leach pads at the Warroo Mine owned by Cuprifex/Aurifex NL, located 40 km ESE of Inglewood, in SE Queensland. Equal weights from each of samples 1,2,4,5,7 & 9 were taken and thoroughly mixed before aliquots were added to shake flasks or bioreactors (Table 2.1). Samples 1,2,4 and 5 were taken by augering from the top level of the pad which is the last ore added for leaching. Sample 7 was taken from the middle level of the pad (level 2), and sample 9 was augered from the oldest level i.e. the first ore leached. Levels of copper, nickel and ferric cyanides were measured to provide a base line value of these metals before the inclusion of the ore into the experiments. Also it was necessary to know the levels of Total and WAD cyanide present in the ore samples.

Table 2.1. Cyanide and metals content (mg kg^{-1}) of the six ore samples constituting the inoculum

Sample Nos.	Total CN	WAD-CN	Cu	Ni	Fe
1	<0.6	<0.2	0.11	<0.1	0.55
2	<0.6	<0.2	0.11	<0.1	0.59
4	32	26	21	<0.1	0.8
5	2	<0.2	0.2	<0.1	0.64
7	10	4	4.2	<0.1	0.9
9	4	<0.2	0.8	<0.1	1.19

2.3 *Experimental designs*

2.3.1 *Experimental design for shake flask cultures*

Shake flask cultures were set up using 250 ml Erlenmeyer flasks containing 100 ml of sterile modified Davis Mineral Salts (DMS) medium (Davis *et al.*, 1959) plus Peptone where appropriate. Details of the medium formulation used throughout the shake flask cultures and bioreactors experiments, are given in Table 2.2. The medium was set at the required pH prior to autoclaving.

At the start of all of the shake flask experiments, the flasks were placed into a Ratek Orbital Mixer cabinet. The mixing rotation was 60 revs min^{-1} and incubation proceeded at 27°C under a black cloth cover.

Table 2.2. Composition of mineral salts (DMS) medium used for growth and isolation of bacteria

Name of chemicals	Quantity (g l^{-1})	Brand name
Potassium dihydrogen orthophosphate	0.2	Univar
di-Sodium hydrogen orthophosphate dihydrate	0.3	Merck
Potassium chloride	1.0	BDH
Magnesium sulfate heptahydrate	0.2	Merck
Calcium chloride dihydrate	0.01	Merck
Bacto Peptone*	1.0	Difco
Bacto Agar**	15.0	Difco
Deionized (Distilled) water	1 l	

*Bacto Peptone was added in the (+P) treatments
** Bacto Agar was only used when a solid medium was required e.g. for isolation of individual bacterial colonies

2.3.2 *Experimental design for bioreactors*

Pretreatment of bioreactors

No pretreatment was given to bioreactor A which contained 1 litre of non sterile DMS medium and 10% leach pad ore. In bioreactor B, 1g l^{-1} peptone was added to the DMS medium plus ore (Photo 2.1.). Both vessels were slowly but continuously stirred with an overhead mechanical stirrer and were shielded from any light. Temperatures in the vessels were keep at 27°C throughout the experimental period. The bacterial populations in both were allowed to grow for 20 days before the first cyanide compound was introduced. Analyses were done on 10 ml subsamples.

Photo 2.1. Bioreactors A and B

Table 2.3. pH of medium in bioreactors during the pretreatment stage (20 days)

Bioreactor	0	1	4	6	14	20
A	8.15	8.00	7.80	7.75	7.80	7.80
B	8.75	8.45	7.45	7.25	8.10	8.35

A slight decrease, 0.35 pH units, occurred in reactor A during the pretreatment stage. In the second reactor (B), a marked decrease of 1.5 pH units was followed by an increase of 1.1units (Table 2.3). At this stage no cyanide complex was present. The fall was probably due to organic acids formed during bacterial growth. Similarly the increase was a consequence of bacteria utilizing the peptone and producing ammonia.

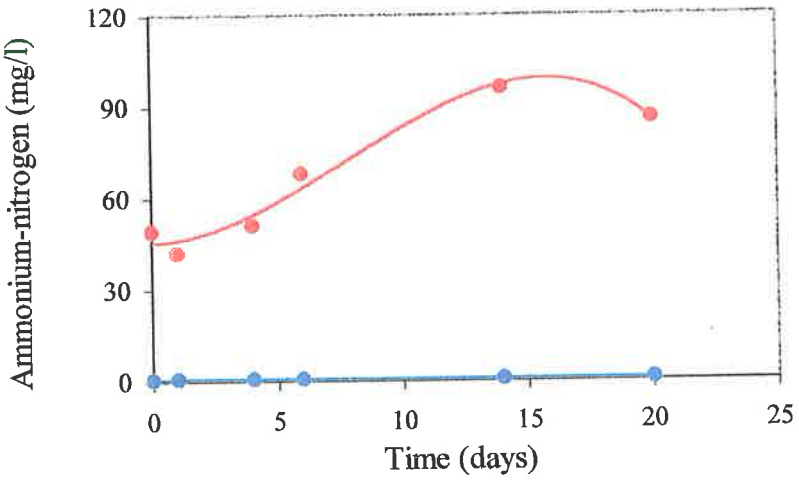

Figure 2.1. Ammonium-nitrogen (mg l^{-1}) produced from peptone added as primer to bioreactor B in 20 days, bioreactor A = ●, bioreactor B = ●.

The maximum level of ammonium-nitrogen production possible from the 1 g l^{-1} of Bacto Peptone used in all of the experiments, is 166.7 mg. This value of total

nitrogen, was measured by a mass spectrograph at CSIRO. However, after 20 days the highest calorimetrically measured value was only 96 mg l^{-1} (Figure 2.1). Having peptone present, enabled the heterotrophic microflora to become dominant in bioreactor B, while in bioreactor A, facultative autotrophic species may have dominated since no organic matter was present.

Subsequently, bioreactors A and B were set up to test the biodegradation of three metal cyanide complexes during two degradation cycles for each complex. One metal cyanide complex was introduced into the bioreactors at a time, starting with copper(I) cyanide followed by sodium tetracyanonickelate and lastly potassium hexacyanoferrate. After the pretreatment, the ore in the bioreactors was allowed to settle for 5 hr. followed by the decanting of the supernatant and the addition of a new batch of DMS medium. A replacement of medium was done before the introduction of each new metal cyanide compound and between the two degradation cycles of each complex.

2.4 *Analyses carried out*

1 pH readings

2 Free cyanide determinations using silver nitrate and rhodanine

3 Spectrophotometry

4 HPLC determinations

5 Ammonium-nitrogen determinations

6 Total cyanide

7 Weak acid dissociable (WAD) cyanide

8 Cyanate determinations

9 Microbiological determinations

a bacterial colony counts and isolations

b broth culturing

c morphological studies

d Fatty Acid Methyl Esters, MIDI FAME

2.4.1 *Instruments, chemicals and procedural details*

1) A Corning pH meter model 120, with a silver/silver chloride (Ag/AgCl) combination electrode was used. At each time of analysis 5 ml of a 0.45 µm filtered solution was checked for pH .

2) Colorimetric analysis for free cyanide was done using 0.0031 M silver nitrate solution (0.531 g l^{-1}) and p-dimethylaminobenzylidene rhodanine. The 5 ml aliquot was titrated with silver nitrate in the presence of 5 drops of rhodanine. A yellow solution was the starting colour. As the solution was titrated with silver nitrate the colour remained yellow while the silver formed a complex with the free cyanide and when no free cyanide remained the excess silver nitrate caused the silver sensitive rhodanine to turn to orange and then red.

Concentration of rhodanine indicator was 0.2 g l^{-1} of p-dimethylaminobenzylidene rhodanine in acetone.

Standard silver nitrate solution was made up by dissolving 4.25 g l^{-1} $AgNO_3$ in distilled water (= 0.025 M). For very low levels of cyanide, a solution of 0.0031 M was used, as suggested by the AMDEL Laboratory. This procedure was a modification from Greenberg *et al.* (1992).

Conversion is 1 ml of standard $AgNO_3$ = 0.0025 g NaCN

The percent of free NaCN concentration in a 5ml aliquot = 0.05 x titre (ml of a 0.025 M $AgNO_3$ solution).

3) Spectrometric scans were done on a Beckman DU 640 unit using a 1 cm quartz cuvette. Before taking the adsorption (O.D.) readings, each sample was filtered through a 0.45 μm syringe tip filter. Each of the three metal cyanides have a characteristic UV or Visible spectrum, by which the amount of complex remaining can be measured.

4) Metallo-cyanide complexes were analysed on a GBC (London, UK) HPLC (Photo 2.2.).

The HPLC unit included:

- GBC LC 1610 Auto Sampler
- GBC LC 1150 HPLC Pump
- GBC LC 1210 UV/VIS Detector
- GBC DI 510 Interface
- LABOC GASTORR GT-104 degassing unit
- The DESQview 386 Quarterdeck operating system plus a data manipulation package, DP900

Software controlling the HPLC and column details

The level of each of the three cyanide compounds remaining in solution were analysed using the HPLC and data system loaded with the DESQview 386 Operating System which runs the DP900 chromatography management software.

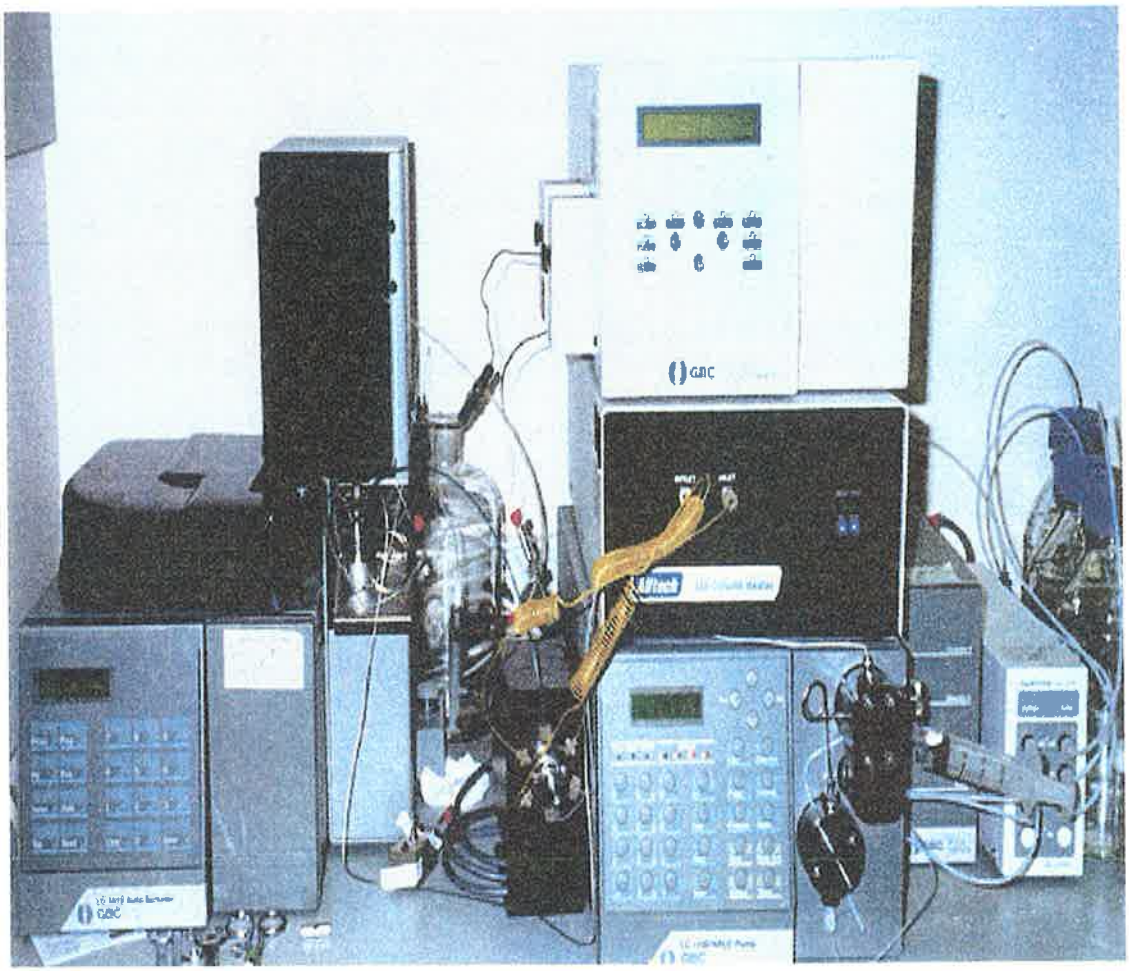

Photo 2.2. HPLC used for analyses

This DP900 system allows complete control of the HPLC by the computer and facilitates easy changes of solvents, run times and sample queues. Also file storage, reprocessing and manipulation of files was done through the DP900 system.
A NOVA-PAK C_{18}, 3.9 mm x 150 mm column was used for the detection of all the three cyanide compounds, each with different eluents and each having a specific UV and/or visible absorption band on the spectrophotometer.
Aliquots of 1.5 ml were taken from each treatment, fortnightly, these samples were filtered through a 0.45 µm tip filter and diluted before two or three 10 µl subsamples were injected into the HPLC. Every time samples were analysed three or four 10 µl injections from a standard solution were included as a 1-point calibration check. An automatic comparison to the appropriate calibration curve, resulted in a value for the residual amount.

5) Equipment used for the Berthelot method to determine ammonium-nitrogen (Photo 2.3.)

- LKB Biochrom Ultraspec 3 UV spectrophotometer
- Gilson Sample Changer model 222
- Diluter model 2101 with sampling tray

After filtration though a 0.45 µm syringe tip filter, 1 ml was diluted until a O.D. reading in the 0.1-2.5 mg l^{-1} range was obtained. The ammonium-nitrogen determinations were done in accordance with the method set out in a publication by Barry (1996), which was based on work by Stainton *et al.* (1977). Aliquots of 1 ml were dispensed into sample tubes with 4 ml of distilled (deionized) water giving a volume of 5 ml per tube. To these add 0.2 ml of the phenol reagent plus

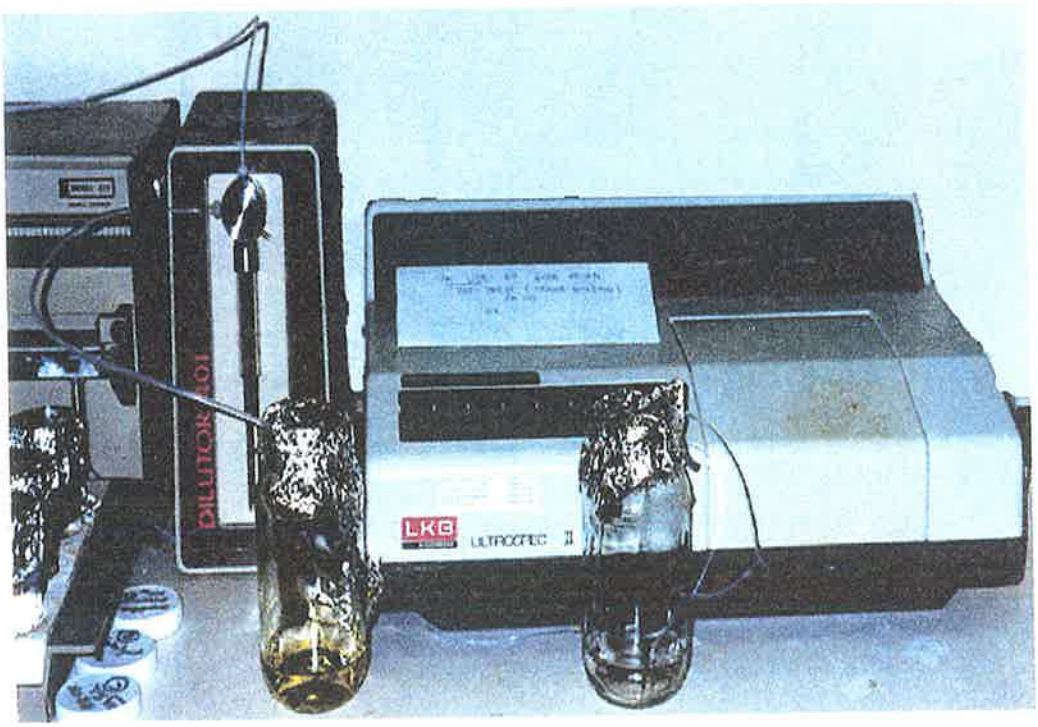

Photo 2.3. LKB equipment used for ammonium-nitrogen determinations

0.2 ml of the catalyst and vortex to mix well. This was followed by the addition of 0.5 ml of the oxidizing reagent and again vortex to mix the solution. After 1 hr, readings were taken at a wavelength of 640 nm.

Accuracy of method was within 3%.

Phenol reagent: 1 g Phenol (C_6H_5OH) in 10 ml 95 % Ethanol.

Catalyst: 0.1 g sodium nitroprusside ($Na_2[Fe(CN)_5NO].2H_2O$) in 20 ml of distilled (deionized) water.

Oxidizing reagent: add I part of the sodium hypochlorite solution to 4 parts of the alkaline solution. (sodium hypochlorite = 44 % dilution of the concentrate 125 g l^{-1} available chlorine); alkaline solution = 4 g trisodium citrate ($Na_3C_6H_5O_7.2H_2O$) plus 0.2 g sodium hydroxide dissolved in 20 ml distilled water).

6) Two methods were used to measure total cyanide during the experimental period.

a) The Alpkem Flow Solution 3000 auto analyser, manufactured by Perstorp Analytical Environmental was used for both the total cyanide and WAD cyanide. The total cyanide (with a detection error of 1.5 mg l^{-1}) is measured by passing the solution together with sulfuric and hypophosphoric acids into a UV digester at wavelength 351 nm, prior to gas diffusion (Steinberg and Jones, 1994). The effect of the UV light is to break the stronger complexes. All of the iron cyanide complexes are measured by this method. Cyanide is separated from the matrix by gas diffusion and then measured colorimetrically by reacting with chloramine T and pyridine-barbituric acid to form a red complex which is measured at 578 nm (Greenberg *et al.*, 1992). All chemicals used were of analytical grade from Aldrich.

Reagents are:

Chloramine-T solution: 1 g chloramine T in 100 ml deionized water

Pyridine-barbituric acid solution: 15 g barbituric acid, 75 ml pyridine, and 15 ml conc. hydrochloric acid made up to 250 ml with deionized water

Acetate buffer: 410 g sodium acetate trihydrate in 500 ml water, adjust to pH 4.5 with glacial acetic acid

Sodium hydroxide dilution solution: 1.6 g NaOH in 1 l distilled water

A stock cyanide solution and a standard cyanide solution are also required (Greenberg *et al.*, 1992).

b) A micro-distillation technique using a manifold designed by McLeod (1993). The operating temperature for the micro-still is 90°C and chemicals used were of analytical grade (McLeod, 1993).

Reagents are;

- Phosphoric acid solution 1: add 300 ml orthophosphoric acid with 60 ml hypophosphorous acid to 1 l distilled water.
- Phosphoric acid solution 2: mix 125 ml orthophosphoric with 25 ml hypophosphoric acids.
- Sodium hydroxide solution: NaOH at 0.02 M.
- DCIC solution 1: prepare a 0.025% dichloro-isocyanurate.
- DCIC solution 2: prepare a 0.1% dichloro-isocyanurate.
- Colour reagent: 3.4 g barbituric acid with 20 ml of 10% sodium hydroxide, at 60-70°C, in 200 ml water, when dissolved, 3.4 g nicotinic acid was added and made up to 250 ml with water.

- Buffer: 13.6 g potassium dihydrogen phosphate with 0.34 g dipotassium hydrogen phosphate in 1 l deionized water.
- Wash solution: NaOH at 0.01M.

This technique, incorporating a 254 nm wavelength UV source, released all of the bound cyanide and gave a measurement for total cyanide (error within 2%).

7) Two methods were also used for the WAD cyanide analyses during the experimental period.

a) WAD cyanide was measured on the Alpkem Flow Solution 3000 auto analyser (detection error of 1.5 mg l^{-1}). An undigested sample was tested by the addition of a solution of sulfuric and hypophosphoric acid prior to gas diffusion. Cyanide is measured by the same method as for Total–CN (Greenberg *et al.*, 1992).

b) The micro-distillation system with the UV source switched off, was used for measurements of WAD cyanides. Only the weakly bound cyanide in the complexes was therefore analysed. This method had an accuracy within 3% (McLeod, 1993).

8) Cyanate (CNO^-) analyses were carried out using the colorimetric method in the Standard Methods 18th Ed. where the cyanate in converted to ammonia by a weak hydrolysis in acid conditions after which the ammonia is measured. By subtracting the ammonia value taken before hydrolysis from the value obtained after hydrolysis and multiplied by three, the cyanate value is obtained (accuracy within 3%).

Reagents: 10 N sodium hydroxide and conc. sulfuric acid diluted by 50%, reaction time 30 min at 90-95°C (Greenberg *et al.*, 1992).

9) Description of microbiological procedures

Microbiological plates were prepared from the DMS plus peptone agar medium (Table 2.2). This formulation was sterilized by steam autoclaving at 100 kPa for 20 mins. At about 50°C a small volume (20 ml) of this medium was dispensed in Gamma ray sterilized plastic Petri Dishes.

a) When the bacterial cells are growing in a liquid medium, a small quantity of solution is spread over the agar surface and the plates are incubated at 27°C to facilitate colony growth. At certain times during the growing period the bacterial colonies are counted. In this way isolation of the different cell types in the original solution can be achieved and the numbers for each are obtained.

b) If bacterial colonies are very slow to grow, they can be inoculated into tubes containing sterile liquid DMS plus peptone medium (Table 2.2) for an allotted time. During incubation growth densities are checked by measuring the turbidity of the liquid cultures at wavelength 590 nm on a spectrophotometer.

c) The morphology of the bacterial colonies was determined from the growth on the DMS plus peptone agar surfaces. The size and shape of the bacterial cells provide useful diagnostic information as does the size, shape and colour of the bacterial colonies.

d) MIDI is a microbiological diagnostic computer software package linked to a Hewlett Packard Gas Chromatograph set up to analyse the fatty acid methyl ester (FAME) profiles of the bacterial strains (Photo 2.4.). After following an established protocol which results in the formation of FAME, samples are analysed

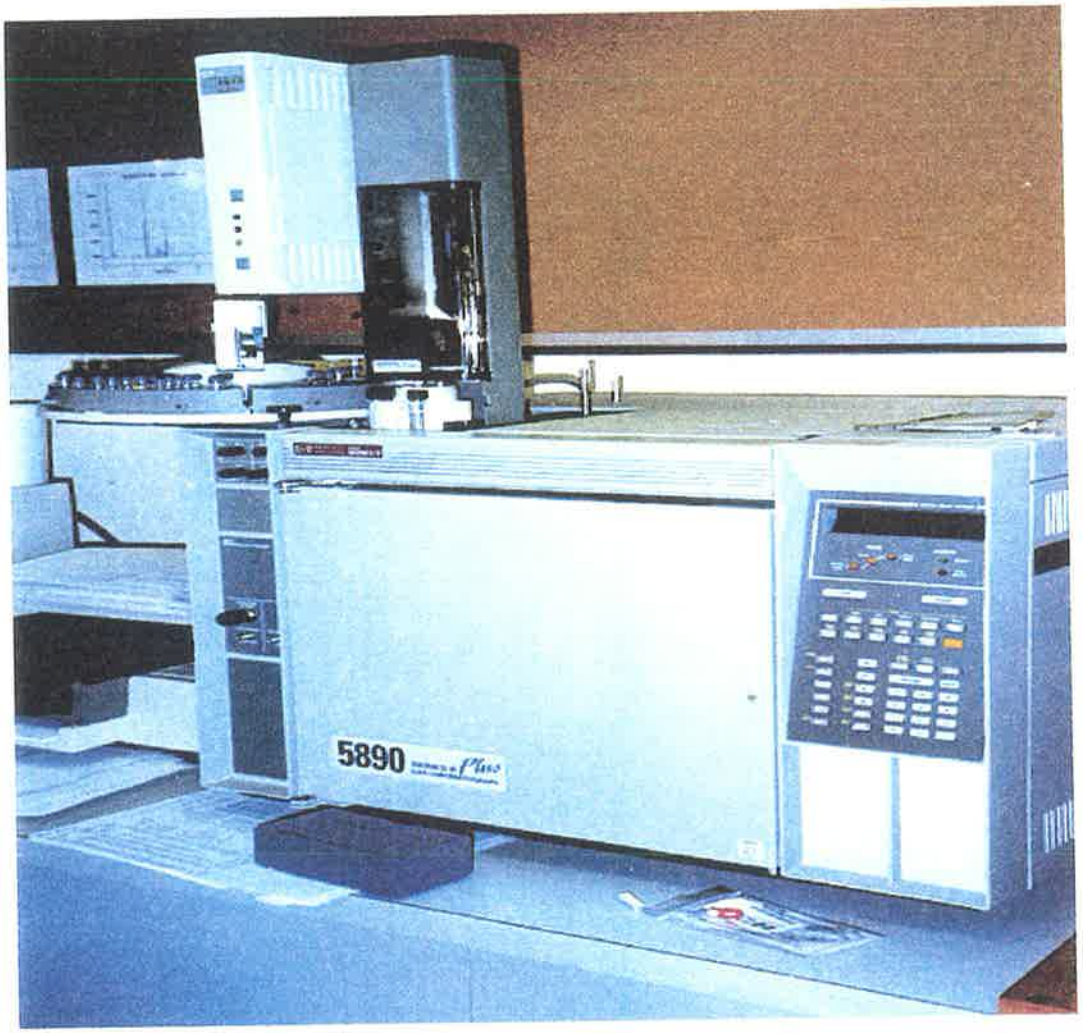

Photo 2.4. Hewlett Packard Gas Chromatograph 5890 used with the MIDI-FAME software

in the gas chromatograph. The fingerprint of the particular bacterial strain is automatically compared to the data base in the software package, a species name and similarity coefficient is given for the unknown bacteria (MIS version 4.0, MIDI: Microbial ID, Inc. Newwark, Delaware, USA 1994).

Preparation of cell extracts

1) Harvesting – removal of cells from culture media (after 24 hr incubation).
2) Saponification – Lysis of the cells to liberate the fatty acids from the cellular lipids.
3) Methylation – Formation of methyl esters of the fatty acids (FAME).
4) Extraction – Transfer of the fatty acids from the aqueous phase to an organic phase.
5) Base wash – Aqueous wash of the organic extract prior to chromatographic analysis.

2.5 *Sample preparation and HPLC settings to produce the calibration curves of the three metal cyanide complexes*

2.5.1 *Copper cyanide trials*

2.5.1.1 *Background*

In this research, the column used was a Waters Assoc. Nova-Pak C_{18} column. Information obtained from chemists analysing metallo-cyanide complexes (Hilton and Haddad, 1986; Huang *et al.*, 1996) indicated that RPII-HPLC separation was an appropriate technique. They showed that accurate detection of copper cyanide, sodium tetracyanonickelate and potassium hexacyanoferrate in an aqueous system,

was possible by this method. Since these compounds had not previously been analysed from bacteriological experiments, various combinations of conditions needed to be determined. Specifically, it was found that the peak formed by peptone injected into the HPLC interfered with the peaks of the metal cyanide compounds. Advice was obtained from Peter Fagan, an analytical chemist with ANSTO, also involved in RPII-HPLC detection of the metal cyanides. The Waters Nova-Pak C_{18} 150 mm x 3.9 mm i.d. column was suggested by P. Fagan (Personal communications).

Four acetonitrile (BDH, HiPerSolv HPLC grade) levels were tried as eluents, 18%, 22%, 25% and 30%. The 25% acetonitrile in water gave the best resolution of the copper(1) cyanide peak which was separate from the peak due to the organic nutrient peptone, at a wavelength of 234 nm. The water used in the preparation of the mobile phases was purified by a Millipore Q filtration unit. Since copper(1) cyanide has a UV spectrum, a scan was done on the Beckman DU 640 unit. This scan showed three peaks, at wavelengths of 207 nm, 221 nm and 234 nm (Figure 2.2). The 207 nm peak overlapped the peptone peak in retention time and was therefore not used. Peak 221 nm (on the shoulder of the peak at 207 nm) was the smallest of the three and would have been the hardest to detect accurately. Therefore, the peak at 234 nm was chosen as the analytical wavelength (Figure 2.3).

In both the shake flasks cultures and bioreactors experiments, the DMS medium (Table 2.2) was used, with and without peptone, and these media were run in the HPLC to assess whether any interference occurred at pH 8 and pH 10. It was

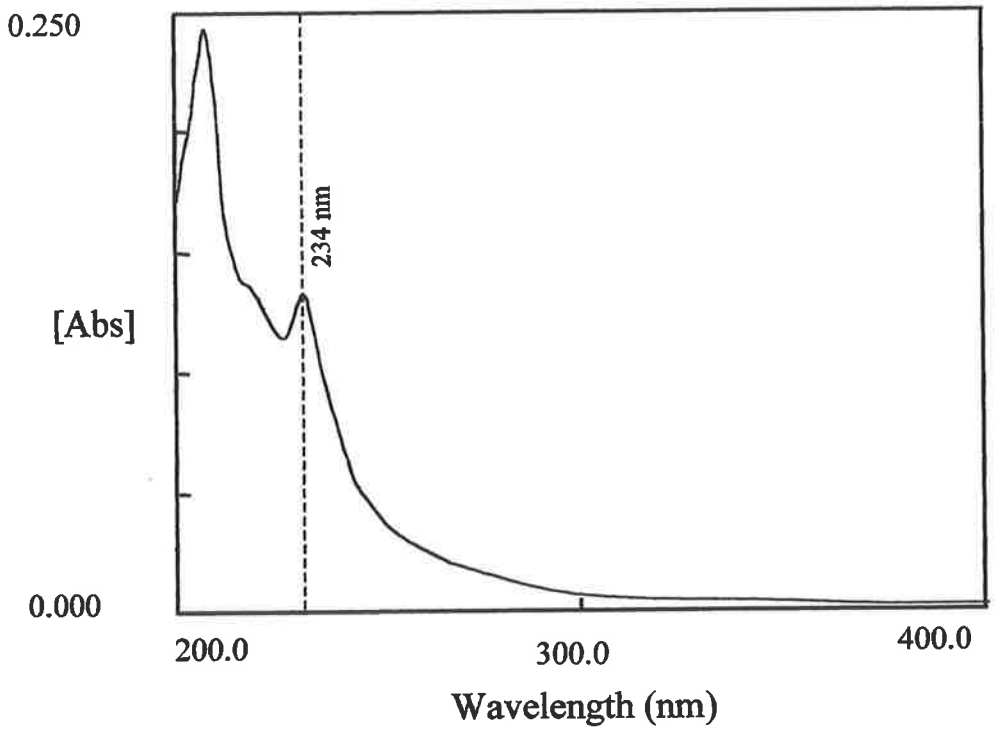

Figure 2.2. Copper(I) cyanide spectrophotometer scan

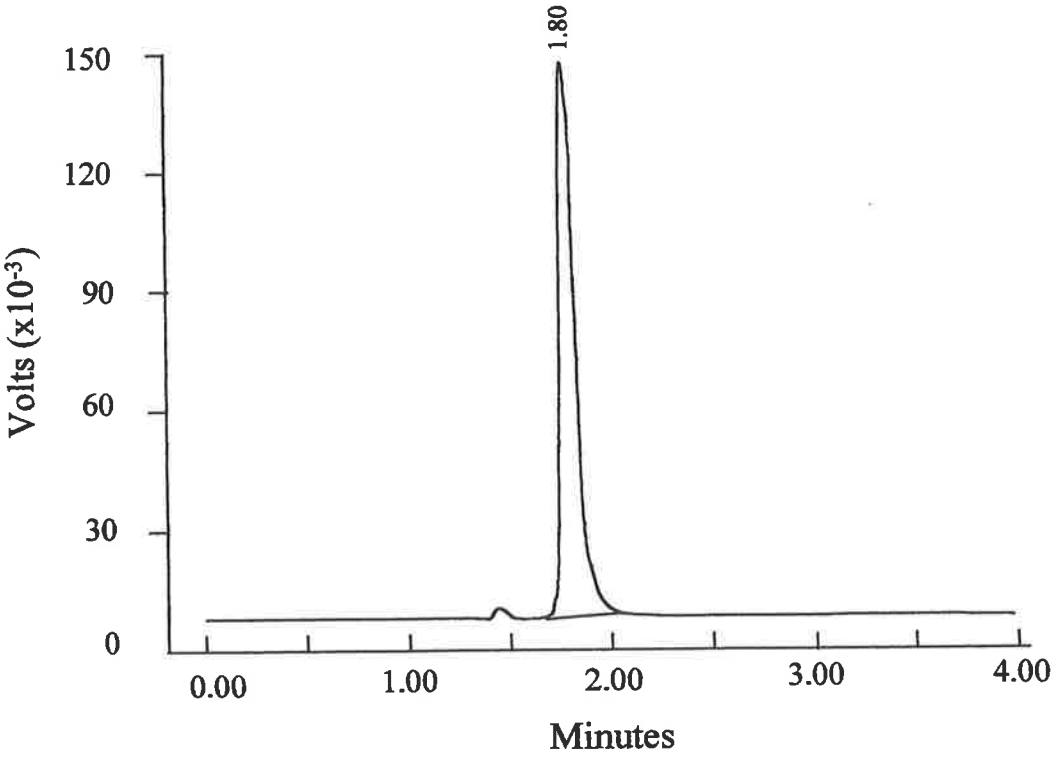

Figure 2.3. Copper(I) cyanide RPII-HPLC peak

necessary to test interactions of the media salts with and without copper(I) cyanide and also the sodium cyanide which was added to stabilize the copper(I) cyanide. Another component tested was the heap pad ore since this could contain some metals that may interfere with the column.

Each sample was filtered through 0.45 μm because foreign particles shorten the life of the column.

As the bacteria grew, an increasing amount of metabolites and biomass collected in the liquid media over the experimental periods. Some samples required two filtrations before injection into the HPLC. A new tip filter and syringe was used for each sample and the two washings with sodium cyanide were also analysed to determine whether any copper cyanide had remained on the filter.

2.5.1.2 *The copper cyanide calibration curve*

The copper(I) cyanide (CuCN) was eluted from the Nova-Pak C_{18} column with acetonitrile in water containing tetrabutylammonium sulfate (TBAS) as the ion-pair reagent in the Reverse Phase Ion-Interaction High Performance Liquid Chromatography (RPII-HPLC) technique. (Huang *et al.,* 1996). All the solutions were prepared using water filtered through a Sartorius AG W-3400 unit.

The wavelength of 234 nm was selected and the column eluent was a 0.45 μm filtered solution of 25% acetonitrile (CH_3CN)/deionized water containing 0.005 M (Aldrich) tetrabutylammonium sulfate, flowing through at a rate of 1 ml min^{-1}.

A calibration curve was constructed using a stock solution of 120 mg l^{-1} CuCN in a 100 mg l^{-1} NaCN solution, each dilution was half the previous concentration;

30 mg l^{-1}; 15 mg l^{-1}; 7.5 mg l^{-1} and 3.75 mg l^{-1}. Triplicate subsamples of 10 µl from each concentration were automatically injected into the HPLC.

The correlation coefficient for this graph was 1.00 (Figure 2.4) which is indicative of a linear relationship (Beer-Lambert Law) between the concentration of the copper(1) cyanide compound and the HPLC response. The resulting chromatogram peaks are measured as areas in mVolts.

Multiple injections of subsamples taken from the experiments were automatically compared to this calibration curve and the amount of CuCN in the samples was calculated in mg l^{-1}.

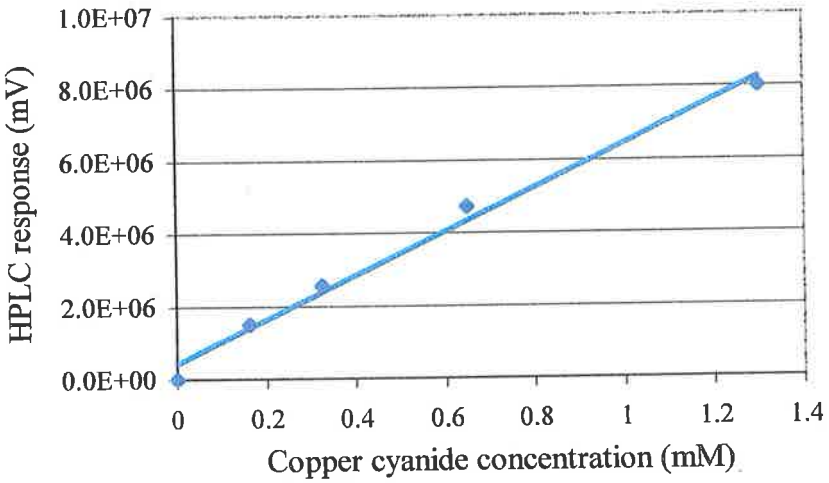

Figure 2.4. Calibration curve obtained with four copper(1) cyanide concentrations

2.5.2 *Sodium tetracyanonickelate trials*

2.5.2.1 *Background*

Reports indicated that the tetracyanonickelate anion could be detected on the Waters Nova-Pak C_{18} 150 mm x 3.9 mm i.d. column (Hilton and Haddad, 1986;

Haddad and Kalambaheti, 1991). The chosen wavelength by both groups was 214 nm but the mobile phase differed. Hilton and Haddad (1986) used acetonitrile in water, 23:77 v/v but Haddad and Kalambaheti (1991) chose methanol-tetrahydrofuran-10 mM phosphate buffer, at 25:1:74 v/v/v. Both groups were separating multiple metal cyano anions.

In the research presented, only the tetracyanonickelate(II) anion was to be traced. A UV scan showed two peaks for sodium tetracyanonickelate, the main peak at 267 nm and a lesser peak at 283 nm (Figure 2.5). Two acetonitrile concentrations in water were tried, 18% and 25% with 0.005 M tetrabutylammonium sulfate. The better separation and peak shape was obtained with 18%. The ingredients of the growth medium (DMS) including the supplement peptone and the heap pad ore were tested with the sodium tetracyanonickelate, at pH 8 and pH 10. Each sample injected into the HPLC was filtered through 0.45 μm, to minimize damage to the column (Figure 2.6).

2.5.2.2 *The sodium tetracyanonickelate calibration curve*

The tetracyanonickelate calibration curve was done using a wavelength of 267 nm, 18% acetonitrile in water and 0.005 M tetrabutylammonium sulfate.

The concentrations of sodium tetracyanonickelate used to set up the calibration curve were 54 mg l^{-1}; 27 mg l^{-1}; 13.5 mg l^{-1}; 6.75 mg l^{-1} and 3.375 mg l^{-1}.

The correlation coefficient for the sodium tetracyanonickelate was 1.00 (Figure 2.7), which indicates a linear relationship between the concentration in mM of the metal cyanide complex and the HPLC response in mVolts.

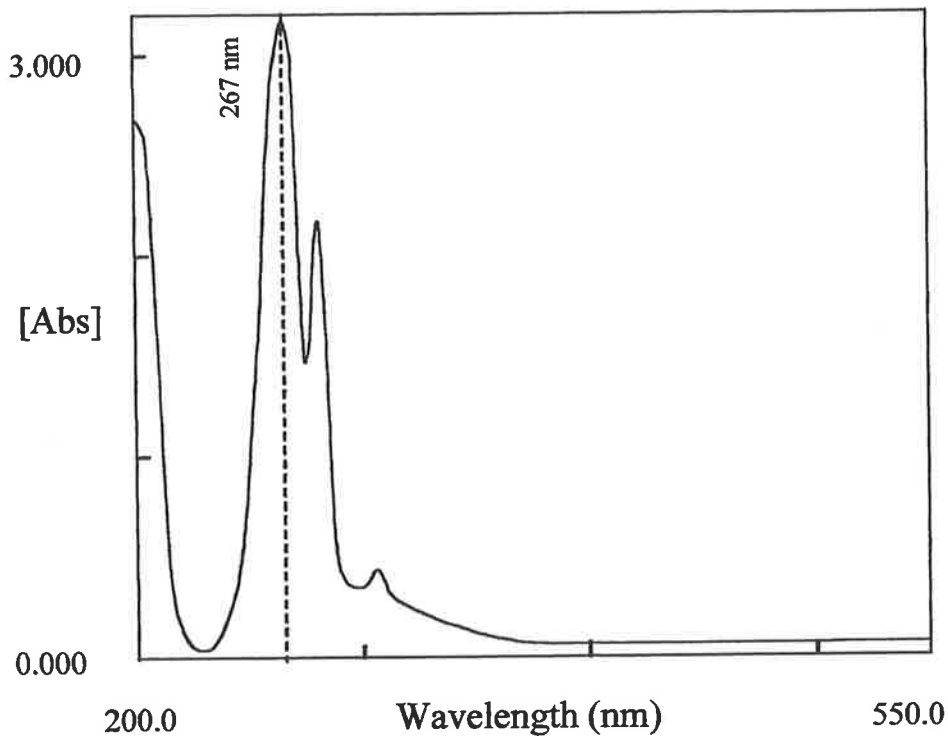

Figure 2.5. Sodium tetracyanonickelate spectrophotometer scan

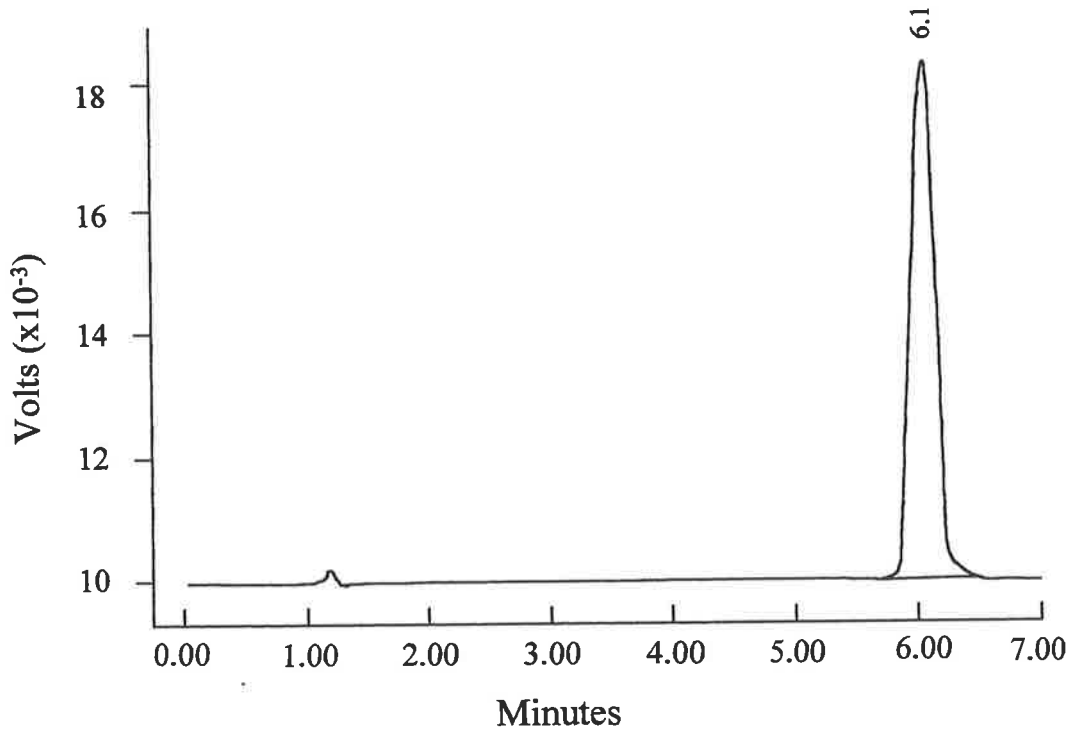

Figure 2.6. Sodium tetracyanonickelate RPII-HPLC peak

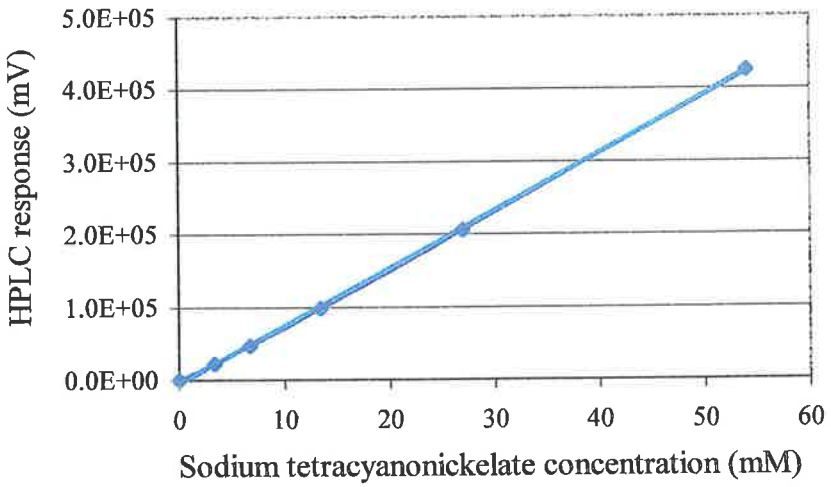

Figure 2.7. Calibration curve obtained with five sodium tetracyanonickelate concentrations

2.5.3 *Potassium hexacyanoferrate trials*

2.5.3.1 *Background*

One of the main concerns when analysing the $Fe(CN)_6^{3-}$ anion, is the presence of the $Fe(CN)_6^{4-}$ anion. The mobile phase chosen needed to separate the FeII and FeIII cyanide complexes and the detection wavelength selected had to discriminate between the two complexes. Some of the wavelengths tried were 214 nm, 316 nm and 413 nm. In papers reporting the chemical analysis of this metal cyanide, the preferred wavelength was 214 nm (Fagan and Haddad, 1991; Hilton and Haddad, 1986; Huang *et al.*, 1996). However, at this wavelength the peptone peak was prominent.

In addition, scans were done on the potassium hexacyanoferrate(III) at pH 8 and pH 10, in the presence of the DMS medium to detect any interference. It was also necessary to carry out some trials with and without the heap pad ore, to test

whether any interaction occurred with the metals in the ore. The visible wavelength range spectrum indicated that potassium ferricyanide had two peaks, one at 316 nm which was almost obscured by the background and another at 413 nm (Figure 2.8). The 413 nm peak was clearly visible and was chosen for further tests (Figure 2.9).

Haddad and Rochester (1988) used 23% acetonitrile in water, and therefore three concentrations 18%, 22% and 25% were chosen for further tests. The mobile phase giving the best peak shape at a convenient retention time was selected. This was the mobile phase of 22% acetonitrile in water.

2.5.3.2 *The potassium hexacyanoferrate*(III) *calibration curve*

In a similar manner to the previous two graphs, a calibration curve was prepared using 200 mg l^{-1} potassium hexacyanoferrate(III) (Merck), dissolved in filtered (0.45μm) deionized water.

The HPLC UV detector was set at a wavelength of 413 nm and elution of the solute was carried out with 22% acetonitrile in water containing 0.005 M tetrabutylammonium sulfate (TBAS). Each RPII-HPLC chromatogram was run for at least 10 mins.

Concentrations used for obtaining the calibration curve were 200 mg l^{-1}, 150 mg l^{-1}, 100 mg l^{-1}, 50 mg l^{-1} and 25 mg l^{-1}.

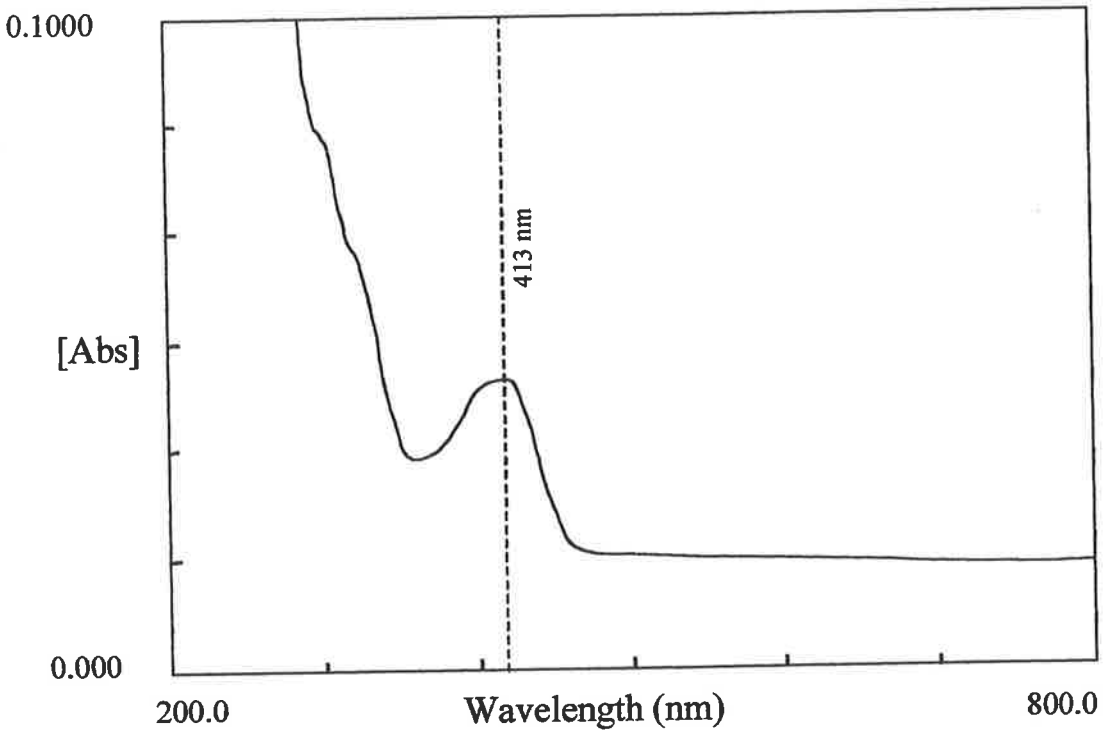

Figure 2.8. Potassium hexacyanoferrate spectrophotometer scan

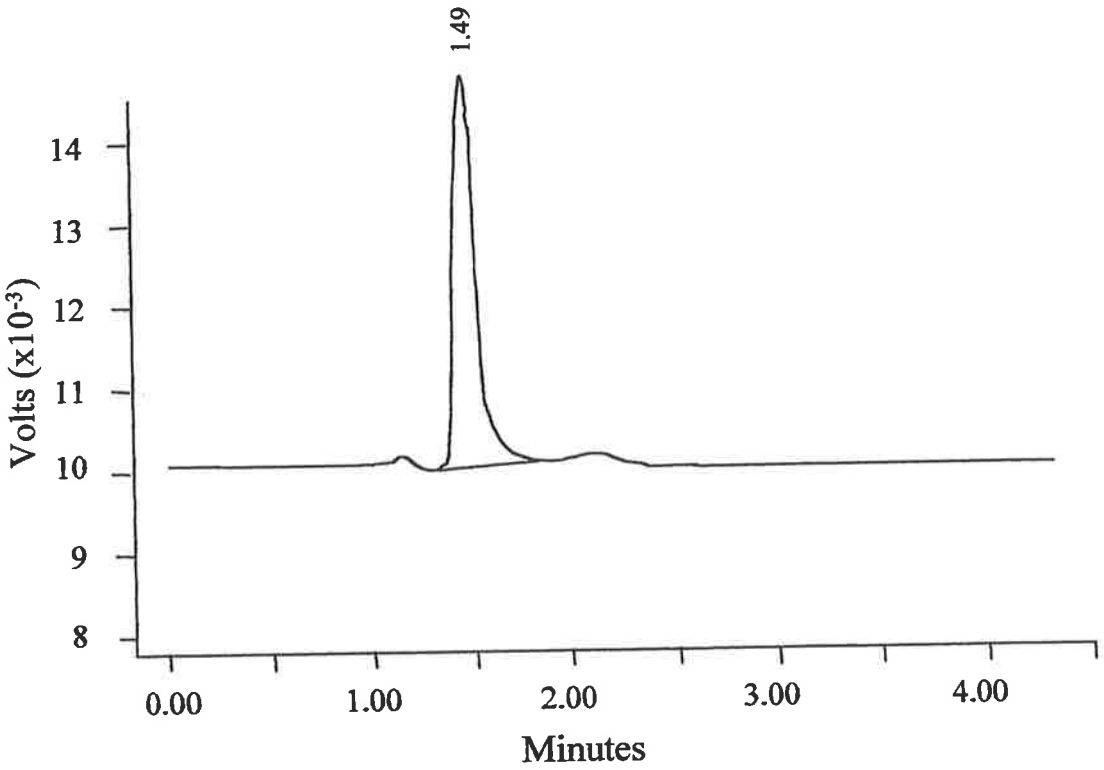

Figure 2.9. Potassium hexacyanoferrate RPII-HPLC peak

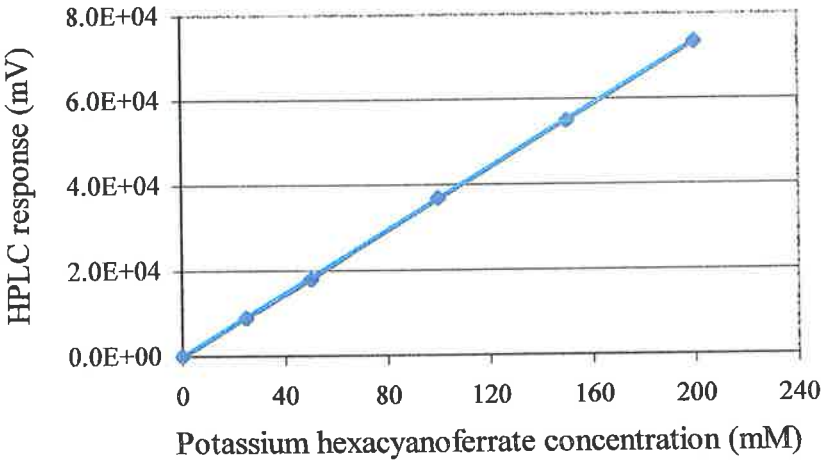

Figure 2.10. Calibration curve obtained with five potassium hexacyanoferrate concentrations

The correlation coefficient for the potassium hexacyanoferrate calibration curve was 1.00 (Figure 2.10), this indicated a linear relationship between the concentration of the compound and the peak produced on the HPLC.

2.6 *Chapter overview*

The RPII-HPLC detection of the metal cyanides of copper, nickel and iron, was often carried out as a mix of 6 or 7 metal cyanides, so conditions selected by some researchers (Hilton and Haddad, 1986; Haddad and Kalambaheti, 1991) had to be capable of separating all of the anions present. However, in the research reported here, the degradation of each of the three metal cyanides, was investigated individually but in the presence of an active biomass.

No reports were found of investigations which followed the degradation of metal cyanides under these circumstances with analyses done by Reversed Phase Ion-Interaction High Performance Liquid Chromatography (RPII-HPLC). Therefore the best conditions had to be selected by experimentation.

The calibration curves of the three metal cyanides, indicated a straight line correlation between concentrations and HPLC peak area responses in each case. Different parameters gave the best results for each of the three cyanide complexes. A concentration of 25% acetonitrile/water and a detection wavelength of 234 nm for copper(1) cyanide, 18% acetonitrile/water and a wavelength of 267 nm for sodium tetracyanonickelate and lastly 22% acetonitrile/water and a detection wavelength of 413 nm for potassium hexacyanoferrate.

The eluent containing 0.005 M tetrabutylammonium sulfate, varied from 18% acetonitrile/water to 25%. For each cyanide complex, 25% acetonitrile in water was tried but did not give the best resolution either for sodium tetracyanonickelate or for potassium hexacyanoferrate.

Chapter 3.0 Biodegradation of copper(I) cyanide in shake flask cultures

3.1 *Introduction*

The cuprocyanide anions, $Cu(CN)_3^{2-}$ and $Cu(CN)_4^{3-}$ are known to undergo degradation. Some species of the Vibrioniaceae, Enterobacteriaceae and Pseudomonadaceae families were found by Boucabeille *et al.* (1994) to biodegrade metal complexed cyanides (Total and Weak Acid Dissociable cyanides). A type culture of *Pseudomonas fluorescens* strain NCIMB 11764 was reported by Rollinson *et al.* (1987) that also utilized $Cu(CN)_4^{2-}$. In addition, $K_3Cu(CN)_4$ was degraded by a *Acinetobacter* sp. strain RFB1 (Finnegan *et al.*, 1991). Moreover, according to Fagan and Haddad (1997), in aqueous solutions copper(I) cyanide can convert to the more soluble cyanide complexes $Cu(CN)_2^-$, $Cu(CN)_3^{2-}$ and $Cu(CN)_4^{3-}$ (Chapter 1).

Overall, the bacterial genera capable of utilizing the cuprocyanides appeared to be few, *Acinetobacter* (Finnegan *et al.*, 1991), *Vibrio*, *Pseudomonas* and species from the Family Enterobacteriaceae (Boucabeille *et al.*, 1994). At the Homestake Mine, minewater with low total cyanides (0.5 to 10 mg l^{-1}) was polished by *Pseudomonas paucimobilis* (Mudder *et al.*, 1984; Altringer and Giddings, 1990) but levels of 20 to 190 mg l^{-1} were present as total cyanides in wastewater studied by Boucabeille *et al.* (1994). Other *Pseudomonas* species tried were *P. putida* (Babu *et al.*, 1996), *P. fluorescens* (Rollinson *et al.*, 1987; Shpak *et al.*, 1995) and *P. diminuta* (Altringer and Giddings, 1990).

Little enzymatic work has been undertaken relating to the copper cyanide anions that are formed in water, with cyanide oxygenase being the only enzyme named and with the formation of ammonia as a by-product (Rollinson *et al.*, 1987).

The degradation of copper(I) cyanide described in this chapter was measured in mineral salts media by using the reverse phase ion-interaction high performance liquid chromatographic technique (Chapter 2). Control treatments under sterile conditions were set up to assess whether any chemical degradation occurred. While the presence or absence of an organic supplement, peptone, showed which environment better degraded the metal cyanide. Measurements of total cyanide, WAD cyanide and cyanate provided data on the degradation pathway.

3.2 *Materials and methods*

The copper (I) cyanide compound ($CuCN_{(S)}$) was purchased from the Aldrich Chemical Co. Although the solubility product (K_s) of this compound is low at $10^{-19.49}$ (Bjerrum *et al.*, 1958), in aqueous solutions there is a conversion to the more soluble cyanide complexes (Chapter 1), $Cu(CN)_2^-$, $Cu(CN)_3^{2-}$ and $Cu(CN)_4^{3-}$, according to Fagan and Haddad (1997).

Testing copper (I) cyanide (CuCN) for biodegradation using shake flask cultures, presented two main problems;

a) the copper cyanide has low solubility and
b) the added organic supplement (peptone) chemically interacted with the copper cyanide.

3.2.1 *Copper(I) cyanide solubility and amino acids studies*

Two experiments were set up, a) to test the behaviour of 150 mg l^{-1} copper cyanide (CuCN) in water over time and b) to determine which amino acids present in the peptone may interact with copper(I) cyanide forming a blue colouration in solution. This blue colour was indicative of a compound that was detected during some HPLC analyses. Eighteen amino acids were individually tested, by adding 10 mg in 50 ml sterile distilled (deionized) water plus copper(I) cyanide and allowed to stand, in the dark.

3.2.2 *Biodegradation of CuCN at pH 8 and pH 10*

Six Erlenmeyer flasks of 250 ml capacity were filled with 100 ml of sterile DMS growth medium at pH 8. The formulation of the DMS medium was given in Chapter 2. Some treatments included the supplement peptone and all of the treatments contained copper(I) cyanide (CuCN). Controls were used to follow abiotic reactions (no bacteria present) and the treatments are set out in Table 3.1, where +P indicated that peptone was added, +B signified that bacteria were present (biotic treatment) and ore meant 0.5 % w/v of finely ground gold ore material was added.

Table 3.1. Treatments used to study degradation of copper(I) cyanide (CuCN) at pH 8

Treatment nos.	Treatments	Description
1	-P-B+ore+CuCN	no peptone plus sterile ore (abiotic)
2&3	-P+B+ore+CuCN	no peptone plus ore (biotic)
4	+P-B+ore+CuCN	peptone plus sterile ore (abiotic)
5&6	+P+B+ore+CuCN	peptone plus ore (biotic)

The DMS medium either with or without peptone was dispensed into the Erlenmeyer flasks which were sealed with aluminium foil and steam autoclaved at 100 kPa for 20 mins. When the liquid had cooled to room temperature, 15 mg copper cyanide (150 mg l^{-1} CuCN) together with 0.5 % w/v ore material were added to each flask. Some ore subsamples had been autoclaved and these treatments became the minus bacteria (-B) controls, nos. 1 & 4 in Table 3.1. Ore samples which were not autoclaved had active bacterial cells present and became the treatments 2,3,5 & 6.

For the pH 10 degradation experiment, a similar design to that described for pH 8 was used. Eight Erlenmeyer flasks were used with the medium adjusted to pH 10 (Table 3.2). Treatment numbers 1, 2, 5 and 6 were set up as controls (-B) using sterilized ore while treatment numbers 3, 4, 7 and 8 were the bacterial inoculated cultures (+B). Copper cyanide, at 150 mg l^{-1}, again was added after the medium was steam autoclaved. All flasks were placed into a Ratek orbital shaker and incubator, rotating at 60 revs min^{-1} and set at 27^0C. The flask cultures for both pH were grown in the dark.

Table 3.2. Treatments used to study degradation of copper(I) cyanide (CuCN) at pH 10

Treatment nos.	Treatments	Description
1&2	-P-B+ore+CuCN	no peptone plus sterile ore (abiotic)
3&4	-P+B+ore+CuCN	no peptone plus ore (biotic)
5&6	+P-B+ore+CuCN	peptone plus sterile ore (abiotic)
7&8	+P+B+ore+CuCN	peptone plus ore (biotic)

3.2.3 *HPLC sample preparation*

Aliquots were taken from the pH 8 and pH 10 experiments and multiple analyses for copper(I) cyanide were done using conditions described in Chapter 2.

3.2.4 *Determination of end products*

At termination of the two shake flask culture experiments, Total-CN, WAD-CN, cyanate and ammonium-nitrogen were measured in duplicate samples (Chapter 2).

3.2.5 *Bacterial counts and isolations*

Plating onto Tryptone Soy Agar (TSA), DMS with peptone and DMS with peptone plus copper(I) cyanide agar media of three appropriate dilutions from each treatment, and allowing the cells to grow into a visible colony, gave a measure of the bacteria present in the various treatments. Once the colonies had grown at 27°C for three weeks, numbers and morphological characteristics were noted. Representative colony types were replated until purity was achieved. Pure cultures were grown on TSA and DMS agar plates and used for MIDI-FAME identifications.

3.3 *Results and discussion*

3.3.1 *Copper(I) cyanide studies*

a) Test results (average of three replicates) for solubility of copper (1) cyanide in sterile water over the 7 days are shown in Table 3.3. An increase in the solubility was measured for copper(I) cyanide with the greatest change occurring between days 4 and 7.

Table 3.3. Solubility of copper(I) cyanide (mg l^{-1}) in water

Days	CuCN
1	2.26
4	6.84
7	41.98

b) The colour reaction only occurred when copper(I) cyanide and the organic supplement, peptone, were present. Varying shades of blue which intensified over 5 days, appeared on mixing the copper cyanide with individual amino acids. This Cu(I)CN-amino acid complex gave a RPII-HPLC response at the same retention time as the copper(I) cyanide. Of the 18 amino acids tested with copper cyanide, DL-α-alanine, DL-α-amino n-butyric acid, DL-asparagine monohydrate, DL-isoleucine, glycine, DL-serine, DL-threonine and DL-valine, produced a pale blue colour in 5 days and 10 others showed shades of blue after 3 weeks. The deepest blue resulted when serine, isoleucine, glycine, threonine and valine, were mixed with copper(I) cyanide. The reactive amino acids were neutral or hydroxy compounds of a simple straight carbon chain structure with a low formula weight.

3.3.2 *Copper cyanide degradation at pH 8 and pH 10*

Flasks were sampled 6 times during the experimental period. At each sampling time, two or three replicate samples were injected into the HPLC for each treatment. Also included as a single point calibration check, were multiple injections of a filtered standard of 60 mg l^{-1} copper cyanide in a 100 mg l^{-1} sodium cyanide solution. This gave an indication of procedural differences between runs on the HPLC.

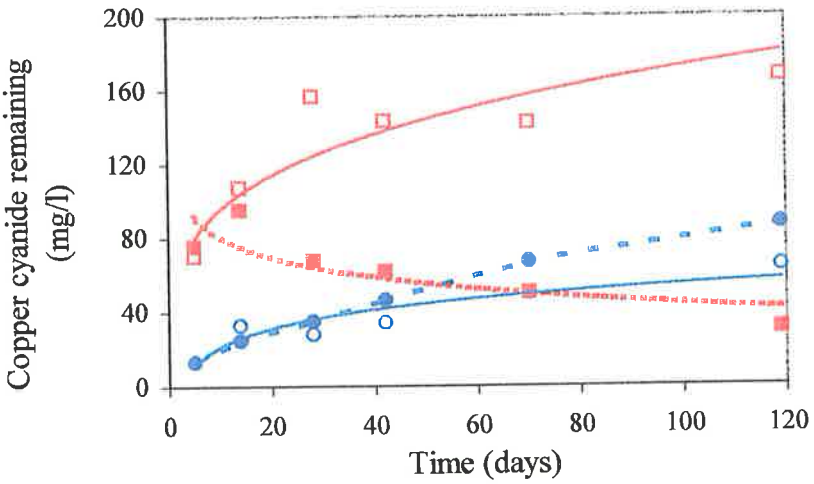

Figure 3.1. Detectable copper cyanide (mg l^{-1}) in solution over time at pH 8. Treatments are ○ =-P-B; ● = -P+B; □ = +P-B and ■ = +P+B, RPII-HPLC data

The control for the minus peptone treatment (-P-B) showed an increase, from 13 mg l^{-1} to 64 mg l^{-1}, in soluble copper(I) cyanide over 119 days (Figure 3.1), as did the bacterial treatment (-P+B) with an increase from 13 mg l^{-1} to 87 mg l^{-1}. The increase in solubility of the copper cyanide may be due to the dissociation of the CN^- ion from some CuCN molecules and the formation of the three Cu(I)-cyanide complexes, $Cu(CN)_2^-$, $Cu(CN)_3^{2-}$ and $Cu(CN)_4^{3-}$ (Chapter 1). No degradation was measured in the absence of peptone.

Copper cyanide was more soluble in the presence of peptone. In the (+P) treatment, at the start of the experiment there was 70 mg l^{-1} copper cyanide in the control treatment with peptone (+P-B) and 75 mg l^{-1} in the bacterial treatment with peptone (+P+B). There was an overall increase in copper(I) cyanide solubility from 13 mg l^{-1} to 75 mg l^{-1} with the addition of peptone.

At pH 8, in the control treatment with peptone there was an increase in solubility from 70 mg l^{-1} to 167 mg l^{-1}, while in the bacterial treatment in the presence of peptone there was a decrease measured from 75 mg l^{-1} at the start of the experiment to 30 mg l^{-1} after 119 days.

Biodegradation of copper(I) cyanide occurred when bacterial cells were given a nutritional component in the medium (+P+B).

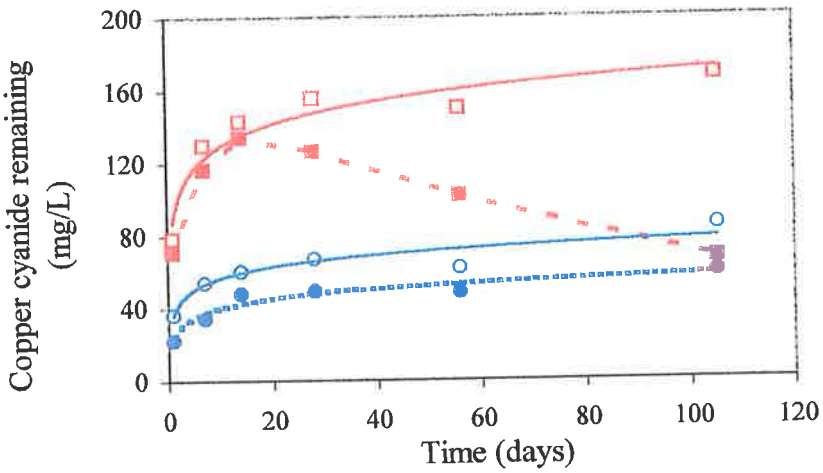

Figure 3.2. Detectable copper cyanide in solution (mg l^{-1}) over time at pH 10. Treatments are ○ =-P-B; ● = -P+B; □ = +P-B ■ = +P+B, RPII-HPLC data

At the higher pH, (Figure 3.2) in the control treatment with no peptone added, the copper(I) cyanide solubility increased from 36 mg l^{-1} to 85 mg l^{-1} in 105 days. The corresponding bacterial treatment increased from 22 mg l^{-1} to 59 mg l^{-1}. No degradation occurred in the 105 days for this treatment. When peptone was added, the control increased from 78 mg l^{-1} to 167 mg l^{-1}, while the bacterial treatment increased from 71 mgl^{-1} at the start of the experiment to a maximum of 134 mg l^{-1} before the degradation process began.

From day 14, a decrease in the level of copper cyanide occurred reaching 67 mg l^{-1} at termination. No utilization of the copper compound was seen without the presence of peptone.

A rise in pH from 8 to 10 resulted in an increase in copper(I) cyanide solubility in the control and in the absence of peptone, from 13 mg l^{-1} to 36 mg l^{-1}. Also a higher maximum value, 134 mg l^{-1}, was obtained for the bacterial treatment in the presence of peptone, at pH 10.

Table 3.4. Degradation rates for copper cyanide (mg l^{-1} day^{-1})

Treatments	pH 8	pH 10
-P-B+CuCN	0	0
-P+B+CuCN	0	0
+P-B+CuCN	0	0
+P+B+CuCN	0.62	0.74

A slightly faster degradation rate, 0.74 mg l^{-1} day^{-1}, was measured for the copper(I) cyanide utilization by the bacteria in the presence of peptone and at the higher pH (Table 3.4). No degradation was found when bacteria were not given the organic supplement, peptone.

3.3.3 *Total-CN, WAD-CN and end products*

In water, copper cyanide can produce moderately strong cyanide compounds (Kurnia *et al.*, 1996) and with weak acids it can be dissociated to form free

cyanide (CN^-). This free cyanide can be measured and provides an indication of the amount that was originally bound to the copper.

After the experimental period of 119 days for the pH 8 experiment and 105 days for pH 10, duplicate subsamples from each culture were analysed for Total-CN and WAD-CN, free cyanide and cyanate (Chapter 2). No free cyanide was detected throughout the test period in either experiment.

Table 3.5. End products (mg l^{-1}) from degradation of copper cyanide (CuCN)

	pH 8			pH 10		
Treatments	**Total CN**	**WAD CN**	**CNO^-**	**Total CN**	**WAD CN**	**CNO^-**
-P-B	40.3	34.6	3.4	46.0	45.5	8.6
+P-B	40.0	34.2	1.2	44.2	26.9	10.4
-P+B	41.5	40.9	2.8	38.3	36.2	6.3
+P+B	<0.1	<0.1	33.0	<0.1	<0.1	1.8

All of the Total-CN and WAD-CN, at the start of the experiment was copper(I) cyanide and when no Total-CN or WAD-CN was detected by termination this indicated that no copper(I) cyanide remained, in solution. The cyanide was converted to the end product cyanate, at pH 8 but not at pH 10 (Table 3.5). All of the copper(I) cyanide remained in the bacterial treatment not containing peptone at pH 8 and approx. 80% was left at pH 10.

3.3.4 *Bacterial counts*

Isolations were done on the pH 8 cultures at 50 days and at 78 days for the cultures at pH 10. Three dilutions from each treatment were plated in duplicates,

onto DMS medium containing peptone and copper cyanide. When required, more dilutions were plated to obtain bacterial numbers between 30 and 300. No bacterial cells were isolated from any of the abiotic treatments at either of the two isolation times, this showed that sterile conditions were maintained throughout the experimental period.

Table 3.6. Bacterial numbers (cfu ml^{-1}) associated with degradation of copper cyanide at pH 8, in the presence or absence of organic material, after 50 days (all counts x 10^6)

Treatments (2 reps)	DMS+P+CuCN
-P+B+CuCN	2.30
+P+B+CuCN	8.75

Bacterial colony counts were almost 4 fold higher in the presence of the organic nutrient (Table 3.6) at pH 8. The peptone enabled the heterotrophic bacterial cells to grow quickly by providing components required in cell structures. In the absence of peptone all components of the cells have to be constructed from basic inorganic elements.

Table 3.7. Bacterial numbers (cfu ml^{-1}) associated with degradation of copper cyanide at pH 10 in the presence or absence of organic material, after 78 days (all counts x 10^6)

Treatments (2 reps)	DMS+P+CuCN
-P+B+CuCN	1.24
+P+B+CuCN	16.0

At pH 10, almost a 13 fold increase in bacterial colony counts was found in the presence of peptone compared to the treatment without the nutrient (Table 3.7).

A rise in pH restricted the growth of the microflora in the absence of peptone to a level almost half that found at pH 8.

At the higher pH, the copper(I) cyanide may have remained in solution for a longer period of time (Figure 3.2) and this greater availability of soluble copper cyanide may have stimulated greater growth of the bacteria. The larger biomass at pH 10 was responsible for the faster rate of degradation compared to that at pH 8.

At the termination of the shake flask culture experiments, bacterial cell counts, in duplicates, were repeated using three microbiological agar media, DMS with peptone (1g l^{-1}) and with and without the addition of copper(I) cyanide at pH 9.4. The latter two media were used to isolate heterotrophs or facultative heterotrophs. The same dilutions were also tested on Tryptone Soy Agar (Difco) at pH 7 for isolation of obligate heterotrophs.

Table 3.8. Bacterial counts (cfu ml^{-1}) at end of shake flask experiment (119 days) cultures grown at pH 8 (all counts x 10^6)

Treatments (2 reps)	TSA	DMS+P	DMS+P+CuCN
-P+B+CuCN	0*	0.02	0.06
+P+B+CuCN	0.80	13.8	60.4

* Also zero colonies at 10^{-1}

All controls, -P-B-ore, -P-B+ore and +P-B+ore remained sterile and no bacterial cells were isolated on any of the three media, at pH 8.

When comparing the DMS+P+CuCN colony counts in Table 3.8 to levels in Table 3.6, it is seen that in the treatment without peptone (-P) there is a large decrease in colony numbers from 2.30 to 0.06 (x 10^6) by the end of the experiment. This contrasted with the large increase from 8.75 to 60.4 (x 10^6) in

the peptone (+P) treatment. In both treatments, the inclusion of copper(I) cyanide in the medium encouraged more bacterial colonies to grow.

The rich isolation medium TSA gave low numbers for the peptone treatment at pH 8, and much lower than the two DMS media. Because the largest number of bacterial colonies were grown on DMS+P+CuCN, this could indicate that the dominant bacterial groups responsible for the degradation process in the shake flask cultures were either facultative autotrophs or heterotrophs.

Table 3.9. Bacterial counts (cfu ml^{-1}) at end of shake flask experiment (105 days) cultures grown at pH 10 (all counts x 10^6)

Treatments (2 reps)	TSA	DMS+P	DMS+P+CuCN
-P+B+CuCN	0*	0.07	0.25
+P+B+CuCN	0*	0.40	42.9

* Also zero colonies at 10^{-1}

At pH 10, all the control treatments -P-B-ore, -P-B+ore and +P-B+ore, remained sterile throughout the experiment (Table 3.9). This was indicated by no bacterial cells being isolated on any of the three media.

Comparison of the results given in Table 3.9 to Table 3.7, for the DMS+P+CuCN medium, showed that a decrease in bacterial counts from 1.24 to 0.25 (x 10^6) occurred by the end of the experiment in the minus peptone treatments at pH 10, but the decline was less than that seen at pH 8. Therefore, in the absence of peptone, the bacterial cells appeared to survive longer at pH 10.

However, the bacterial numbers shown for the peptone treatment at termination, were greater at pH 8 (Table 3.8) than at pH 10 (Table 3.9). Since there was a

greater bacterial biomass when peptone was added, a build up of metabolites could have caused a greater detrimental effect at pH 10 than at pH 8.

Final isolation counts for the cultures minus peptone, were higher at pH 10 than pH 8, this was reversed for cultures containing peptone. No colonies grew on the rich TSA medium.

3.3.5 *Bacterial isolations*

Very little colony diversity was found by using MIDI-FAME on isolates from the various treatments. At pH 8, 43 bacterial colonies, chosen on the basis of diverse colour, shape and other microbiological criteria, were prepared in duplicates for FAME analysis. At the two times when isolations were done on the DMS containing peptone and copper cyanide (DMS+P+CuCN) pH 8 medium, the main species identified was *Pseudomonas stutzeri* and some *Bacillus pumilus* colonies. These species were isolated in the absence and presence of peptone. No bacterial colonies could be regrown and purified from the pH 10 cultures.

3.4 *Chapter overview*

Bacterial break down of the copper cyanide complexes formed in water was only measurable when the nutrient, peptone, was present. This occurred at both pH 8 and pH 10. When isolations were carried out at the mid-point (50 days) from each shake flask culture, bacterial numbers (cfu ml^{-1}) were high in the plus peptone treatment and low in cultures with no nutrient. Similarly at termination, bacterial numbers were much lower in the no peptone cultures, with a ca. 38 fold decrease from the counts taken at 50 days. In the same period, for the peptone cultures counts increased by a factor of 6.9 at pH 8.

By the end of the experiment at pH 10 there was a ca. 5 fold decrease in bacterial numbers in the treatment not containing peptone, but an increase by a factor of 2.7 for the treatment with added peptone. Overall smaller fluctuations in bacterial numbers were found when peptone was available.

At termination of the experiment, Total-CN and WAD-CN measurements confirmed that only the microflora assisted by the presence of peptone (+P) degraded the cyanide bound in the copper complex (Table 3.5). The heterotrophic species active in the peptone treatment converted cyanide to cyanate at the lower pH, but no cyanate was found at pH 10. Therefore the production of cyanate may be pH sensitive. When 150 mg l^{-1} CuCN was added to the medium, the theoretical value of CN^- was 43.6 mg l^{-1} and 47% of this was converted to cyanate. Analyses carried out at end of the experiments, showed that no copper remained in solution in the peptone plus bacteria treatments, at either pH 8 or 10. During the reaction where cyanide was removed by the bacteria, the copper cyanide complex was converted to copper hydroxide which precipitated out of solution.

Figures 3.1 and 3.2, show that the presence of peptone helped to increase the solubility of copper(I) cyanide. Although initially copper(I) cyanide had very low solubility, there was an increase in solubility over the experimental period. The species isolated, *Pseudomonas stutzeri* and *Bacillus pumilus*, have the ability to survive in a mineral salts medium and use cyanide as a carbon source. Facultative chemolithotrophic species are known to be members of the genus Bacillus and have the capability of growing autotrophically in an oxygen/carbon

dioxide/hydrogen or oxygen/carbon monoxide atmosphere. However, if an appropriate carbon and energy source is provided, these species grow heterotrophically. Since both *P. stutzeri* and *B. pumilus* were isolated from the no peptone and plus peptone treatments, they may be exhibiting facultative autotrophic characteristics or they may be growing heterotrophically on impurities in the agar.

Rollinson *et al.* (1987) showed that copper cyanide, $Cu(CN)_4^{2-}$, could act as a source of nitrogen to *Pseudomonas fluorescens* strain NCIMB 11764. The active enzyme was cyanide oxygenase which was induced not only by free cyanide but also by the complexed cyanide. In the Rollinson *et al.* (1987) paper, it was reported that ammonia was formed. The enzyme activated therefore was cyanide dioxygenase (Dubey and Holmes, 1995).

In the presence of peptone and at pH 8, the end product was cyanate (Table 3.5) but at pH 10, cyanate was not found. Ammonia was not determined during the experiment. At pH 8 therefore, the enzyme cyanide monoxygenase was active but at pH 10, the enzyme cyanide dioxygenase was induced since no cyanate was detected (Dubey and Holmes, 1995).

An unnamed enzyme from a *Acinetobacter* sp. was able to degrade a range of inorganic cyanide salts including $Cu(CN)_4^{3-}$ but the optimal growth concentration was only 5 mg l^{-1} (Finnegan *et al.*, 1991). This enzyme was present whether cyanide containing substances were included in the growth medium or not, and therefore was expected to be a constitutive enzyme. Unfortunately no by-products were reported.

The opposite was found in the results presented in this chapter. Tables 3.8 and 3.9 show that when the cyanide compound was added to one of the isolation media, there was a large increase in bacterial numbers. This may indicate that there was a specific enzyme induced as a response to the cyanide present and this enzyme aided greater cell growth.

Research has been done on cell-free extracts from *Pseudomonas putida* and the enzymes were able to convert cyanides in minewater to ammonia (Babu *et al.*, 1996). The specific activity of the extract was measured in the presence of copper cyanide amongst other metal cyanides and was found to be more active at pH 9.5 than at pH 7.5. This was in agreement with the present research where a better degradation rate was obtained at pH 10 (Table 3.4).

Wastewater at gold mines can have high levels of copper cyanide present as WAD-CN (Boucabeille *et al.*, 1994), therefore it is necessary to identify conditions best suited to achieve the degradation of this pollutant. Altringer and Giddings (1990), have found that the cyanide degrading bacteria in their system would not remove the copper. Some of the research into degradation of copper cyanide has been incomplete in that details were not given as to experimental procedure or end products. Encouraging results were obtained by some groups, only to have the work discontinued before complete degradation pathways were obtained.

Overall few reports are available for the study of copper(I) cyanide degradation.

Chapter 4.0 Degradation of copper(I) cyanide in bioreactors

4.1 *Introduction*

Copper cyanide was briefly studied by Rollinson *et al.* (1987) as part of research on another metal cyanide complex. The cyanocupric anion in that study was $Cu(CN)_4^{2-}$. Others have analysed the degradation of the $Cu(CN)_4^{3-}$ anion by *Pseudomonas fluorescens* (Shpak *et al.*, 1995) and by an *Acinetobacter* sp. (Finnegan *et al.*, 1991). Copper was also investigated as part of the Total-CN and WAD-CN where degradation was attributed to the genera *Vibrio*, *Enterobacter*, and *Pseudomonas* (Boucabeille *et al.*, 1994). In the reviewed papers the only enzyme implicated in the degradation of copper cyanide, was cyanide oxygenase (Rollinson *et al.*, 1987).

The most quoted system, where bioremediation of mine wastewater has operated for over 18 years, is at the Homestake Gold Mine in South Dakota, USA (Mudder *et al.*, 1984). At this mine, the combined influent wastewater contained only low levels of the copper cyanide complex (0.1 to 1.5 mg l^{-1}) as a fraction of the WAD-CN. The bacterium *Pseudomonas paucimobilis* degraded the WAD-CN, to less than the proposed permit limits of 0.18 mg l^{-1}.

In this chapter, degradation of copper(I) cyanide was studied using bioreactors to assess whether a larger system using non sterile growth medium, could facilitate the break down processes.

4.2 *Materials and methods*

Spinner Flasks (BELLCO) of 1 litre capacity were set up as bioreactors A and B. Non sterile growth medium of 1 litre DMS was inoculated with 10% w/v leach pad gold ore material (Chapter 2). Both bioreactors were shielded from light, continuously stirred and maintained at 27°C.

Bioreactor B had a pretreatment with 1g l^{-1} peptone being added and lasting 20 days to encourage a different bacterial consortium than in bioreactor A (Chapter 2). All points on the graphs constructed from the RPII-HPLC data were checked two or three times by repeated analyses.

4.2.1 *First degradation cycle*

In each bioreactor A and B, 150 mg l^{-1} of copper (I) cyanide compound (CuCN) was added to the DMS medium and the degradation monitored over 28 days. Once degradation was complete, the ore was allowed to settle and the supernatant was removed.

4.2.2 *Second degradation cycle*

A second litre of DMS spiked with another aliquot of CuCN (150 mg l^{-1}) was then added to each bioreactor. This degradation cycle lasted 33 days during which pH, levels of copper cyanide and ammonium-nitrogen concentrations were measured.

4.2.3 *Determination of end products*

Subsamples of 10 ml were taken at set intervals over the incubation period during both degradation cycles and duplicate samples were tested for pH, free cyanide, Total-CN, WAD-CN and ammonium-nitrogen (Chapter 2).

4.2.4 *Bacterial counts and isolations*

Bacterial isolations were not performed during this first degradation cycle.

After the second addition of copper cyanide, isolations were carried out on the surfaces of three agar media, TSA, DMS containing 1 g l^{-1} peptone (DMS+P), and DMS with 1g l^{-1} peptone plus 300 mg l^{-1} copper(I) cyanide (DMS+P+CuCN) at 14 and 28 days. Three appropriate bacterial dilutions were spread onto the agars, in duplicates. After isolation, bacterial colonies were counted, described and purified by repeated plating.

When pure cultures were obtained, the MIDI-FAME protocol was carried out on duplicates of 25 colonies in order to identify the bacterial species isolated.

4.3 *Results and discussion*

4.3.1 *Degradation of copper cyanide, two cycles*

4.3.1.1 *First degradation cycle*

Overall changes in pH were slight during the first cycle with a small decrease of 0.2 units in bioreactor A and 0.15 units in bioreactor B. By day 2 the pH increased in both and by day 28 had stabilized to 8.25 in bioreactor A and 8.60 in bioreactor B. In bioreactor A, copper(I) cyanide remaining in solution was seen to increase up to day 7, after which a steady decrease occurred (Figure 4.1). Bioreactor B appeared to have a residue of peptone from the pretreatment stage and again the solubility of copper(I) cyanide increased when peptone was present, giving a maximum value of 67 mg l^{-1} compared to that in bioreactor A of 39 mg l^{-1} (Chapter 3).

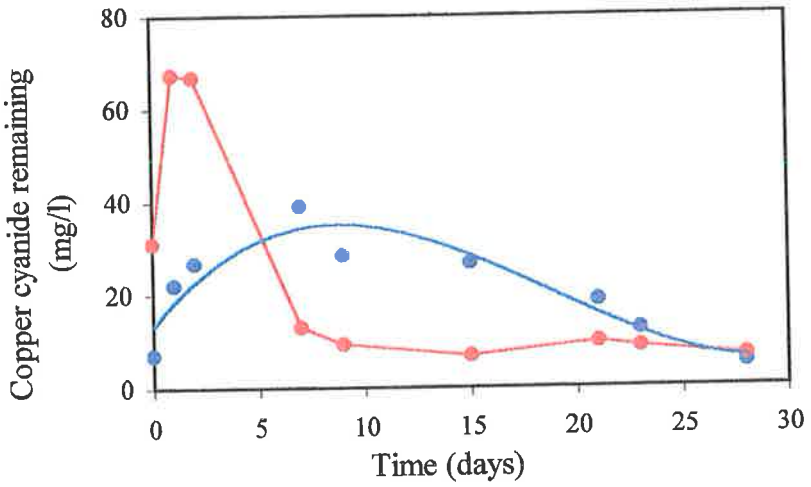

Figure 4.1. Degradation of CuCN, cycle 1, bioreactor A = ●, bioreactor B = ●, RPII-HPLC data

In bioreactor A, the rate of degradation was 1.6 mg l^{-1} day^{-1} and in bioreactor B, a much higher rate of 8.3 mg l^{-1} day^{-1} was obtained. By day 9, the bacteria had utilized 87% of the compound, in bioreactor B.

Replicated analyses at termination of the first degradation cycle, gave a level of copper of <0.2 mg l^{-1} at pH 8.3 for bioreactor A, and for bioreactor B a final Cu concentration in solution equal to 3.3 mg l^{-1} at pH 8.6. When using the RPII-HPLC technique, the final concentration (at 28 days) of copper cyanide, remaining in solution was down to 5.0 mg l^{-1} for bioreactor A and 6.5 mg l^{-1} for bioreactor B.

4.3.1.2 *Second degradation cycle*

Results in Table 4.1 show that the value of copper as soluble copper cyanide in the media was associated with a lowering of pH. As copper cyanide levels in the solution decreased due to degradation, after 7-10 days the pH increased due to the formation of ammonium-nitrogen.

Table 4.1. Cu values (mg l^{-1}) vs pH in bioreactors over 33 days, cycle 2

Time (days)	Bioreactor A Cu mg l^{-1}	pH	Bioreactor B Cu mg l^{-1}	pH
0	3.1	8.25	5.7	8.45
1	10.7	8.15	23.2	8.30
5	17.6	7.90	20.2	8.20
7	21.6	7.95	0.5	8.25
10	19.2	7.95	0.6	8.35
12	15.7	8.05	0.5	8.25
14	10.8	8.15	0.3	8.35
17	4.3	8.15	0.3	8.30
22	1.5	8.20	0.4	8.30
26	0.3	8.20	0.3	8.25
28	0.3	8.20	0.2	8.25
33	0.3	8.25	0.3	8.25

This was more noticeable in bioreactor A where high copper was found in solution for a longer time since degradation proceeded at a slower rate. No difference in pH was seen in the two bioreactors by 33 days.

Results similar to those presented in Figure 4.1, were obtained for the second degradation study of copper cyanide (Figure 4.2) using the RPII-HPLC technique.

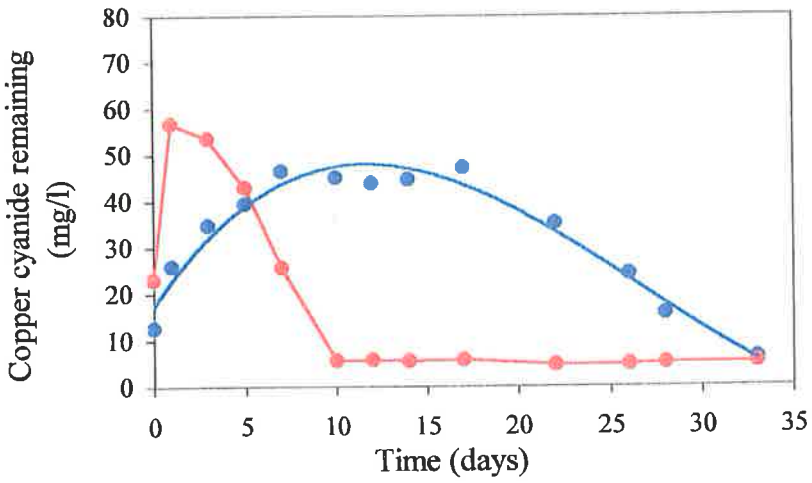

Figure 4.2. Degradation of CuCN, cycle 2, bioreactor A = ●, bioreactor B = ●, RPII-HPLC data

The maximum amount of copper cyanide measured in solution, in bioreactor A was 20.5% higher in the second cycle but 15% lower for bioreactor B.

In the second cycle, the degradation rate in bioreactor A was 2.7 mg l^{-1} day^{-1}, this was a 69% increase from 1st cycle, but bioreactor B indicated a slowing down in the degradation process, giving a degradation rate of 5.7 mg l^{-1} day^{-1} which was a 31% decrease from cycle 1. In bioreactor B, by day 10, 89% of the copper cyanide in solution had degraded. This compared well with the 87% by day 9 in the cycle 1. The final concentration in bioreactor A was 6 mg l^{-1} at 33 days, compared with 5 mg l^{-1} at 28 days, in cycle 1 while bioreactor B contained 5 mg l^{-1} at 33 days and 6.5 mg l^{-1} at 28 days, in the 1st cycle.

In the two cycles, copper cyanide was degraded down to the same level, in both bioreactors ie. 5-6 mg l^{-1}. In 1980, the National Academy of Science estimated the safe level for adults to be 2-3 mg l^{-1} (Smith *et al.*, 1988) and for aquatic

ecosystems levels at 5-10 μg l^{-1} (ANZECC, 1992), therefore, the degradation periods would have to be extended until this level is reached.

4.3.2 *Total-CN, WAD-CN and end products*

4.3.2.1 *Ammonium-nitrogen production during the first degradation cycle*

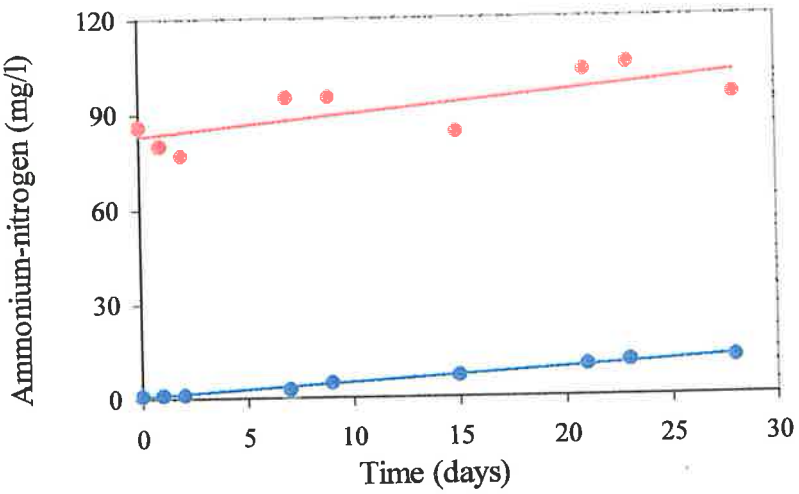

Figure 4.3. Ammonium-nitrogen produced from degradation of copper cyanide, cycle 1, bioreactor A = ●, bioreactor B = ●.

During the 28 day experimental period, analysis of duplicate samples indicated a slow increase in ammonium-nitrogen and this was noted in both bioreactors. Bioreactor A, increased from 1 mg l^{-1} to 12 mg l^{-1} while bioreactor B increased from 80 mg l^{-1} to 96 mg l^{-1} (Figure 4.3). The high starting value for bioreactor B was the residual ammonium-nitrogen produced by the bacteria in the pretreatment stage (Chapter 2).

4.3.2.2 *Total-CN, WAD-CN and end products during the second degradation cycle*

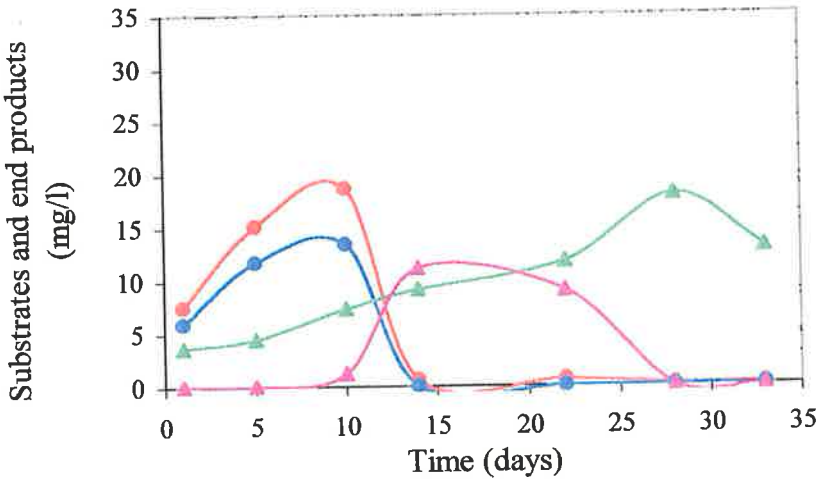

Figure 4.4. End products from the degradation of copper cyanide (mg l^{-1}) in bioreactor A (2^{nd} cycle), Total-CN = ●, WAD-CN = ●, ammonium-nitrogen = ▲, cyanate = ▲

As Total-CN and WAD-CN levels decreased, a rapid rise in cyanate levels was found. Conversion of the cyanide to ammonium-nitrogen was steady to a maximum of 18 mg l^{-1} at 28 days (Figure 4.4). The ammonium-nitrogen appeared to be decreasing by day 33. This may be due to the nitrification.

$$CuCN \rightarrow CNO^{-} \rightarrow CO_2 + NH_3 \qquad (1)$$

During the course of the Total cyanide and WAD cyanide degradation there was a conversion to cyanate which was in turn hydrolysed to ammonium-nitrogen and carbon dioxide (reaction 1).

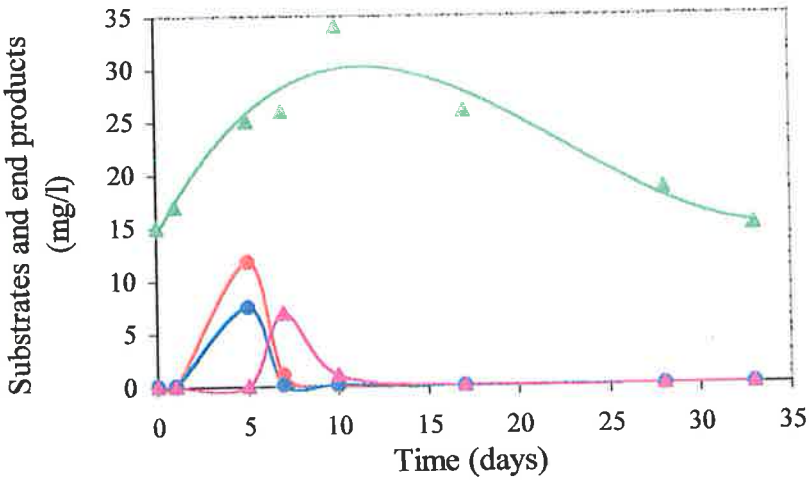

Figure 4.5. End products from the degradation of copper cyanide (mg l^{-1}) in bioreactor B (2^{nd} cycle), Total-CN = ●, WAD-CN = ●, ammonium-nitrogen = ▲, cyanate = ▲

Total cyanide (Total-CN) and weak acid dissociable cyanide (WAD-CN) disappeared very early in the second degradation cycle, as did cyanate which could only be measured for a short time (Figure 4.5). Levels for all three were lower than in bioreactor A.

Hydrolysis to the ammonium-nitrogen occurred quickly and increased from 15 mg l^{-1} at 1 hr to 34 mg l^{-1} at 10 days. After day 10, the level of ammonium-nitrogen slowly decreased. As in bioreactor A, ammonium-nitrogen was itself converted to other compounds. The disappearance of ammonium-nitrogen during the second cycle began earlier than in the first degradation cycle.

4.3.3 *Bacterial counts*

Bacteria were not isolated during the 1^{st} degradation cycle, since some residual

peptone from the preliminary stage in bioreactor B may have been present which would distort any numbers obtained.

Table 4.2. Bacterial isolation (cfu ml^{-1}) from CuCN, cycle 2 (all counts x 10^6)

Bioreactors	Days	TSA	DMS+P	DMS+P+CuCN
Bioreactor A	14	0.1	1.9	0.5
Bioreactor A	28	0.5	3.6	8.6
Bioreactor B	14	7.0	25.0	11.0
Bioreactor B	28	5.8	26.0	25.0

Mid-way through the experiment, bacterial counts were very low in bioreactor A compared to bioreactor B, in the three media. By the end of the experimental period of 28 days, the number of colony forming units (cfu ml^{-1}) in the DMS agar containing peptone plus copper cyanide, had increased in bioreactor A (Table 4.2). Bioreactor B showed high numbers throughout the test period, however, for the DMS+P+CuCN agar the increase in the 14 to 28 days period was less pronounced than in bioreactor A. At 28 days in bioreactor A, the bacterial numbers in the DMS+P+CuCN medium, was only ca. ⅓rd that counted in bioreactor B.
The greater bacterial mass in bioreactor B promoted a faster degradation process than was occurring in bioreactor A, as supported by data shown in Figures 4.2 and 4.5.

4.3.4 *Bacterial isolations*

As in the shake flask cultures (Chapter 3), low bacterial species diversity was again

assessed by isolations from the bioreactors. In bioreactor A, some *Pseudomonas stutzeri* colonies were seen at 28 days. In the 14 day isolations for bioreactor B, *P. stutzeri* was dominant. This changed to a shared dominance by *P. stutzeri* and *Bacillus pumilus* by 28 days. In all of the isolations done, a pin point, white colony (Chapter 9) was found which could not be identified by the MIDI technique.

4.4 *Chapter overview*

In both degradation cycles, chemical reactions occurred between the copper(I) cyanide and water with the possible formation of more soluble compounds, for example $Cu(CN)_2^-$, $Cu(CN)_3^{2-}$ and $Cu(CN)_4^{3-}$ (Fagan and Haddad, 1997).

Degradation proceeded more rapidly in bioreactor B, accelerated by a greater biomass, and was completed by 7 and 10 days, whereas in bioreactor A completion was reached at 28 and 33 days in the two cycles. Although copper(I) cyanide had a very low solubility (Chapter 3), there appeared to be a reaction when peptone was present which caused an increase in the solubility.

In the first degradation cycle, very low production of ammonium-nitrogen in bioreactor A was indicative of the slow degradation of copper cyanide (Figure 4.1). Measurements of ammonium-nitrogen carried out in bioreactor B, indicated some residual peptone since the degradation of the copper cyanide alone could not produce the high levels of ammonium-nitrogen that were measured. No other end products were measured since the residual peptone made this an atypical situation.

In the second cycle, bioreactor A (Figure 4.4) showed high levels of Total-CN, WAD-CN and cyanate which persisted for a longer period than in bioreactor B, as well as a slower build-up of ammonium-nitrogen. Again the microflora in bioreactor B were more active, which is indicated in Figure 4.5 where little Total-CN, WAD-CN and cyanate were measured. However, there was a rapid rise in ammonium-nitrogen with a maximum value at 10 days.

In both Figures 4.4 and 4.5, the data showed there was utilization of the Total-CN and WAD-CN to form cyanate. Subsequently, cyanate was hydrolysed to CO_2 and ammonium-nitrogen to complete the metabolic pathway. Two enzymes are presumed responsible for this conversion; first the cyanide monoxygenase oxidized the cyanide to cyanate which subsequently was used as a substrate for the enzyme cyanase with the formation of ammonium-nitrogen and carbon dioxide.

Isolations carried out on the 3 different media, at mid-point and at end of the experiment (Table 4.2) showed low bacterial numbers in bioreactor A after 14 days, but a rapid increase by 28 days. The addition of copper(I) cyanide in the isolation agar markedly increased bacterial counts. In bioreactor B, the highest number of bacterial colonies was present by 28 days on DMS+P agar (no copper(I) cyanide). At 14 days, the inclusion of copper(I) cyanide into the isolation agar seemed to suppress numbers but by 28 days numbers were almost at the DMS+P agar level.

By this time, all of the copper(I) cyanide had been degraded in bioreactor B. Generally higher numbers (cfu ml^{-1}) were counted at both isolation times on all 3 media in bioreactor B.

The inclusion of copper(I) cyanide in the isolation agar medium promoted a 2.4 fold increase in the number of bacterial colonies from bioreactor A at 28 days, compared to a slight decrease in bioreactor B.

Isolations showed that very few types of bacteria adapted to an environment with copper cyanide as a nitrogen or carbon source. The two main species identified by MIDI-FAME were *Pseudomonas stutzeri* and *Bacillus pumilus*, as in Chapter 3.

The bacterium *Pseudomonas stutzeri* can grow well on mineral salts media with ammonia as the sole nitrogen source (Williams *et al.*, 1989) and glucose as the carbon and energy source. The glucose was substituted by cyanide as the energy source in the bioreactors. The other bacterium, *Bacillus pumilus*, is known to tolerate and utilize cyanide (Williams *et al.*, 1989).

Although there was little diversity in the microflora, very rapid degradation of copper(I) cyanide was measured in bioreactors with or without the pretreatment. There appears to be no report in the literature that has shown the disappearance of copper(I) cyanide and the concurrent appearance of the end products cyanate and ammonium-nitrogen. In addition, no paper was found that associated the degradation process to bacterial numbers and species in bioreactors under a non sterile system as well as naming some end products.

Chapter 5.0 Biodegradation of sodium tetracyanonickelate in shake flask cultures

5.1 *Introduction*

Bacterial degradation of nickel cyanide complexes has been reported by a number of authors (Rollinson *et al.,* 1987; Silva-Avalos *et al.*, 1990) where the bacterium *Pseudomonas fluorescens*, strain NCIMB 11764, was shown to utilize cyanide as a source of nitrogen when glucose was supplied as the carbon source (Rollinson *et al.*, 1987). The authors also showed that tetracyanonickelate degradation was associated with the induction of the cyanide oxygenase enzyme which, however, could not be induced in the presence of ammonia.

In another study, *Pseudomonas putida,* BCN3 (Silva-Avalos *et al.,* 1990), degraded potassium tetracyanonickelate, $K_2(NiCN_4)$, which was used as the sole nitrogen source, also in the presence of glucose. The work of Silva-Avalos *et al.* (1990), showed that the degradation of K_2NiCN_4 led to the formation of $NiCN_2$ but this only occurred when ammonia was absent. Another group (Shpak *et al.*, 1995) used a strain of *Pseudomonas fluorescens* to degrade a range of different metal cyanide complexes. They concluded that a possible mechanism for degradation of metal cyanide complexes may be a shift in the chemical equilibrium of the dissociation of complex anions which takes place at the expense of the utilization of cyanide ligands by the degrading strain (Shpak *et al.*, 1995).

Shake flask experiments were prepared to evaluate the ability of indigenous bacteria on the ore to degrade sodium tetracyanonickelate at pH 8 and 10. The metabolic pathway was investigated, giving an insight to the types of enzymes that were active in the degradation. Estimation of the bacterial population and identification of the species present were also carried out.

5.2 *Materials and methods*

5.2.1 *Biodegradation of* Na_2NiCN_4 *at pH 8 and pH 10*

A sodium tetracyanonickelate solution was prepared by adding 0.5 mM nickel sulfate (Analar) and 2 mM sodium cyanide (Merck) in 1 litre of deionised water (reaction 1).

$$NiSO_4 + 4NaCN \rightarrow Na_2NiCN_4 + Na_2SO_4 \qquad (1)$$

Solutions were sterilized by filtration using a 0.45 μm pore size membrane and then added to autoclaved Davis Mineral Salts medium (DMS) with and without peptone (Chapter 2). The medium was prepared at double strength and 50 ml was dispensed per Erlenmeyer flask along with 50 ml of the filter sterilized sodium tetracyanonickelate solution also at double strength. Warroo ore was added at the same weight as in the copper cyanide experiments (Chapter 3).

After preparation, the sodium tetracyanonickelate (final concentration of 0.5 mM), shake flasks cultures were inoculated with ore (0.5% w/v). Controls consisted of the DMS plus and minus peptone media with sterile ore (autoclaved) added. Sterile ore was added to determine whether abiotic adsorption or chemical reactions occurred between the metal cyanide complexes and the ore material.

Treatments were prepared in duplicate for each set of culture conditions. Flasks were shaken slowly (60 rpm) and incubated at 27°C. Treatment detail and experimental design were the same as for the shake flask cultures at pH 10 in Chapter 3.

For each analysis, 8 ml of supernatant solution was removed and filtered through a 0.45 μm pore size membrane. The filtrate was used for quantitative chemical analysis and pH measurements. Ammonium-nitrogen was determined by the Berthelot colour reaction (Stainton *et al.,* 1977) using a LKB Biochrom Ultraspec 3 UV/visible spectrophotometer (Chapter 2). The degradation of the sodium tetracyanonickelate complex was monitored over time by RPII-HPLC and microbial numbers were determined by inoculating agar plates containing specific mineral salts plus metal cyanide and/or peptone.

5.2.2 *HPLC sample preparation*

All analyses during the course of the experiments, were done under the same conditions as for the preparation of the calibration curve (Chapter 2). For the sodium tetracyanonickelate calibration curve a wavelength of 267 nm was used and the eluent was 18 % acetonitrile in water with 0.005 M tetrabutylammonium sulfate (TBAS).

5.2.3 *Determination of end products*

During the two experiments, ammonium-nitrogen was measured six times using the method described in Chapter 2. At the end of the test period, 77 days, the level of cyanate and any remaining free cyanide, Total-CN and WAD-CN was also measured. All analyses were replicated two or three times.

5.2.4 *Bacterial counts and isolations*

The shake flask cultures were processed twice to obtain colony forming units (cfu ml^{-1}), at 55 and 77 days. Subsamples were taken from each culture and plated onto three isolation media, TSA, DMS+P and DMS+P+Na_2NiCN_4, at 55 days. But at 77 days, no TSA was used. Three appropriate dilutions, differing by 10^{-1}, were plated out in duplicates on each media and incubated at 27°C. Bacterial colonies were counted weekly.

At the end of the growth period, colony types were described and replated to assess the purity of the bacterial colony. When purity was achieved, duplicates of 49 cultures from pH 8 and 36 from pH 10 were prepared by the MIDI-FAME protocol for identification.

5.3 *Results and discussion*

5.3.1 *Sodium tetracyanonickelate pH studies,* Na_2NiCN_4

The pH determinations of the cultures at pH 8 and pH 10 are shown in Tables 5.1 and 5.2 respectively.

Table 5.1. Changes in pH during sodium tetracyanonickelate biodegradation at pH 8, over 77 days

Treatments	1	6	21	42	56	77
-P-B+ore	7.40	7.40	7.60	7.65	7.60	7.55
+P-B+ore	7.40	7.40	7.60	7.65	7.65	7.65
-P+B+ore	7.40	7.45	7.85	8.15	8.10	8.05
+P+B+ore	7.45	8.15	8.75	8.75	8.75	8.70

Little change occurred in the pH during the experiment in the control treatments

(minus bacteria). A slow rise of 0.75 (Table 5.1) was seen in the bacterial treatment without peptone (-P+B) but when the treatment contained both bacteria and peptone (+P+B) the pH rose by 1.30 units. During bacterial growth there was some slow formation of ammonium-nitrogen from the degradation of the cyanide complex which was responsible for the slight pH increase in the absence of peptone. When peptone was present a greater amount of nitrogen became available for the formation of ammonium-nitrogen and this caused a greater rise in pH.

Table 5.2. Changes in pH during sodium tetracyanonickelate biodegradation at pH 10, over 77 days

Treatments	1	7	21	42	56	77
-P-B+ore	9.55	8.35	8.40	8.35	8.35	8.35
+P-B+ore	9.50	8.70	8.60	8.50	8.50	8.45
-P+B+ore	9.65	8.70	8.45	8.35	8.35	8.35
+P+B+ore	9.70	8.95	8.80	8.90	8.85	8.85

In the experiment where the initial pH was 10 (Table 5.2), there was a general decrease in the pH in the controls with a maximum drop of 1.2. Even in the bacterial treatments there was a trend downwards but the slowest decline was in the bacterial treatments with peptone present, these only decreased by 0.90 pH units. In the two experiments, although the initial pH was 8 and 10, the final pH for the bacterial treatments ended approximately in the same range, 8.05-8.35 for minus peptone and 8.70-8.85 with peptone.

5.3.2 *Degradation of sodium tetracyanonickelate*

5.3.2.1 *Degradation at pH 8*

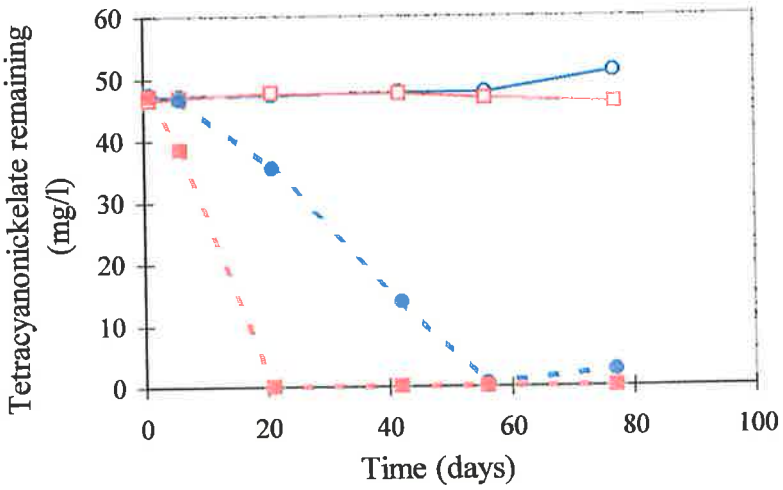

Figure 5.1. Degradation (mg l^{-1}) of sodium tetracyanonickelate at pH 8. Treatments are ○ =-P-B; ● = -P+B; □ = +P-B and ■= +P+B, RPII-HPLC data

Biological degradation of sodium tetracyanonickelate proceeded rapidly in the presence of peptone at pH 8 with a rate of 2.29 mg l^{-1} day^{-1}. Without peptone, the rate was 0.86 mg l^{-1} day^{-1}, this being almost three times slower than with peptone (Figure 5.1). No chemical changes were observed in the sterile control treatments.

5.3.2.2 *Degradation at pH 10*

At pH 10 (Figure 5.2), degradation only occurred when peptone was present but with a rate of only 0.62 mg l^{-1} day^{-1}, which was much slower than that at pH 8.

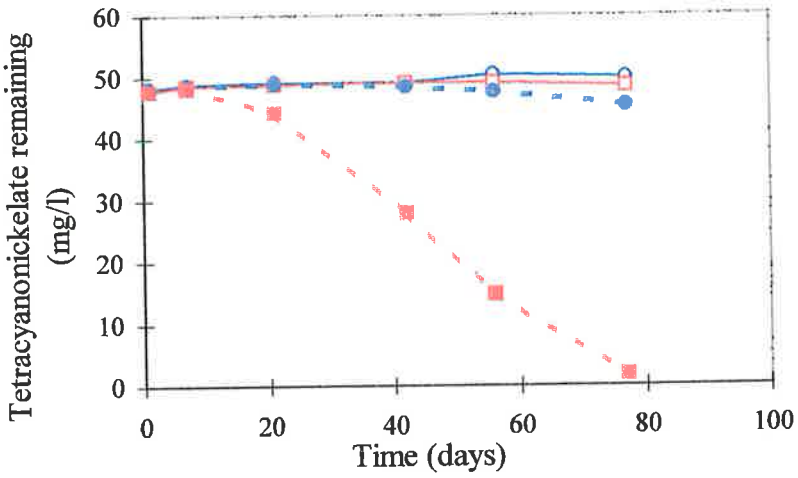

Figure 5.2. Degradation (mg l^{-1}) of sodium tetracyanonickelate at pH 10. Treatments are O =-P-B; ● = -P+B; □ = +P-B and ■ = +P+B, RPII-HPLC data

Sodium tetracyanonickelate degradation may have been just starting in the minus peptone treatment at 77 days. As in the pH 8 experiment the sterile control treatments remained unchanged throughout the test period.

5.3.3 *Total-CN, WAD-CN and end products*

5.3.3.1 *Ammonium-nitrogen at pH 8*

From the plus peptone treatment ammonium-nitrogen production was initiated very quickly rising to the maximum of 18 mg l^{-1} by 41 days (Figure 5.3). By 21 days all of the tetracyanonickelate had degraded (Figure 5.1), and from this point on, most of the ammonium-nitrogen was formed.

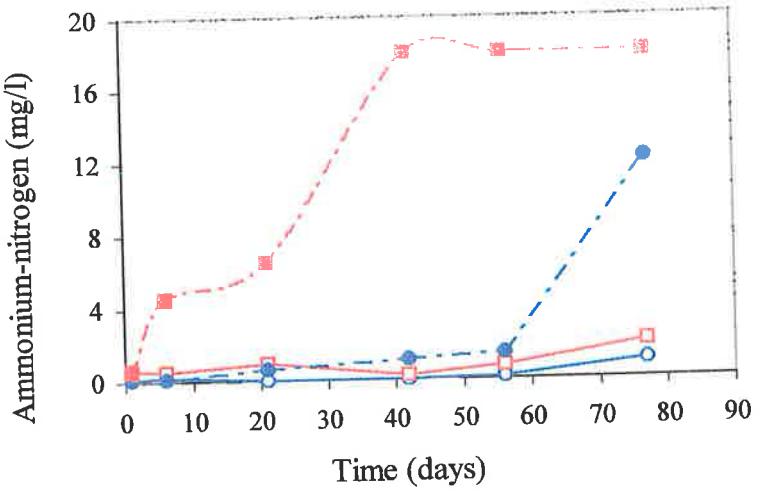

Figure 5.3. Ammonium-nitrogen produced during sodium tetracyanonickelate degradation at pH 8. Treatments are O =-P-B; ● = -P+B; □ = +P-B and ■ = +P+B.

In the minus peptone cultures the ammonium-nitrogen level was not seen to rise till the 56th day, at which point all of the tetracyanonickelate had been degraded (Figure 5.1). Although the maximum value of ammonium-nitrogen, for the minus peptone cultures, was 12.1 mg l^{-1} at 77 days it was rising rapidly and probably would have gone higher.

5.3.3.2 *Ammonium-nitrogen at pH 10*

In Figure 5.4, the level of ammonium-nitrogen started to increase for the plus peptone culture, on the 21st day of the experiment and only reached 18 mg l^{-1} on the 77th day. As soon as the degradation of tetracyanonickelate began ammonium-nitrogen was detected.

Only a trace of ammonium-nitrogen was measured in the minus peptone treatment.

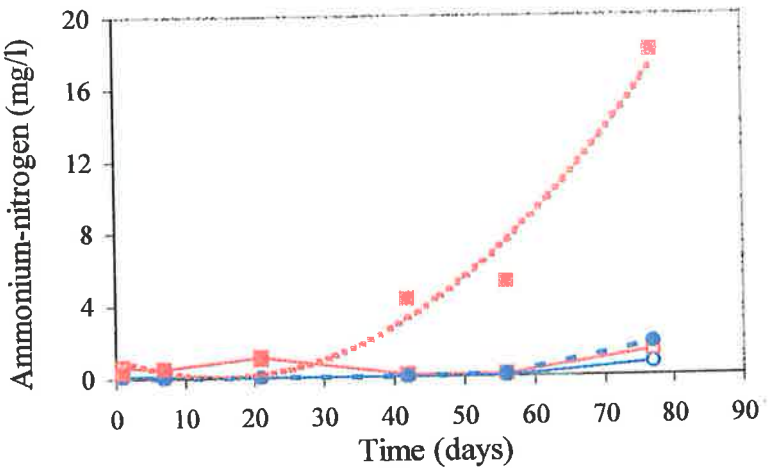

Figure 5.4. Ammonium-nitrogen produced during sodium tetracyanonickelate degradation at pH 10. Treatments are ○ =-P-B; ● = -P+B; □ = +P-B and ■ = +P+B.

5.3.3.3 *Values for Total-CN, WAD-CN and end products*

Table 5.3. End products from degradation of sodium tetracyanonickelate at pH 8

Treatments	Total-CN	WAD-CN	CNO^-	Ammonium-N
-P-B	30.8	26.8	0.9	1.0
+P-B	27.1	26.4	1.1	2.0
-P+B	<0.1	<0.1	14.4	12.1
+P+B	<0.1	<0.1	25	18.0

All of the Total-CN and WAD-CN were degraded by the bacteria present in the pH 8 experiment. Both cyanate and ammonium-nitrogen were found as end products (Table 5.3).

When 0.5 mM Na_2NiCN_4 solution was used the maximum CN^- possible is 52 mg l^{-1} and the possible ammonium-nitrogen 28 mg l^{-1}. In the plus peptone

cultures, ammonium-nitrogen could be produced from the peptone as well as from the tetracyanonickelate(II) but in the absence of peptone, the nitrogen was formed only from the cyanide compound. In this treatment, the total amount of nitrogen accounted for, both in the cyanate and ammonium at termination, was 16.9 mg l^{-1} which corresponds to 31.4 mg l^{-1} CN^-.

Table 5.4. End products from degradation of sodium tetracyanonickelate at pH 10

Treatment	Total-CN	WAD-CN	CNO^-	Ammonium-N
-P-B	32.6	28.4	1.1	0.7
+P-B	30.4	27.8	3.0	1.4
-P+B	27.6	27.5	<0.3	1.8
+P+B	0.9	1.2	<0.3	18.0

A small amount of degradation was observed in the absence of peptone at the higher pH (Table 5.4) but almost all of the Total-CN and WAD-CN were degraded in the presence of peptone. Only ammonium-nitrogen was measured as an end product at the termination of the experiment at 77 days. No free cyanide or cyanate was detected.

5.3.4 *Bacterial counts*

Bacterial counts were carried out on days 55 and 77 during the degradation of sodium tetracyanonickelate at pH 8 and pH 10.

Sterility was maintain throughout the experiment (Table 5.5), as evidenced by no isolation of bacterial colonies from the controls on any of the three media. On

each of the agars the highest isolation counts were associated with the peptone nutrient being present in the treatment.

Table 5.5. Bacterial colony counts (cfu ml^{-1}) during sodium tetracyanonickelate degradation at pH 8 for two isolation times (all counts x 10^6)

Treatments	TSA	DMS+P		DMS+P+$Na_2Ni(CN)_4$	
	55 days	55 days	77 days	55 days	77 days
-P-B+ore	0*	0*	0*	0*	0*
+P-B+ore	0*	0*	0*	0*	0*
-P+B+ore	1.0	0.8	1.7	5.7	3.6
+P+B+ore	16.9	8.3	18.9	20.0	25.5

* Also zero colonies at 10^{-1}

Of the three isolation media, the best was that which contained sodium tetracyanonickelate. This showed that the cells had adapted to this cyanide compound.

By the end of the experimental period of 77 days bacterial numbers had decreased in the no peptone cultures. At this time no tetracyanonickelate was available as an energy source since it had all been degraded (Figure 5.1). There were only slight increases in numbers in the plus nutrient (peptone) cultures when isolations were carried out using agar containing sodium tetracyanonickelate (Table 5.5). Counts for the peptone cultures were high at 55 days and higher at 77 days. This showed that the bacterial population being isolated from the shake flask cultures containing peptone, did not require the presence of sodium tetracyanonickelate since this had all been degraded by 21 days (Figure 5.1).

Table 5.6. Bacterial colony counts (cfu ml^{-1}) during sodium tetracyanonickelate degradation at pH 10 for two isolation times (all counts x 10^6)

Treatments	TSA	DMS+P		DMS+P+$Na_2Ni(CN)_4$	
	55 days	55 days	77 days	55 days	77 days
-P-B+ore	0*	0*	0*	0*	0*
+P-B+ore	0*	0*	0*	0*	0*
-P+B+ore	0.8	0.3	0.4	1.1	4.7
+P+B+ore	NA**	115.0	187.0	50.0	174.0

* Also zero colonies at 10^{-1} **NA indicates data is not available

High levels of bacteria were found in the peptone treatment (Table 5.6) at pH 10, much higher than at pH 8. This occurred because the isolations were carried out during the very active phase of the break down process where the tetracyanonickelate was present (Figure 5.2) up to the last (77^{th}) day. Overall the bacterial numbers for the no peptone cultures were lower in all three media except at 77 days on the DMS+P+$Na_2Ni(CN)_4$ agar when compared to numbers at pH 8. Very high numbers were found for plus peptone cultures in the presence and absence of sodium tetracyanonickelate (Table 5.6). Whereas the microflora from the no peptone shake flask cultures responded to the incorporation of sodium tetracyanonickelate in the agar, giving numbers more than 10 times greater, by the end of the experiment. This did not occur in the plus peptone cultures where the highest numbers were from the DMS+P agar, at both isolation times.

5.3.5 *Bacterial isolations*

No bacterial colonies were identified from either of the experimental pH in the

minus peptone (-P) treatments. A very small (pin-point) type of bacterial colony, as in Chapter 3, could be seen on the surface of the DMS isolation agar but this colony could not be regrown.

This pin-point colony type was also found in the cultures containing peptone at both pH 8 and 10. In addition, at pH 8 from the plus peptone (+P) treatment, a few colonies of *Pseudomonas stutzeri*, *Bacillus firmus*, *Bacillus sphaericus* and *Sphingomonas paucimobilis* (Williams *et al.*, 1989) were identified. A lower diversity grew from the pH 10 cultures, with only a few colonies of *P. stutzeri* and *Bacillus filicolonicus* being named using the MIDI-FAME technique. A representative of each bacterial species isolated during the shake flask experiments were individually tested for the degradation of sodium tetracyanonickelate and results are reported in Chapter 9.

5.4 *Chapter overview*

At pH 8, sodium tetracyanonickelate biodegradation occurred in the presence and absence of peptone (Figure 5.1). The rate of cyanide degradation was, however, higher in the presence of peptone 2.29 mg $l^{-1}day^{-1}$ compared to 0.86 mg $l^{-1}day^{-1}$ when without peptone. With added peptone, degradation of tetracyanonickelate(II) was complete by day 21, whereas it took 56 days for complete degradation of tetracyanonickelate in the absence of peptone. This clearly indicated the difference made by adding a carbon and nitrogen source (peptone).

The beneficial value of adding peptone was also reflected in the increased bacterial numbers. In the presence of peptone, microbial numbers were

approximately an order of magnitude higher, 10^7 cell ml^{-1} compared to 10^6 cells ml^{-1} without added peptone.

At pH 10, degradation of tetracyanonickelate was only observed when peptone was a supplement in the inoculated cultures (Figure 5.2). The degradation rate in these cultures was measured at 0.62 mg $l^{-1}day^{-1}$.

As a result of sodium tetracyanonickelate degradation ammonium-nitrogen concentration increased over the incubation period (Figure 5.3). In the presence of peptone, the ammonium-nitrogen was formed from peptone plus cyanide degradation and ammonium-nitrogen concentrations reached a maximum value of 18 mg l^{-1} within 41 days (Figure 5.3). In the absence of peptone, ammonium-nitrogen concentrations increased gradually over the incubation period and by day 77 had reached a concentration of 12.1 mg l^{-1}. Ammonium-nitrogen production only began when the tetracyanonickelate had been totally degraded (Figure 5.3) i.e. at day 56.

When peptone was present, the concentration of ammonium-nitrogen (Figure 5.4) was lower at pH 10 (18 mg l^{-1} at day 77) compared to pH 8 (18 mg l^{-1} at day 41), due to the slower degradation rate of sodium tetracyanonickelate and utilization of peptone at the higher pH. For this treatment, production of ammonium-nitrogen began concurrently with the start of degradation of the cyanide complex.

The WAD-CN concentrations were reduced to below detection limits at pH 8 in both the absence and presence of peptone. An accumulation of cyanate varied between the two bacterial treatments 14.4 mg l^{-1} without peptone and 25 mg l^{-1} with peptone added (Table 5.3). The break down of tetracyanonickelate proceeded through cyanate as an intermediary product then this degraded further to

ammonium-nitrogen (Figures 5.1 and 5.3). Over 60 % of the nitrogen in the added tetracyanonickelate was accounted for in the cyanate and ammonium-nitrogen, in the absence of peptone.

At pH 10, no cyanate was detected. This finding indicated that a slower degradation pathway operated in the absence of peptone. For the peptone cultures because no cyanate was found, a different pathway was activated and consequently the direct formation of end products such as carbon dioxide and ammonia resulted.

Possibly the enzyme responsible for cyanate hydrolysis (i.e. Cyanase) was not activated in the bacteria at pH 8 due to higher ammonia concentrations leading to its repression or the cyanase enzyme was activated but later inhibited by high concentrations of ammonia.

At pH 10, cyanate was not present at termination of the experiment. Therefore, either cyanate was not formed or it was hydrolysed to ammonium-nitrogen and carbon dioxide by cyanase.

The results in this chapter contrasts with the work of Silva-Avalos *et al.* (1990) who found that tetracyanonickelate was not degraded in the presence of ammonia which was added as ammonium sulfate. The data presented here, however, showed that at pH 8 in the presence and absence of peptone, cyanate was formed and that under certain conditions, tetracyanonickelate was degraded at the same time that ammonium-nitrogen was formed. Therefore, tetracyanonickelate did degrade in the presence of ammonia when it was a by-product.

Chapter 6.0 Degradation of sodium tetracyanonickelate in bioreactors

6.1 *Introduction*

Reports from various research groups have shown that some work was carried out on the biodegradation of tetracyanonickelate (Chapter 5). The only commercial process in North America (and possibly in the World) uses *Pseudomonas paucimobilis* (*Sphingomonas paucimobilis*) as the biodegrading organism and this is operating at the Homestake Gold Mine. The system was commissioned in 1984 (Dubey and Holmes, 1995). At this mine, the influent wastewater contained a very low level of nickel cyanide, 0.1-0.5 mg l^{-1}, as part of the Total-CN and WAD-CN. The proposed permit limits for Total-CN and WAD-CN were 1.8 mg l^{-1} and 0.18 mg l^{-1} respectively (Mudder *et al.*, 1984). Very little degradation was required to reach the permit limits.

A bacterial strain *Pseudomonas fluorescens* NCIMB 11764 was used to show utilization of the nitrogen in potassium cyanide and potassium tetracyanonickelate (Harris and Knowles, 1983a; Rollinson *et al.*, 1987). Another group (Silva-Avalos *et al.*, 1990) has shown that for their biodegrading organism, *Pseudomonas putida* BCN3, reduction of the substrate and growth occurred in parallel. However, degradation by *P. putida* did not occur when ammonia was present. Few other genera or species have been investigated for metal cyanide degradation.

One litre BELLCO vessels were used as bioreactors A and B under non sterile conditions, for the degradation of sodium tetracyanonickelate in the research for this chapter. These bioreactors contained the same ore that had been introduced during the pretreatment preparation (Chapter 2) before starting the two copper cyanide experiments (Chapter 4). No additional ore or peptone was added in this study. The biomass attached to the ore particles, was the same as that used for the copper(I) cyanide degradation experiments. After the two copper(I) cyanide degradation cycles a newly prepared batch of DMS medium containing sodium tetracyanonickelate was added to each bioreactor.

6.2 *Materials and methods*

6.2.1 *Degradation of sodium tetracyanonickelate*

Having completed two degradation cycles with copper cyanide (Chapter 4), the medium was replaced with new DMS at pH 9.4, spiked with 0.5 mM sodium tetracyanonickelate (Na_2NiCN_4). As in Chapter 5, sodium tetracyanonickelate was prepared by adding 0.5 mM nickel sulfate (Analar) and 2 mM sodium cyanide (Merck) to a litre of DMS medium at pH 9.4 (reaction 1). The DMS medium containing sodium tetracyanonickelate was left non sterile for the degradation studies in the two bioreactors.

$$NiSO_4 + 4NaCN \rightarrow Na_2NiCN_4 + Na_2SO_4 \qquad (1)$$

The two degradation cycles were monitored over a period of 56 days for the 1st cycle and 27 days for the 2nd cycle.

6.2.2 *Determination of end products*

During the test period, 10 ml aliquots were removed at set times and filtered through a 0.45 μm syringe tip filter. The pH, free cyanide, Total-CN, WAD-CN, ammonium-nitrogen and cyanate concentration were determined as detailed in Chapter 2. Free cyanide was determined according to the procedure described in Standard Methods for the Examination of Water and Wastewater (Greenberg *et al.*, 1992). Briefly, filtrates were titrated with silver nitrate (0.0031 M) after the addition of *p*-dimethylaminobenzylidene rhodanine.

Total cyanide and WAD cyanide were analysed using an Alpkem Flow Solution 3000 Auto Analyzer (Perstorp Analytical Environmental) according to the procedure described in Standard Methods for the Examination of Water and Wastewater (Greenberg *et al.*, 1992). Ammonium-nitrogen was measured using the Berthelot colour reaction. Cyanate (CNO^-) was converted to ammonium and analyzed using an ammonia ion selective electrode supplied by Orion (Greenberg *et al.*, 1992). All analyses were duplicated and if necessary multiple repeats were done.

6.2.3 *Bacterial counts and isolations*

The cultures in the bioreactors were assessed for colony numbers at 50 days in cycle 1, and at 7 and 27 days for cycle 2. Three agars were used for the growth of the bacterial cells, from duplicate subsamples, DMS plus sodium tetracyanonickelate; DMS with peptone and DMS with peptone and sodium tetracyanonickelate, all at pH 9.4 (Na_2NiCN_4 at the same level as in the bioreactors). The Midi-FAME bacterial identification was done on 31 colonies in duplicates.

6.3 *Results and discussion*

6.3.1 *Degradation of sodium tetracyanonickelate, two cycles*

The first cycle determining degradation of sodium tetracyanonickelate lasted 56 days (Figure 6.1) and the second cycle was monitored for 27 days (Figure 6.2).

Table 6.1. Changes in pH during sodium tetracyanonickelate degradation, first cycle (56 days)

Bioreactors	0	2	6	14	20	27	34	41	48	56
A	8.40	8.25	8.20	8.05	8.10	8.10	8.20	8.30	8.40	8.35
B	8.45	8.05	8.05	8.20	8.35	8.35	8.35	8.35	8.30	8.30

There was very little fluctuation in the pH in the first degradation cycle conducted in the bioreactors (Table 6.1). A movement of 0.35 units for bioreactor A and 0.40 in bioreactor B over the 56 days of monitoring. In an aerobic system, the tetracyanonickelate(II) remains very stable over a large pH range (Osseo-Asare *et al.*, 1984). Therefore no natural degradation via chemical processes was expected.

Table 6.2. Changes in pH during sodium tetracyanonickelate degradation, second cycle (27 days)

Bioreactors	0	1	3	5	7	10	14	20	27
A	8.55	8.45	8.40	8.35	8.25	8.25	8.25	8.25	8.40
B	8.50	8.25	8.20	8.20	8.40	8.40	8.40	8.40	8.35

During the second degradation cycle (Table 6.2) both bioreactors A and B had shifts of 0.30 pH units over the 27 days of the experiment.

6.3.1.1 *First degradation cycle*

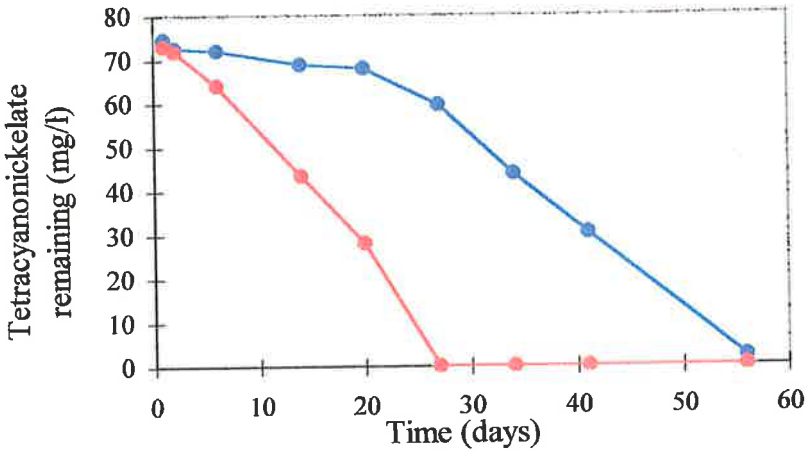

Figure 6.1. Degradation of sodium tetracyanonickelate (mg l^{-1}) over 56 days, bioreactor A = ●, bioreactor B = ●, RPII-HPLC data

In the first cycle, the break down processes monitored by multiple injection of filtered samples into the HPLC, took 20 days to begin in bioreactor A but after this lag period the degradation rate was rapid. The first degradation progressed quicker in bioreactor B, with no lag period and a degradation rate of 2.9 mg l^{-1} day^{-1} compared with 1.9 mg l^{-1} day^{-1} in bioreactor A. All of the tetracyanonickelate(II) had been utilized by day 27 in bioreactor B (Figure 6.1) but this took 56 days in bioreactor A.

6.3.1.2 *Second degradation cycle*

In Figure 6.2, the plotted data indicated that in both bioreactors, the degradation processes started quickly. In bioreactor A, all of the metal cyanide had disappeared by 27 days and in bioreactor B by 10 days.

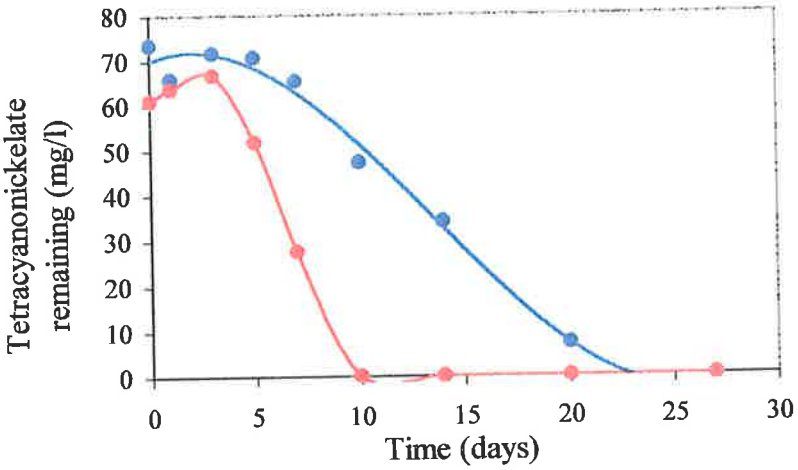

Figure 6.2. Degradation of sodium tetracyanonickelate (mg l^{-1}) over 27 days, bioreactor A = ●, bioreactor B = ●, RPII-HPLC data

The degradation rates for bioreactors A and B were 3.2 mg l^{-1} day^{-1} and 9.6 mg l^{-1} day^{-1} respectively. A 3.3 fold increase in the rates of degradation was measured in bioreactor B during the second cycle when compared to the first cycle and in bioreactor A, a 1.7 fold increase was measured.

6.3.2 *Total-CN, WAD-CN and end products*

6.3.2.1 *First degradation cycle*

A steady decrease for Total-CN and WAD-CN, was seen in Figure 6.3. Total-CN showed a 0.7 mg l^{-1} day^{-1} rate of degradation and WAD-CN gave a slightly lower rate of 0.6 mg l^{-1} day^{-1}.

In bioreactor A, the maximum level of cyanate was observed from 14 to 20 days followed by a steady decrease to zero. At 20 days, ammonium-nitrogen levels

began to rise to reach 27 mg l^{-1} by 56 days, where the maximum value possible was 28 mg l^{-1}.

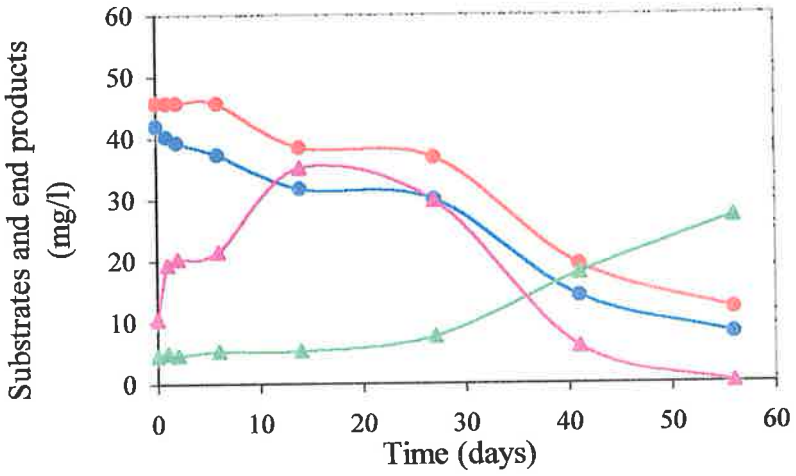

Figure 6.3. Degradation of sodium tetracyanonickelate (mg l^{-1}) in bioreactor A. Total-CN = ●; WAD-CN = ●; Ammonium-nitrogen = ▲ and cyanate = ▲

At day 14, Total-CN, WAD-CN and cyanate was present but no ammonium-nitrogen. By day 56, some Total-CN and WAD-CN was still measured but no cyanate and a high level of ammonium-nitrogen. Degradation of the cyanide progressed through the cyanate and onto ammonium-nitrogen (reaction 2).

$$CN^- \rightarrow CNO^- \rightarrow CO_2 + NH_4^+ \qquad (2)$$

In bioreactor B, Total and WAD cyanides disappeared quickly with a degradation rate for each of 1.4 mg l^{-1} day^{-1} (Figure 6.4). The production of cyanate began almost immediately reaching 30 mg l^{-1}, the highest level by day 2 and remained high throughout the test period.

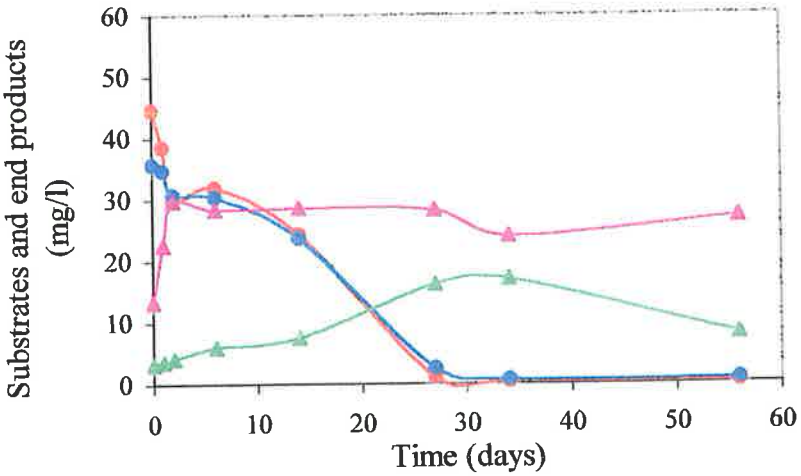

Figure 6.4. Degradation of sodium tetracyanonickelate(mg l^{-1}) in bioreactor B. Total-CN = ●; WAD-CN = ●; Ammonium-nitrogen = ▲ and cyanate = ▲

A low level of ammonium-nitrogen was found during most of the experiment, with a maximum of 17 mg l^{-1} at 34 days. Also some nitrification or assimilation of ammonium-nitrogen occurred after day 34 since the level decreased to 8 mg l^{-1} by 56 days.

During the first 27 days the cyanide was oxidized to cyanate. After day 27, no tetracyanonickelate remained and the cyanate stayed almost at a constant level till day 56. The cyanate was not converted to ammonium-nitrogen. Two processes were operating in this bioreactor; the tetracyanonickelate oxidation to cyanate (reaction 3) which was analogous to findings by Harris and Knowles (1983b) and the tetracyanonickelate conversion to ammonium-nitrogen and CO_2 (reaction 4) again following the work with cyanide by Harris and Knowles (1983a).

$$CN^- + O_2 + H^+ + NADPH \rightarrow CNO^- + NADP^+ + H_2O \qquad (3)$$

$$CN^- + O_2 + 2H^+ + NADPH \rightarrow NH_4^+ + CO_2 + NADP^+ \qquad (4)$$

6.3.2.2 *Second degradation cycle*

As in the first cycle, duplicate samples were taken at 8 different times for analyses.

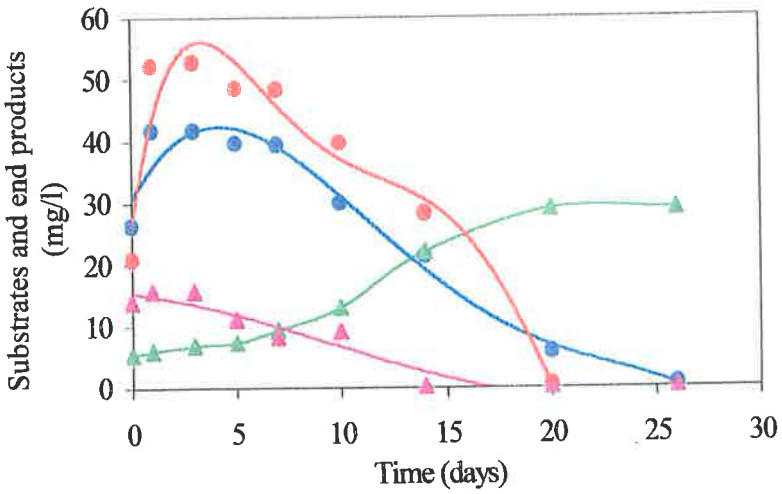

Figure 6.5. Degradation of sodium tetracyanonickelate (mg l^{-1}) in bioreactor A. Total-CN = ●; WAD-CN = ●; Ammonium-nitrogen = ▲ and cyanate = ▲.

During the second degradation cycle, reactions in bioreactor A were similar to those in the first. There was a rapid loss of Total-CN to complete degradation in 20 days with a 3.1 mg l^{-1} day^{-1} rate of degradation, and WAD-CN reached zero by 27 days (Figure 6.5) giving a degradation rate of 1.7 mg l^{-1} day^{-1}. This was accompanied by a low production of cyanate peaking at 16 mg l^{-1} at day 3 and not detected after 14 days. A gradual but steady increase in the amount of ammonium-nitrogen was seen over the duration of the experiment, 27 days.

The highest amount of ammonium-nitrogen measured was 29 mg l^{-1} at 20 days which accounted for all of the nitrogen originally in the tetracyanonickelate. The degradation process again progressed from tetracyanonickelate to cyanate with a further conversion to ammonium-nitrogen (reaction 5).

$$CN^- \rightarrow CNO^- \rightarrow CO_2 + NH_4^+ \qquad (5)$$

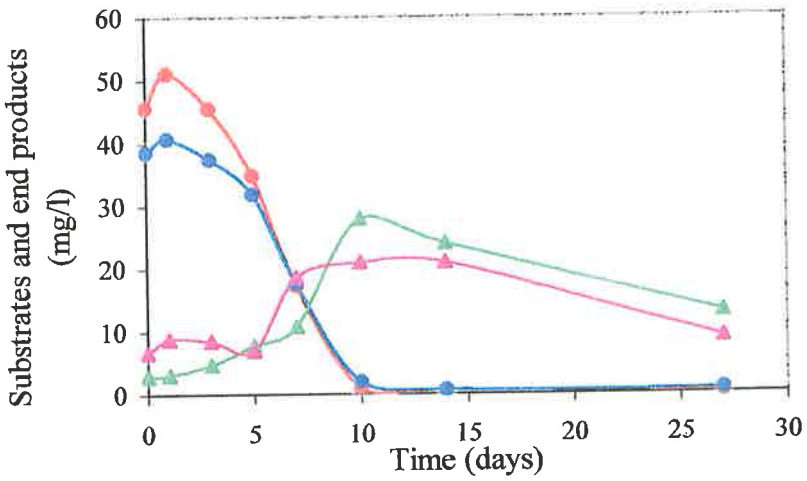

Figure 6.6. Degradation of sodium tetracyanonickelate (mg l^{-1}) in bioreactor B. Total-CN = ●; WAD-CN = ●; Ammonium-nitrogen = ▲ and cyanate = ▲.

By 10 days, both the Total and WAD cyanide levels had reached zero in bioreactor B in the second cycle (Figure 6.6). Rates of degradation for Total-CN and WAD-CN were 5.7 mg l^{-1} day^{-1} and 4.6 mg l^{-1} day^{-1} respectively. The production of cyanate and ammonium-nitrogen occurred simultaneously in the bioreactor with ammonium-nitrogen reaching a maximum of 28 mg l^{-1} at day 10, after which a slow decrease to 13 mg l^{-1} occurred by the end of the experiment, indicating that nitrification was occurring.

Cyanate reached a maximum of 21 mg l^{-1} at day 10 and decreased down to 9 mg l^{-1} by day 27. The cyanate was converted to ammonium-nitrogen but very rapidly may have been incorporated into amino acids and proteins in the increasing biomass (Figure 6.6).

Again two processes occurred in bioreactor B, the oxidation of tetracyanonickelate to cyanate (reaction 3) and the formation of ammonium-nitrogen from the tetracyanonickelate (reaction 4), as in the first degradation cycle.

6.3.3 *Bacterial counts*

In the first degradation cycle of sodium tetracyanonickelate, bacteria were counted, at day 50. But in the second cycle, isolations were carried out twice, at 7 and 27 days.

Table 6.3. Bacterial colony counts (cfu ml^{-1}) from sodium tetracyanonickelate degradation, first cycle (all counts x 10^6)

Bioreactors	DMS+P	DMS+ Na_2NiCN_4	DMS+P+Na_2NiCN_4
A	0.87	4.70	6.40
B	35.0	48.0	150.0

The inclusion of the test metal cyanide, Na_2NiCN_4, in the isolation agar, was of greater benefit to the microflora in bioreactor A, giving a 5.4 fold increase in the number from the agar containing peptone only (Table 6.3). For bioreactor A, the best performance in terms of colony forming units (cfu ml^{-1}) occurred when both the sodium tetracyanonickelate and peptone were present. Highest counts were again obtained on the agar containing peptone and sodium tetracyanonickelate when inoculated with a sample from bioreactor B.

Overall at the end of the first degradation cycle, there was a 23 fold increase in bacterial numbers in bioreactor B compared to A, when using the most complex isolation agar. For both bioreactors the least beneficial agar was DMS+P which showed that the bacteria were receiving some benefit from the tetracyanonickelate(II).

Table 6.4. Bacterial colony counts (cfu ml^{-1}) from sodium tetracyanonickelate degradation at 7 and 27 days, second cycle (all counts x 10^6)

Bioreactors	Days	DMS+P	DMS+Na_2NiCN_4	DMS+P+Na_2NiCN_4
A	7	3.10	7.50	9.50
	27	2.80	4.10	15.0
B	7	16.0	52.0	49.0
	27	16.0	130.0	170.0

Similar results to those obtained for cycle 1, were seen for the second degradation cycle. Bioreactor A showed high bacterial counts on the DMS+Na_2NiCN_4 agar at day 7, taken during the rapid degradation of the cyanide complex. Moreover, the counts had decreased by 45% at day 27 when all of the cyanide had been degraded. Highest colony numbers were usually obtained when peptone and sodium tetracyanonickelate were added to the isolation agar (Table 6.4).

But in bioreactor B by the end of the test period, 27 days, there was a 11 fold increase in bacterial numbers when compared to bioreactor A.

Looking at the final colony numbers in the two degradation cycles, there was a 2.3 fold increase for bioreactor A in the second cycle compared with a 1.1 fold increase for bioreactor B.

6.3.4 *Bacterial isolations*

After purification of the bacteria isolated during the first cycle, identification of the bacterial species was done using the MIDI-FAME system. By this method many *Pseudomonas stutzeri* were found in bioreactor A. Two other main bacterial types were isolated; one can be described as a white pin-point colony consisting of Gram negative, oxidase positive and catalase positive cocci and the other a brown large colony, which was made up of Gram positive, oxidase positive and catalase positive rods. In bioreactor B, pin-point colonies were many, as well *as P. stutzeri, Sphingomonas paucimobilis* and *Bacillus filicolonicus.*

In the second cycle, the microflora in bioreactor A changed from many pin-point colonies, many *P. stutzeri*, some *S. paucimobilis* and a few *Bacillus* colonies of the species *B. cereus*, *B. filicolonicus* and *B. pumilus* at 7 days to many pin-point colonies, many *P. stutzeri*, some *S. paucimobilis* and many *B. pumilus.*

Similarly in bioreactor B , the main types were pin-point colonies, *P. stutzeri*, *B. pumilus*, *S. paucimobilis* and *B. filicolonicus.*

Pure cultures of species isolated during these experiments were retested for sodium tetracyanonickelate degradation and results are reported in Chapter 9.

6.4 *Chapter overview*

Biodegradation of sodium tetracyanonickelate occurred readily in bioreactor B where the heterotrophic bacteria were very active. For both degradation cycles bioreactor B performed better with rates of 2.9 mg l^{-1} day^{-1} and 9.6 mg l^{-1} day^{-1}. Although in bioreactor A, the rate of degradation increased in the second cycle from 1.9 mg l^{-1} day^{-1} to 3.2 mg l^{-1} day^{-1}, it lagged behind bioreactor B. Similarly when bacterial counts were done, bioreactor B had the highest levels (cfu ml^{-1}).

The degradation process in bioreactor A, proceeded with the formation of cyanate and the subsequent conversion of this to ammonium-nitrogen. This sequence was measured for both degradation cycles. The pathway defined during the experiments suggest that the main enzymes operating were cyanide monoxygenase to form cyanate (Harris and Knowles, 1983b) and the enzyme, cyanase decomposing cyanate to CO_2 and NH_3 (Cohen and Oppenheimer, 1977).

In bioreactor B, there was a simultaneous formation of both cyanate and ammonium-nitrogen. Cyanate was formed early and remained high throughout the 56 days. As in bioreactor A, cyanate was formed by the cyanide monoxygenase enzyme. A slow rate of formation of ammonium-nitrogen to 30 days was noted followed by a gradual bacterial utilization of this intermediary either through nitrification or assimilation.

During the second cycle, a similar pattern of degradation was seen for bioreactor A as in the first cycle. However, the degradation of the tetracyanonickelate(II) was faster as indicated by the rapid decrease in the amount of Total and WAD cyanide. Again cyanate formed early but not at the same time as the ammonium-nitrogen. By 14 days all of the cyanate had been hydrolysed. The ammonium-nitrogen peaked at day 20 and remained high till the end of the experiment.

In bioreactor B, the utilization of the cyanide was very rapid with all of the Total and WAD cyanide degraded by day 10. In this reactor both cyanate and ammonium-nitrogen formed simultaneously followed by a slow decrease for both. Therefore, concurrent to the production of cyanate by cyanide monoxygenase, the

enzyme cyanide dioxygenase produced carbon dioxide and ammonia by an alternate pathway (Harris and Knowles, 1983a).

Subsequently, nitrification of the ammonium-nitrogen was also noted in bioreactor B during both cycles with the probable formation of other nitrogenous end products, e.g. nitrite or nitrate or utilized in the formation of amino acids and proteins during cell growth.

Patterns for the end products suggested that different microflora existed in the two bioreactors although this was not strongly supported by the bacterial isolations. The formation of ammonium-nitrogen was consistently higher in bioreactor A, where the degradation of the end product cyanate coincided with an increase in ammonium-nitrogen. This pattern was not seen in bioreactor B, where cyanate either remained high or decreased at the same time as the ammonium-nitrogen. In bioreactor B, cyanate may have undergone hydrolysis to ammonium-nitrogen at a slow steady rate while the ammonium-nitrogen was either assimilated or converted to nitrite or nitrate.

Biodiversity in the two reactors appeared to be similar with the dominant species found in both being *P. stutzeri*, *S. paucimobilis* (formally *Pseudomonas paucimobilis*), *B. filicolonicus* and *B. pumilus*. Researchers (Skowronski and Strobel, 1969; Meyers *et al.*, 1991) have reported the ability of *B. pumilus* to utilize cyanide. In their work, utilization of cyanide was measured both by using bacterial cells (Skowronski and Strobel, 1969) and cell-free extracts of an enzyme which was not named (Meyers *et al.*, 1991). In more recent work Guilfoyle (1996), at the Parker centre, Perth WA, identified *B. pumilus* as the cyanide

detoxifying bacterium in his research and has started working on the purification of a *Bacillus* enzyme, cyanide dihydratase.

The two most often cited bacterial genera, for bioremediation have been *Pseudomonas* and *Bacillus* and species from these two genera were again involved in the biodegradation of sodium tetracyanonickelate.

Chapter 7.0 Biodegradation of potassium hexacyanoferrate in shake flask cultures

7.1 *Introduction*

The solubility of potassium hexacyanoferrate(III), $K_3Fe(CN)_6$, is known to be high, 330 g l^{-1} at 4°C (Smith and Mudder, 1991). Aqueous solutions of this complex are thermodynamically stable when protected from light and are chemically stable over time. The knowledge that potassium ferricyanide is a strong complex has usually led researchers to dismiss this complex as a problem when degradation of metal cyanide complexes are discussed (Hoecker and Muir, 1987). However, due to both the frequent occurrence of this complex and the high percentage (47%) of cyanide (CN^-) present in the molecule, it has the potential to be hazardous if all of the cyanide does not remain bound.

Some researchers have investigated the stability of this complex in nature (Pablo *et al.*, 1997; Cherryholmes *et al.*, 1985) to assess if in fact it was environmentally safe. Tests for free cyanide gave positive results after only 2 to 4 days in both studies and this release of cyanide in aqueous solutions was attributed to bacterial activity. In one study, hexacyanoferrate(III) solutions were seeded with two bacterial cultures, *Pseudomonas aeruginosa* (ATCC 27883) and *Escherichia coli* (ATCC 259277) and both were found to release cyanide from the anion (Cherryholmes *et al.*, 1985).

Like all metal cyanide complexes, the stability of potassium ferricyanide depends on the pH, Eh, light and other constituents present (Osseo-Asare *et al.*, 1984).

For this chapter, the rates of degradation for potassium hexacyanoferrate were determined using shake flask cultures at two pH levels. Throughout the two experiments an assessment of cyanate and ammonium-nitrogen levels was carried out in order to define the degradation pathway taken under different experimental conditions. Bacterial species present during the breakdown of the hexacyanoferrate(III) were isolated and identified by the MIDI-FAME technique.

7.2 *Materials and methods*

A solution at 200 mg l^{-1} potassium hexacyanoferrate(III) (Merck), dissolved in the DMS medium (Chapter 2), was prepared and 100 ml was dispensed into each of 8 Erlenmeyer flasks. These flasks, with the addition of sterile or non sterile Warroo ore, as appropriate, and with some containing peptone (1 g l^{-1}), became the shake flask cultures. Two experiments were set up, one at pH 8 and the other at pH 10 (Chapter 2) and both were monitored over 58 days. All of the culture flasks were kept under a black cover throughout the experimental period.

Parameters for the RPII-HPLC analyses were the same as for the calibration curve viz: detection wavelength of 413 nm and an eluent consisting of 22% acetonitrile in water and containing 0.005 M tetrabutylammonium sulfate (TBAS).

7.2.1 *Biodegradation of $K_3Fe(CN)_6$ at pH 8 and pH 10*

The same DMS medium formulation and the same number of treatments as for sodium tetracyanonickelate (Chapter 5) was used. An addition of the nutrient, peptone, was included as with the previous experiments and again 0.5% Warroo gold heap leach pad ore provided the bacterial inoculum. If the treatment was required to be abiotic, the ore was sterilized by steam autoclaving at 100 kPa for

20 min before inclusion into the growth medium. All the ingredients including potassium hexacyanoferrate, ($K_3Fe(CN)_6$), were added and pH adjusted to pH 8 or pH 10, before the medium was autoclaved. Flasks were sampled 6 times during the test period and each time two or three aliquots from each treatment were filtered through a 0.45 μm filter, these were then injected into the HPLC. Details of the treatments for the shake flask cultures at pH 8 and pH10 are given in Table 7.1.

Table 7.1. Description of the treatments

Treatments	Description
-P-B+ore+$K_3Fe(CN)_6$	no peptone plus sterile ore (abiotic)
-P+B+ore+$K_3Fe(CN)_6$	no peptone plus ore (biotic)
+P-B+ore+$K_3Fe(CN)_6$	peptone plus sterile ore (abiotic)
+P+B+ore+$K_3Fe(CN)_6$	peptone plus ore (biotic)

All of the treatments were prepared in the DMS medium containing 200 mg l^{-1} of potassium hexacyanoferrate(III). Some had an addition of peptone (+P) while others did not (-P). The bacterial cells were attached to the ore particles and by the addition of the ore the cultures were inoculated (Table 7.1). Autoclaving the ore resulted in a sterile environment for the abiotic treatments. It was important to add sterile ore to the appropriate cultures, so that a consistent background of chemical compounds could be maintained.

7.2.2 *Determination of end products*

Aliquots of 8 ml were filtered through 0.45 μm filters for Total-CN, WAD-CN and cyanate analyses which were done in duplicates at the end of the test period of 58 days.

Descriptions of the tests done are detailed in Chapter 2. The ammonium-nitrogen levels were determined six times during each of the two experiments with duplicates for each treatment (Chapter 2).

7.2.3 *Bacterial counts and isolations*

Isolations of bacteria were only carried out at the end of the experimental period. Four agar preparations were used TSA (Tryptic Soy Agar) at pH 7.2, DMS with $K_3Fe(CN)_6$, DMS with peptone and DMS containing $K_3Fe(CN)_6$ plus peptone. All of the DMS agars were adjusted to pH 9.4. Appropriate dilutions were plated in duplicates, onto the four agars and were incubated at 27°C in an incubator. At weekly intervals, for 4 weeks, colony forming units (cfu) on each of the agar plates were counted. After the incubation period bacterial colonies were described and purified. Representative colony types of 22 bacteria were then prepared as duplicates for gas chromatographic determinations of the fatty acids methyl esters (FAME) using the MIDI Inc. software.

7.3 *Results and discussion*

7.3.1 *Potassium hexacyanoferrate(III) pH studies*, $K_3Fe(CN)_6$

Table 7.2. Changes in pH over 58 days during degradation of potassium hexacyanoferrate, at pH 8

Treatments	1	8	15	22	35	58
-P-B+ore	7.80	7.60	7.70	7.65	7.70	7.70
+P-B+ore	7.90	7.75	7.80	7.75	7.80	7.75
-P+B+ore	7.90	7.70	7.75	7.75	7.75	7.60
+P+B+ore	7.85	8.45	8.70	8.75	8.80	8.70

Treatment notations are explained in Chapter 2. Negligible changes in pH were observed in the abiotic treatments (controls) and this was also true for the bacterial treatment where peptone was omitted (-P+B). A gradual rise in pH of 0.95 pH units, due to bacterial growth was observed in the cultures with added peptone (Table 7.2). Bacterial production of ammonium-nitrogen during cell growth was responsible for the pH rise and in this treatment ammonium-nitrogen could be formed from biodegradation of both peptone and the hexacyanoferrate(III).

Table 7.3. Changes in pH over 58 days during degradation of potassium hexacyanoferrate at pH 10

Treatments	1	9	16	23	35	58
-P-B+ore	9.55	8.30	8.20	8.25	8.20	8.15
+P-B+ore	9.60	8.65	8.55	8.55	8.45	8.45
-P+B+ore	9.60	8.30	8.30	8.30	8.25	8.20
+P+B+ore	9.70	8.10	8.55	8.75	8.85	8.90

Over the first 9 days at the higher pH, a drop of 0.95-1.60 was seen in the four treatments, this was probably due to a chemical interaction between reagents in the DMS medium and the potassium hexacyanoferrate. The results of these interactions were solutions in equilibrium at an approximate pH of 8.30 (Table 7.3). Only in the bacterial treatment containing peptone was a slow rise in pH seen between 9 and 58 days, reaching pH 8.90.

7.3.2 *Degradation of potassium hexacyanoferrate*

7.3.2.1 *Degradation at pH 8*

During the experiment, replicate samples were taken 6 times from the shake flasks cultures.

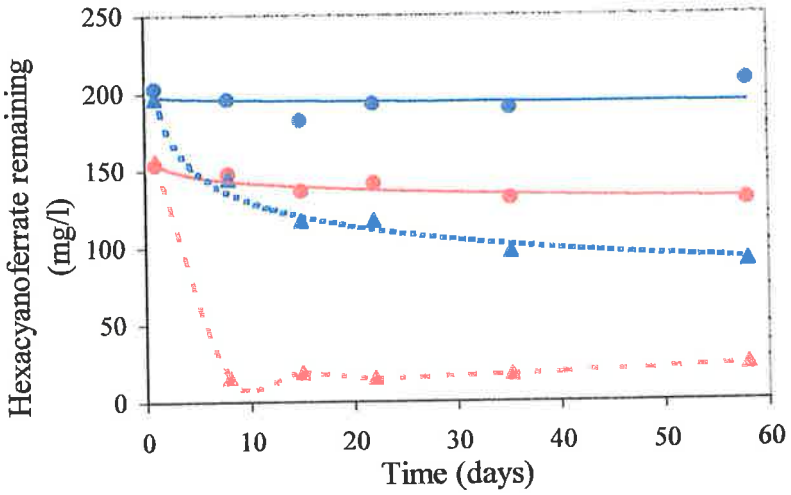

Figure 7.1. Degradation of potassium hexacyanoferrate in shake flask cultures at pH 8, -P-B = ●; -P+B = ▲ ; +P-B = ● and +P+B = ▲, RPII-HPLC data

From the results (Figure 7.1), it was seen that a reaction caused a decrease in the initial value of potassium hexacyanoferrate for two treatments (+P-B and +P+B) when peptone was present at pH 8. Over time this reaction appeared to cease since in the abiotic control treatment (+P-B), there was little further reduction of the level of potassium hexacyanoferrate found in the samples tested (Figure 7.1). It is possible that the peptone was involved in the reduction of the FeIII complex to the more stable FeII complex. Almost immediately, a sharp drop was seen with the bacteria present in the peptone added cultures, giving a degradation rate of 17.5 mg $l^{-1}day^{-1}$.

A very steady but slower decrease in the ferricyanide levels was found when peptone was absent, with a degradation rate of 1.86 mg $l^{-1}day^{-1}$. No decrease in the amount of hexacyanoferrate was observed in the abiotic cultures (-P-B) which acted as a control.

7.3.2.2 *Degradation at pH 10*

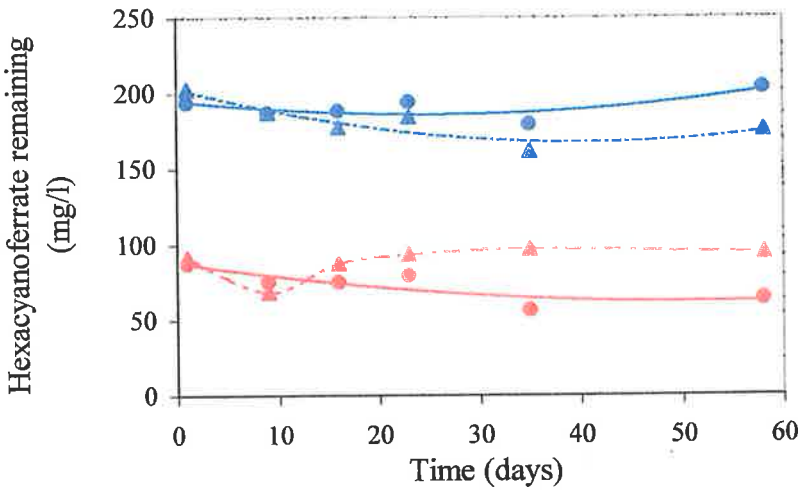

Figure 7.2. Degradation of potassium hexacyanoferrate in shake flask cultures at pH 10, -P-B = ●; -P+B = ▲ ; +P-B = ● and +P+B = ▲, RPII-HPLC data

The peptone appeared to have a greater interaction with the potassium hexacyanoferrate at pH10 (Figure 7.2), leaving less than 50% of the hexacyanoferrate available for bacterial degradation. Moreover, the pH did not remain at 10 but stabilised at pH 8.5, any degradation occurring would have been measured. In this experiment no degradation of potassium ferricyanide was observed even when peptone was added. A very low degradation rate of 0.47 mg l^{-1} day^{-1} was found for cultures without peptone.

7.3.3 *Total-CN, WAD-CN and end products*

7.3.3.1 *Ammonium-nitrogen at pH 8*

The ammonium-nitrogen level in the added peptone treatment reached a value of 99 mg l^{-1} at 58 days compared to 8.3 mg l^{-1} when peptone was absent (Figure 7.3).

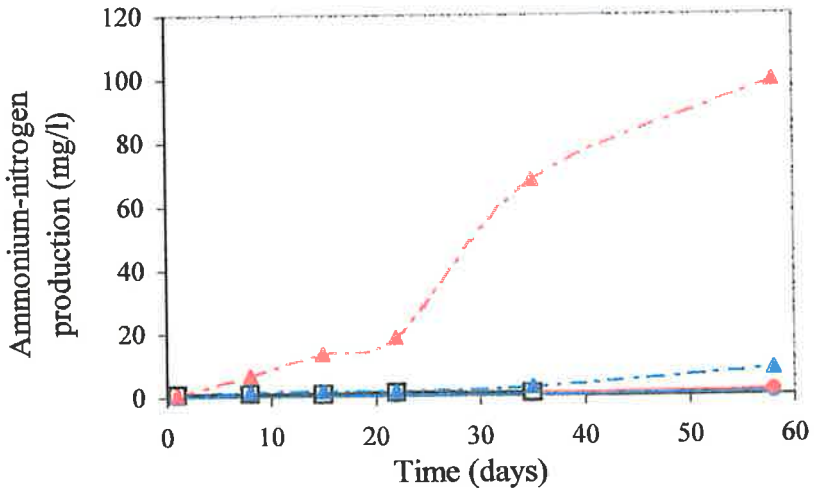

Figure 7.3. Ammonium-nitrogen (mg l^{-1}) produced from potassium hexacyanoferrate degradation at pH 8, -P-B = ●; -P+B = ▲; +P-B = ● and +P+B = ▲.

With the amount of potassium ferricyanide added, the possible maximum concentration for ammonium-nitrogen is 51 mg l^{-1} when only ferricyanide is present but if peptone is also added, the possible maximum ammonium-nitrogen concentration is 218 mg l^{-1}.

Comparing results in Figure 7.3 with the degradation data in Figure 7.1, it was found that all of the hexacyanoferrate was degraded by day 8 in the cultures with added peptone. At this stage only a small amount of ammonium-nitrogen was

present. By day 58 there was 99 mg l^{-1} in the cultures which accounted for 45% of the maximum possible for ammonium-nitrogen.

In the cultures without peptone 16% of the hexacyanoferrate was degraded by day 58 with little ammonium-nitrogen being measured.

7.3.3.2 *Ammonium-nitrogen at pH 10*

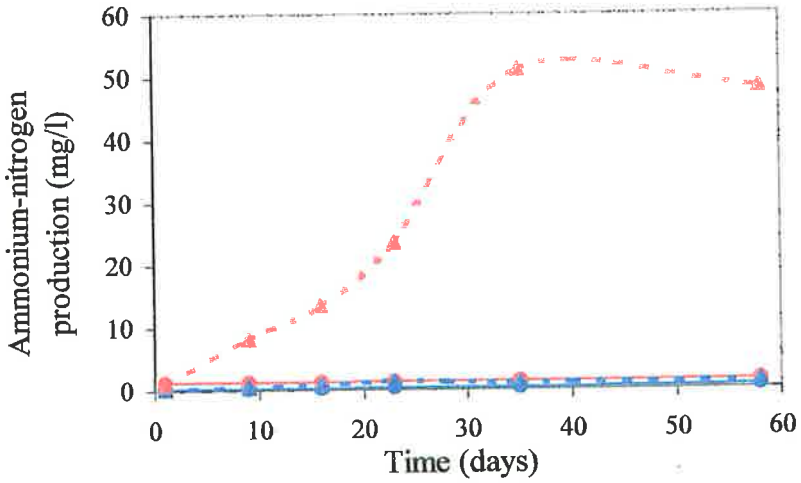

Figure 7.4. Ammonium-nitrogen (mg l^{-1}) produced from potassium hexacyanoferrate degradation at pH 10, -P-B = ●; -P+B = ▲; +P-B = ● and +P+B = ▲.

Since at pH 10 half of the added potassium hexacyanoferrate appeared to be bound to the peptone and little was seen to degrade, the ammonium-nitrogen measured during the experiment originated from the peptone added in this treatment (Figure 7.4). The maximum value of ammonium-nitrogen measured was 51 mg l^{-1} for the plus peptone and 1 mg l^{-1} for the minus peptone treatment.

7.3.3.3 *Values for Total-CN, WAD-CN and cyanate*

When 200 mg l^{-1} of K_3FeCN_6 was used, the maximum possible level of CN^- that can be formed is 95 mg l^{-1}. The measurable amount of Total-CN remaining at

pH 8 was about 1/5th that found in the control for the plus peptone treatment (Table 7.4).

Table 7.4. Cyanide and cyanate levels (mg l^{-1}) from degradation of potassium hexacyanoferrate, at pH 8, after 58 days

Treatments	Total-CN	WAD-CN	CNO^-
-P-B	105.5	6.1	9.4
+P-B	95.3	7.0	10.5
-P+B	44.5	3.1	3.3
+P+B	20.6	2.7	116.4

In the no peptone cultures almost half of the Total-CN was still present at termination which correlated well with the data in Figure 7.1. The conversion of cyanide to cyanate accounted for most of the potassium hexacyanoferrate added. Strong metal cyanide complexes like potassium hexacyanoferrate(III) are not dissociated by weak acids and therefore have low levels of WAD-CN. Free cyanide from the potassium hexacyanoferrate is also included in the Total-CN (Hoecker and Muir, 1987).

Table 7.5. Cyanide and cyanate levels (mg l^{-1}) from degradation of potassium hexacyanoferrate, at pH 10, after 58 days

Treatments	Total-CN	WAD-CN	CNO^-
-P-B	104.9	5.0	9.4
+P-B	101.4	4.3	9.6
-P+B	90.7	4.0	13.5
+P+B	95.0	3.8	1.7

The pH 10, cultures gave little indication of any degradation process occurring. Most of the Total-CN was still present at day 58 when peptone was added. Only a low level of cyanate was detected in the absence of peptone therefore most the cyanide remained bound in the hexacyanoferrate complex (Table 7.5). From Figure 7.2, it appeared that almost 50% of the hexacyanoferrate was not available, however, the data in Table 7.5 showed that it was present. This supports the theory that a K_3FeCN_6 /peptone compound was formed which bound the hexacyanoferrate and as a consequence the iron cyanide anion was not detected by the HPLC.

7.3.4 *Bacterial counts*

Table 7.6. Bacterial colony counts (cfu ml^{-1}) from potassium hexacyanoferrate(III) cultures at pH 8, after 58 days (all counts x 10^6)

Treatments	TSA	DMS+P	DMS+K_3FeCN_6	DMS+P+K_3FeCN_6
-P-B+ore	0*	0*	0*	0*
+P-B+ore	0*	0*	0*	0*
-P+B+ore	3.10	7.20	14.6	14.9
+P+B+ore	25.2	41.0	41.5	72.0

* Also zero colonies at 10^{-1}

Colony counts (cfu ml^{-1}) for the TSA plates were always the lowest. These bacteria, taken from the shake flasks pH 8 cultures and growing on TSA, were dependent on the organic components available in the TSA (Table 7.6). Growth requirements appeared to be better met by the DMS plus $K_3Fe(CN)_6$ agar and best met by the DMS agar containing potassium hexacyanoferrate plus peptone.

In using the medium without peptone, the bacteria were able to manufacture all of the cell components only using the inorganic salts and the efficiency of the bacteria in this regard was demonstrated by the equally high colony forming units (cfu ml^{-1}) counted on the DMS plus $K_3Fe(CN)_6$ and the DMS with $K_3Fe(CN)_6$ plus peptone agars.

The majority of the bacteria isolated from the pH 8 cultures, grew in the temperature range for mesophiles and demonstrated facultative heterotrophic characteristics.

Table 7.7. Bacterial colony counts (cfu ml^{-1}) from potassium hexacyanoferrate(III) cultures at pH 10, after 58 day (all counts x 10^6)

Treatments	TSA	DMS+P	DMS+K_3FeCN_6	DMS+P+K_3FeCN_6
-P-B+ore	0*	0*	0*	0*
+P-B+ore	0*	0*	0*	0*
-P+B+ore	3.05	4.45	12.0	15.3
+P+B+ore	52.5	51.4	134.0	154.0

* Also zero colonies at 10^{-1}

Since it was shown that very little potassium hexacyanoferrate was degraded at pH 10 (Table 7.5), the bacterial counts (cfu ml^{-1}) given in Table 7.7, indicated that the bacteria isolated had mainly utilized the peptone in the shake flask cultures. In the cultures without peptone, degradation may just be starting by 58 days.

7.3.5 *Bacterial isolations*

Bacterial species isolated from pH 8 cultures in the absence of peptone, were characterised by the Fatty Acids Methyl Esters (FAME) technique. In the treatment without peptone, pin-point white colonies, *Bacillus pumilus*,

Bacillus sphaericus and a few *Cellulomonas cellulans* colonies were found. While in the presence of peptone, again pin-point white colonies, *Bacillus filicolonicus* and *Bacillus sphaericus* were identified.

The types of isolates were very similar at pH 10 with pin-point white colonies, *B. pumilus* and *B. sphaericus* plus a few colonies of *Pseudomonas stutzeri* when peptone was not added. In the presence of peptone, *B. pumilus*, *B. filicolonicus* and *B. sphaericus* with a few pin-point white colonies and *B. cereus* were found.

Table 7.8. Bacterial isolations from potassium hexacyanoferrate(III), at pH 8, after 58 days

Minus Peptone Treatment	Level	Plus Peptone Treatment	Level
Bacillus pumilus	+++	*Bacillus filicolonicus*	+++
B. sphaericus	+++	*B. sphaericus*	++
Pin-point, white	++	Pin-point, white	++
Cellulomonas cellulans	+		

Many colonies = +++, some = ++ and a few = +

The most efficient combination of bacterial species operated in the pH 8, plus peptone cultures. Isolations subsequently revealed the presence of only three bacterial species with *Bacillus filicolonicus* being the only species not found in the minus peptone treatment (Table 7.8). However, this *Bacillus* species was also isolated in the pH 10 culture along with *B. pumilus*, *B. sphaericus* and the pin-point white colony where degradation was not recorded. *Cellulomonas cellulans* which is unlikely to be responsible for, or to contribute to, any breakdown of the ferricyanide, was only isolated from the active pH 8 cultures in the absence of peptone.

A more likely candidate for degradation was *Pseudomonas stutzeri* which was isolated from the minus peptone treatment at pH 10 culture (Table 7.9) where some degradation had occurred (Table 7.5). This bacterium is known to contain the gene encoding for cyanidase which catalyses the hydrolysis of cyanide to formate or ammonia (Watanabe *et al.*, 1998). But the enzyme cyanidase appeared to be ineffective when the cyanide was bound to the iron.

Representatives of these bacterial species were further tested for hexacyanoferrate(III) degradation and results are reported in Chapter 9.

Table 7.9. Bacterial isolations from potassium hexacyanoferrate(III), at pH 10, after 58 days

Minus Peptone Treatment	Level	Plus Peptone Treatment	Level
Bacillus pumilus	+++	*Bacillus pumilus*	+++
B. sphaericus	+++	*B. sphaericus*	+++
Pin-point, white	+++	*B. filicolonicus*	+++
Pseudomonas stutzeri	+	*B. cereus*	+
		Pin-point, white	+

Many colonies = +++, some = ++ and a few = +

7.4 *Chapter overview*

Some unexpected reactions occurred in the growth media which may have complicated the results. By comparison with the control treatments, the degradation rates could still be calculated. At the pH 8, degradation occurred quickly in the cultures with added peptone but a steady degradation process was also measured in the absence of peptone. When the pH was raised to 10, it remained high for a few days only, followed by a drop to pH 8.5. The initial

higher pH appeared enough to slow down the break down process almost completely.

At termination, the Total-CN values at pH 8 for both with and without peptone treatments, were low. This confirmed that degradation had occurred with the formation of cyanate in one case. The end product cyanate was found at a high level in the plus peptone cultures at pH 8.

Ammonium-nitrogen, the other end product measured at pH 8, was detected only for the treatment containing peptone. In the treatments containing 1 g l^{-1} peptone, the maximum possible ammonium-nitrogen is 51 mg l^{-1} from the K_3FeCN_6 and 167 mg l^{-1} from the peptone giving a total of 218 mg l^{-1}. The major part of the ammonium-nitrogen, 99 mg l^{-1}, originated from the peptone-nitrogen since almost all of the cyanide utilized by the bacteria was converted to cyanate.

Cyanate was formed from the CN^- in the potassium hexacyanoferrate complex. The 116.4 mg l^{-1} cyanate measured (Table 7.4) accounted for 77% of the cyanide in the hexacyanoferrate complex with 22% still bound as Total-CN. The favoured pathway for degradation of hexacyanoferrate was cyanide to cyanate with very little being converted to ammonium-nitrogen. Therefore the main enzyme was cyanide monoxygenase (Dubey and Holmes, 1995).

In all of the bacterial treatments some of the nitrogen may go undetected since many components of the bacterial cell (amino acids and proteins including enzymes) require nitrogen for their formation and the nitrogen therefore becomes incorporated into the biomass.

In the absence of the added nutrient the bacteria produced neither cyanate nor ammonium-nitrogen and it is concluded that other metabolic pathways producing different end products may have been operating. Ammonia and formate were end products from cyanide decomposition, in research by Basheer *et al.* (1992). Also there is evidence for multiple pathways from cyanide to yield four metabolites, carbon dioxide, formamide, formate and ammonia (Kunz *et al.*, 1992).
At the higher pH, little degradation of hexacyanoferrate occurred.

The bacterial species identified were almost uniform between treatments and pH, with the genus *Bacillus* being represented by *B. cereus*, *B. filicolonicus*, *B. pumilus* and *B. sphaericus*. Only one species of *Pseudomonas*, *P. stutzeri* and one species of *Cellulomonas*, *C. cellulans* were isolated. The ability to degrade cyanide has most frequently been attributed to the genus *Pseudomonas* (Harris and Knowles, 1983a; Silva-Avalos *et al.*, 1990) but some researchers have named *Bacillus* as the main genus responsible for degradation (Boucabeille *et al.*, 1994). Their work showed that wastewaters from a gold mine contaminated with cyanide was polished mainly by *Bacillus* species and tests confirmed that 85% of their isolated species were *Bacillus*.

The suite of bacteria isolated from the degradation experiments of potassium ferricyanide therefore contains many species belonging to genera that have been implicated in releasing cyanide from this complex. These species have been tested individually for degradation of potassium hexacyanoferrate and results are reported in Chapter 9.

Chapter 8.0 Degradation of potassium hexacyanoferrate(III) in bioreactors

8.1 *Introduction*

Although the stability constant of ferricyanide is very high (10^{42}) it is affected by soil composition, pH and total cyanide (Total-CN) content according to Meeussen *et al.* (1990). In soils of low clay content and low pH there are high levels of positive exchange sites available which can strongly attenuate the ferricyanide (Mehling and Broughton, 1989). High levels of iron cyanide could be detected in groundwater in soils with a pH greater than 4, reported Meeussen *et al.* (1992), since the mobility of iron cyanide can increase with a rise in soil alkalinity. Other researchers (Cherryholmes *et al.*, 1985) found free cyanide in aqueous solutions of potassium ferricyanide after a few days and bacteria were shown to be responsible for this release.

Overall in the literature, few references can be found that implicate this very stable metal cyanide complex as a source of free cyanide from degradation processes, but researchers Pablo *et al.* (1997) found that free cyanide was possible from potassium ferricyanide. After 48 hr, they recorded 55.9 $\mu g\ l^{-1}$ of free cyanide from 406 $\mu g\ l^{-1}$ Total-CN. This level was higher than the LOEC (Lowest Observed Effect Concentration) at which a deleterious effect is observed in the test organisms *Chlamys asperrimus*. At the LOEC of 45 $\mu g\ l^{-1}$ CN^-, 50% of the embryos of this scallop were adversely affected.

Cyanide compounds like potassium ferricyanide which has a very high percentage of cyanide, 47% of the molecular weight in this case, can be removed from wastewaters by bacteria.

In this chapter, two bioreactors were monitored for the degradation of potassium hexacyanoferrate under non sterile conditions (Chapter 2). The RPII-HPLC technique was again used to determine the amount of complex still remaining (Chapter 2). Bacterial numbers were counted and metabolic products e.g. cyanate and ammonium-nitrogen, were measured.

8.2 *Materials and methods*

The BELLCO vessels (1 L) continued to be used as batch fed bioreactors, following from the degradation of sodium tetracyanonickelate (Chapter 6) and copper(I) cyanide (Chapter 4). At all times, the bioreactors were clad in aluminium foil to prevent any light reaction with the cyanide complex. The ore inoculum introduced during the pretreatment preparation (Chapter 2) before the start of the two copper cyanide experiments (Chapter 4), was maintained without any further addition of ore in either of the bioreactors or peptone in bioreactor B. Bacterial cells adhering to the ore particles were again the degrading organisms.

8.2.1 *Degradation of potassium hexacyanoferrate*

8.2.1.1 *First Degradation cycle*

Only inorganic salts at pH 9.4 were used in the non sterile DMS medium (Chapter 2) containing 200 mg l^{-1} potassium ferricyanide which was added to bioreactors A and B. Two stirrers suspended over the bioreactors stirred the media at 300 r.p.m.

throughout the first degradation cycle thereby incorporating a high oxygen level.

8.2.1.2 *Second degradation cycle*

At the end of the first cycle, the stirrers were stopped and the suspended ore material was allowed to settle overnight. The supernatant in each bioreactor was syphoned off leaving behind the ore material. Another 1 litre of medium at pH 9.4 was added to each bioreactor.

After the breakdown of one stirrer, the replacement could only be used at the lower speed of 200 r.p.m, therefore this became the new speed for both stirrers.

8.2.2 *HPLC sample preparation*

There were 16 sampling occasions during the first cycle of 68 days and 9 samplings in the second cycle of 82 days. Each time multiple injections were taken from each bioreactor for analysis. All the HPLC settings were the same as those used for the calibration curve (Chapter 2) and the shake flask culture experiments (Chapter 7).

8.2.3 *Determination of end products*

Measurements of cyanate and ammonium-nitrogen were carried out 10 times in duplicates during the two test periods of 68 days and 82 days. Methods for all of the analyses are recorded in Chapter 2. Remaining Total-CN and WAD-CN in duplicate subsamples were also checked 10 times during each of the two degradation cycles.

8.2.4 *Bacterial counts and isolations*

Serial dilutions were plated onto three isolation agars, DMS with $K_3Fe(CN)_6$, DMS with peptone and DMS containing peptone and $K_3Fe(CN)_6$, all at pH 9.4 ($K_3Fe(CN)_6$ at the same level as in bioreactors). Duplicate aliquots from three appropriate dilutions were spread on the agar surfaces and cultures were incubated at 27°C. After weekly inspections for 4 weeks, 30 bacterial colonies were described and purified, before duplicates were processed by the MIDI Inc. protocol for identification (Chapter 2).

8.3 *Results and discussion*

8.3.1 *Potassium hexacyanoferrate*(III) *pH studies*

Table 8.1. Changes in pH during degradation of potassium hexacyanoferrate, over 68 days in cycle 1

Bioreactors	1	5	10	15	27	41	55	68
A	8.35	8.05	7.95	8.00	8.05	8.05	8.05	8.00
B	8.20	8.05	8.05	8.10	8.15	8.20	8.20	8.15

The pH remained stable after the initial drop from pH 9.4, with a 0.4 pH unit drop in bioreactor A and only 0.15 pH unit fall in bioreactor B over 68 days (Table 8.1). As with the degradation of the tetracyanonickelate anion, little movement in pH was seen. Potassium ferricyanide degradation did not result in ammonium-nitrogen production (Figure 8.3), therefore the pH remained around pH 8.

In the second degradation cycle lasting 82 days (Table 8.2) there was a pH change of 0.4 and 0.5 units for bioreactors A and B respectively. Because no noticeable break down of the complex was measured or production of ammonium-nitrogen, the pH changes were very small.

Table 8.2. Changes in pH during degradation of potassium hexacyanoferrate, over 82 days in cycle 2

Bioreactors	0	4	15	21	34	49	57	82
A	8.30	7.95	7.85	7.90	7.90	7.90	7.90	8.00
B	8.45	8.00	8.00	8.00	7.95	8.00	8.00	8.10

8.3.2 *Degradation of potassium hexacyanoferrate, two cycles*

8.3.2.1 *First degradation cycle*

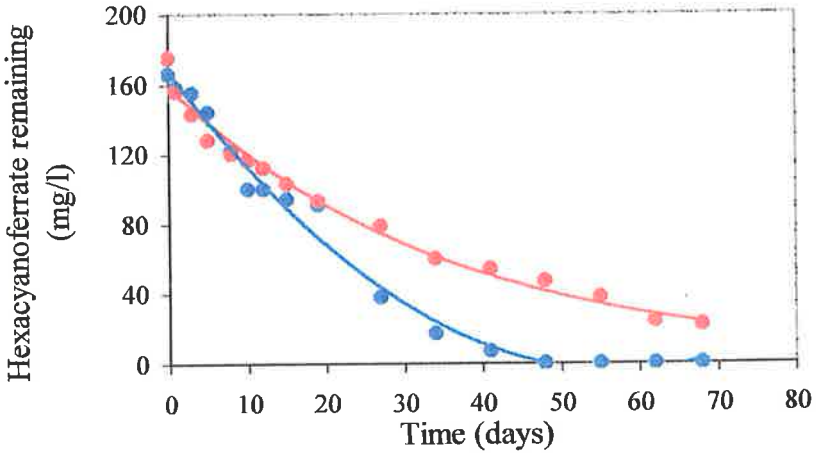

Figure 8.1. Degradation of potassium hexacyanoferrate, cycle 1, bioreactor A = ●, bioreactor B = ●, RPII-HPLC data

A steady degradation rate of 3.65 mg l^{-1} day^{-1} for potassium ferricyanide was observed in bioreactor A with bioreactor B having a slower degradation rate of 2.25 mg l^{-1} day^{-1} (Figure 8.1). At termination, the final value for bioreactor B was 22 mg l^{-1}. It was unexpected that bioreactor A would out perform bioreactor B, as the opposite situation had previously occurred. The pretreatment with peptone had been carried out on bioreactor B, almost 10 months prior to the introduction of the potassium hexacyanoferrate (Chapter 2), whereas no pretreatment was carried out

on bioreactor A. This pretreatment difference between the two bioreactors had resulted in a consistently better performance in bioreactor B.

The original microflora that grew with the assistance of the added peptone in bioreactor B had, after 10 months, utilized all of their essential requirements and now had reached a static or even a degenerative stage.

8.3.2.2 *Second degradation cycle*

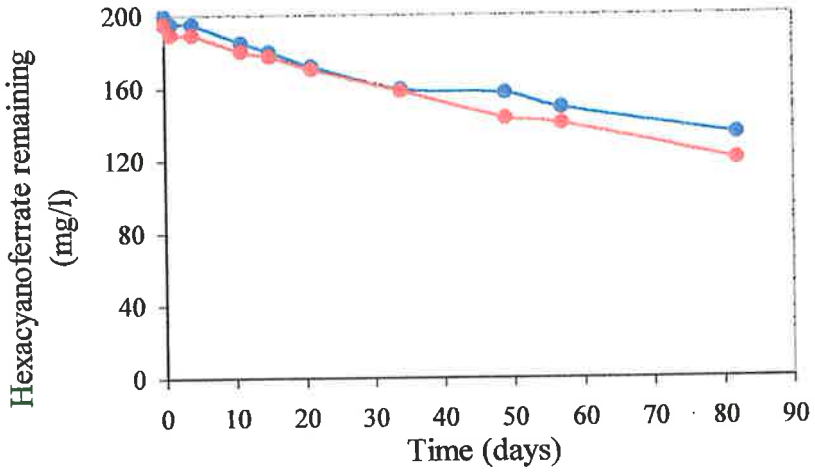

Figure 8.2. Degradation of potassium hexacyanoferrate, cycle 2, bioreactor A = ●, bioreactor B = ●, RPII-HPLC data

In the second cycle (Figure 8.2), both bioreactors gave lower degradation rates, 0.74 mg l^{-1} in bioreactor A and 0.84 mg l^{-1} in bioreactor B, and respectively this was only $1/5^{th}$ and $1/4^{th}$ of the previous rates during the first cycle.

Possible causes for this inactivity in the two bioreactors could have been; a) lack of sufficient oxygen caused by the reduced r.p.m. of the stirrers, b) a build-up of by-products and c) a suite of different organisms becoming dominant. A change in microflora was confirmed by identifying the species isolated during the two degradation cycles.

8.3.3 *Total-CN, WAD-CN and end products*

8.3.3.1 *First degradation cycle*

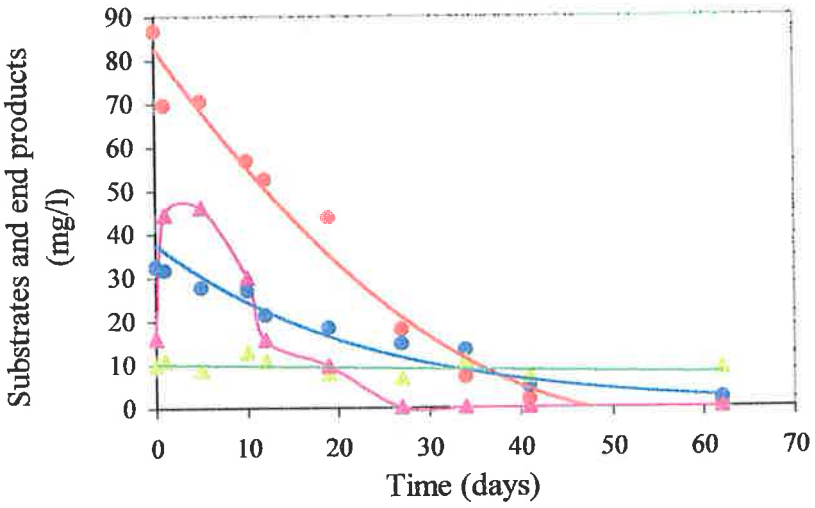

Figure 8.3. End products from degradation of potassium hexacyanoferrate in bioreactor A, cycle 1, Total-CN = ●; WAD-CN = ●; Ammonium-nitrogen = ▲, cyanate = ▲

Total cyanide (Total-CN) was not detected in bioreactor A after 45 days in the first cycle and only small traces of WAD-CN (Figure 8.3). This correlates well to Figure 8.1 where all of the $K_3Fe(CN)_6$ was degraded by 48 days. The degradation rate for Total-CN was 2.1 mg l^{-1} day^{-1}. Since potassium ferricyanide is a strong metal complex, the cyanide is strongly bound and cannot be dissociated by weak acids. This is reflected in low levels of WAD-CN.

The level of ammonium-nitrogen did not increase as had been the case with previous metal cyanide complexes, but remained low throughout the 62 days. The end product cyanate increased to a maximum of 46 mg l^{-1} by day 5, but at 27 days no measurable amount was left. The degradation rate for cyanate was 2.1 mg l^{-1} day^{-1}.

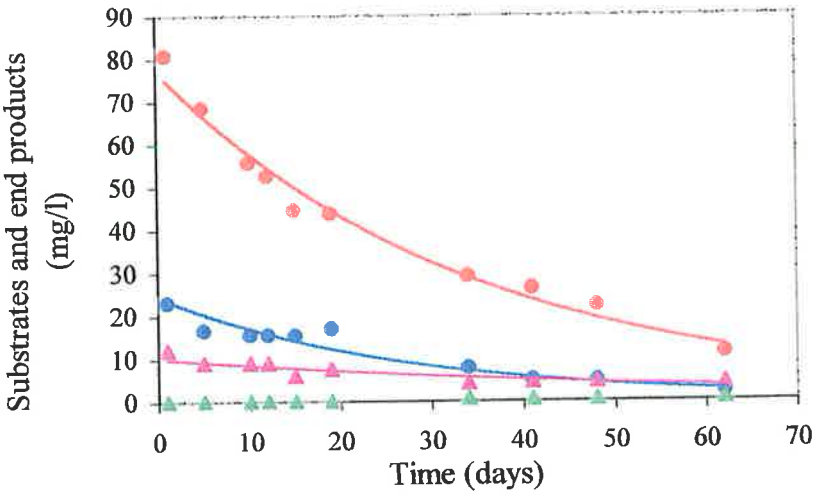

Figure 8.4. End products from degradation of potassium hexacyanoferrate in bioreactor B, cycle 1, Total-CN = ●; WAD-CN = ●; Ammonium-nitrogen = ▲, cyanate = ▲

Similar trends to bioreactor A were observed in bioreactor B with Total-CN decreasing steadily over 62 days from 81 mg l^{-1} to 11 mg l^{-1}, and a degradation rate of 1.1mg l^{-1} day^{-1} (Figure 8.4). The WAD-CN was low throughout as was ammonium-nitrogen. Cyanate level was also very low decreasing from 12 mg l^{-1} to 4 mg l^{-1}. The main difference between the two bioreactors was that no peak of cyanate was noted during the degradation of potassium hexacyanoferrate in bioreactor B.

8.3.3.2 *Second degradation cycle*

Very little activity was measured during the second cycle in bioreactor A (Figure 8.5). Over 82 days there was a slow decline of 32% in the level of Total-CN but no WAD-CN, ammonium-nitrogen or cyanate were found.

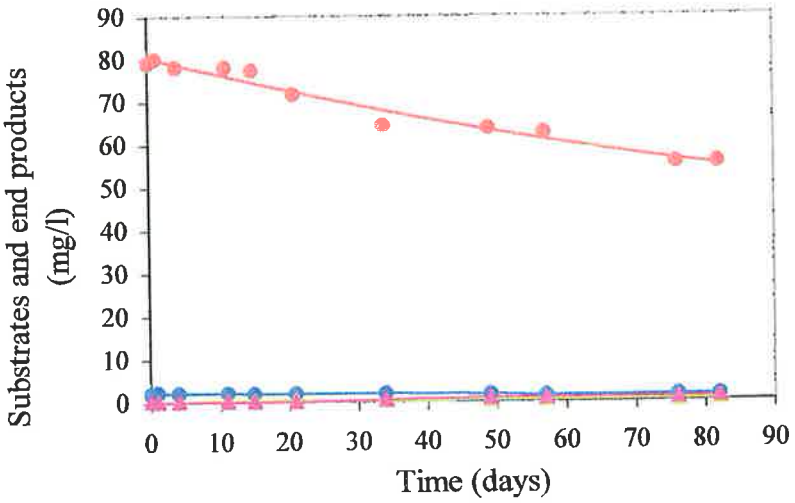

Figure 8.5. End products from degradation of potassium hexacyanoferrate in bioreactor A, cycle 2, Total-CN = ●; WAD-CN = ●; Ammonium-nitrogen = ▲, cyanate = ▲

A slow level of decomposition still occurred, as shown by the 0.32 mg l^{-1} day^{-1} degradation rate for Total-CN but the end products for this degradation were neither ammonium-nitrogen nor cyanate.

Again in bioreactor B the only result was a decrease in value of Total-CN (Figure 8.6). As in bioreactor A, a slow degradation rate of 0.45 mg l^{-1} day^{-1} was calculated from the 43% decomposition of the Total-CN. In this second cycle, no WAD-CN, ammonium-nitrogen or cyanate was detected, but other end products that were not determined may have been present.

As Figure 8.2 showed, there was slightly greater degradation in bioreactor B, i.e 43% in bioreactor B and 32% in bioreactor A.

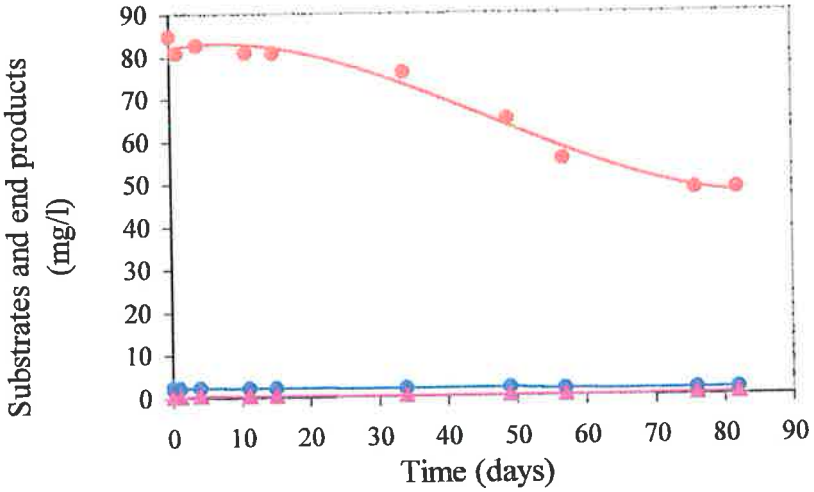

Figure 8.6. End products from degradation of potassium hexacyanoferrate in bioreactor B, cycle 2, Total-CN = ●; WAD-CN = ●; Ammonium-nitrogen = ▲, cyanate = ▲

8.3.4 *Bacterial counts*

In the first cycle, bacterial isolations were carried out three times during the test period of 68 days and three agars were used, DMS with $K_3Fe(CN)_6$, DMS plus peptone and DMS with peptone and $K_3Fe(CN)_6$.

The first set of isolations at 27 days, showed that the DMS plus $K_3Fe(CN)_6$ agar gave the best results (Table 8.3). As the test period increased to 48 and 68 days, DMS with peptone and $K_3Fe(CN)_6$ produced the highest bacterial numbers for bioreactor A.

A notable rise in bacterial numbers (Table 8.3) was evident when $K_3Fe(CN)_6$ was included instead of peptone, showing that the bacteria had adapted to and required this cyanide compound for optimal growth.

Table 8.3. Bacterial colony counts (cfu ml^{-1}) from bioreactor A during cycle 1 (all counts x 10^6)

Bioreactor A	DMS+P	DMS+ $K_3Fe(CN)_6$	DMS+P+$K_3Fe(CN)_6$
27 days	11.9	74.0	66.0
48 days	18.6	143.0	187.0
68 days	28.8	214.0	370.0

Bioreactor B followed a similar pattern to A, with the DMS plus $K_3Fe(CN)_6$ agar giving the best bacterial counts at 27 days (Table 8.4) but at 48 and 68 days, DMS with peptone and $K_3Fe(CN)_6$ provided the best results.

Table 8.4. Bacterial colony counts (cfu ml^{-1}) from bioreactor B during cycle 1 (all counts x 10^6)

Bioreactor B	DMS+P	DMS+ $K_3Fe(CN)_6$	DMS+P+$K_3Fe(CN)_6$
27 days	4.3	100.0	81.0
48 days	9.3	117.0	142.0
68 days	23.2	360.0	430.0

By 68 days, bioreactor B gave the highest counts of bacterial colonies on the most complex agar, although degradation was proceeding at a slower rate than in bioreactor A.

In the second degradation cycle in bioreactor A (Table 8.5), there is an increase in bacterial numbers between 21 and 62 days on all three agars but a sharp drop has occurred by 82 days which is the end of the experimental period.

Table 8.5. Bacterial colony counts (cfu ml^{-1}) from bioreactor A during cycle 2 (all counts x 10^6)

Bioreactor A	DMS+P	DMS+ $K_3Fe(CN)_6$	DMS+P+$K_3Fe(CN)_6$
21 days	17.0	68.0	62.0
62 days	30.6	72.0	68.0
82 days	2.1	13.5	17.6

In the second cycle, the first two isolation times at 21 and 62 days showed that the DMS plus $K_3Fe(CN)_6$ agar was the best for the microflora in bioreactor A. Even in the best performing agar (DMS+P+$K_3Fe(CN)_6$), the bacterial count was a 21 fold decrease at 82 days compared to that found at termination in the first cycle. Therefore, at termination in the second cycle, there remained only about 5% of the bacteria found at the end of the first cycle. By the end of the experiment all indicators showed that the system was no longer functioning well.

Table 8.6 shows that a decline in bacterial number in bioreactor B had occurred from 27 days to 82 days. Although the inclusion of $K_3Fe(CN)_6$ was beneficial when compared to the presence of peptone (DMS+P), the best numbers were generally still found on the DMS with peptone plus $K_3Fe(CN)_6$ agar.

Table 8.6. Bacterial colony counts (cfu ml^{-1}) from bioreactor B during cycle 2 (all counts x 10^6)

Bioreactor B	DMS+P	DMS+ $K_3Fe(CN)_6$	DMS+P+$K_3Fe(CN)_6$
21 days	3.5	55.0	67.0
62 days	1.5	23.0	22.0
82 days	0.1	35.0	46.0

In the second cycle at termination there remained only 11% of the bacterial numbers counted at the conclusion of the first cycle, on the best performing agar DMS+P+$K_3Fe(CN)_6$. Again all indicators showed that the bioreactors were starting to fail after almost 12 months of constant use.

8.3.5 *Bacterial isolations*

In both bioreactors A and B, at the three sampling times, many pin-point white colonies were present during cycle 1.

Table 8.7. Levels of bacterial species isolated during degradation of potassium hexacyanoferrate in bioreactor A, cycle 1

Name	27 days	48 days	68 days
Bacillus firmus	+	+	++
Bacillus sphaericus	+	++	++
Bacillus thuringiensis	+	++	-
Cellulomonas cellulans	-	-	+++
Pseudomonas stutzeri	+++	+	++
Pin-point, white colony	+++	+++	++
Sphingomonas paucimobilis	+	++	+++

Many colonies = +++, some = ++ and a few = +

In bioreactor A at 27 days, many *Pseudomonas stutzeri* colonies were present but by 48 days, more *Sphingomonas paucimobilis* were found (Table 8.7). In the last sampling at 68 days, the dominant species were *S. paucimobilis* and *Cellulomonas cellulans*. The bacterial species *Sphingomonas paucimobilis* was the only species that steadily increased in number over the test period. This may indicate that

S. paucimobilis found the initial hexacyanoferrate level inhibitory but increased in number as the amount of hexacyanoferrate was reduced through degradation or that it grew efficiently on by-products released from other microorganisms. The unidentified pin-point, white colony was present at a high level throughout the test period of the first degradation cycle.

The degradation of the hexacyanoferrate was completed by day 48 (Figure 8.1), therefore the isolations at 27 and 48 days were a true indication of the bacterial species involved in the degradation processes. By 68 days, all of the hexacyanoferrate had been decomposed and therefore some of the species found must have grown after the cyanide complex had disappeared e.g. *Cellulomonas cellulans.*

Table 8.8. Levels of bacterial species isolated during degradation of potassium hexacyanoferrate in bioreactor B, cycle 1

Name	27 days	48 days	68 days
Bacillus cereus	++	+	+
Bacillus sphaericus	+	++	+
Bacillus thuringiensis	+	+	+
Cellulomonas cellulans	-	+++	++
Pseudomonas stutzeri	++	++	++
Pin-point, white colony	++	+	+++
Sphingomonas paucimobilis	+	+	+

Many colonies = +++, some = ++ and a few = +

Samples from bioreactor B at 27 days did not have a dominant bacterial species (Table 8.8). At 48 days, the main species was *C. cellulans* but in the last isolations

at 68 days, pin-point white colonies were mostly isolated. The bacterium, *Pseudomonas stutzeri* was again consistently present at quite a high level.

Table 8.9. Levels of bacterial species isolated during degradation of potassium hexacyanoferrate in bioreactor A, cycle 2

Name	21 days	62 days	82 days
Acinetobacter radioresistans	++	+++	-
Bacillus pumilus	-	-	++
Micrococcus kristinae	++	++	+
Pin-point, white colony	++	+	+++
P. putida	++	-	+
Sphingomonas paucimobilis	++	+	-

Many colonies = +++, some = ++ and a few = +

In the second cycle at 21 days, some new species were found in bioreactor A (Table 8.9). Isolations at 62 days showed that *Acinetobacter radioresistans* was dominant but by 82 days the pin-point white colony was present in the greatest numbers.

As the test period advanced and as the bioreactor progressively became more inactive the bacterium *S. paucimobilis* disappeared from the isolations.

In bioreactor B (Table 8.10), species changed from quite a high level of *P. stutzeri, B. pumilus, Acinetobacter radioresistans* and the small white colony at 21 days to a low level of *S. paucimobilis, P. stutzeri, B. cereus* and *B. pumilus* at 62 days.

Table 8.10. Levels of bacterial species isolated during degradation of potassium hexacyanoferrate in bioreactor B, cycle 2

Name	21 days	62 days	82 days
Acinetobacter radioresistans	++	-	-
Amycolatopsis orientalis	-	-	++
Bacillus cereus	-	+	-
Bacillus pumilus	++	+	++
Micrococcus kristinae	-	+	++
Pin-point, white colony	++	++	+
Pseudomonas stutzeri	++	+	-
Sphingomonas paucimobilis	-	+	-

Many colonies = +++, some = ++ and a few = +

The last testing at 82 days, gave *B. pumilus*, *Micrococcus kristinae* and *Amycolatopsis orientalis* as the main species but no isolations of *S. paucimobilis*, *P. stutzeri* and *B. cereus*. All of these species were retested for potassium hexacyanoferrate decomposition in Chapter 9.

8.4 *Chapter overview*

The metal cyanide potassium hexacyanoferrate(III), also known as potassium ferricyanide, was degraded by the suite of microorganisms present in the two bioreactors A and B during the first degradation cycle. This complex, with a cyanide content responsible for 47% of the molecular weight has usually been ignored in degradation experiments because it is regarded as a very stable metal cyanide. In a paper by Hoecker and Muir (1987), the authors wrote that the cyanide species of most concern were those giving free CN^- and weak or

moderate cyanocomplexes. They disregarded strong complexes like potassium ferrocyanide and potassium ferricyanide.

However, research conducted in The Netherlands (Meeussen *et al.*, 1992) has disputed the idea that iron cyanide complexes can be regarded as safe in the environment. Their work showed that these complexes rapidly decomposed to free cyanide when exposed to light. Others (Pablo *et al.*, 1997) have measured sufficient amounts of free cyanide from potassium ferricyanide to have an effect on their test organism, *Chlamys asperrimus*.

The breakdown of potassium ferricyanide by bacteria is also shown by the results of the first degradation cycle described in this Chapter. Low levels of the end products, ammonium-nitrogen and cyanate, were found to be present but no free cyanide was detected throughout the test period. This led to the conclusion that other end products must have been produced e.g. formamide, nitrite or nitrate (Kunz *et al.*, 1992).

By the second degradation cycle, the bioreactors system appeared to become unsuitable for the degrading suite of bacteria and very different species were isolated. There ensued a drop in bacterial numbers and a decrease in familiar species.

Representatives from all species isolated were purified and retested for degradation of the potassium ferricyanide as individual cultures (Chapter 9).

The two bioreactors by this stage had been in continuous use for 12 months under

non sterile conditions before the degradation system slowed down. Apart from the initial pretreatment of adding 1 g l^{-1} of peptone in bioreactor B, only inorganic salts were used throughout the 12 months in both vessels. It is interesting to note that the degradation of a strong cyanide complex, potassium hexacyanoferrate(III) could be achieved in bioreactor A where only inorganic salts and the indigenous ore bacteria were present and in bioreactor B where only 1 g l^{-1} peptone was added 12 months prior.

Chapter 9.0 Degradation of the three metal cyanides by purified bacterial species

9.1 *Introduction*

Removal of toxic cyanides by bacteria was investigated by Finnegan *et al.* (1991) where the *Acinetobacter* strain RFB1 was found to grow on 13 cyanide compounds including copper and ferric cyanide complexes. Other work carried out in South Africa (Meyers *et al.*, 1991) and Perth, Australia (Guilfoyle, 1996) with the species *Bacillus pumilus* (Sneath *et al.*, 1986) found that degradation of cyanide had occurred. In 1993, Meyers *et al.*, reported that *B. pumilus* was very efficient in utilizing cyanide to form the by-products formate and ammonia in a single-step reaction but the enzyme responsible could not hydrolyse cyanate or thiocyanate. The cyanide degrading enzyme from *B. pumilus* CI was named cyanide dihydratase with maximum activity at pH 8.6 in the presence of trivalent metal ions i.e Fe^{3+}.

Overall, cyanide degradation studies have concentrated on the genus *Pseudomonas* with a strain of *P. fluorescens* (BKM B-5040) reported to have degraded complexes in the order of their stability (Shpak *et al.*, 1995), but no enzyme was named. Another strain often cited is *P. fluorescens* biotype II, NCIMB 11764 (Rollinson *et al.*, 1987) which utilized tetracyanonickelate ($Ni(CN)_4^{2-}$) as a nitrogen source with the aid of the enzyme cyanide oxygenase. Also in a study with *Pseudomonas putida*, it was found that this bacterium could degrade potassium tetracyanonickelate (Silva-Avalos *et al.*, 1990).

The decomposition involved an oxygenase but the pathway was unclear. Unidentified enzymes from *P. putida* were also able to reduce cyanates to ammonia (Babu *et al.*, 1996).

The bacterial studies reported in this chapter, include some of the species already found in the literature plus many not previously known to degrade cyanide compounds. Included in the group were *Acinetobacter radioresistens*, *Amycolatopsis orientalis*, *Arthrobacter viscosus*, 9 species of *Bacillus*, 3 species of *Pseudomonas* and 4 unnamed strains. The degradation pathways varied depending on the cyanide compound present, indicating that a variety of enzymatic systems were involved.

Growth patterns of 31 bacterial species previously isolated from incubations with the three metal cyanides, were followed over varying time periods. Pure cultures were inoculated into media containing each of the three metal cyanide compounds (copper(I) cyanide, sodium tetracyanonickelate and potassium hexacyanoferrate(III). These experiments showed which bacterium best degraded each cyanide compound and by which enzymatic pathway this breakdown occurred. Because one of the by-products of degradation was cyanate, a test was carried out to follow the conversion of sodium cyanate to ammonium-nitrogen thereby indicating whether the enzyme cyanase was induced.

9.2 *Materials and methods*

During the execution of the degradation studies of three metal cyanide compounds in shake flask cultures and bioreactors, bacterial colonies were isolated and stored (Chapters 3,4,5,6,7 & 8). From this bacterial collection, representative members of each species were purified and characterised by the MIDI-FAME system.

These bacterial species (Table 9.1) were then individually retested for growth and degradation of the three cyanide compounds, copper(I) cyanide, sodium tetracyanonickelate and potassium hexacyanoferrate(III). The source of each strain used in these experiments is listed in Table 9.1. Numbers (1,2 and 3) in column 1 assist in clarifying the origin of the cultures in subsequent referencing.

9.2.1 *Design of the degradation experiments*

Each of the three cyanide complexes was added to the DMS media, adjusted to pH 9.4, in the same concentration as in the shake flask cultures and bioreactors experiments. The medium containing copper(I) cyanide (Chapters 3 and 4) was sterilized by filtration through a 0.45 μm membrane while the medium for sodium tetracyanonickelate (Chapters 5 and 6) was steam autoclaved at 100 kPa for 20 mins., as was the potassium hexacyanoferrate medium (Chapters 7 and 8). All of the three media were supplemented with the nutrient peptone, at 1 g l^{-1} for copper(I) cyanide and potassium hexacyanoferrate but at 0.1 g l^{-1} for the sodium tetracyanonickelate medium since at the higher peptone level no degradation of the tetracyanonickelate was found to occur.

Each culture tube was filled with 7.5 ml of the appropriate medium and three replicates were inoculated with each of the 31 bacterial isolates. Also included in each degradation study were three uninoculated tubes which became the abiotic controls. Each bacterial species that was to be used in the experiments was grown on DMS agar plates for 5 days, after which a 2 mm loopful was taken to inoculate each of the three replicates and this was repeated for each of the 31 bacterial species. The tube cultures were sloped, placed under a black cover and shaken at 75 r.p.m. during the 27°C incubation until the termination date when all cultures were filtered through a 0.45 μm membrane before any analyses were done.

Table 9.1. Source of the bacterial isolates

Bacterial species	Metal	P	Experiment	Type	pH
Bacillus sphaericus (2)	Cu	+	2	Bio	9
Bacillus sphaericus (3)	Cu	-	2	Bio	9
Bacillus cereus (1)	Ni	+	1	SFC	8
Bacillus cereus (2)	Ni	+	1	SFC	8
Bacillus circulans	Ni	+	1	SFC	10
Bacillus filicolonicus	Ni	+	1	SFC	10
Bacillus firmus	Ni	+	1	SFC	10
Bacillus globisporus	Ni	+	1	SFC	10
Bacillus polymyxa	Ni	+	1	SFC	10
Bacillus pumilus (1)	Ni	+	1	SFC	10
Bacillus pumilus (2)	Ni	+	1	SFC	10
Bacillus pumilus (3)	Ni	+	1	SFC	10
Bacillus thuringiensis (1)	Ni	+	1	SFC	8
Bacillus thuringiensis (2)	Ni	+	2	Bio	9
Bacillus thuringiensis (3)	Ni	+	1	SFC	8
Cellulomonas cellulans (1)	Ni	+	1	SFC	8
Cellulomonas cellulans (2)	Ni	+	1	SFC	8
Pseudomonas stutzeri (1)	Ni	-	2	Bio	9
Acinetobacter radioresistens (1)	Fe	-	1	Bio	9
Acinetobacter radioresistens (2)	Fe	-	2	Bio	9
Amycolatopsis orientalis	Fe	-	1	Bio	9
Arthrobacter viscosus	Fe	+	1	Bio	9
Bacillus sphaericus (1)	Fe	-	1	Bio	9
Micrococcus kristinae	Fe	+	2	Bio	9
Pseudomonas putida	Fe	-	2	Bio	9
Pseudomonas stutzeri (2)	Fe	+	1	Bio	9
Sphingomonas paucimobilis	Fe	-	1	Bio	9
Unnamed (No. 157)	Fe	-	1	Bio	9
Unnamed (No.161)	Fe	+	1	Bio	9
Unnamed (No. 94)	Fe	-	1	SFC	8
Unnamed (No.101)	Fe	+	1	SFC	8

Metal indicates the metal cyanide complex from which the strain was isolated.

P, + or -, indicate the presence or absence of peptone.

Experiment 1 or 2, indicates the degradation cycle from which the bacterium was isolated.

Type means isolated from shake flask cultures (SFC) or bioreactors (Bio)

pH shows the initial pH (to the nearest unit) of the metal cyanide medium.

Numbers (1,2 and 3) in column 1are aids to subsequent referencing

9.2.2 *Test for cyanate utilization*

Ingredients in the sodium cyanate medium (Taussig, 1960) used to test whether any of the bacterial isolates have the capability to utilize the cyanate to form ammonium and carbon dioxide, are set out in Table 9.2. This medium was sterilized by filtering through a 0.45 μm membrane and dispensed in 7.5 ml aliquots into culture tubes. Again each bacterial species was replicated three times.

Table 9.2. Formulation for the cyanate medium (pH 7)

Name of chemical*	Formula	g l^{-1}
Potassium dihydrogen phosphate	KH_2PO_4	0.2
Disodium hydrogen phosphate	Na_2HPO_4	0.3
Potassium chloride	KCl	1.0
Magnesium sulfate	$MgSO_4.7H_2O$	0.2
Calcium chloride	$CaCl_2$	0.01
Glucose	$C_6H_{12}O_6$	1.0
Sodium cyanate	NaCNO	0.65

*All of the chemicals were of analytical grade.

9.2.3 *Analyses carried out on cultures*

At termination, cultures grown on each of the three metal cyanides were tested for ammonium-nitrogen and cyanate. Also the pH was taken and cultures were scanned in the UV and visible wavelength ranges, to assess the level of any residual metal complexes. For the sodium cyanate there is no typical absorbance therefore no scans were done on these cultures. Before each analysis the cultures were filtered through a 0.45 μm membrane.

9.3 *Results and discussion*

9.3.1 *Copper(I) cyanide degradation*

The best copper(I) cyanide degrading bacterium was found to be a strain of *Bacillus sphaericus*, isolated from the first degradation experiment in the bioreactor A containing potassium hexacyanoferrate. At the end of the 37 days, *Sphingomonas paucimobilis* was the second best bacterium tested, followed by no. 161, *B. polymyxa*, no. 101, and *B. circulans* (Figure 9.1). Interestingly, the two best copper(I) degrading bacterial strains were isolated from the same source. While both nos. 161 and 101 were purified from the hexacyanoferrate(III) degradation experiments, *B. polymyxa* and *B. circulans* were isolated from the sodium tetracyanonickelate degradation study at pH 10 (Table 9.1).

Levels of ammonium-nitrogen for *Amycolatopsis orientalis* and *Sphingomonas paucimobilis* were high but could not be attributed to degradation of the copper(I) cyanide since the growth medium contained 1 g l^{-1} peptone which added 167 mg l^{-1} nitrogen to the system (Figure 9.2). Cyanate was not detected after 37 days of incubation (Figure 9.3), it may have been produced earlier in the experiment, followed by hydrolysis to form ammonia and carbon dioxide. This pathway would employ the two enzymes cyanide monoxygenase and cyanase. However, another reaction (1) may have occurred under the influence of the enzyme cyanide dioxygenase. Some bacteria are known to degrade hydrogen cyanide by the action of cyanide dioxygenase (Harris and Knowles, 1983a) and the by-products formed by certain bacteria in this study would suggest that this reaction had taken place.

$$\text{CN-metal complex} + O_2 + 2H^+ + \text{NADPH} \rightarrow CO_2 + NH_3 + NADP^+ \qquad (1)$$

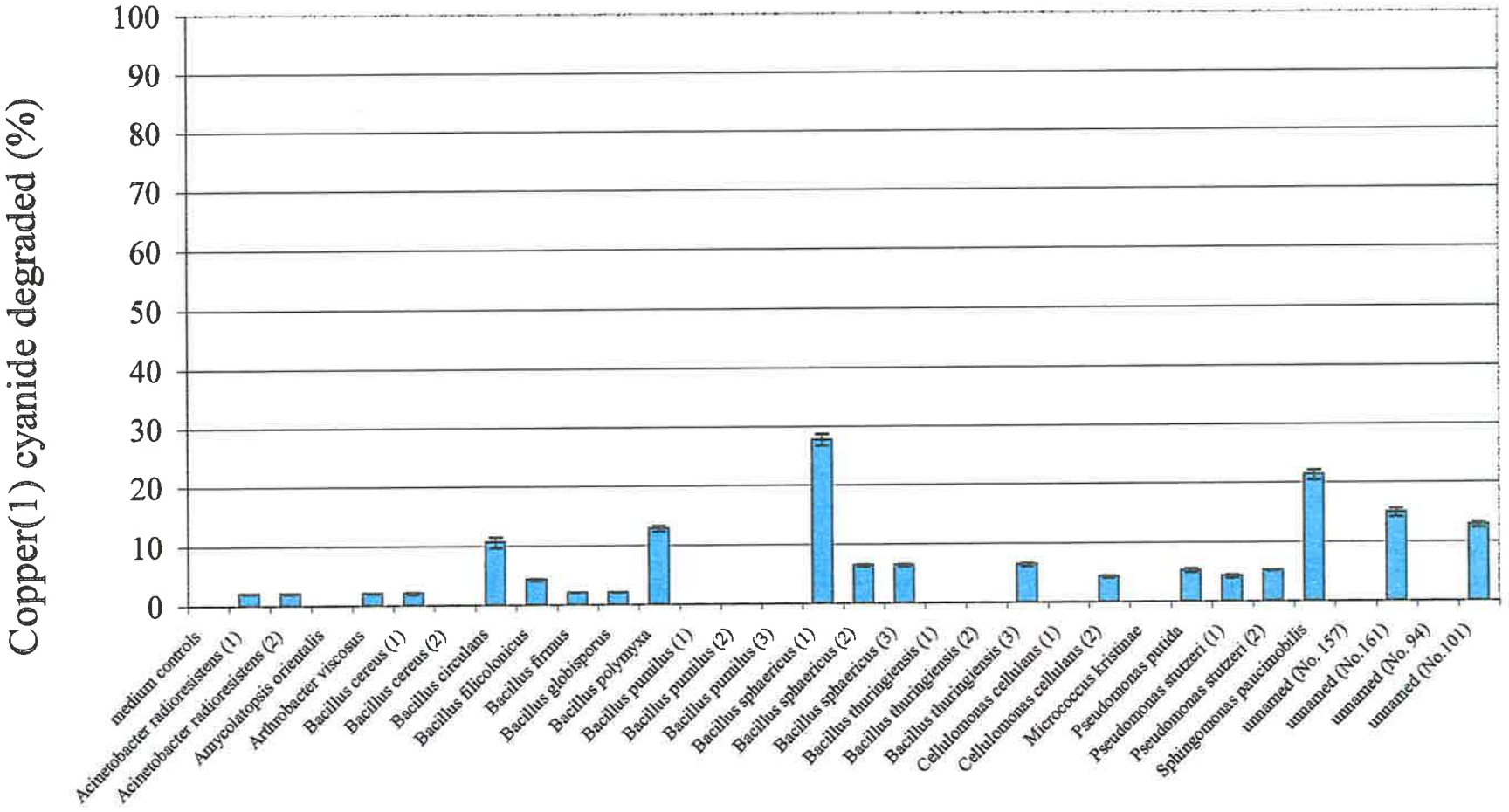

Figure 9.1. Copper(I) cyanide degraded by each bacterial species (av. of 3 reps.)

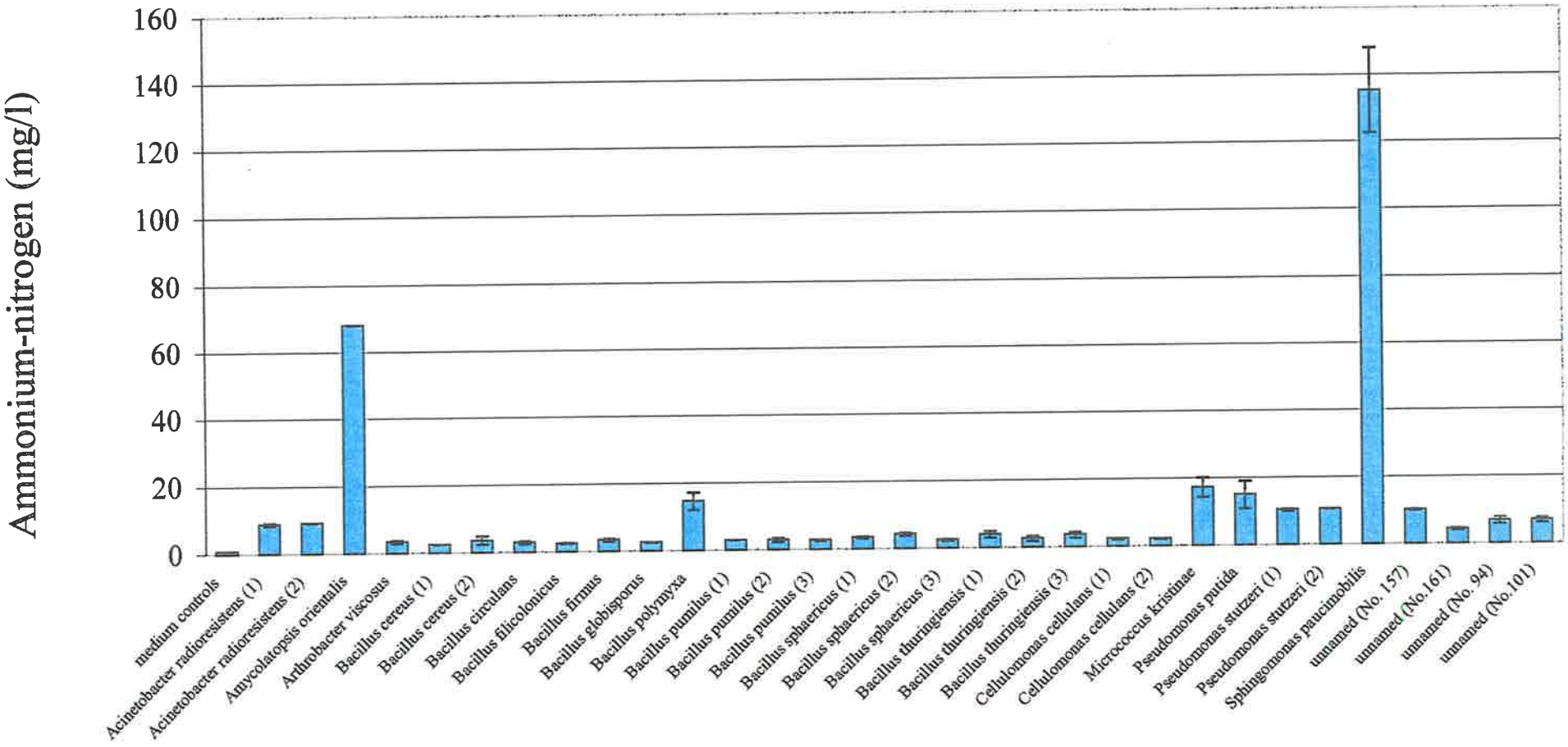

Figure 9.2. Ammonium-nitrogen production from copper(I) cyanide by each bacterial species (av. of 3 reps.)

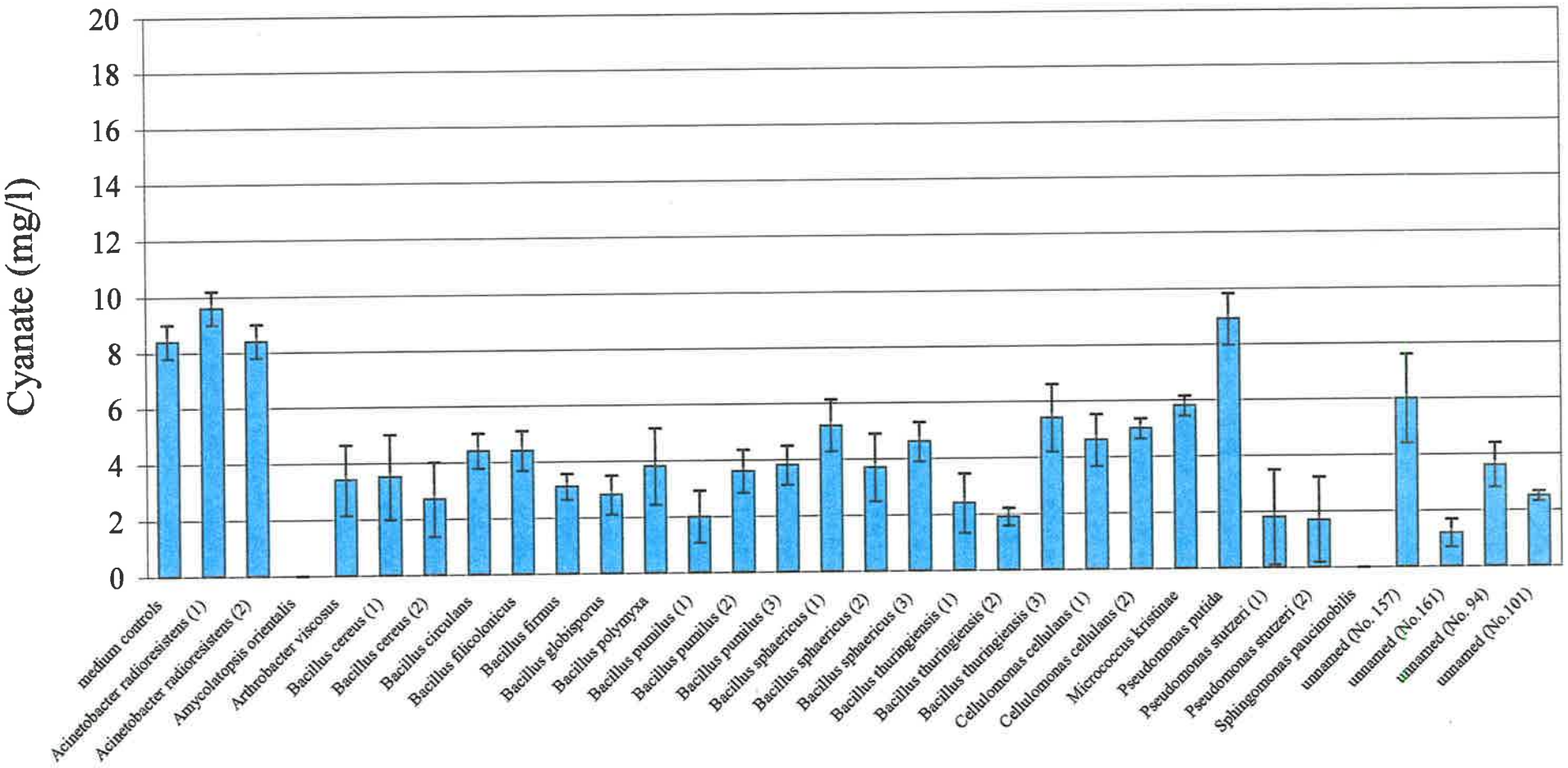

Figure 9.3. Cyanate production from copper(I) cyanide by each bacterial species (av. of 3 reps.)

Table 9.3. Copper(I) cyanide degraded by the six most active bacteria (av. of 3 reps.)

Name	Cu(I)CN degraded (mg l^{-1})	Theoretical N from Cu(I)CN degraded (mg l^{-1})	CNO^--N (mg l^{-1})	NH_4^+-N (mg l^{-1})
Bacillus sphaericus (1)	41.6	6.5	1.7	3.5
Sphingomonas paucimobilis	32.0	5.0	0	135.3
No. 161	22.4	3.5	0.4	4.3
Bacillus polymyxa	19.2	3.0	1.3	14.7
No. 101	19.2	3.0	0.8	6.8
B. circulans	15.9	2.5	1.4	3.0

The measured ammonium-nitrogen and cyanate presented in Table 9.3, exceeded the theoretical ammonium-nitrogen possible from the degraded copper(I) cyanide complex for 5 of the 6 species listed. In the added 150 mg l^{-1} copper(I) cyanide, the available nitrogen is 23.5 mg l^{-1}. The *Bacillus sphaericus* strain showed a high degradation (27.7%) which corresponded to 41.6 mg l^{-1} of the copper(I) cyanide with a level of 6.5 mg l^{-1} nitrogen. The nitrogen in the cyanate plus the ammonium accounted for 5.2 mg l^{-1} out of the possible theoretical value of 6.5 mg l^{-1} but some of this ammonium-nitrogen may have originated in the peptone. It was also possible that some of the cyanide was converted by the enzyme cyanide hydratase to formamide (Kunz *et al.*, 1992), a by-product not measured in this research (reaction 2).

The bacterium *Sphingomonas paucimobilis* utilized 21.3% of the copper(I) cyanide with no cyanate being produced only ammonium-nitrogen. Therefore most of the 135.3 mg l^{-1} ammonium-nitrogen measured, originated from the peptone.

$$\underset{\text{formate}}{CO_2H} + NH_3 \xleftarrow{+2H_2O} \text{CN-metal complex} \xrightarrow{+O_2} NH_3 + CO_2 \qquad (2)$$

$$\downarrow + H_2O$$

$$HCONH_2$$
formamide

9.3.2 *Sodium tetracyanonickelate degradation*

The majority of the bacterial isolates gave low levels of degradation of sodium tetracyanonickelate with only five strains being effective (Figure 9.4). In the group of five (Table 9.4) two were outstanding, the unnamed isolate no. 94 showing 66.3% degradation of the compound and no. 101 responsible for 79.9% degradation. The bacterium *Sphingomonas paucimobilis* which was isolated from bioreactor A in the presence of potassium hexacyanoferrate during the first degradation cycle, only degraded 12.5% of the sodium tetracyanonickelate. The species *B. globisporus* which gave a 8.5% degradation, originated from the added peptone treatment in the shake flask experiment at pH 10 containing sodium tetracyanonickelate.

The two unnamed isolates no. 94 and no. 101, were very similar in appearance both 1 mm diameter, cream coloured and with a shiny colony surface on tryptone soy agar (TSA). These two strains which are Gram positive cocci, catalase test positive and also have almost identical MIDI-FAME fingerprints, therefore appear to belong to the same species. Both strains were isolated from the shake flask culture experiment with potassium hexacyanoferrate at pH 8. Number 94 originated in cultures not containing peptone and number 101 from cultures with added peptone.

Production of cyanate was not detected from the degradation of sodium tetracyanonickelate by any of the 31 species tested.

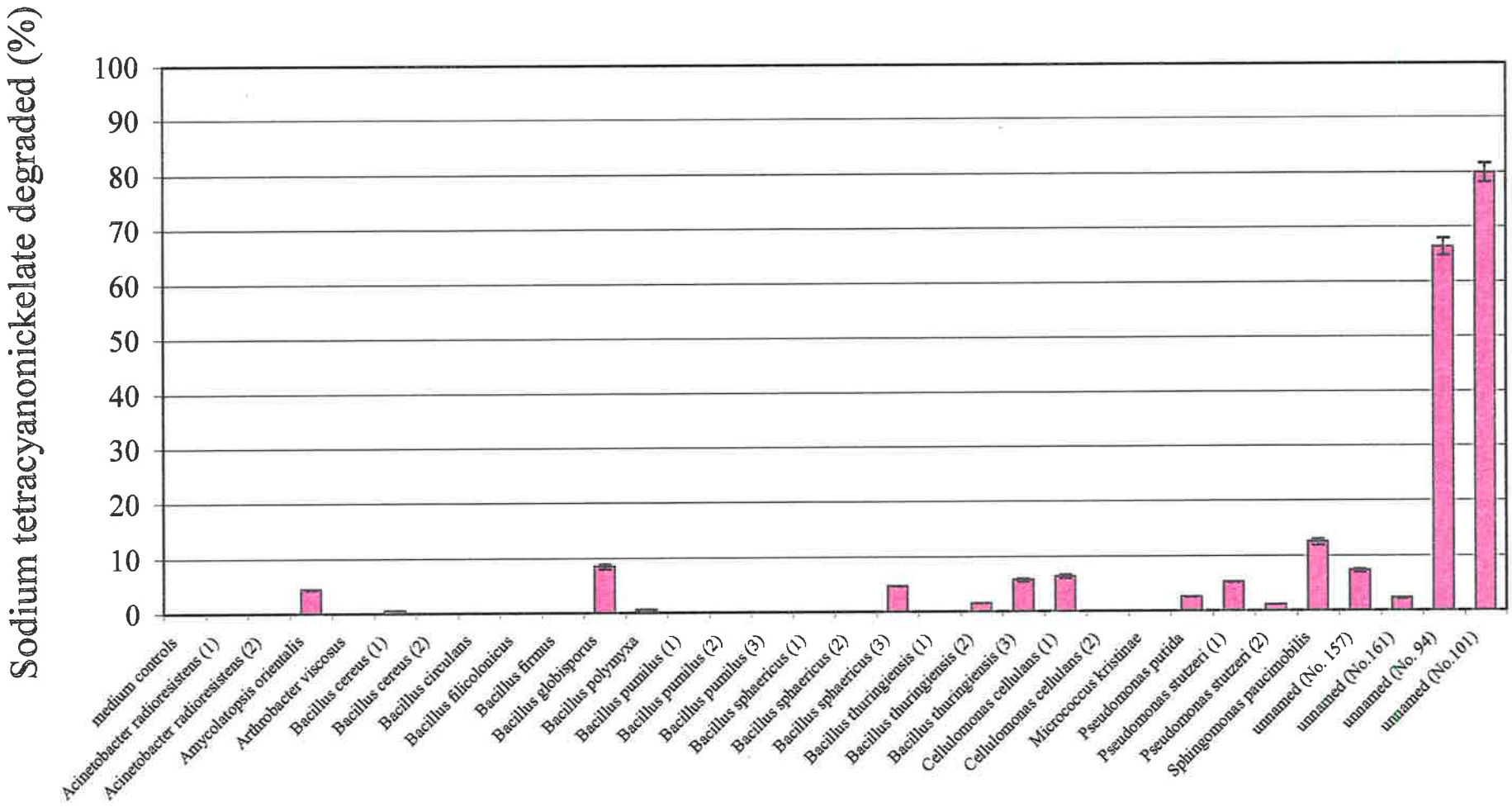

Figure 9.4. Sodium tetracyanonickelate degraded by each bacterial species (av. of 3 reps.)

Table 9.4. Sodium tetracyanonickelate degraded by the five most active bacteria (av. of 3 reps)

Name	$Na_2Ni(CN)_4$ degraded (mg l^{-1})	Theoretical N from Na_2NiCN_4 degraded (mg l^{-1})	CNO^--N (mg l^{-1})	NH_4^+-N (mg l^{-1})
Unnamed (No.101)	83.5	22.5	0	23.8
Unnamed (No. 94)	69.3	18.7	0	25.7
Sphingomonas paucimobilis	13.1	3.5	0	17.6
Bacillus globisporus	8.9	2.4	0.4	8.0
Unnamed (No. 157)	7.6	2.1	0	4.5

In Table 9.4, the two unnamed bacterial strains nos. 94 and 101 markedly reduced the level of sodium tetracyanonickelate. The most active bacterial strains to utilize the possible 28 mg l^{-1} nitrogen in the sodium tetracyanonickelate during the 27 day growth period, were nos. 94 and 101. Much lower levels of degradation were measured for the other three strains in Table 9.4, i.e. *Sphingomonas paucimobilis*, *Bacillus globisporus* and the unnamed strain no.157.

With no cyanate but high levels of ammonium-nitrogen being measured for nos. 94 and 101, this may indicate that the enzyme cyanide dioxygenase was again active in the sodium tetracyanonickelate degradation (Figure 9.5). Either no, or only traces of, cyanate was found for any of the 31 species tested (Figure 9.6).

9.3.3 *Potassium hexacyanoferrate(III) degradation*

Most of the bacterial species were found to degrade potassium hexacyanoferrate, except for the unnamed strain no. 161 (Figure 9.7). The inability of no. 161 to degrade hexacyanoferrate was unexpected since it was isolated from the potassium hexacyanoferrate degradation in bioreactor B (Table 9.1).

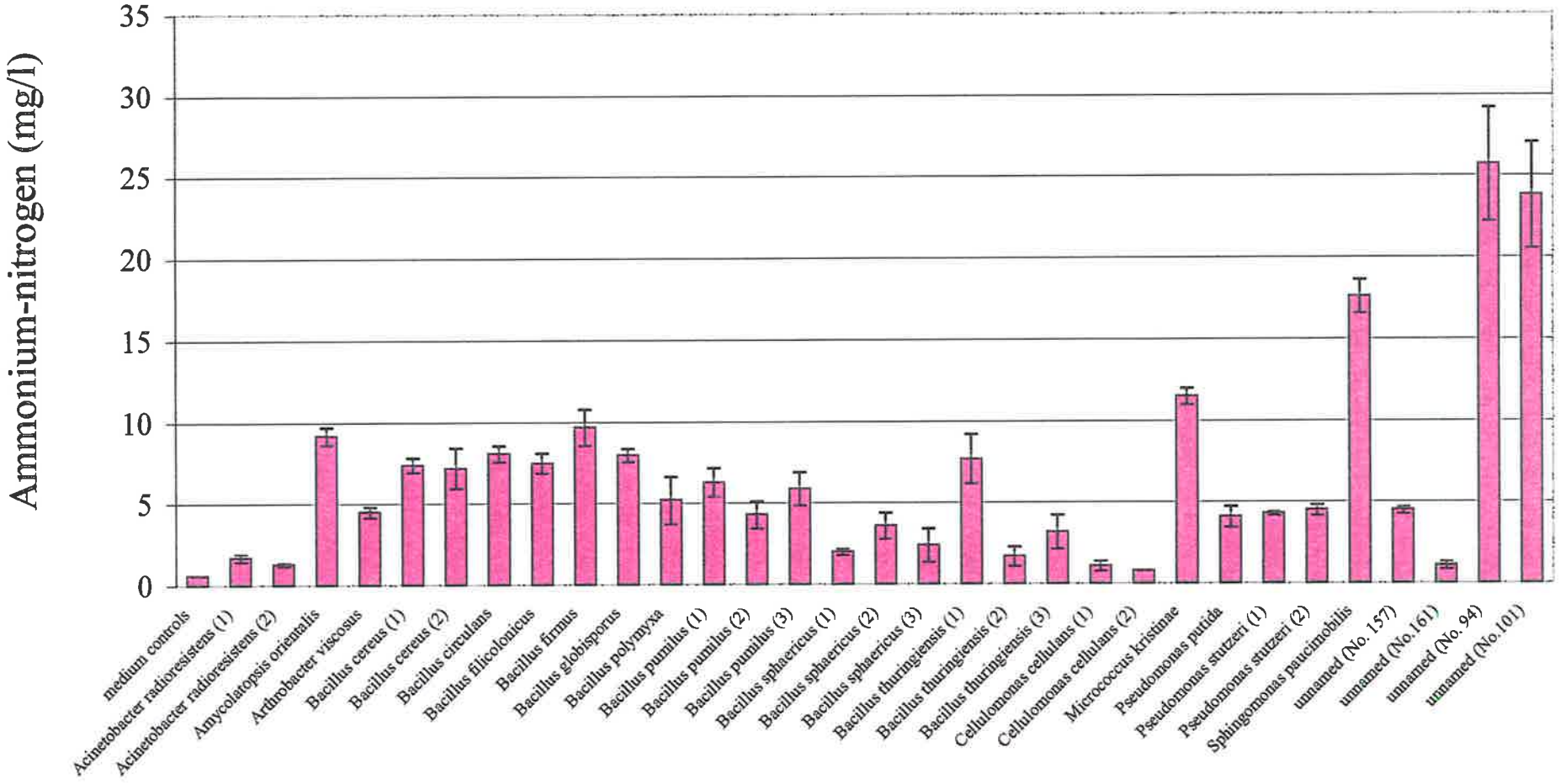

Figure 9.5. Ammonium-nitrogen production from sodium tetracyanonickelate by each bacterial species (av. of 3 reps.)

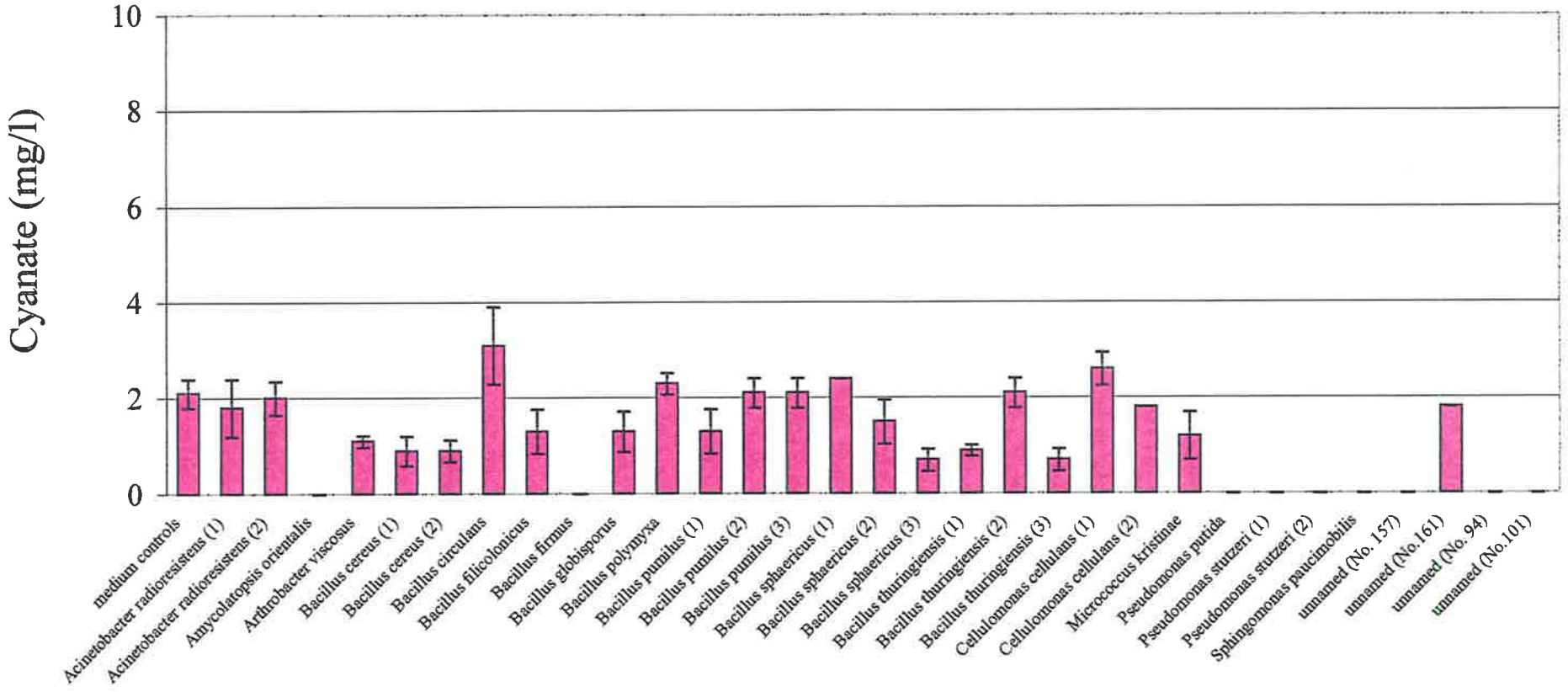

Figure 9.6. Cyanate production from sodium tetracyanonickelate by each bacterial species (av. of 3 reps.)

This strain formed a small pink colony, regularly edged with a shiny surface on TSA, and is a Gram negative, catalase positive, coccus.

After 21days incubation, the ten best bacterial strains, to degrade potassium hexacyanoferrate were *B. sphaericus* (1), *P. putida*, two strains of *Cellulomonas cellulans*, *Amycolatopsis orientalis*, no. 101, no. 157, *B. pumilus* (1), no. 94 and *Micrococcus kristinae*. All ten bacterial strains originated either from sodium tetracyanonickelate or potassium hexacyanoferrate degradation experiments, at different initial pH and from shake flasks or bioreactors cultures (Table 9.1).

In Table 9.5, the amounts (mg l^{-1}) of potassium hexacyanoferrate degraded by the individual bacterial species during a 21day study, are listed. With the exceptions of the two *Acinetobacter radioresistens* strains and the unnamed strain no. 161, the other 28 bacterial species degraded this metal cyanide efficiently to obtain theoretical levels of nitrogen in the 36.1 to 49.5 mg l^{-1} range from the possible 51 mg l^{-1}. The species *Amycolatopsis orientalis* and *Sphingomonas paucimobilis*, having both high levels of ammonium-nitrogen and cyanate, derived the ammonium-nitrogen from the utilization of the cyanide substrate but much of the ammonium-nitrogen also originated from the 166.7 mg l^{-1} nitrogen in the peptone (Figure 9.8).

The two strains of *Cellulomonas cellulans* also degraded the substrate effectively, however, very little ammonium-nitrogen and no cyanate was detected in these two cultures. It would appear that other degradation pathways were in operation for these two bacteria giving by-products not measured during this study.

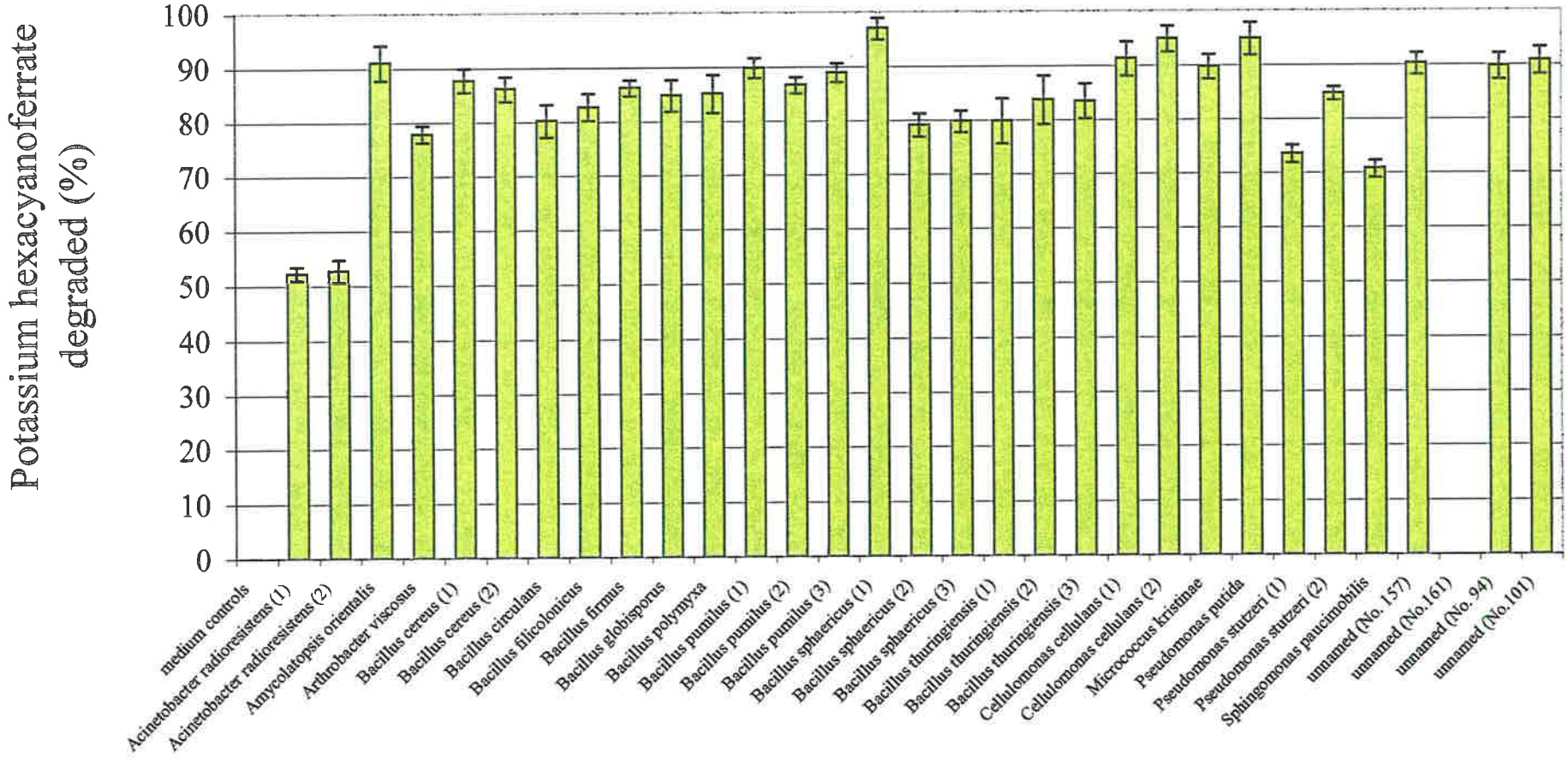

Figure 9.7. Potassium hexacyanoferrate degraded by each bacterial species (av. of 3 reps.)

Table 9.5. Potassium hexacyanoferrate(III) degraded by the 31 bacterial strains (av. of 3 reps)

Name	$K_3Fe(CN)_6$ degraded (mg l^{-1})	Theoretical N from $K_3Fe(CN)_6$ degraded (mg l^{-1})	CNO^--N (mg l^{-1})	NH_4^+-N (mg l^{-1})
Bacillus sphaericus (1)	194	49.5	0	30.3
Pseudomonas putida	189.8	48.4	0	27.0
Cellulomonas cellulans (2)	189.8	48.4	0	5.0
Amycolatopsis orientalis	182.2	46.5	11.0	89.5
Cellulomonas cellulans (1)	182	46.4	0	2.3
Unnamed (No.101)	181.6	46.3	0	32.2
Unnamed (No. 157)	180.6	46.1	0	31.8
Bacillus pumilus (1)	179.8	45.8	6.7	49.0
Unnamed (No. 94)	179.6	45.8	1.3	30.0
Micrococcus kristinae	179.6	45.8	4.6	50.3
Bacillus pumilus (3)	178	45.4	0	43.3
Bacillus cereus (1)	175.6	44.8	1.0	63.5
Bacillus pumilus (2)	173.4	44.2	5.1	43.5
Bacillus firmus	172.6	44.0	3.9	57.2
Bacillus cereus (2)	172.4	44.0	7.2	80.3
Bacillus polymyxa	170.4	43.5	9.5	56.3
Bacillus globisporus	169.8	43.3	0	61.8
Pseudomonas stutzeri (2)	169.6	43.2	0	26.5
Bacillus thuringiensis (2)	167.6	42.7	1.3	54.8
Bacillus thuringiensis (3)	167	42.6	3.0	54.5
Bacillus filicolonicus	165.6	42.2	1.0	50.3
Bacillus circulans	160.6	41.0	2.4	44.3
Bacillus sphaericus (3)	159.8	40.7	2.0	35.0
Bacillus thuringiensis (1)	159.8	40.7	0	53.7
Bacillus sphaericus (2)	158.4	40.4	0	38.8
Arthrobacter viscosus	155.8	39.7	1.0	50.8
Pseudomonas stutzeri (1)	147.2	37.5	0	23.3
Sphingomonas paucimobilis	141.6	36.1	11.8	80.0
Acinetobacter radioresistens (2)	105.6	26.9	0	10.0
Acinetobacter radioresistens (1)	104.6	26.7	0	10.5
Unnamed (N. 161)	0	0	0	4.3

* Numbers (1, 2 and 3) in column 1 refer to origin of cultures (Table 9.1)

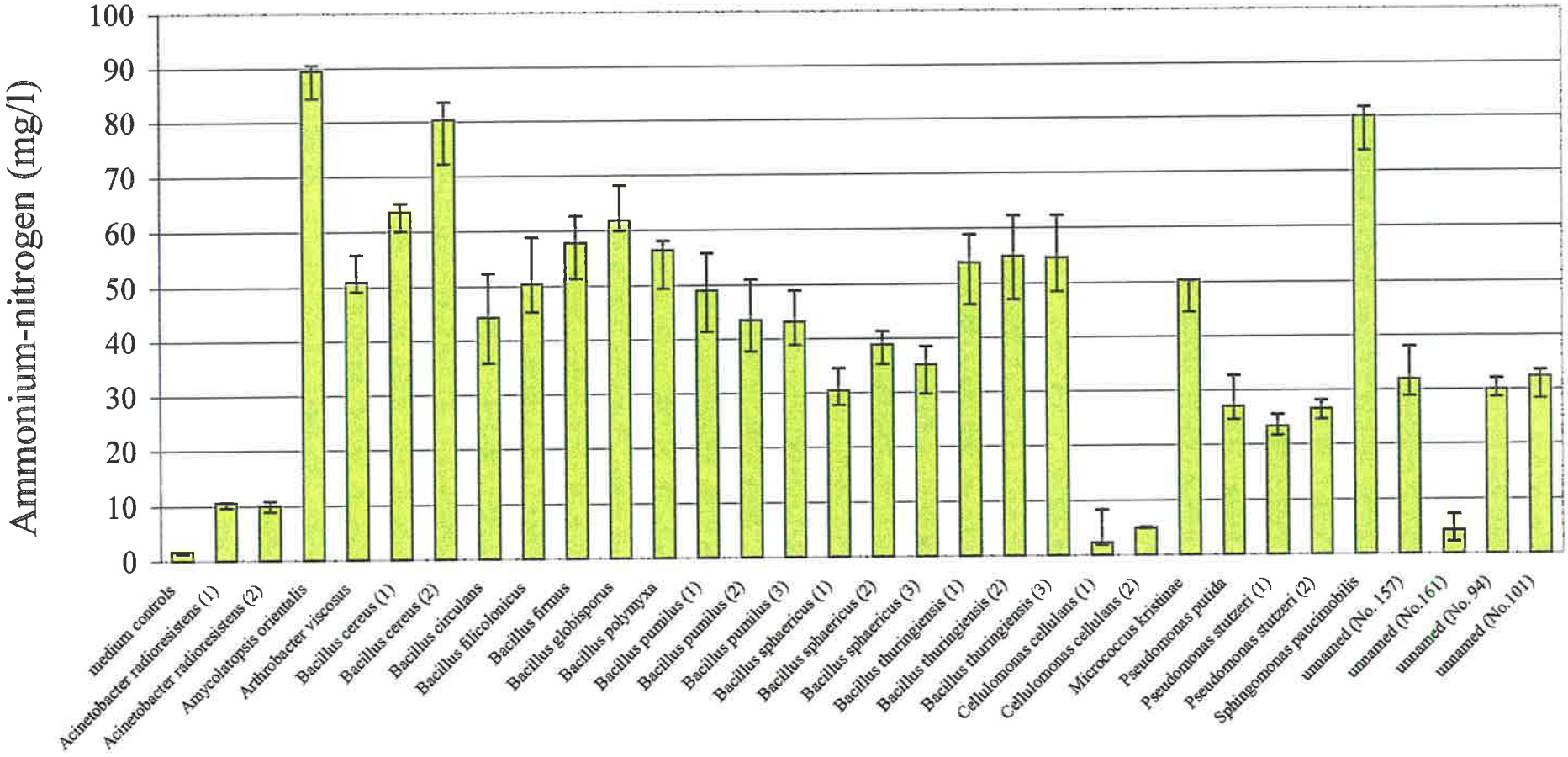

Figure 9.8. Ammonium-nitrogen production from potassium hexacyanoferrate by each bacterial species (av. of 3 reps.)

Comparatively, low levels of ammonium-nitrogen and very low to no cyanate were measured for the *Pseudomonas* species (*P. putida* and *P. stutzeri*) plus the unnamed strains nos. 157, 94 and 101 (Figure 9.8). The unnamed bacterium no. 157 had similar characteristics to nos. 94 and 101, i.e. was Gram positive, catalase positive cocci. In the *Bacillus* group, 11 species out of the 16 strains included in the experiment required additional ammonium-nitrogen from the peptone. The five strains that may not have used the peptone-nitrogen, were the three *B. sphaericus* species and two of the three *B. pumilus*. These five *Bacillus* strains may have only used the hexacyanoferrate for their nitrogen requirements.

In the production of cyanate the highest levels were measured for the two bacteria *Sphingomonas paucimobilis* with a level of 35.4 mg l^{-1} and *Amycolatopsis orientalis* with 33 mg l^{-1}, followed by *Bacillus polymyxa* with 28.5 mg l^{-1} (Figure 9.9). No cyanate was found for the three *Pseudomonas* strains, but the *Bacillus* group covered a wide range of cyanate values of 28.5 mg l^{-1} for *B. polymyxa* to 0 mg l^{-1} for *B. sphaericus* (1) as shown in Figure 9.9. Almost all of the bacterial strains, 26 out of 31, gave moderate to very high levels (greater than 15 mg l^{-1}) of ammonium-nitrogen and 11 from the same group produced quite high levels (greater or equal to 6 mg l^{-1}) of cyanate. Therefore in this degradation, the enzymes activated may have been cyanide monoxygenase and cyanase. In work using a *Pseudomonas* sp. to degrade hydrogen cyanide, Harris and Knowles (1983b) found that the enzyme cyanide monoxygenase converted cyanide to cyanate. Furthermore, because the work by Taussig (1960) showed that cyanate could be utilized by an enzyme called cyanase to form carbon dioxide and ammonia, the potassium hexacyanoferrate could have been

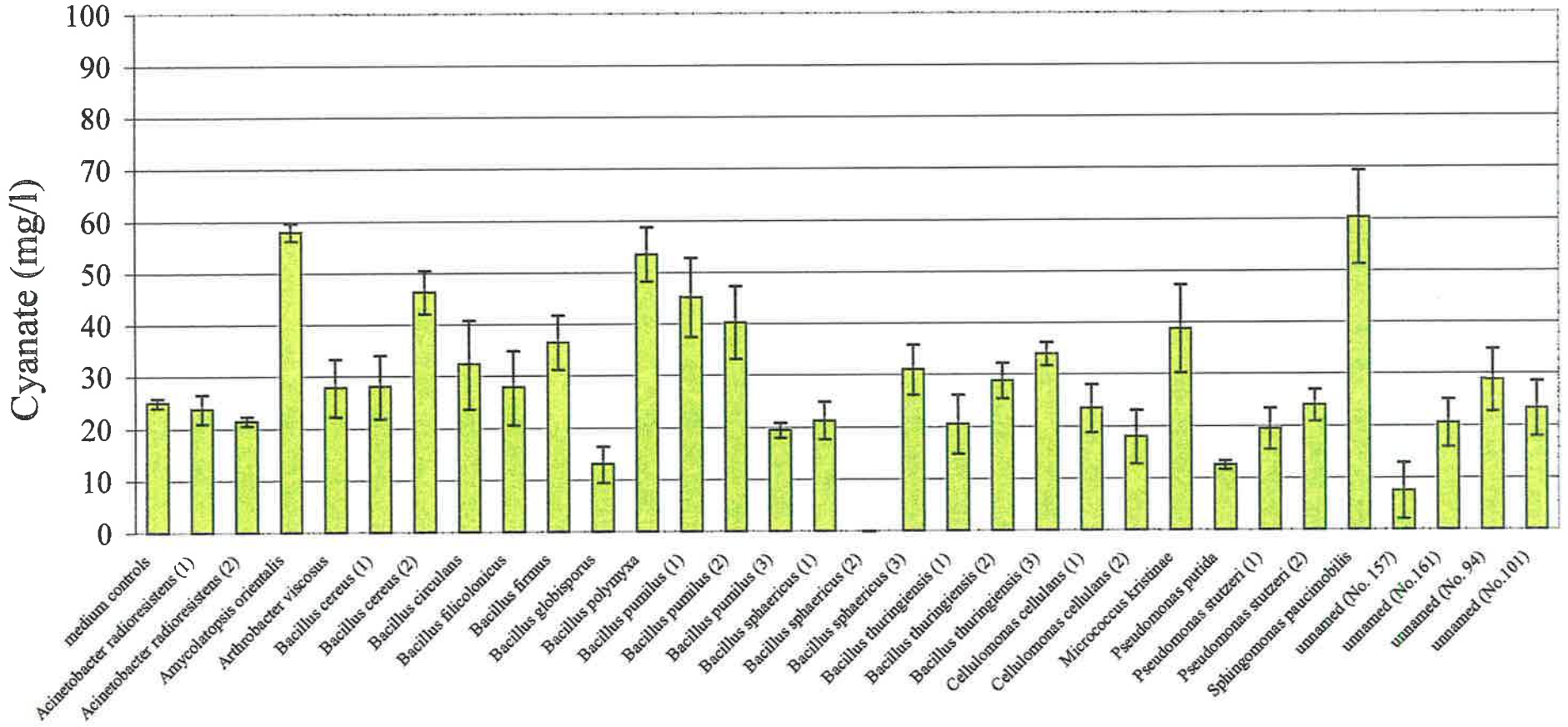

Figure 9.9. Cyanate production from potassium hexacyanoferrate by each bacterial species (av. of 3 reps.)

degraded by a combination of the two enzymes, cyanide monoxygenase (reaction 3) and cyanase (reaction 4).

$$\text{metal complex-CN} + O_2 + H^+ + \text{NADPH} \rightarrow CNO^- + H_2O + NADP^+ \quad (3)$$

$$\text{metal complex-}CNO^- + H_2O \rightarrow CO_2 + NH_3 \quad (4)$$

9.3.4 *Sodium cyanate utilization*

In the medium used for this test (Table 9.2) which lasted for 13 days, nitrogen was only present in the form of cyanate (CNO^-) with glucose added as the organic carbon source (Taussig, 1960). Peptone was not used in this test since a carbon source without nitrogen was required.

Cyanate was an end product of some metal cyanide degradation processes by certain bacterial species while others utilized the cyanate to form ammonia and carbon dioxide. From the results, it appeared that few of the bacterial species had the ability to facilitate the conversion of the cyanate.

As well, little ammonium-nitrogen was produced from sodium cyanate by any of the 31 bacterial strains tested (Figure 9.10). But some increase in the level of ammonium-nitrogen was found for three *Bacillus* species, *B. circulans*, *B. filicolonicus* and *B. firmus,* three *Pseudomonas* spp., *P. putida* and the two *P. stutzeri* (Palleroni *et al.*, 1984) and three unidentified species nos. 94, 101 and 157.

With a possible maximum nitrogen of 140 mg l^{-1}, from the 0.65 g l^{-1} sodium cyanate in the medium (Figure 9.11), the bacterial species for which this amount of nitrogen could be accounted, were *B. circulans*, *B. firmus*, the two *P. stutzeri*

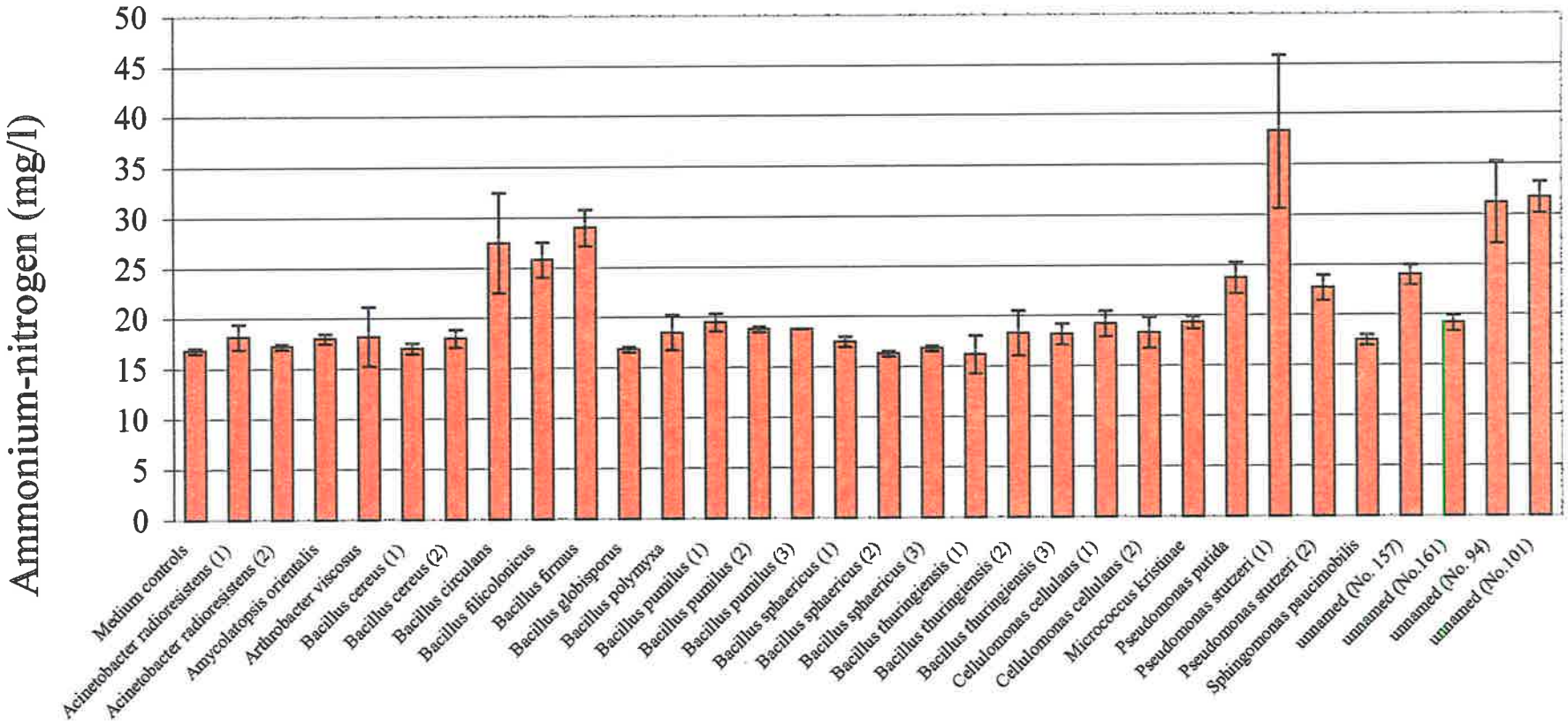

Figure 9.10. Ammonium-nitrogen formed from sodium cyanate by each bacterial species (av. of 3 reps.)

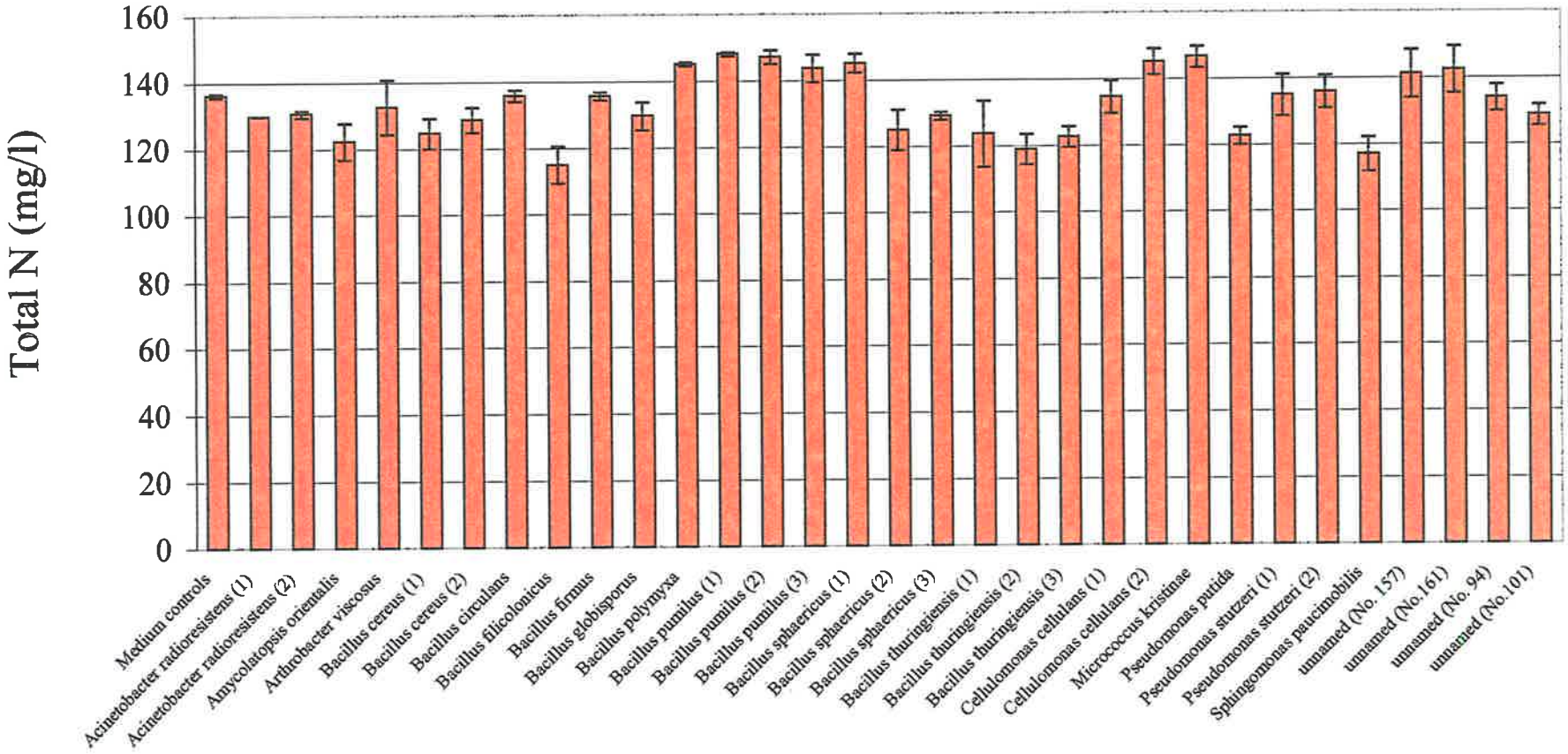

Figure 9.11. Nitrogen balance, including cyanate-nitrogen, for each bacterial species (av. of 3 reps.)

strains and nos. 94, 101 and 157. A total nitrogen value was calculated for each bacterial species by adding the nitrogen residual in the medium which was still bound in the cyanate anion, to the ammonium-nitrogen produced by the bacteria (Figure 9.11).

For the other strains e.g. *Amycolatopsis orientalis*, one of the *B. cereus* species, *B. filicolonicus*, one of the *B. sphaericus* strains (isolated from the copper cyanide study, Table 9.1), the three *B. thuringiensis* strains, *P. putida* and *Sphingomonas paucimobilis*, the total nitrogen value (Figure 9.11) did not reach the 140 mg l^{-1}. It is suspected that these 9 bacterial strains formed end products not investigated in this study e.g. nitrite, nitrate, formate or formamide.

9.4 *Chapter overview*

In the degradation studies for copper(I) cyanide, only for *Bacillus sphaericus* (1) which reduced the copper(I) cyanide by 27.7% could all of the ammonium-nitrogen (3.5 mg l^{-1}) have originated in the cyanide compound (Table 9.3). *Sphingomonas paucimobilis* (Palleroni, 1984) showed 21.3% degradation but most of the measured ammonium-nitrogen was found to come from the peptone. For *Amycolatopsis orientalis* a high ammonium-nitrogen level was found, however, because there was no degradation of the original copper(I) cyanide, the ammonium-nitrogen had resulted from the utilization of the nitrogenous components in the peptone.

None of the 31 bacterial species produced more than a trace of the end product cyanate. More commonly, the bacterial strains listed in Table 9.3, degraded some copper(I) cyanide but also metabolised the nitrogen in the peptone to form

ammonium-nitrogen. The main constituents of peptone are amino acids and peptides which can readily be used by soil bacteria with the activation of the enzymes proteases and amidases (Tate, 1995). In this study, the *B. sphaericus* (Sneath *et al.*, 1986) could have employed the enzyme cyanide dioxygenase or cyanidase for conversion of cyanide to ammonium-nitrogen. While the remaining group of bacteria would have had amidase, protease and cyanide dioxygenase enzymes operating during the conversion of cyanide, and the organic-nitrogen from the peptone, to ammonium-nitrogen (Dubey and Holmes, 1995).

In studies with sodium tetracyanonickelate, two unnamed bacterial strains nos. 94 and 101were the most efficient with 66% and 80% of the added cyanide being utilized. Since only ammonium-nitrogen was formed, a very effective cyanide dioxygenase system was operating in these two bacterial strains.
Three other species, *Sphingomonas paucimobilis, B. globisporus* and the no. 157 were found to utilize this complex to form ammonium-nitrogen but not cyanate, thereby indicating that in the presence of this compound cyanide dioxygenase was again active.

In most of the remaining species presented in Figure 9.5, a mix of amidase and protease enzymes were operating but none of the enzymes involved in cyanide degradation. This was true for all of the species where no degradation of the cyanide complex had occurred. In these species only the enzymes able to use the nitrogen in the peptone were active. When compounds copper(I) cyanide or sodium tetracyanonickelate were degraded, very little or no cyanate was detected, therefore the pathway for conversion of the bound cyanide to ammonium-nitrogen and carbon dioxide was via the enzyme cyanide dioxygenase.

Other pathways have been described where cyanide monoxygenase converted the cyanide to cyanate and the enzyme cyanase hydrolysed the cyanate to ammonium-nitrogen (Dubey and Holmes, 1995). It was postulated that the degradation of potassium hexacyanoferrate(III) followed this alternate pathway.

Contrary to some work reported in the literature, 30 species out of the 31 strains tested, did degrade potassium hexacyanoferrate with the most efficient being one of the three *B. sphaericus* strains.

Large amounts of cyanate (greater or equal to 6.0 mg l^{-1}) were produced by 10 strains and for these, high levels of ammonium-nitrogen (higher than 40 mg l^{-1}) were also measured which indicated that the enzymes cyanide monoxygenase and cyanase were active. Most of these 10 bacterial strains that had degraded the potassium hexacyanoferrate(III) also utilized some peptone to form ammonium-nitrogen.

Fourteen of the bacterial strains degraded potassium hexacyanoferrate(III) to only produce ammonium-nitrogen while another 16 species formed both cyanate and ammonium-nitrogen. In the 14 species, the enzyme cyanide dioxygenase could have been activated to form ammonium-nitrogen and in the 16 bacterial strains, both the cyanide monoxygenase and cyanase would have operated. It was found that 4 strains out of the 14, produced only traces of ammonium-nitrogen and no cyanate, therefore, other by-products such as formate, nitrate or formamide could have formed since degradation of the cyanide complex had taken place.

In some species which could use both the metal cyanide and the peptone, a mix of amidase, protease, cyanide dioxygenase and cyanase enzymes would be contributing to the formation of the measured ammonium-nitrogen.

The degradation of sodium cyanate study was included to assess which of the 31 species had the ability to express the cyanase enzyme and thereby convert the bound cyanate-nitrogen to ammonium-nitrogen. Only 9 bacteria species were found to degrade this compound efficiently, *Amycolatopsis orientalis*, one of the two *B. cereus* strains, *B. filicolonicus*, one of the *B. sphaericus* strains, the three *B. thuringiensis* strains, *P. putida* and *Sphingomonas paucimobilis.* However, because no ammonium-nitrogen was measured for 7 out of the 9 species, i.e. *Amycolatopsis orientalis*, the *B. cereus* strain, the *B. sphaericus* strain, the three *B. thuringiensis* strains and *Sphingomonas paucimobilis*, there was a shortfall in the total nitrogen value for these species (Figure 9.11) i.e. the value 140 mg l^{-1} was not reached.

It is suggested that either a nitrogen product other than ammonium-nitrogen was formed by unknown enzymes in these species or that the cyanase enzyme was active and the ammonium-nitrogen produced was assimilated into new cell components.

For two of the nine strains i.e. *B. filicolonicus* and *P. putida*, a small amount of the end product ammonium-nitrogen was indicative that the cells contained the enzyme cyanase. Furthermore, *B. filicolonicus* and *P. putida* were also below the possible 140 mg l^{-1} nitrogen level, suggesting that in addition to the ammonium-nitrogen another end product was formed, or some assimilation had again occurred.

The enzyme cyanase appeared difficult to induce and the utilization of cyanate was a rare event rather than a common occurrence.

If the bacterium *Amycolatopsis orientalis* is studied for each of the three metal cyanides, it is found that this species did not degrade copper(I) cyanide, slightly degraded sodium tetracyanonickelate but efficiently broke down potassium hexacyanoferrate. In the degradation of sodium tetracyanonickelate the enzyme employed was cyanide dioxygenase but for potassium hexacyanoferrate the active enzymes were cyanide monoxygenase and cyanase.

While *Sphingomonas paucimobilis* had the ability to degrade all three cyanide compounds, this was again achieved by using two pathways. Both copper(I) cyanide and sodium tetracyanonickelate were degraded by the enzyme cyanide dioxygenase while the degradation of potassium hexacyanoferrate required the enzymes cyanide monoxygenase and cyanase.

Chemically, the strongest complex of the three tested was potassium hexacyanoferrate(III) and two pathways were used in the degradation process by most bacterial species, the first being an oxidation of the cyanide to cyanate, and the second hydrolysis to ammonium-nitrogen and carbon dioxide.

Because most of the individual bacterial strains could not degrade cyanate, it is therefore likely that the following three enzymatic systems were operating:

1) The conversion of the metal cyanides to carbon dioxide and ammonium-nitrogen,
2) The production of cyanate and

3) The degradation of the metal cyanides to formate, formamide or nitrate as suggested by Kunz *et al.*, (1992).

Alternatively, the behaviour of bacterial cells in a study where degradation of the cyanate was measured using a chemical (sodium cyanate) added to the medium may not be the same as when the cyanate itself is a by-product of bacterial decomposition of a cyanide compound.

Chapter 10.0 Comparative behaviour of the three metal cyanides

10.1 *Introduction*

Utilization of cyanides by microorganisms has been the subject of continuous research for many years. In 1955, Pettet and Ware reported the isolation of a cyanide utilizing bacterium that was Gram positive and filamentous. Winter (1963) also isolated a Gram positive organism that probably was an Actinomycete. Unfortunately, some cultures which had been active cyanide degraders subsequently lost this function (Knowles and Bunch, 1986). After some attempts, Harris and Knowles, (1983a) isolated a Gram negative, oxidase positive *Pseudomonas fluorescens* strain that used cyanide as a nitrogen source. This later became *Pseudomonas fluorescens* NCIB (also NCIMB) 11764, a popular choice for many researchers.

Throughout the period that cyanide degradation was investigated, only a few metal cyanides were studied. While Pettet and Mills (1954) found that sewage bacteria were able to degrade cyanide complexes of cadmium, zinc and copper, no bacterial species were identified in the study. Even fewer papers have reported detailed research on metal cyanides, e.g. tetracyanonickelate(II) by Rollinson *et al.* (1987) and zinc, copper, silver and iron cyanide complexes by Shpak *et al.* (1995). Both of these papers reported research using a *Pseudomonas fluorescens* strain.

Overall, studies have rarely evaluated the toxicity or degradability of the metal cyanide complexes by bacteria.

The research in Chapters 3 to 8 was conducted to assess the extent to which the three chosen metal cyanide compounds, copper(I) cyanide, sodium tetracyanonickelate and potassium hexacyanoferrate(III), could be degraded by naturally occurring bacterial species residing on the gold ore particles. Parameters tested included, using sterile and non sterile mineral salts solutions as growth media, various pH levels, addition of an organic supplement, i.e. peptone, chemical vs biological reactions and shake flask cultures vs bioreactors. In Chapter 9, many bacterial species previously isolated from the various degradation experiments were tested as individual strains on the same three metal cyanide compounds. Since few of the original isolates were pseudomonads, only three cultures a *P. putida* and two *P. stutzeri* strains formed part of the tests in Chapter 9.

A comparison of the degradation rates for the three metal cyanides under various conditions, as well as the degradation products, is presented in this chapter. The ability of a diversity of species to utilize each of the cyanide complexes is also summarized.

10.2 *Discussion of the degradation rates in the two systems*

10.2.1 *Shake flask cultures*

Table 10.1. Degradation rates (mg l^{-1} day^{-1}) for the three metal cyanides

Metal cyanide	pH 8		pH 10	
	- P	+ P	- P	+ P
Cu(I)CN	0	0.62	0	0.74
$Na_2Ni(CN)_4$	0.86	2.29	0	0.62
$K_3Fe(CN)_6$	1.86	17.5	0.47	0

-P indicates absence and +P indicates presence of peptone

In 5 out of the 6 sets of results in Table 10.1, it is seen that the inclusion of peptone in the DMS media was beneficial to the utilization of the metal cyanide by the microflora present. Even for the sparingly soluble copper(I) cyanide, degradation occurred when the bacterial growth was assisted by the organic material, peptone. For copper(I) cyanide, in the more alkaline environment, the degradation rate was not significantly different from the degradation at pH 8.

Better rates of degradation for sodium tetracyanonickelate and potassium hexacyanoferrate were measured for the more acid pH range in the presence of the organic supplement. Most of the heterotrophic bacterial species were encouraged by the presence of peptone and better utilized the bound cyanide at pH 8.

10.2.2 *Bioreactors*

The ability of bioreactor B to outperform bioreactor A in 5 of the 6 sets of results (Table 10.2) is attributed to the pretreatment when peptone had been added to the DMS medium. For copper(I) cyanide, in the second cycle, the performance

improved in bioreactor A as the microflora slowly adapted to the growth conditions, but deteriorated in bioreactor B where the residual organic nutrient had by now been depleted. Similar results were obtained for bioreactor A when sodium tetracyanonickelate was introduced into the system, with an adaptive behaviour in both bioreactors. For this metal cyanide, however, bioreactor B gave better degradation rates than bioreactor A in both cycles.

Interestingly, for the strong metal cyanide potassium ferricyanide, the better bioreactor was A, in the 1st cycle with a deterioration observed in the second cycle.

In the bioreactors, degradation rates generally were much higher than those in the shake flask cultures. In both bioreactors, a fouling process began in the 1st cycle and intensified during the second cycle.

Table 10.2. Degradation rates (mg l^{-1} day^{-1}) for the three metal cyanides

Metal cyanide	Bioreactor A		Bioreactor B	
	Cycle 1	Cycle 2	Cycle 1	Cycle 2
Cu(I)CN	1.6	2.7	8.3	5.7
$Na_2Ni(CN)_4$	1.9	3.2	2.9	9.6
$K_3Fe(CN)_6$	3.6	0.74	2.2	0.84

10.3 *By-products from the degradation of metal cyanides*

10.3.1 *Shake flask cultures*

Ammonium-nitrogen was only determined for sodium tetracyanonickelate and potassium hexacyanoferrate at the end of the experimental period.

Table 10.3. Ammonium-nitrogen (mg l^{-1}) production from two metal cyanides in the absence of peptone at termination

Metal cyanide	pH 8	pH 10
$Na_2Ni(CN)_4$	12.1	1.8
$K_3Fe(CN)_6$	8.3	1.0

In the case of both metal cyanides, there remained a higher level of ammonium-nitrogen at pH 8 (Table 10.3). No determinations for the treatment where peptone was added are shown since ammonium-nitrogen can be formed from the nitrogenous components in the peptone. Low levels of ammonium-nitrogen were found at pH 10, for the two metal cyanide complexes (Table 10.3), this correlated well with the low degradation rates calculated (Table 10.1).

Table 10.4. Cyanate (mg l^{-1}) production from three metal cyanides at termination

Metal cyanide	pH 8		pH 10	
	- P	+ P	- P	+ P
Cu(I)CN	2.8	33.0	6.3	1.8
$Na_2Ni(CN)_4$	14.4	25.0	<0.3	<0.3
$K_3Fe(CN)_6$	3.3	116.4	13.5	1.7

-P indicates absence and +P indicates presence of peptone

Cyanate is formed as a consequence of metal cyanide degradation, either as an intermediate or end product. Further hydrolysis can occur at certain times when the appropriate bacterial enzymes are active. At pH 8, more cyanate was detected when peptone was present for each of the three metal cyanides at the end of the test period (Table 10.4), with potassium hexacyanoferrate producing the greatest

amount. Data at pH 10 showed different trends for each of the metal complexes, with almost no cyanate measured for sodium tetracyanonickelate either in the presence or the absence of peptone (Chapter 5). Although, a low degradation rate of 0.62 mg l^{-1} day^{-1} was measured when peptone was present (Table 10.1).

The small amounts of cyanate shown for copper(I) cyanide at pH 8 and pH 10 without peptone (Chapter 3) and for potassium hexacyanoferrate at pH 10 with peptone represented only a background level (Chapter 7) since no degradation occurred in these three treatments (Table 10.1).

10.3.2 *Bioreactors*

Table 10.5. Ammonium-nitrogen (mg l^{-1}) production from three metal cyanides

	Bioreactor A				Bioreactor B			
Metal cyanide	**Cycle 1**		**Cycle 2**		**Cycle 1**		**Cycle 2**	
	#Max.	**##End**	**Max.**	**End**	**Max.**	**End**	**Max.**	**End**
Cu(I)CN	12	12	18	13	96*	96*	34	15
$Na_2Ni(CN)_4$	27	27	29	29	17	8	28	13
$K_3Fe(CN)_6$	13	9	0	0	0	0	0	0

*Residual ammonium-nitrogen from pretreatment also present
#Max. indicate the maximum value measured
##End shows the value at termination

The value of 96 mg l^{-1} is suspect due to the pretreatment when 1 g l^{-1} peptone was added to bioreactor B (Table 10.5) and the ammonium-nitrogen measured therefore may have been produced from residual peptone. Ammonium-nitrogen always was an end product for both copper(I) cyanide and sodium

tetracyanonickelate. For potassium hexacyanoferrate this is only true in the first cycle in bioreactor A.

The sets of data (Table 10.5) where a decrease occurred between the maximum value of ammonium-nitrogen and the value at the end, indicated that some ammonium-nitrogen was further utilized by the bacterial cells.

All three metal cyanide compounds were degraded to some extent in both cycle (Table 10.2) and in most, ammonium-nitrogen was produced (Table 10.5). However, for potassium hexacyanoferrate(III), no ammonium-nitrogen was detected in bioreactor A during the second cycle or for bioreactor B, in either cycle. This suggested that the low levels of ammonium-nitrogen available from the degradation processes were assimilated by the bacterial species for cellular growth.

Table 10.6. Cyanate (mg l^{-1}) production from three metal cyanides

	Bioreactor A				Bioreactor B			
Metal cyanide	**Cycle 1**		**Cycle 2**		**Cycle 1**		**Cycle 2**	
	#Max.	**##End**	**Max.**	**End**	**Max.**	**End**	**Max.**	**End**
Cu(I)CN	*ND	*ND	11	0	*ND	*ND	7	0
$Na_2Ni(CN)_4$	35	0	16	0	30	27	21	9
$K_3Fe(CN)_6$	46	0	0	0	12	4	0	0

*ND means test was not done for this cycle
#Max. indicate the maximum value measured
##End shows the value at termination

Overall, more cyanate was produced from the degradation processes in bioreactor A (Table 10.6). The highest level was found for potassium hexacyanoferrate, indicating that degradation had occurred at a fast rate (Table 10.2) in bioreactor A

during the first cycle, reaching a maximum at day 5 and followed by a rapid utilization or conversion (Chapter 8) to other products. The high level of cyanate formed from potassium hexacyanoferrate was related to the fact that this complex contained the greatest amount of cyanide, (0.474 M) or 95 mg l^{-1} (Chapter 7) which could be oxidized to cyanate.

Levels of cyanate decreased rapidly after the maximum for the two bioreactors during the degradation of copper(I) cyanide (Chapter 4). A decrease was also measured during the utilization of sodium tetracyanonickelate in both bioreactors (Chapter 6), although this was more pronounced in bioreactor A where all of the cyanate was utilized. Similarly a decrease to zero for cyanate was observed for potassium hexacyanoferrate during the first cycle in bioreactor A (Chapter 8). In all cases where a value was available, the cyanate was completely utilized by the bacterial consortium in bioreactor A. This indicated that the pretreatment in bioreactor B when peptone was added (Chapter 2) had encouraged a biomass which only slowly converted cyanate to other products. Moreover, the complete utilization of cyanate in bioreactor A may indicate that the enzyme cyanase was efficiently operating in this system.

10.4 *Bacterial counts*

10.4.1 *Shake flask cultures*

Isolations were carried out during the experiments for copper(I) cyanide and sodium tetracyanonickelate (Table 10.7) but were only done at the end of the experiments for potassium hexacyanoferrate.

Table 10.7. Bacterial colony counts (cfu ml^{-1}) associated with the two metal cyanides during the experimental periods (all counts x 10^6)

Metal cyanide	pH 8		pH 10	
	- P	+ P	- P	+ P
Cu(I)CN	2.30	8.75	1.24	16.0
$Na_2Ni(CN)_4$	5.70	20.0	1.10	50.0

-P indicates absence and +P indicates presence of peptone

Highest bacterial numbers were counted on the DMS agar in the presence of the organic material (+P) for the four sets of data shown in Table 10.7.

It is seen in Table 10.7, that adding peptone encouraged a higher level of biomass during the experiments at the two pH levels and for each of the two metal cyanides tested.

Table 10.8. Bacterial colony counts (cfu ml^{-1}) associated with the three metal cyanides at termination (all counts x 10^6)

Metal cyanide	pH 8		pH 10	
	- P	+ P	- P	+ P
Cu(I)CN	0.06	60.4	0.25	42.9
$Na_2Ni(CN)_4$	3.6	25.5	4.7	174.0
$K_3Fe(CN)_6$	14.9	72.0	15.3	154.0

-P indicates absence and +P indicates presence of peptone

Including peptone in the DMS agar always encouraged greater bacterial numbers for each of the three metal cyanides at either pH (Table 10.8). Lowest counts were generally obtained when copper was added, except at pH 8 with peptone. Much higher levels of bacteria were found at pH 10 plus organic matter, for both sodium tetracyanonickelate and potassium hexacyanoferrate, a moderately strong and a

very strong complex respectively. In Table 10.1, the data indicated that little degradation of the sodium tetracyanonickelate and no degradation of potassium hexacyanoferrate had occurred by the end of the experimental period at pH 10. Therefore most of the bacteria counted were organotrophic and merely grew on the peptone.

10.4.2 *Bioreactors*

No isolations were carried out for copper(I) cyanide during the first cycle since the counts in bioreactor B would have been distorted because of the pretreatment and a small amount of residual peptone may have been present.

Table 10.9. Bacterial colony counts (cfu ml^{-1}) associated with the three metal cyanides during the experimental periods (all counts x 10^6)

Metal cyanide	Bioreactor A		Bioreactor B	
	Cycle 1	Cycle 2	Cycle 1	Cycle 2
Cu(I)CN	ND*	0.5	ND*	11.0
$Na_2Ni(CN)_4$	ND*	9.5	ND*	49.0
$K_3Fe(CN)_6$	66.0	62.0	81.0	67.0

*ND means counts not done

The data for the three metal cyanides during the second cycles, showed that bioreactor B always performed better, as indicated by numbers on the DMS agar plates, and of the three cyanide substrates, potassium hexacyanoferrate supported the best bacterial growth (Table 10.9) with copper(I) cyanide being the least attractive.

Table 10.10. Bacterial colony counts (cfu ml^{-1}) associated with the three metal cyanides at termination (all counts x 10^6)

Metal cyanide	Bioreactor A		Bioreactor B	
	Cycle 1	Cycle 2	Cycle 1	Cycle 2
Cu(I)CN	ND*	8.6	ND*	25.0
$Na_2Ni(CN)_4$	6.4	15.0	150.0	170.0
$K_3Fe(CN)_6$	370.0	17.6	430.0	46.0

*ND means counts not done

Copper(I) cyanide counts were not done at termination of the first cycle due to the presence of residual peptone in bioreactor B. As seen in Table 10.10, the lowest counts on the DMS agar were again found in the copper(I) cyanide experiments, with bioreactor B supporting higher counts than bioreactor A. For all three metal cyanides bioreactor B performed better. Very high numbers of bacteria were present in both bioreactors during the experiment with potassium hexacyanoferrate and were particularly high by the end of the first cycle but numbers dropped sharply by the end of the second cycle. Again, in the bioreactors with potassium hexacyanoferrate, most of the biomass consisted of non cyanide degrading species since a low degradation rate was calculated for this cycle.

10.5 *Degradation by individual bacterial strains*

Natural bacteria isolated from the gold ore were tested on copper(I) cyanide, sodium tetracyanonickelate and potassium hexacyanoferrate DMS plus peptone media (Chapters 9).

An evaluation of the behaviour of the 31 bacterial strains showed that few species could readily degrade all three metal cyanides (Table 10.11). This characteristic

was found, however, in *Sphingomonas paucimobilis* and an unnamed strains (No. 101). A fatty acids methyl esters fingerprint of each bacterium shows the difference between the two strains (Figure 10.1).

The bacterium *Sphingomonas paucimobilis* was able to utilize the metal cyanides with the following rates of degradation, 6.74 mg l^{-1} of potassium hexacyanoferrate day^{-1}, 0.86 mg l^{-1} of copper(I) cyanide day^{-1} and 0.48 mg l^{-1} of sodium tetracyanonickelate day^{-1} while the sequence for no.101 was 8.65 mg l^{-1} of potassium hexacyanoferrate day^{-1}, 3.09 mg l^{-1} of sodium tetracyanonickelate day^{-1} and 0.52 mg l^{-1} of copper(I) cyanide day^{-1}.

In both cases, potassium hexacyanoferrate was degraded most efficiently. In fact 30 strains out of the 31 tested (the exception being no. 161) rapidly utilized the potassium ferricyanide with very high degradation levels.

For the copper cyanide compound, 20 bacterial strains, and when the tetracyanonickelate was added, 16 species gave a degradation value.

It was found that the incubation times for both the copper(I) cyanide and sodium tetracyanonickelate in the pure culture studies were long enough to observe any utilization of the compounds. Although for the hexacyanoferrate anion, the duration of the pure culture experiment was only 21 days compared to 68 and 82 days in the bioreactors, the percent degradation obtained was extremely high (Table 10.11). This suggested that the bioreactors, especially in the second degradation cycle during the hexacyanoferrate work (Chapter 8), had stopped functioning well.

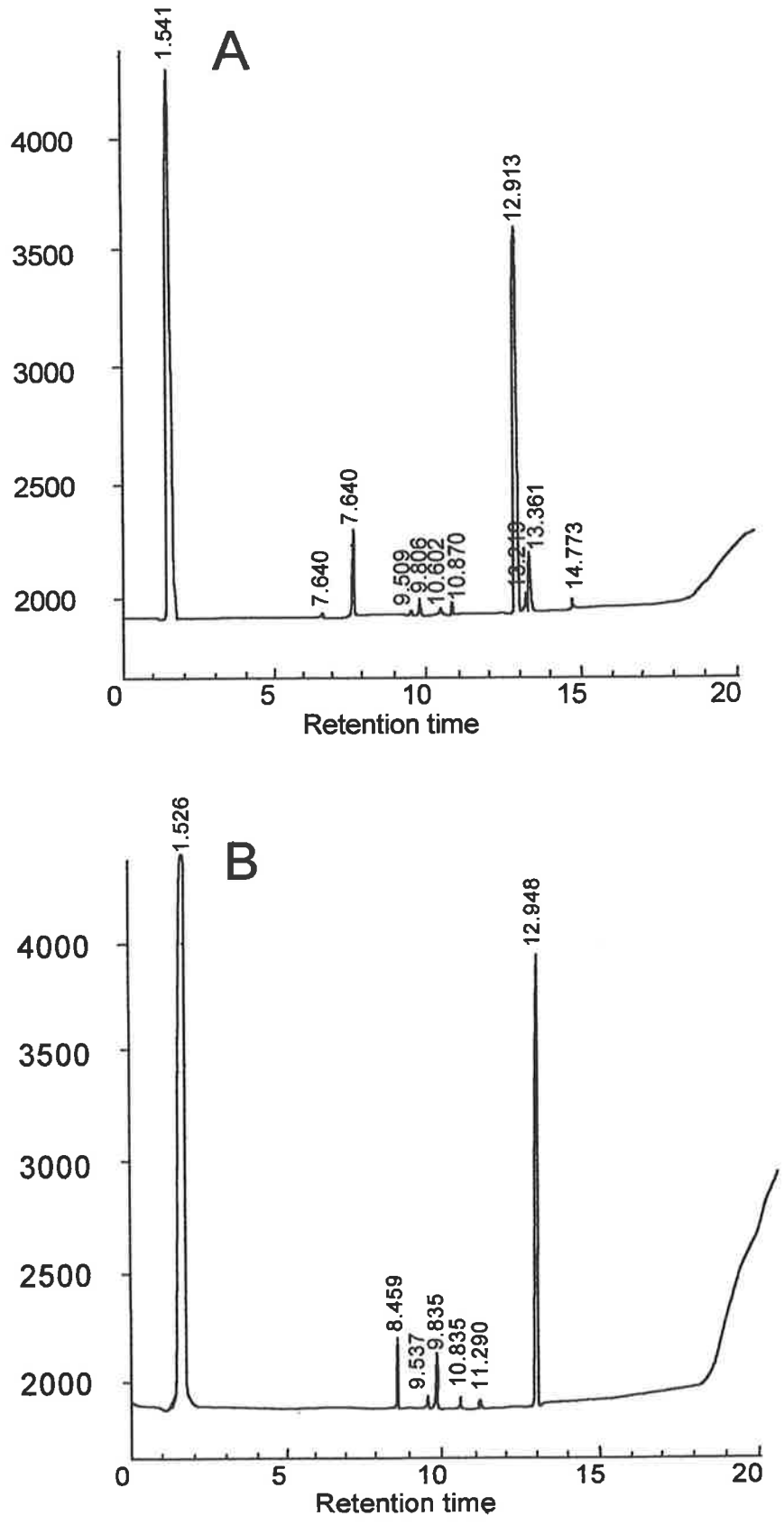

Figure 10.1. MIDI-FAME profiles for the unidentified bacterium no. 101 (A) and *Sphingomonas paucimobilis* (B).

Table 10.11. Degradation (%) of three metal cyanides by 31 bacterial species

Bacterial species	Cu(I)CN	Na_2NiCN_4	K_3FeCN_6
Acinetobacter radioresistens (1)	2	0	52
Acinetobacter radioresistens (2)	2	0	53
Amycolatopsis orientalis	0	5	91
Arthrobacter viscosus	2	0	78
Bacillus cereus (1)	2	1	88
Bacillus cereus (2)	0	0	86
Bacillus circulans	11	0	80
Bacillus filicolonicus	4	0	83
Bacillus firmus	2	0	86
Bacillus globisporus	2	9	85
Bacillus polymyxa	13	1	85
Bacillus pumilus (1)	0	0	90
Bacillus pumilus (2)	0	0	89
Bacillus pumilus (3)	0	0	87
Bacillus sphaericus (1)	28	0	97
Bacillus sphaericus (2)	6	0	79
Bacillus sphaericus (3)	6	5	80
Bacillus thuringiensis (1)	6	0	80
Bacillus thuringiensis (2)	0	2	84
Bacillus thuringiensis (3)	0	6	84
Cellulomonas cellulans (1)	0	6	91
Cellulomonas cellulans (2)	4	0	95
Micrococcus kristinae	0	0	90
Pseudomonas putida	5	3	95
Pseudomonas stutzeri (1)	4	5	74
Pseudomonas stutzeri (2)	5	1	85
Sphingomonas paucimobilis	21	13	71
Unnamed (No. 157)	0	7	90
Unnamed (No.161)	15	2	0
Unnamed (No. 94)	0	66	90
Unnamed (No.101)	13	80	91

* Numbers (1, 2 and 3) in column 1 refer to origin of cultures (Table 9.1)

10.6 *Evaluation of the enzyme activity*

Four principle enzymes have been studied in connection with cyanide degradation by bacteria:

1) Cyanide monoxygenase (Harris and Knowles, 1983b)

$$CN^- \rightarrow CNO^-$$

2) Cyanide dioxygenase (Harris and Knowles, 1983a)

$$CN^- \rightarrow NH_4^+ + CO_2$$

3) Cyanase (Taussig, 1960; Raybuck, 1992)

$$CNO^- \rightarrow CO_2 + NH_4^+$$

4) Cyanidase (Ingvorsen *et al.*, 1991; Watanabe *et al.*, 1998)

$$CN^- + H_20 \rightarrow HCOOH + NH_3$$

Degradation of metal cyanide complexes is believed to follow similar pathways as degradation of the simple cyanides, e.g. sodium cyanide, with the same enzymes responding to the presence of the bound cyanide.

In Chapter 9, it was reported that sodium cyanate was not degraded by the majority of the pure cultures. This signifies that the enzyme cyanase was generally slow or not active in the pure cultures.

Nonetheless cyanate was formed during the degradation processes in the shake flask and bioreactors, but in most cases subsequently disappeared from the

cultures. Therefore cyanase was active in promoting the hydrolysis of cyanate. In addition, ammonium-nitrogen was detected in the absence of any organic matter, therefore cyanide dioxygenase, cyanidase (Watanabe *et al.*, 1998) or cyanase was activated in the system. No analyses for formic acid and formamide were done in this work but Kunz *et al.* (1992) found formate and formamide in their study. It is possible that these end products were also formed, but were not measured in the experiments reported in this chapter.

10.7 *Chapter overview*

At the conclusion of the degradation studies in the shake flask cultures, conducted with the three metal cyanides, the peptone treatments gave better results. This included good rates of degradation mostly at pH 8, however, higher bacterial counts were usually found at pH 10. Bacterial numbers indicated that the species present preferred the availability of some organic supplement. Higher bacterial counts at pH 10 showed that many strains were alkalophilic but did not degrade metal cyanides.

In the presence of peptone at pH 8, higher cyanate levels were measured which complemented the higher degradation rates. This signified that high levels of cyanate were produced and persisted at pH 8 when peptone was present. At pH 10, this did not occur, indicating that either cyanate was not produced or was quickly converted to carbon dioxide and ammonium-nitrogen. However, at pH 10, a higher level of cyanate was found in the absence of peptone for copper(I) cyanide and hexacyanoferrate(III). Less ammonium-nitrogen was measured at

pH 10 than at pH 8, in the absence of peptone for tetracyanonickelate and hexacyanoferrate. There usually appeared to be an inverse relationship between detecting the formation of ammonium-nitrogen and cyanate, in the absence of peptone for both pH 8 and 10.

The batch bioreactors A and B, differed in that a pretreatment was given to bioreactor B. This was done to promote both a greater biomass and a different consortium of microorganisms. In bioreactor A, for copper(I) cyanide and sodium tetracyanonickelate(II) the second cycle was better, but for potassium hexacyanoferrate(III) the first cycle gave higher degradation rates. Also in bioreactor A, the degradation rates for copper(I) cyanide and tetracyanonickelate(II) complemented the ammonium-nitrogen results with slightly higher values in the second cycle. When analysing hexacyanoferrate the degradation rate was better in the first cycle and this coincided with a higher cyanate value. This trend was also observed for bioreactor B, with the only difference being that the copper(I) cyanide degradation was higher in the first cycle.

In would appear that a different composition of organisms was established in bioreactor B, and that this difference was lessened over subsequent experiments.

Representative bacterial strains, both from the shake flask and bioreactors, showed that many were able to degrade one or two of the metal cyanides, however, only two degraded all three (Table 10.11).

The very high results measured for the hexacyanoferrate(III) degradation

suggested that the available iron (Fe^{+++}) may have enhanced enzyme activity. The Fe^{++}/Fe^{+++} cations are part of the redox system by which the cytochrome oxidases assist in cell respiration (Smith *et al.*, 1987).

Interestingly, the activity of cyanide dihydratase, an enzyme characterised from a strain of *Bacillus pumilus,* was found to be enhanced in the presence of Fe^{+++} (Meyers *et al.*, 1993).

Chapter 11.0 General discussion and conclusions

11.1 *General discussion*

Aerobic degradation by microorganisms of simple cyanides e.g. NaCN, KCN has been the subject of continual research for many years with the involvement of both fungal and bacterial species.

Fungi are eukaryotes with protective features such as chlamydospores and have assimilatory processes that assist the cells to resist the deleterious effects of cyanide. Incorporation of this toxic material by a basidiomycete snow mould was shown by Strobel (1967) where cyanide was included mainly into the amino acid, alanine. Other fungi including *Rhizopus nigricans* also converted cyanide to alanine (Allen and Strobel, 1966). Further studies with radioactive carbon, have indicated the formation of glutamic (Strobel, 1967) and α-aminobutyric acids (Allen and Strobel, 1966). In addition, it was found that cyanide could be quantitatively converted to formamide by the spores or mycelia of *Stemphylium loti* and *Gloeocercospora sorghi* (Fry and Munch, 1975). These studies showed that in some fungal genera, the enzyme cyanide hydratase utilized the components of cyanide (carbon and nitrogen) thereby detoxifying the anion and removing any harmful effects.

Slowly the focus of research was diverted to bacteria for the detoxification of cyanides. In the early years, pioneer researchers such as Harris and Knowles (1983a) began to report that some bacterial species had the ability to tolerate cyanide.

Due to the reactivity of the cyanide anion, its use is increasing in industrial processes. Photography, electroplating and gold mining are just a few of the procedures that rely on cyanide and each contributes to cyanide containing wastes.

All of the chemical reactions in mine tailings have not been clearly defined. Many possible reactions are suspected between metals and cyanide, but also between the cyanide released from these complexes and intermediary ammonium compounds, with the eventual formation of carbon dioxide and ammonia. Further nitrification of ammonia producing nitrite and nitrate is also possible.

Many hundreds of thousands of dollars are spent yearly in most gold producing countries to recover the cyanide from waste material, to confine these wastes in impoundment areas and to detoxify the tailings. The toxicity of tailings material has been reported in Chapter 1, with case studies of the devastating results when tailings are inadvertently released. Although new improved chemical technologies for the destruction of cyanide and metal cyanides are continually being cited, the only biodegradation method for these compounds that is cited frequently, is operating at the Homestake Mine.

Biodegradation may take a longer time than chemical destruction but after it is completed, the waters can safely be used for irrigation or for addition to wetlands. Metals cyanides are converted to metal hydroxides from which the metals can be retrieved and sold. The toxicity of waste materials after biodegradation will be lower and after the initial cost for infrastructure construction, the maintenance costs would be reduced.

Biomass is self generating, given a moderate pH environment and for a faster regeneration the only requirement is some organic matter. From this modest beginning, cyanide and metal cyanides can be rendered harmless.

The United Nations, since the 1950's, has encouraged recycling and recovery of metals. An expert working group convened by the United Nations Industrial Development Organisation (UNIDO) in 1969 wrote that it was advisable, *"To optimize the use of material by utilizing where practicable and environmentally sound, the residues from production processes"*. It went further and recommended that *"States should encourage industry to exercise environmental responsible care through hazardous waste reduction"* (Brett and Kadushkin, 1996).

11.2 *Outcomes from the shake flask culture system*

The initial pH of the system was important mainly for the degradation rates of sodium tetracyanonickelate and potassium hexacyanoferrate(III). Greater degradation at pH 8 was measured regardless of the presence or absence of peptone.

Generally the tailings in the impoundment areas at gold mine sites are at pH 10-11, however, as the tailings 'age', the pH is slowly lowered. This is a natural process due to the infusion of carbon dioxide from the air and from the acidic end products, e.g. organic acids from bacterial growth.

Consequently, the pH moves towards the neutral point. This movement in pH can now be said to encourage metal cyanide degradation.

Although at times, a greater number of alkalophiles were counted at pH 10, they were not as effective as the bacterial consortium acting at pH 8. The addition of peptone did not alter this finding.

The two intermediate or end products measured in this research were ammonium-nitrogen and cyanate. Both of these were generally at a higher level at pH 8 by the termination date of the experiment and this was due to the greater level of degradation that had occurred at this pH. It is believed that the main enzymes involved were cyanide monoxygenase, cyanide dioxygenase and cyanidase with cyanase also present, but only at low levels.

11.3 *Outcomes from the bioreactor system*

The results from the bioreactor experiments, clearly indicated that the pretreatment with a small amount (1 g l^{-1}) peptone was beneficial. Generally, the microorganisms in bioreactor B degraded the three metal cyanides faster than bioreactor A where no such pretreatment was given. This was essentially the response to the organic supplement since both bioreactors were set up at pH 9.4. The level of the bacterial population coincided with better degradation rates in bioreactor B both during and at termination of the two degradation cycles.

Levels of ammonium-nitrogen at the end of the test period were higher in bioreactor A for two metal cyanides, sodium tetracyanonickelate and potassium hexacyanoferrate(III), but lower for copper(I) cyanide. Conversely, the cyanate level was zero for all three compounds in bioreactor A.

When the enzyme cyanase was active, the cyanate became the substrate for hydrolysis to carbon dioxide and ammonium-nitrogen. Research presented in

Chapter 9, however, showed that only a few bacterial species, tested as individual pure cultures, could induce the enzyme cyanase.

Complete disappearance of the cyanate did occur in bioreactor A, therefore cyanase was active in this system. In bioreactor B, enzymatic systems possibly involving cyanide monoxygenase, cyanide dioxygenase and cyanidase operated simultaneously producing both ammonium-nitrogen and cyanate. Very little cyanase could have been activated since this conversion was slow.

There is some evidence that the cyanase gene (designated *cynS*) is repressed by ammonium-nitrogen (Harano *et al.*, 1997). This could explain the overall low levels of cyanase in the work reported here since ammonium-nitrogen was usually high during the degradation experiments.

Not withstanding that faster degradation rates were noted in bioreactor B, a more polished effluent was possible from bioreactor A since all of the cyanate was utilized.

11.4 *Activity of individual bacterial species*

Little inhibition of bacteria was detected by the hexacyanoferrate(III) ion. Almost 97% of the species tested quickly utilized this cyanide complex. This may be due to the requirement for iron in the cytochrome c oxidase which is active in aerobic respiration. Similarly for the copper(I) cyanide, with 65% of the strains utilizing this cyanide compound to some extent. Again the copper is a co-factor in the reduction-oxidation processes required in aerobic respiration. Only 52% of the bacteria were involved in degrading the tetracyanonickelate(II) ion. The nickel cation, out of the three metals, is the least necessary for enzymatic activity.

Toxicity of heavy metals towards microorganisms, is well documented and the three metals tested are in the sequence least to most toxic, iron, nickel and copper.

11.5 *Conclusions drawn from the research presented*

1) **The three metal cyanides, copper(I) cyanide, sodium tetracyanonickelate and potassium hexacyanoferrate(III), can be degraded. A slightly alkaline environment of pH 8 is usually preferred by the bacterial consortium. This pH environment can be easily and safely maintained in field studies with the addition of an alkaline substance e.g. lime, to the liquid mixture.**

2) **Degradation of copper(I) cyanide, sodium tetracyanonickelate and potassium hexacyanoferrate(III) can proceed without the addition of any organic matter. However, degradation is accelerated by the inclusion of an organic supplement e.g. peptone. One of the bioreactors, with the biomass grown on 0.1% peptone, remained effective for over 10 months. Therefore, it is apparent that an efficient and inexpensive bacterial system can be designed for the degradation of metal cyanides.**

3) **All three metal cyanides were only degraded by two bacterial species, *Sphingomonas paucimobilis* and an unidentified strain no. 101. These two species were also active in the consortium which degraded the three metal cyanides in the shake flasks and bioreactors.**

   The bacterial species *Sphingomonas paucimobilis* and no. 101 adapted well to an environment of heavy metals, cyanide, cyanate and ammonium-

nitrogen while also competing effectively with other bacteria, for resources.

When constructing bioreactors for the degradation of multiple cyanide pollutants, inoculation with a consortium of specific bacterial species is recommended. This will ensure that a large component of the biomass can degrade more than one cyanide complex efficiently.

4) **Although only 6% of the bacterial species tested, degraded all three metal cyanides, potassium hexacyanoferrate(III), a chemically stable metal cyanide complex, was degraded by 97% of the bacterial species tested. The large amount of cyanide complexed as hexacyanoferrates can easily be utilized by bacteria, thereby removing this common pollutant from the environment.**

5) **Introduction of peptone leads to an accumulation of cyanate.**

When no peptone was available, cyanate was formed but was always further hydrolysed to ammonium-nitrogen and carbon dioxide by the enzyme cyanase.

Of the 31 individual bacterial species tested, 9 species could induce the activity of the cyanase enzyme.

In the absence of any organic matter and with the right mix of bacterial species, the final waste solution is more environmentally acceptable since the final product ammonium-nitrogen can easily be removed by other microbes through assimilation or utilized by plants, e.g. in a wetland system.

The conclusions presented in this final chapter show that toxic pollutants currently stored in tailings dams can be converted to beneficial plant nutrients by specific soil microbes. The inclusion of appropriately designed bioreactors at the end of the gold extraction process would achieve this desired result.

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