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Enrichment cultures and microcosm experiments for cyanide degrading bacteria

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Abstract

Cyanides, encompassing compounds with a cyano-group ($-C\equiv N$), are significant environmental pollutants originating from various anthropogenic sources. High levels of cyanides in surface- and groundwaters pose severe hazards to the aerobic lifeforms that feed in these environments by inhibiting cellular respiration. Despite numerous studies on cyanide contamination and biological cyanide degradation at the laboratory level, there is a notable gap in applying bioremediation techniques at field scale. This study aimed to lay the foundation for field-scale bioremediation by establishing enrichment cultures of indigenous bacteria capable of degrading cyanide collected from two cyanide-polluted legacy contamination sites in Vienna, Austria. A suite of experiments was conducted to establish the enrichment cultures and track their viability. The main experiments included trials with six different mineral salt media (MSM) to find optimal conditions for bacterial growth. Indigenous mixed bacteria sourced from bacterial traps exposed to contaminated groundwater were cultured in the best-performing MSM, and their vitality was tracked using optical density measurements and colony counts. Results indicate limited yet promising success in cultivating viable cyanide-degrading bacterial cultures. Enrichment cultures derived from the bac-trap material showed low cell density, extended lag phases and weak initial growth, but the cultures' ability to grow on plates with agar and potassium cyanide as sole nutrient sources implies the potential degradation capabilities of the bacteria. The groundwork laid by this study provides a foundation for continued research and development in cyanide bioremediation, emphasizing the need for further research to bridge the gap between laboratory and field-scale applications.

Zusammenfassung

Cyanide, Verbindungen mit einer Cyano-Gruppe ($-C\equiv N$), sind bedeutende Umweltschadstoffe, welche aus verschiedenen anthropogenen Quellen stammen können. Hohe Cyanidgehalte in Oberflächen- und Grundwasser stellen ein erhebliches Gefahrenpotential für aeroben Lebewesen dar, da sie die Zellatmung hemmen. Trotz zahlreicher Studien zur Cyanidkontaminationen und biologischen Cyanidabbau in Laboren gibt es noch kaum Feldstudien zur Anwendung im Bereich der Bioremediation von kontaminierten Standorten. Ziel dieser Arbeit war es, die Grundlage für die Bioremediation im Feldmaßstab zu schaffen, indem Anreicherungskulturen von Bakterien etabliert wurden, die Cyanid abbauen können und aus zwei cyanidkontaminierten Altlastenstandorten in Wien, Österreich, stammen. Eine Reihe von Experimenten wurde durchgeführt, um die Anreicherungskulturen zu etablieren und ihre Lebensfähigkeit zu verfolgen. Die Hauptexperimente umfassten Versuche mit sechs verschiedenen Mineralsalzmedien (MSM), um die optimalen Bedingungen für das Bakterienwachstum zu finden. Autochtone Bakterienkulturen die mittels Aufwuchskörpern („bac-traps“) in Grundwassermessstellend der kontaminierten Standorte gesammelt worden waren, wurden in dem am besten geeigneten MSM kultiviert und ihre Vitalität mittels optischer Dichtemessungen und Koloniezählungen überwacht. Die Ergebnisse zeigen bedingte, aber vielversprechenden Erfolg bei der Kultivierung lebensfähiger cyanidabbauender Bakterienkulturen. Anreicherungskulturen aus dem Bac-Trap-Material zeigten eine geringe Zelldichte, verlängerte Lag-Phasen und schwaches Initialwachstum. Die Fähigkeit der Kulturen, auf Platten mit Agar und Kaliumcyanid als einzige Nährstoffquelle zu wachsen, impliziert jedoch die potenziellen Abbaukapazitäten der Bakterien. Die erarbeiteten Grundlagen bieten eine Basis für weiterführende Forschung und Entwicklung im Bereich der Cyanid-Bioremediation und zeigen die Notwendigkeit weiterer Forschungen, um die Lücke zwischen Labor- und Feldanwendungen zu schließen.

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List of Abbreviations

CN	Cyanide
HCN	Hydrogen Cyanide
MGP	Manufactured Gas Plant
MSM	Mineral Salt Medium
OD	Optical Density
SAD	Strong-acid-dissociable
TSA	Tryptic Soy Broth Agar
WAD	Weak-acid-dissociable

1. Introduction

Cyanides is a collective term covering all compounds containing a cyano-group, a carbon atom triple-bonded to a nitrogen atom ($\text{-C}\equiv\text{N}$). Cyanides can exist as a gaseous, liquid, and solid species, often complexing with metal cations in nature. Cyanide is known for its toxicity, which is caused by the high reactivity of the so-called free cyanide, a term that includes both hydrogen cyanide (HCN) and the cyanide anion (CN^-) (ATSDR, 2006). HCN is highly soluble in water, but also highly volatile. While certain metal-cyanide complexes are inherently toxic, the environmental impact of most cyanide species arises from their tendency to release free cyanide through dissociation (Gensemer et al., 2006).

Cyanide contamination of surface- and groundwaters is extremely toxic to the aerobic lifeforms that feed in these environments. CN^- can not only form complexes with metal cations in natural waters but also with various metallic species present in enzymatic structures. For example, cyanide can react with the ferric heme iron found in the cytochrome c oxidase structure, thereby inhibiting the respiration of the cell and impeding its function. (Jones et al., 1984; Massey & Edmondson, 1970). The inability of microbial communities to perform their functions is disastrous to the environment. Cyanide is lethal to aquatic life in $\mu\text{g/L}$ concentrations and to birds, mammals and humans in sub-mg/kg bodyweight doses (ATSDR, 2006; Cipollone et al., 2006; Donato et al., 2007; Gensemer et al., 2006).

Cyanides predominantly enter the environment through human activities, stemming from various anthropogenic sources such as gold mining, electroplating, fossil fuel combustion — especially coal combustion — petroleum refining, iron and steel production, steel hardening, plastic production and burning, incineration of household waste, water and wastewater treatment, vehicle exhaust, cigarette smoking, dissolution of road salts, biomass burning, and the application of pesticides, fungicides, and herbicides in agriculture and some food and feed production (Bruno et al., 2023; Dzombak, Ghosh, & Wong-Chong, 2006; Jaszczak et al., 2017; Lottermoser, 2010). Legacy contamination is prevalent in numerous sites across the USA and Europe, with manufactured gas plants (MGPs) being a common locus (Young & Theis, 1991).

Despite the predominantly anthropogenic origin of environmental cyanides, a variety of organisms, including plants, insects, fungi, and bacteria, contribute to cyanide production: Plants use the bitter taste of cyanogenic glucosides to act as a defence against pathogens and herbivores, while certain bacteria possess the capability to degrade cyanide, utilizing the carbon and nitrogen as resources (Baxter & Cummings, 2006a). Because cyanides are found widely in nature, natural

degradation pathways readily exist. In soils, the cyanide ion is rapidly complexed by trace metals, microbially metabolized or lost through volatilization when the pH is lower than 11.5 (Gagnon et al., 2004; Nakles et al., 2006; Oudjehani et al., 2002; Özel et al., 2010). Nevertheless, anthropological sources often accumulate cyanide to environmentally deteriorating degrees, necessitating remediation efforts.

Cyanide contamination is a widespread issue observed in soils and groundwaters across various industrial sites, both currently active and legacy contamination sites. Currently active industries are subject to present-day strict environmental regulations, whereas legacy contamination sites rarely had to comply with environmental standards. Former manufactured gas plants (MGP) are common legacy contamination sites. Kuppusamy et al. (2017) estimate almost 4,000 MGP sites in Europe and 36,000-55,000 in the USA. The gas manufacturing process produced spent oxide-box material as residue, which contained mostly iron sulphides, elemental sulphur and double-iron-cyanide solid complexes such as Prussian Blue and Turnbull's Blue (Ghosh, Meeussen, et al., 2006). The spent oxide-box material was repurposed as fill material both onsite and offsite, contaminating the soil and groundwater at the deposition sites. The sulphur species in the deposited material have rendered the soils extremely acidic, and the solid cyanides give the contaminated soils an intense blue colour.

It's crucial to understand the different forms and systematics of cyanides present in the polluted environment to be able to effectively manage the contamination. Prussian Blue and Turnbull's Blue are extremely stable in dark, acidic soils like those of former MGPs (Ghosh, Dzombak, & Luthy, 1999; Mansfeldt et al., 1998; Meeussen et al., 1994; Meeussen, Keizer, Van Riemsdijk, et al., 1992; Theis et al., 1994). The blue solid-phase cyanides dominate cyanide speciation in these sites and therefore serve as the primary source of dissolved cyanide species, in this case, the strong iron-cyanide complexes hexacyanoferrate II and III (Ghosh, Dzombak, Luthy, et al., 1999). Strong complexes have a high stability constant, i.e., they are relatively stable and dissociate only in strongly acidic conditions ($\text{pH} < 2$). An estimated 10 % or less of the aqueous cyanide at former MGP sites exist as other types of weak cyanide complexes, and free cyanide constitutes only a fraction of the pollution (Ghosh, Dzombak, Luthy, et al., 1999; Theis et al., 1994). The mobility of the pollutant determines its bioavailability and distribution in the environment, thus representing its ecotoxicity. The main mechanisms influencing the cyanide species mobility are colloidal transport for the solid-phase cyanides and dissolution-precipitation and sorption-desorption processes for the aqueous cyanide species (Mansfeldt et al., 2004; Meeussen et al., 1994; Sut et al., 2015; Theis & West, 1986)

Legacy contamination sites like former MGP sites often have cyanide pollution levels exceeding defined actionable threshold values. The harmful effects of cyanide contamination in soils,

groundwater, and water bodies warrant effective remediation measures. Remediation methods are generally categorized as physical, chemical, or biological. Natural attenuation is also possible. While biological remediation aims to degrade the cyanide compounds with plants, microbes or fungi, physical and chemical treatments can also recover and recycle cyanide for reuse (Botz et al., 2016; Mekuto et al., 2016). Bioremediation has been reported to be cost-effective compared to conventional physicochemical methods and faster than natural attenuation (Kumar et al., 2017; Özel et al., 2010). However, while many successes in laboratory conditions have been achieved, studies presenting field-scale bioremediation results are lacking.

Microbial degradation makes use of a diverse mixture of indigenous soil bacteria adapted to degrade cyanide through prolonged exposure (Mudder et al., 1991). Bioremediation begins by testing the degradative potential of the indigenous soil bacteria by establishing enrichment cultures. This method aims to enrich those microorganisms with biodegradative capabilities and assess their performance (Zaidi & Imam, 2008). In this study, bacterial cultures were obtained from groundwater and bac-trap samples collected from two former Manufactured Gas Plant (MGP) sites in Vienna, characterized by elevated levels of cyanide in the groundwater. The final objective of this work was to establish active enrichment cultures from the collected indigenous bacteria and track their vitality and performance for future application in degrading polluted sites.

2. State of Knowledge

2.1. Systematics of cyanide compounds

Cyanide is a collective term covering all compounds containing the CN functional group, a carbon atom triple-bonded to a nitrogen atom. The inorganic cyanide anion CN^- and the hydrogen cyanide (HCN) are known as free cyanide. It, and many other cyanide species, are acknowledged for their potent toxicity (ATSDR, 2006). The toxicological significance of a particular cyanide species is determined by its dissociative properties, i.e. its tendency to release free cyanide (Ghosh, Dzombak, & Wong-Chong, 2006). Individual cyanide species have distinct toxicity and reactivity profiles, which influence their transport and fate mechanisms in natural environments, and eventually also their susceptibility to different treatment approaches – be they physical, chemical or biological. Cyanide species exist in gaseous, liquid, and solid states, some of which are introduced in Figure 1:

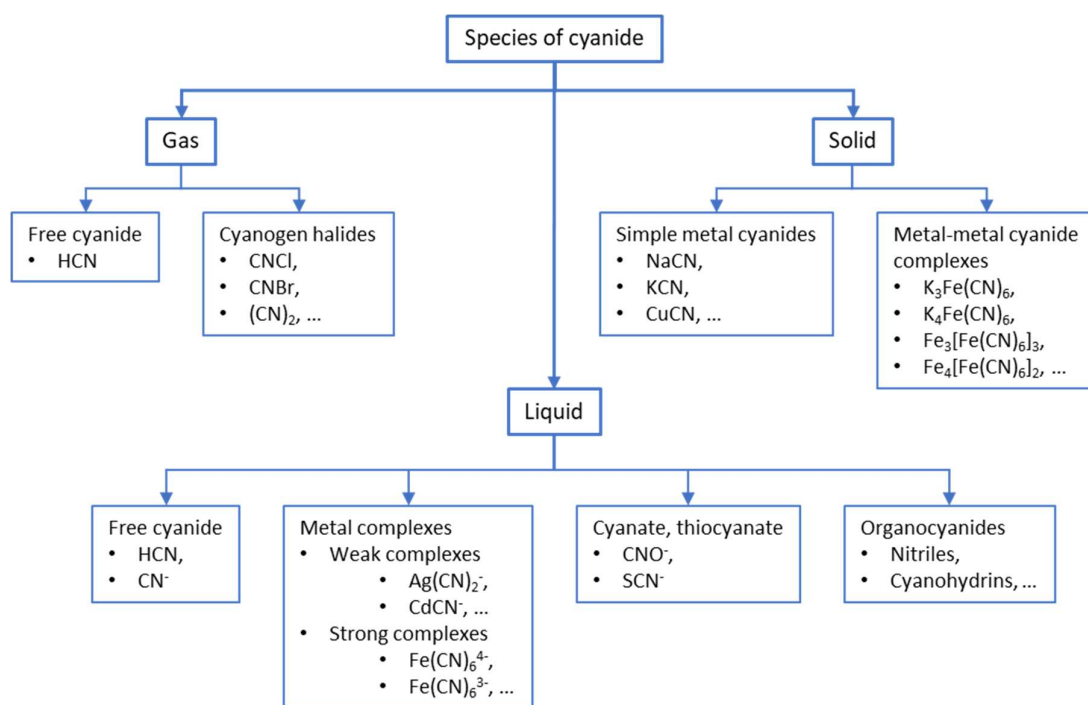


Figure 1: Common species of cyanide in gas, liquid and solid phases. Adapted from (Ghosh, Dzombak, & Wong-Chong, 2006)

2.1.1. Gaseous cyanide species

The gaseous phases of cyanide include hydrogen cyanide (HCN) as free cyanide, cyanogen ((CN)₂), and cyanogen halides, such as cyanogen chloride (CNCl) and cyanogen bromide (CNBr). HCN is the most common gaseous cyanide, it is colourless and has a faint odour of bitter almonds (National Center for Biotechnology Information, 2024). HCN has a high vapour pressure (742 mm Hg at 25°C) and Henry's Law constant (1.33×10^{-4} atm-cu m/mole), both values indicating a very high volatility. HCN can be found in the atmosphere in pptv concentrations, where its largest source is biomass burning with minor contributions from industrial activities, tobacco smoking, vehicular exhausts and natural HCN formation (Bruno et al., 2023; Jaszczak et al., 2017).

Other species of cyanide that can be found as gases are highly volatile CNCl and CNBr, which are by-products of water disinfection and wastewater treatment (Ghosh, Dzombak, & Wong-Chong, 2006; Jiang et al., 2022). However, CNCl and CNBr quickly degrade in water by hydrolysis at high pH. Additionally, volatile (CN)₂ and CNCl have been reported to be found in gold mine leachates, however, their persistence in the environment is uncertain (Johnson, 2015).

2.1.2. Aqueous cyanide species

Aqueous cyanide species can be broadly classified into four distinct categories, as depicted in Figure 1: free cyanide, metal-complexed cyanides (further divided into weak and strong complexes by their dissociation affinity), cyanite and thiocyanate, and organocyanides (Ghosh, Dzombak, & Wong-Chong, 2006). Within a solution, free cyanide is considered as both HCN and the cyanide anion CN⁻. HCN is highly soluble in water, undergoing dissociation according to the following equation (Dzombak, Ghosh, & Young, 2006):



The cyanide anion, CN⁻, exclusively exists as a soluble anion, whereas HCN manifests in both aqueous and gaseous forms. HCN is the dominating species at pH levels below 9.2, posing a considerable risk due to its quick volatilization and toxicity. The cyanide anion serves as a versatile ligand that reacts with various transition metal cations to form metal-cyanide complexes (Dzombak, Ghosh, & Young, 2006). These complexes are further categorized into weak and strong variants, based on the strength of the CN-metal bond. Weak complexes are less reactive than free cyanide and undergo dissociation under mildly acidic conditions (pH 4-6) – they are commonly termed weak-acid-dissociable (WAD) complexes. Strong complexes are relatively stable and dissociate in strongly acidic conditions (pH

<2), commonly termed strong-acid-dissociable (SAD) complexes. The general formula for metal-cyanide dissociation can be expressed as:



where M = Metal cation, x = number of cyanide groups, and n = ionic charge of the species. Table 1 provides an overview of some prevalent aqueous metal-cyanide complexes, along with their associated stability constants:

Table 1: Common aqueous metal-cyanide complexes and their stability constants. Adapted from (Kumar et al., 2017)

Cyanide species	IUPAC name	Strength of the CN-metal bond	Stability constant (logK at 25°C)
[Co(CN) ₆] ³⁻	Hexacyanocobaltate (III)	Strong	64
[Fe(CN) ₆] ³⁻	Hexacyanoferrate (III)	Strong	43.6
[Pd(CN) ₄] ²⁻	Tetracyanopalladate (II)	Strong	42.4
[Pt(CN) ₄] ²⁻	Tetracyanoplatinate (II)	Strong	40
[Hg(CN) ₄] ²⁻	Tetracyanomercurate (II)	Strong	39
[Au(CN) ₂] ²⁻	Dicyanoaurate (I)	Strong	37
[Fe(CN) ₆] ⁴⁻	Hexacyanoferrate (II)	Strong	35.4
[Ni(CN) ₄] ²⁻	Tetracyanonickelate (II)	Weak	30.2
[Cu(CN) ₄] ³⁻	Tetracyanocuprate (I)	Weak	23.1
[Cu(CN) ₃] ²⁻	Tricyanocuprate (I)	Weak	Not available
[Ag(CN) ₂] ⁻	Dicyanoargentate (I)	Weak	20.5
[Zn(CN) ₄] ²⁻	Tetracyanozincate (II)	Weak	19.6
[Cd(CN) ₄] ²⁻	Tetracyanocadmium (II)	Weak	17.9

The rest of the aqueous cyanide species, cyanate, thiocyanate and organocyanides, are rarely found in environmental settings. Cyanate (CNO⁻) requires a strong oxidizing agent and a catalyst before it converts from free cyanide to CNO⁻ or the protonated version, HOCN (Mudder et al., 1991). Thiocyanate (SCN⁻) forms in a reaction of free cyanide and polysulfide (S_xS²⁻) or thiosulfate (S₂O₃²⁻), two reactive forms of sulphur commonly found in nature. Even though thiocyanate can form naturally, most of it is generated through industrial processes (Luthy, 1981). In soil and water systems, thiocyanate complexes with essentially all metals of interest (Burgess, 2004). Both cyanate and thiocyanate are substantially less toxic than free cyanide (Mudder et al., 1991). Organocyanides have a cyano group covalently bonded to a hydrocarbon group, most commonly to an alkyl group to

form nitriles (Dzombak, Ghosh, & Young, 2006). Organocyanides are produced synthetically for chemical solvents and naturally by a wide variety of plants, some algae, bacteria and fungi, as well as a few arthropod species. Cyanides can be found in substantial amounts in some seeds and fruit stones, e.g., bitter almonds, apricots, apples, and peaches (ATSDR, 2006). About 2,000 plant species produce cyanogenic glucosides as a defence against pathogens and herbivores (Larsen et al., 2004). Despite the toxicity of cyanide, many microorganisms have developed diverse metabolic pathways to detoxify the cyanide and utilize carbon and nitrogen as nutrients (Ebbs et al., 2006).

2.1.3. Solid cyanide species

In systems where an abundance of metals and cyanide coexists, they can react to form a range of solid compounds (Ghosh, Dzombak, & Wong-Chong, 2006). These solid forms can be broadly classified into two categories: simple metal-cyanide solids, characterized by high solubility, and metal-metal-cyanide complexes, which have varying degrees of solubility, as depicted in Table 2. Examples of simple metal-cyanide solids include KCN, NaCN, and CuCN, all of which are extremely soluble, readily releasing the toxic cyanide anion in water. The simple metal-cyanide solids dissociate following Equation 2 $2M(CN)_x^{n-} \rightarrow M^+ + xCN^-$

(Equation 2. In contrast, the more complex metal-metal-cyanide solids exhibit only moderate to minimal dissociation, forming stable precipitates. Due to the abundance of iron in the environment, cyanide often forms iron-iron solids, such as Prussian Blue and Turnbull's Blue (Chatwin et al., 1988; Ghosh, Dzombak, & Luthy, 1999; Ghosh, Dzombak, Luthy, et al., 1999; Mansfeldt et al., 1998; Meeussen, Keizer, Van Riemsdijk, et al., 1992; Theis et al., 1994). Complex metal-metal-cyanide compounds release cyanide in the form of dissolved metal-cyanide species, following two general dissociation formulas:



Where A = alkali or alkaline earth metal cation, M = transition metal, x = number of alkali metal cations, y = number of cyanide groups, n = number of water molecules in the solid structure, z = number of metal-cyanide complexes and m = ionic charge.

Table 2: Common solid metal-cyanide and metal-metal-cyanide complexes and their solubility products. For consistency, solubility products are presented as completely dissociated species. Adapted from (Dzombak, Ghosh, & Young, 2006)

Cyanide species	IUPAC name (common name)	Dissociation equation	Solubility product (logK _{sp} at 25°C)
NaCN	Sodium cyanide	2	-2.29
KCN	Potassium cyanide	2	-1.44
AgCN	Silver cyanide	2	16.22
CuCN	Copper(I) cyanide	2	19.49
Pb ₂ Fe(CN) ₆	Lead(II) hexacyanoferrate(II)	4	27.59
Cd ₂ Fe(CN) ₆	Cadmium hexacyanoferrate(II)	4	28.22
Zn ₂ Fe(CN) ₆	Zinc hexacyanoferrate(II)	4	29.93
K ₄ Fe(CN) ₆	Potassium hexacyanoferrate(II)	3	48.82
K ₃ Fe(CN) ₆	Potassium hexacyanoferrate(III)	3	54.64
Mn ₂ Fe(CN) ₆	Manganese(II) hexacyanoferrate(II)	4	59.03
Cu ₂ Fe(CN) ₆	Dicopper hexacyanoferrate	4	61.42
KZn _{1.5} Fe(CN) ₆	Potassium zinc hexacyanoferrate(II)	3	66.81
K ₂ Cu ₂ Fe(CN) ₆	Potassium-copper hexacyanoferrate(II)	3	72.51
K ₂ Mn ₃ (Fe(CN) ₆) ₂	Potassium-manganese hexacyanoferrate(II)	3	121.00
Fe ₃ [Fe(CN) ₆] ₂	Iron(III) hexacyanoferrate(II) (Turnbull's Blue)	4	177.40
K ₄ Ni ₄ (Fe(CN) ₆) ₃	Nickel-potassium hexacyanoferrate(II)	3	183.55
Ag ₄ Fe(CN) ₆	Silver(I) hexacyanoferrate(II)	4	193.91
Fe ₄ [Fe(CN) ₆] ₃	Iron(II) hexacyanoferrate(III) (Prussian Blue)	4	263.60

2.1.4. Behaviour of cyanide in water and soil

Aqueous and solid species of cyanides and free cyanide can be found in nature mostly due to industrial and anthropological activities, sources including gold and silver mining, electroplating, fossil fuel combustion — especially coal combustion — petroleum refining, iron and steel production, steel hardening, plastic production and burning, incineration of household waste, water and wastewater treatment, vehicle exhaust, cigarette smoking, dissolution of road salts, biomass burning, and the application of pesticides, fungicides, and herbicides in agriculture and some food and feed production (Bruno et al., 2023; Dzombak, Ghosh, & Wong-Chong, 2006; Jaszczak et al., 2017; Lottermoser, 2010). The extensive use of cyanide in industry has led to the contamination of many industrial sites, warranting research about its behaviour in the environment to facilitate treatment methods.

In soils and waters, free cyanide seldom stays biologically available, because it is either rapidly complexed by trace metals or organic carbon, microbially metabolized or lost through volatilization (Gagnon et al., 2004; Nakles et al., 2006; Oudjehani et al., 2002; Özel et al., 2010). WAD cyanide anions either dissociate or they can adsorb to common soil and sediment components that are positively charged under acidic conditions, such as iron, aluminium, silicon and manganese oxides and clays (Alesii & Fuller, 1976; Theis & Richter, 1980; Theis & West, 1986). Because iron is an

abundant element in the environment and process waters, iron–cyanide complexes are the most significant SAD metal–cyanide complex contaminants, the most common of which are hexacyanoferrate II and III (Dzombak, Ghosh, & Young, 2006). However, the hexacyanoferrates dissociate rapidly when exposed to light (Broderius & Smith, 1980; Fuller et al., 1986; Gaspar & Beck, 1983; Meeussen, Keizer, & Haan, 1992). In dark conditions underground, they can dissociate in strongly acidic conditions or adsorb similarly to weak metal-cyanide anions (Alesii & Fuller, 1976; Bushey & Dzombak, 2004; Theis & West, 1986). Ultimately, the dissociation of all aqueous cyanide complexes depends on their stability constants, pH, temperature, redox potential, and other environmental factors.

The same goes for solid metal-metal-cyanide complexes, which dissociate incompletely according to Equations 3 and 4. The most common cyanide solids, Prussian Blue and Turnbull's Blue, are scarcely soluble in acidic to neutral pH and under moderately oxic to anoxic conditions (Ghosh, Dzombak, & Luthy, 1999; Mansfeldt et al., 1998; Meeussen et al., 1994; Meeussen, Keizer, Van Riemsdijk, et al., 1992; Theis et al., 1994). The same studies proved that under alkaline and highly oxic conditions, these compounds are relatively soluble.

2.2. Cyanide at contaminated sites with a focus on former gas plants

Cyanide contamination of surface- and groundwaters is extremely toxic to the aerobic lifeforms that feed in these environments. While certain metal-cyanide complexes possess inherent toxicity (e.g., $\text{Ag}(\text{CN})_2^-$), the environmental impact of most cyanide species arises from their tendency to release free cyanide through dissociation (Gensemer et al., 2006). The toxicity is caused by the high reactivity of the cyanide anion: it can form complexes with various metallic species present in enzymatic structures, thus impeding their functions and inhibiting cellular growth (Jones et al., 1984; Massey & Edmondson, 1970). For example, cyanide can irreversibly react with the ferric heme iron found in the cytochrome c oxidase structure, thereby inhibiting the respiration of the cell. The inability of microbial communities to perform their functions is disastrous to the environment. Cyanide is also lethal to aquatic life in $\mu\text{g/L}$ concentrations and to birds, mammals and humans in sub-mg/kg bodyweight doses (ATSDR, 2006; Cipollone et al., 2006; Donato et al., 2007; Gensemer et al., 2006). Humans can be exposed to cyanides from contaminated sites by breathing air, drinking (ground)water collected near the contamination, or touching the polluted soil or water. The inherent toxicity of cyanide necessitates the control and remediation of anthropologically contaminated environments.

Cyanide contamination is a widespread issue observed in soils and groundwaters across various industrial sites, both currently active and legacy contamination sites. These include but are not limited to electroplating facilities, manufactured gas plants (MGPs), and sites associated with metal mining and ore heap leaching (Ghosh, Meeussen, et al., 2006). The cyanide-rich by-products and residues generated by these industrial processes mixed in environments with an abundance of iron predominantly result in the formation of solid-phase iron-cyanide compounds. However, certain locations, such as gold mine tailings or electroplating spill sites, have a more complex composition, featuring not only iron-cyanide compounds but also other weak-acid-dissociable (WAD) cyanide complexes, such as copper-, gold- and nickel-cyanides. This variability is dependent on site-specific factors, as demonstrated by Bakatula & Tutu (2016), Tran et al. (2022) and Zhang et al. (2015). The distribution and speciation of cyanide within an industrial site are influenced by the nature of the industrial processes employed and the prevailing environmental conditions (Ghosh, Meeussen, et al., 2006). This study focuses on former MGP sites, and so, in this chapter, the characteristics of cyanide contamination in former MGP sites are described.

Former MGP sites are commonly contaminated with sulphur, tar and iron-cyanide complexes, i.e. the by-products of the gas purification process (Ghosh, Dzombak, Luthy, et al., 1999; Ghosh, Meeussen, et al., 2006; Hayes et al., 1996; Theis et al., 1994). During the coal gasification process, hydrogen cyanide (HCN) and hydrogen sulphide (H_2S) were generated from the compounds inherent in the coal. The gas purification process involved passing the gas through containers filled with either wood shavings or crushed blast-furnace slag saturated with a chemically active form of hydrated iron oxide. A portion of the iron oxide then reacted with HCN to produce primarily solid-phase iron-cyanide complexes, such as Prussian Blue and Turnbull's Blue. However, most of the iron oxide reacted with H_2S , resulting in the formation of iron sulphides, which diminished the absorbent capacity of the iron oxide. The spent oxide-box material could undergo regeneration several times before necessitating replacement with fresh material. Eventually, the spent oxide-box material, containing iron sulphides, elemental sulphur and double-iron-cyanide solid complexes, was repurposed as fill material both onsite and offsite, contaminating the soil and groundwater at the deposition sites. The Prussian Blue present in the fill material gives the contaminated soils an intense blue colour.

So, the predominant cyanide contaminants found at former Manufactured Gas Plant (MGP) sites primarily consist of iron-cyanides, notably the double-iron-cyanide solid compounds Prussian Blue and Turnbull's Blue. The contamination dynamics are primarily dependent on the dissolution and mobility of these compounds and their dissolution by-products, alongside site hydrology and geology. The dissolution of the cyanide solids is significantly influenced by pH and redox potential

(Ghosh, Dzombak, & Luthy, 1999; Kjeldsen, 1999; Meeussen et al., 1994, 1995; Meeussen, Keizer, Van Riemsdijk, et al., 1992). Due to the sulphuric content of the deposited oxide-box residuals, the soils at the deposition grounds produce sulfuric acid via oxidation of the sulphur species which causes the soil to acidify (Rennert, 2003). The double-iron-cyanide salts are minimally soluble under acidic to neutral pH conditions (Ghosh, Dzombak, & Luthy, 1999; Meeussen, Keizer, Van Riemsdijk, et al., 1992). As a result, these solid compounds dominate in cyanide-impacted soils within the acidic to neutral pH range. Figure 2 illustrates the predominance diagram of the double-iron-cyanide solids concerning pH and redox potential within a groundwater system with goethite presence (Ghosh, Dzombak, & Luthy, 1999).

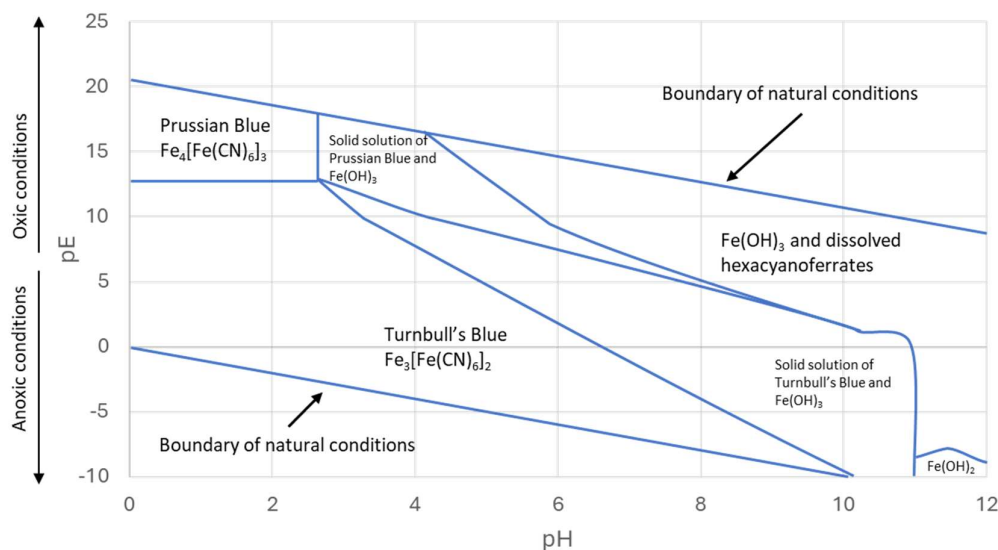
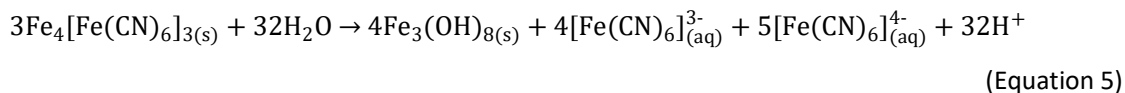


Figure 2: Predominance diagram of Prussian Blue and Turnbull's Blue as a function of pH and pE in groundwater in a system with goethite present. (Ghosh, Dzombak, & Luthy, 1999; further adapted from Ghosh, Meeussen, et al., 2006)

Eventually, Prussian Blue and Turnbull's Blue undergo dissociation in water, releasing iron oxides and hexacyanoferrates II and III, for example as follows (Sut et al., 2015):



For such incomplete dissolution of Prussian Blue, the solubility product constant is $\log K_{sp} = 84.5$ (Meeussen, 1992). Despite the markedly low solubility of solid double-iron-cyanide compounds, their dissolution products have been detected in contaminated MGP sites' groundwaters (Meeussen

et al., 1995; Rennert, 2003). As depicted in Figure 3, hexacyanoferrate III prevails under moderately oxidic to highly oxidic conditions, whereas hexacyanoferrate II dominates under anoxic conditions. These hexacyanoferrates, as elaborated in Chapter 2.1.4, are categorized as Strong Acid Dissociable (SADs) substances and can further decompose into iron cations and exceedingly toxic free cyanide anions under strongly acidic conditions or when exposed to light. The mobility of the hexacyanoferrates is mainly determined by the outlined dissolution systematics as well as precipitation, sorption and desorption (Mansfeldt et al., 2004; Meeussen et al., 1994; Theis & West, 1986).

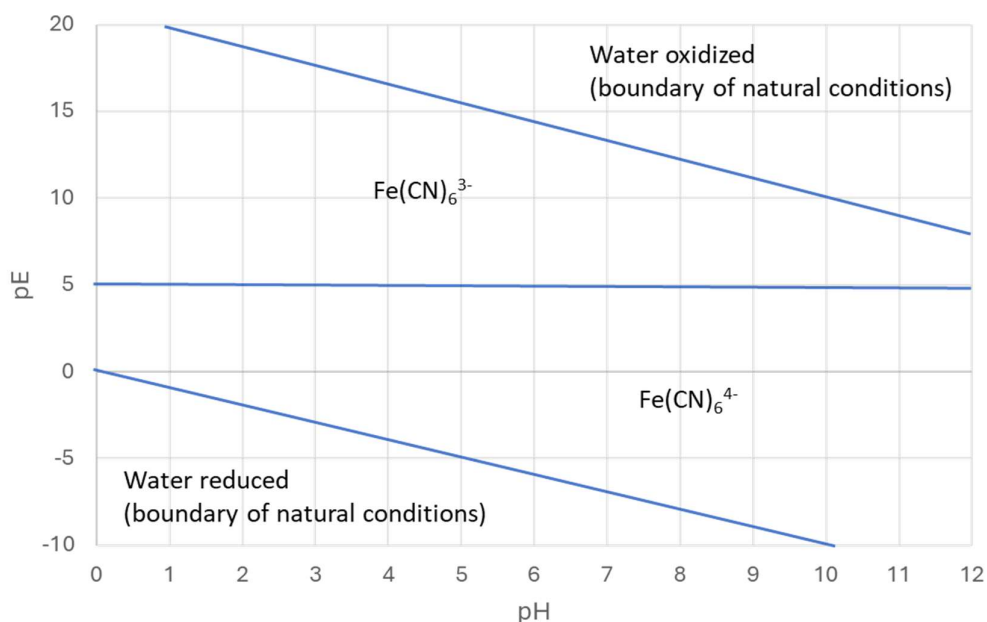


Figure 3: Predominance diagram of dissolved iron–cyanide complexes. (Ghosh, Meeussen, et al., 2006)

The sulfuric content of deposited oxide-box residuals induces soil acidification, protonating soil particle surfaces, thereby forming positively charged surfaces onto which negatively charged dissolved hexacyanoferrates can adsorb (Rennert, 2003). As pH decreases, the adsorption of iron-cyanide complexes on iron and aluminium oxides and clay minerals, which are all positively charged under acidic conditions, increases (Alesii & Fuller, 1976; Bushey & Dzombak, 2004; Rennert, 2003; Theis & West, 1986). Consequently, neutral and alkaline soils have a lower retention capacity of the iron-cyanide species than acidic soils (Alesii & Fuller, 1976; Kang et al., 2010). Additionally, natural organic matter serves as a noteworthy adsorbent for the hexacyanoferrates across a range of pH values (Rennert, 2003). Organic carbon is also an effective adsorbent for free cyanide, which adsorbs weakly to oxide minerals (Rennert, 2003; Theis & West, 1986). Another potential retention

mechanism involves the precipitation of dissolved iron-cyanide complexes, facilitated by the presence of iron generated through the oxidation of iron sulphide.

In summary, at former MGP sites, due to the acidity of the soil, the solid form cyanides Prussian Blue and Turnbull's Blue dominate the cyanide speciation. Given that these cyanide solids serve as the primary source of dissolved cyanide species, their dissolution products, the SADs hexacyanoferrate II and III, are the most prominent aqueous cyanide species (Ghosh, Dzombak, Luthy, et al., 1999). An estimated 10 % or less of the cyanide at such sites exists as weak metal-cyanide complexes, with free cyanide constituting only a fraction thereof (Ghosh, Dzombak, Luthy, et al., 1999; Theis et al., 1994). The main mechanisms influencing the cyanide species mobility have been considered as the dissolution-precipitation and sorption-desorption processes (Mansfeldt et al., 2004; Meeussen et al., 1994; Theis & West, 1986). However, owing to the exceedingly low dissolution of cyanide solids in acidic soils, colloidal transport may emerge as a primary process, as suggested by Sut et al. (2015). Other noteworthy mechanisms, such as oxidation-reduction and microbial decomposition also play significant roles, to be discussed further in Chapter 2.4.

2.3. Physical and chemical remediation of cyanides

The harmful effects of cyanide contamination in soils, groundwater, and water bodies warrant effective control and remediation measures. Industries that produce cyanide-containing waste, such as gold and silver mining or iron and steel production, are subject to regulations to prevent environmental cyanide pollution. Additionally, legacy contamination sites like former MGP sites can have cyanide pollution levels exceeding defined actionable threshold values. Given the extensive application of cyanide across industries and strict environmental regulations, many treatment methodologies have been developed. These approaches generally fall into three categories: physical, chemical, or biological. Natural attenuation is also possible. While biological remediation aims to degrade the cyanide compounds with plants, microbes or fungi, physical and chemical treatments can also recover and recycle cyanide for reuse (Botz et al., 2016; Mekuto et al., 2016).

Most of the destructive cyanide treatment techniques are based on the oxidation of cyanide molecules (Botz et al., 2016). Through the introduction of oxidizing agents, cyanide (CN^-) is converted into cyanate (OCN^-), subsequently undergoing hydrolysis to form ammonia (NH_4^+) and ultimately nitrogen gas (N_2). For example, chlorine has historically been the most widely applied oxidizer, although it has been replaced by more efficient and cost-effective processes:



Chlorine can also be used to oxidize thiocyanate:



Other common oxidizing agents include ozone, hydrogen peroxide, sulphur dioxide, and Caro's acid (Botz et al., 2016; Dash et al., 2009; Mekuto et al., 2016; Yadav et al., 2022). The oxidation can also be assisted by photolysis with the help of a photocatalyst such as titanium dioxide or a nano-thin film. The different oxidizers naturally create different by-products, which can be difficult and expensive to deal with. In addition to destructive oxidizing methods, cyanide can be recollected with simple distillation and adsorption methods, or by employing more complicated methods such as reverse osmosis, electrodialysis, acidification-volatilization-neutralization, sulfidation-acidification-recycling-thickening, gas-filled membrane absorption, or iron-cyanide precipitation. However, often cyanide is simply diluted with water before released to the environment, or left to volatilize in open water bodies.

While physical and chemical treatment methods for cyanide contamination are well established, they often have limitations such as the inability to degrade SAD cyanides, reliance on costly reagents or equipment, ongoing maintenance needs, the generation of undesirable by-products, etc. In contrast, biological remediation offers an attractive alternative that is not only efficient and cost-effective but also environmentally friendly (Kumar et al., 2017). Bioremediation is discussed further in the next Chapter (2.4).

2.4. Bioremediation of cyanides

While the conventional chemical and physical remediation methods are widely used in cyanide-utilizing industries, bioremediation offers a proven alternative (Dash et al., 2009; Ibrahim et al., 2015; Kumar et al., 2017; Malmir et al., 2022; Mekuto et al., 2016; Razanamahandry et al., 2017). Bioremediation has been reported to be cost-effective compared to traditional methods and faster than natural attenuation (Kumar et al., 2017; Özel et al., 2010). Bioremediation – *i.e.* biological degradation of pollutants – is broadly categorized into phytoremediation, involving degradation mediated by plants, and microbial remediation, which involves degradation by bacteria and fungi.

Both processes make use of enzymatic degradation pathways that convert cyanides into less toxic end products. This study concentrates on microbial degradation by bacteria.

2.4.1. Cyanide biodegradation pathways

Microbial degradation makes use of a diverse mixture of indigenous soil bacteria adapted to degrade cyanide and thiocyanide through prolonged exposure (Mudder et al., 1991). The degradation of metal cyanide complexes corresponds to their chemical stability: SAD cyanide species like iron and cobalt cyanides are very resistant to biodegradation, whereas free and simple cyanides are readily degradable and can act as an excellent source of nitrogen for bacterial growth (Akcil, 2003; Dash et al., 2009; Ibrahim et al., 2015; Kumar et al., 2017; Razanamahandry et al., 2017).

There are four common pathways for microbes to degrade cyanide in the environment: hydrolytic, oxidative, reductive and substitution/transformation reactions. Certain microorganisms may utilize more than one pathway for cyanide degradation (Ezzi & Lynch, 2005; Raybuck, 1992). In hydrolytic reactions, cyanide interacts with water under enzymatic influence to yield formate or formamide along with ammonia (Johnson, 2015). The enzymes, reactions, and associated bacteria are outlined in the following table:

Table 3: Hydrolytic pathways of enzymatic cyanide degradation. (Alvillo-Rivera et al., 2021; Dash et al., 2009; Ibrahim et al., 2015; Kumar et al., 2017; Razanamahandry et al., 2017).

Enzyme	Chemical reaction	Bacteria
Cyanide hydratase	$\text{HCN} + \text{H}_2\text{O} \rightarrow \text{HCONH}_2$	<i>Pseudomonas fluorescens</i> , <i>Micromonospora braunna</i>
Cyanidase	$\text{HCN} + 2\text{H}_2\text{O} \rightarrow \text{HCOOH}$	<i>Alcaligenes xylosoxidans</i> , <i>Alcaligenes xylosoxidans</i> , <i>Bacillus pumillus</i> , <i>Pseudomonas stutzeri</i>
Nitrile hydratase	$\text{R-CN} + \text{H}_2\text{O} \rightarrow \text{RCONH}_2$	<i>Pseudomonas (marginales, putida)</i> , <i>Corynebacterium sp</i> , <i>Rhodococcus rhodochrous</i> , <i>Brevibacterium sp</i> , <i>Klebsiella sp</i> , <i>Pseudonocardia thermophila</i>
Nitrilase	$\text{R-CN} + 2\text{H}_2\text{O} \rightarrow \text{R-COOH}$	<i>Klebsiella (sp, ozaenae)</i> , <i>Arthrobacter sp.</i> , <i>Pseudomonas sp</i> , <i>Norcadia sp.</i> , <i>Acinetobacter sp.</i> , <i>Rhodococcus rhodochrous</i> , <i>Alcaligenes faecalis</i> ,

Hydrolytic reactions involving nitriles are denoted with R, representing either an aliphatic or aromatic group. Cyanide hydratase is primarily found in fungal species, whereas cyanidase is predominantly produced by bacteria. Enzymes utilizing nitriles have been identified across a range of fungal, plant, and bacterial species. Nitrilases and nitrile hydratases can modify both aliphatic and

aromatic nitriles into their respective acid or amide forms (Ebbs et al., 2006; Gupta et al., 2010). However, they have lower substrate specificity than cyanide hydratase and cyanidase. Cyanide hydratase and nitrile hydratase directly cleave the carbon-nitrogen triple bond, resulting in the formation of formamide. Nitrilase and cyanidase, on the other hand, convert cyanide into ammonia and carboxylic acid, which are subsequently utilized in their metabolic processes.

In oxidative reactions facilitated by the presence of NADPH (nicotinamide adenine dinucleotide phosphate), cyanide undergoes a reaction with dioxygen (O₂) to yield ammonia (NH₃) and carbon dioxide (CO₂) (Ebbs et al., 2006). Table 4 provides an overview of the enzymes, reactions, and associated bacteria involved in these oxidative processes.

Table 4: Oxidative pathways of enzymatic cyanide degradation. (Alvillo-Rivera et al., 2021; Dash et al., 2009; Ibrahim et al., 2015; Kumar et al., 2017).

Enzyme	Chemical reaction	Bacteria
Cyanide monooxygenase	$\text{HCN} + \text{O}_2 + \text{H}^+ + \text{NAD(P)H} \rightarrow \text{HOCN} + \text{NAD(P)}^+ + \text{H}_2\text{O}$	<i>Pseudomonas sp.</i>
Cyanide dioxygenase	$\text{HCN} + \text{O}_2 + 2\text{H}^+ + \text{NAD(P)H} \rightarrow \text{CO}_2 + \text{NH}_3 + \text{NAD(P)}^+$	<i>Pseudomonas (fluorescens, cereus), Bacillus (cereus, pumillus), Escherichia coli</i>
Cyanase	$\text{HCN} + \text{O}_2 + \text{H}^+ + \text{NADPH} \rightarrow \text{CO}_2 + \text{NH}_3 + \text{NADP}^+$	<i>Escherichia coli, Rhodococcus rhodochrous, Pseudomonas pseudoalcaligenes</i>

Cyanide monooxygenase is responsible for converting cyanide into cyanate, which subsequently undergoes further conversion to NH₄ and CO₂ via the same pathway as cyanate derived from thiocyanate (refer to Table 7) (Ebbs et al., 2006). Cyanide dioxygenase directly generates ammonia and carbon dioxide. Cyanase is found in a variety of organisms including bacteria, fungi, plants, and animals, and its primary function is to provide protection against cyanate poisoning.

The reductive pathway initiates with the activation of the nitrogenase enzyme, which catalyzes reductive reactions following the transfer of a pair of electrons (Table 5):

Table 5: Reductive pathway of enzymatic cyanide degradation. (Alvillo-Rivera et al., 2021; Dash et al., 2009; Ibrahim et al., 2015; Kumar et al., 2017).

Enzyme	2-step chemical reaction		Bacteria
Nitrogenase	$\text{HCN} + 2\text{H}^+ + 2\text{e}^- \rightarrow$	$\text{CH}_2=\text{NH} + \text{H}_2\text{O} \rightarrow \text{CH}_2=\text{O}$	<i>Methanogenes</i> , <i>Klebsiella oxytoca</i> , <i>Rhodopseudomonas gelatinosa</i> , <i>Streptomyces thermoautotrophicus</i> , <i>Herbaspirillum seropedicae</i> , <i>Azotobacter sp.</i> , <i>Azospirillum sp.</i> , <i>Rhodospirillum rubrum</i>
		$\text{CH}_2=\text{NH} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{CH}_3-\text{NH} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{CH}_4 + \text{NH}_3$	

Anaerobic conditions are typically conducive to the reductive degradation of cyanide. The enzyme nitrogenase operates under these conditions, utilizing HCN and yielding methane and ammonia as end products. A study by Kao et al. (2003) demonstrated that bacterial strains belonging to the genus *Klebsiella* are capable of degrading both cyanide and thiocyanate.

The substitution/transfer pathway, also known as the assimilatory pathway, is catalyzed by cyanoalanine synthase and utilizes O-acetylserine as a substrate (Table 6):

Table 6: Substitution/transformation pathways of enzymatic cyanide degradation. (Alvillo-Rivera et al., 2021; Dash et al., 2009; Ibrahim et al., 2015; Kumar et al., 2017).

Enzyme	Chemical reaction	Bacteria
Cyanoalanine synthase	$\text{Cysteine} + \text{CN}^- \rightarrow \beta\text{-cyanoalanine} + \text{H}_2\text{S}$	<i>Pseudomonas sp.</i> , <i>Bacillus megaterium</i> , <i>Chromobacterium violaceum</i> , <i>Escherichia coli</i> , <i>Citrobacter freundii</i> , <i>Enterobacter sp.</i> , <i>Escherichia coli</i>
	$\text{O-acetylserine} + \text{CN}^- \rightarrow \beta\text{-cyanoalanine} + \text{CH}_3\text{COO}^-$	
Cyanide sulphurtransferase	$\text{CN}^- + \text{S}_2\text{O}_3^{2-} \rightarrow \text{SCN}^- + \text{SO}_3^{2-}$	<i>Thiobacillus denitrificans</i> , <i>Bacillus subtilis</i> , <i>Bacillus stearothermophilus</i> , <i>Azotobacter vinelandii</i> , <i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i> , <i>Ferrobacillus ferrooxidans</i> , <i>Thermobacillus denitrificans</i> , <i>Desulfotomaculum nitrificans</i>

As indicated in the table above, the assimilatory pathways operate independently of oxygen (O_2) or NAD(P)H, and do not release carbon dioxide (CO_2) during the reaction. These reactions serve to protect cells from cyanide toxicity by assimilating cyanide and provide an additional nitrogen source for the growth of the bacteria (Ebbs et al., 2006).

Thiocyanate is commonly regarded as a product of the biological transformation of cyanide rather than as a substrate for degradation. Nevertheless, bacterial degradation of thiocyanate has been documented, with researchers proposing two possible pathways (Table 7):

Table 7: Thiocyanate biodegradation pathways by enzymatic cyanide degradation. (Ebbs et al., 2006; Ibrahim et al., 2015; Kumar et al., 2017)

Enzyme	Chemical reaction		Micro-organism
Carbonyl pathway (thiocyanate hydrolase)	$\text{SCN}^- + 2\text{H}_2\text{O} \rightarrow \text{COS} + \text{NH}_3 + \text{OH}^-$		<i>Proteobacteria</i> , <i>Thioalkalimicrobium</i> sp, <i>Thioalkalivibrio</i> sp, <i>Acremonium strictum</i> , <i>Thiobacillus thioparus</i> , <i>Thiobacillus thiocyanoxidans</i> , <i>Pseudomonas stutzeri</i> , <i>Ralstonia eutropha</i> , <i>Bosea thioxidans</i> , <i>Sphingomonas paucimobilis</i>
Cyanate pathway (cyanase)	$\text{SCN}^- + 3\text{H}_2\text{O} + 2\text{O}_2 \rightarrow \text{CNO}^- + \text{HS}^- \rightarrow$	$\text{HS}^- + 2\text{O}_2 \rightarrow \text{SO}_4^{2-} + \text{H}^+$	
		$\text{CNO}^- + 3\text{H}^+ + \text{HCO}_3^- \rightarrow \text{NH}_4^+ + 2\text{CO}_2$	

The prevailing evidence primarily supports a cyanate pathway, wherein thiocyanate undergoes conversion to cyanate, followed by the subsequent generation of ammonia and carbon dioxide (Ebbs et al., 2006). Thiocyanate is used as source of carbon, nitrogen and occasionally sulphur. Thiocyanate degradation has been observed across various physicochemical conditions, including highly alkaline soda lake sediments and soils.

2.4.2. Factors affecting cyanide biodegradation

For successful biodegradation, the presence of a microorganism able to degrade the contaminants in the polluted environment is crucial. Typically, the microorganisms engaged in the biological treatment of cyanide and thiocyanate include a mixture of commonly found indigenous soil bacteria. Through prolonged exposure, these bacteria have adapted to the treatment of cyanides (Mudder et al., 1991). Each of the following factors influences the biodegradation process:

- *pH*: cyanide degrading bacteria typically thrive within a pH range of 6-8, and the degrading enzymes at pH 6-9. Thiocyanate degradation is optimal at a higher pH of 10 (Akciil & Mudder, 2003). Extreme pH levels can significantly impede the biodegradation process. However, many researchers prefer conducting experiments with alkaliphilic microorganisms that can tolerate pH > 9.2 to avoid HCN volatilization (Alvillo-Rivera et al., 2021).

- *Temperature*: Cyanide degrading enzymes are typically produced by mesophilic microorganisms isolated from soils, their optimum temperature ranging from 20 to 40 °C (Akciil et al., 2003; Babu et al., 1992; Baxter & Cummings, 2006b; Dursun & Aksu, 2000; Kao et al., 2003; Ubalua, 2010).
- *Oxygen availability*: oxygen is consumed in several of the cyanide degrading pathways, which makes it a significant factor in the microbial cyanide degradation process (Baxter & Cummings, 2006b).
- *Cyanide concentration, its bioavailability and solubility*: Naturally, cyanide can only be degraded when it is in the reach of the degrading microorganisms and not precipitated as stable species or adsorbed to the soil interface. On the other hand, too high CN concentration can be harmful to the degrading microorganisms. For example, in a study by Kao et al. (2006), high concentrations of acetonitrile were proven toxic to *Klebsiella oxytoca*, and in another study by Alfani et al. (2001), high concentrations of acrylonitrile caused irreversible damage to *Brevibacterium imperialis* CBS 489–74.
- *Nutrient availability*: Availability of carbon has been identified as a significantly limiting factor in microbial remediation of metal-cyanides. Lack of carbon may hinder especially the remediation of industrially contaminated soils (Baxter & Cummings, 2006b; Dash et al., 2009; Kao et al., 2006). When carbon is abundant in the system, phosphate is the limiting factor for cyanide biodegradation (Watts et al., 2017).
- *Other pollutants*: The presence of other inorganic and organic pollutants at contaminated sites can potentially affect the cyanide bioremediation process either positively or negatively. High concentrations of co-contaminants can influence cyanide degradation by altering the composition of the indigenous microbial population, favoring or inhibiting the growth of desired organisms (Baxter & Cummings, 2006b; Kao et al., 2006). In a study by (Ubalua, 2010), xenobiotics were observed to serve as a nutrient source for bacteria involved in cyanide biodegradation.

2.4.3. Cyanide biodegradation technologies

There are few documented field-scale studies solely focused on cyanide degradation. Some well-established processes exist, for example industrial and municipal wastewater treatment plants (WWTPs) and the gold mining industry have utilized microbial treatment methods for decades (Wong-Chong & VanBriesen, 2006). WWTPs predominantly use suspended growth systems, including batch, semi-batch, and sequencing batch reactors and activated sludge systems, alongside

attached growth systems such as trickling filters, rotating biological contactors, and fluidized bed reactors. These systems are adept at targeting various synthetic compounds, including aromatic hydrocarbons, halogenated solvents, BTEX compounds, and cyanide. In the gold-mining industry, commercial-scale biodegradation has been adopted in a few sites in the USA, Canada and Australia since the 1980s to treat the free and WAD cyanides in the mining effluents (Akcil, 2003). These sites include two Homestake mines in Lead, South Dakota and in British Columbia, Canada, the Green Spring Gold Operation in Ely, Nevada and the Peak Gold Mine in Western NSW, Australia (Wong-Chong & VanBriesen, 2006). Within these facilities, an aerobic attached growth biological treatment is employed to degrade cyanide, thiocyanate, cyanate, ammonia, and metals from tailings impoundment decant solution before their discharge into natural waters (Akcil, 2003). Despite the successful implementation of biological treatment at these select facilities, its widespread adoption within the gold mining industry remains limited.

Remediation efforts at former Manufactured Gas Plant (MGP) sites seldom concentrate solely on cyanide due to the presence of numerous other contaminants such as PAHs, heavy metals, phenols, ammonium, nitrite and sulphates polluting the site (Thavamani et al., 2011; Wehrer et al., 2011). The presence of other pollutants, especially toxic heavy metals and metalloids, impedes the biological degradation of organic pollutants (Feng & Ni, 2024). The diversity of the polluting compounds, the different compound properties, the multitude of processes determining the fate of the pollutants over often decades, the heterogeneous random structure of the subsurface and the fluctuating groundwater conditions make the contamination situation at former MGPs extremely complex (Wehrer et al., 2011). Efforts aimed at remediating former MGP sites often prioritize addressing PAHs due to their prevalence. Kuppusamy et al. (2017) provide a comprehensive overview of successful MGP remediation examples in their publication. Established MGP remediation methods include incineration and in-situ thermal desorption (ISTD), solvent extraction/soil washing, chemical oxidation (ISCO) and bioremediation methods such as land farming, biostimulation, bioaugmentation, composting/biopiling and phytoremediation. Emerging remediation technologies include electrokinetic remediation, enzyme-mediated bioremediation, multi-process phytoremediation and vermiremediation. As for current trends, bioremediation followed by its integration with other approaches have gained a recognition as feasible remediation technologies. A step further, emerging cyanide bioremediation methods can not only facilitate cyanide degradation but also yield value-added products during the process, such as biogas, co-factors of neurotransmitters and electricity, making biodegradation even more attractive as a remediation method (Malmir et al., 2022).

2.4.4. Enrichment cultures and culturing of degrading bacteria/microorganisms

Prior to testing degradation processes in situ, the degradative potential of the indigenous bacteria must be evaluated. This often involves the establishment of enrichment cultures, a method aimed at isolating microorganisms with biodegradative capabilities and assessing their performance (Zaidi & Imam, 2008). In the context of this study, the target pollutant for degradation is free cyanide. Cyanide is introduced as the sole nitrogen source into a culture medium containing indigenous mixed microorganism populations, along with mineral salts and a carbon source to supply essential nutrients for bacterial growth. In the case of this study, bacterial cultures were obtained from groundwater samples collected from two study sites, a former Manufactured Gas Plant (MGP) site and a site of a chemical industry in Vienna, characterized by elevated levels of cyanide in the groundwater. Only bacteria with cyanide-degrading abilities can utilize nitrogen from cyanide and thrive within a medium otherwise lacking nitrogen, becoming the dominant population. These dominant populations are then isolated and purified for further analysis. Purified microorganisms are subsequently inoculated into a separate medium containing a known quantity of cyanide as the sole nitrogen source, enabling monitoring of cyanide degradation over time. This study aimed to enrich cyanide-degrading bacteria from the indigenous populations and establish a microcosm test to assess their degrading capabilities.

Given that conditions in pure culture settings differ from those found in the natural environment, findings derived from pure culture studies may not accurately reflect real-world scenarios. Additionally, it is noteworthy that only an estimated 1% of bacteria can be successfully cultured under laboratory conditions. Cyanide analysis presents many challenges, which will be elaborated upon in the next Chapter. Despite the challenges, numerous studies have reported successful cyanide degradation under laboratory conditions using various microorganisms cultivated in different types of bioreactors, including batch, packed bed, fixed bed batch and continuous reactors, as documented in review papers from Alvillo-Rivera et al. (2021), Dash et al. (2009), Ibrahim et al. (2015) and Razanamahandry et al. (2017).

2.5. Analytical methods of cyanide quantification

Cyanide analysis methods aim to measure groups of compounds with similar chemical traits and report them as a single value. To measure total cyanide, the method should capture the values of free cyanide, weak-acid-dissociable (WAD), and strong-acid-dissociable (SAD) cyanide. This section focuses on analysis methods relevant to groundwater samples from former MGP sites, as opposed to, for example, samples from soil, air, food, plants, or humans. It will also address interferences

resulting from sampling, storage, and sample preparation from contaminated groundwaters. To measure total cyanide, WAD and SAD must be liberated for measurement by UV-radiation and/or distillation, which in turn brings about its own set of interferences. In fact, the U.S. EPA Solutions to Analytical Chemistry Problems with Clean Water Act Methods (2007) states that “next to oil and grease, cyanide is the pollutant for which the most matrix interferences have been reported to EPA”.

2.5.1. Sampling, storage and sample preparation

The accuracy of determining cyanide levels depends on the precedence of sampling, storage, and sample preparation, particularly in complex matrices such as contaminated groundwaters. These matrices often contain interfering substances that can lead to errors in the analysis results. Commonly encountered interferences are listed in Table 8. In acidic groundwaters from former MGP sites, light exposure and the presence of various sulphur compounds are the most significant factors that interfere with the analysis. Cyanide samples collected from environmental matrices are typically preserved using NaOH ($\text{pH} > 11$) to prevent the loss of HCN through volatilization and the precipitation of metal-cyanide complexes. Additionally, the samples are cooled to 4°C and stored in the dark to limit the degradation of free cyanide through biological means. The standard methods outlined by Ghosh, Dzombak, Drop, et al. (2006) also recommend filtering samples with high iron content to remove any colloidal or suspended iron oxides that could form iron-cyanide solids during distillation.

Table 8: Interferents in the determination of cyanide ions during sampling, storage and preparation
(Jaszczak et al., 2017; OI Analytical, 2009)

Interferent	Description of interference
Oxidizers	React with cyanide in solution rapidly decreasing the free cyanide concentration. Oxidizers can co-exist with cyanide.
Chloramines	React with the sample at pH>10 increasing the free cyanide concentration.
Sulphide	Reacts with cyanide to form thiocyanate, decreasing the free cyanide concentration. The reaction is especially rapid if metal sulphides, such as lead sulphide, are present. The reaction is slow without metal sulphides.
Native sulphur (colloidal sulphur)	Reacts with cyanide to form thiocyanate decreasing the free cyanide concentration. The reaction is very fast with colloidal sulphur.
Sulphite	Reacts with strong cyanide complexes at pH>10 decreasing the free cyanide concentration. The reaction is almost immediate at pH>12.
Light	Reacts with strong metal-cyanide complexes releasing free cyanide.
Ascorbic acid	Reacts with cyanide decreasing the cyanide concentration. Sample holding time when ascorbic acid is added is less than 48 hours. In some samples, ascorbic acid can react with ammonia or other nitrogen sources and increase the cyanide concentration.
Formaldehyde	Reacts with cyanide decreasing the concentration. In some samples, formaldehyde reacts with ammonia or other nitrogen sources and increases the cyanide concentrations.

2.5.2. Total cyanide

Given that most analytical techniques are tailored towards the quantification of free cyanide, the presence of bound cyanides introduces complexities, as they do not directly contribute to the measurement of free cyanide. Any substances that bind cyanide can potentially interfere with the measurement process. To obtain accurate measurements of total cyanide, it is critical to free the bound cyanides, which is often accomplished through distillation methods (Ghosh, Dzombak, Drop, et al., 2006). The conventional distillation method involves the addition of a strong acid to the water sample, resulting in a pH below 2, followed by heating the solution to 125°C. This process breaks down weak acid-dissociable (WAD) and most strong acid-dissociable (SAD) cyanides, converting the formed cyanide ions (CN^-) to hydrogen cyanide (HCN) gas, which is then distilled from the sample and absorbed in a sodium hydroxide (NaOH) solution. However, it is noteworthy that certain compounds, such as cyanates, thiocyanates, most organocyanides, and especially strong metal-cyanide complexes like cobalt and platinum cyanide, may not undergo complete decomposition under conventional distillation conditions. Therefore, supplementary pre-distillation treatments, such as UV irradiation, are indispensable for the efficient recovery of these complexes. In this method, SAD cyanide undergoes decomposition under UV-B light (312-400 nm) at pH 3.8. To prevent the conversion of thiocyanate into cyanide, UV light wavelengths below 290 nm are filtered out. The HCN is then separated by distillation at 125°C or by gas diffusion at 30-40°C across a hydrophobic

membrane. Finally, the absorber solution is analysed to ascertain the concentration of total cyanide present. Throughout the distillation process, potential sources of error can arise, whether through manual or automated setups. Additionally, many substances possess the capacity to interfere with the results, as summarized in Table 9.

Table 9: Interferents in the determination of cyanide ions during sample processing (OI Analytical, 2009).

Interferent	Processing technique	Description of interference
Oxidizers	Distillation	Reacts with cyanide decreasing its concentration.
Sulphide	Distillation, Gas diffusion	Distils into absorber solution and reacts with CN forming thiocyanate. Passes through diffusion membrane.
Sulphite (or Sulphur Dioxide)	Distillation	Distils into absorber solution and reacts with cyanide decreasing its concentration.
Thiosulfate and other oxidized sulphur species (except sulphate)	Distillation	Decompose to form native sulphur and sulphur dioxide. React with cyanide decreasing its concentration. Sulphur Dioxide distils into an absorber solution and reacts with cyanide decreasing its concentration.
Thiocyanate	Distillation, UV-irradiation	Decomposes to sulphur dioxide and reacts with cyanide decreasing its concentration. Sulphur dioxide distils into an absorber solution. Can react at < 280 nm to form cyanide
Thiocyanate + Nitrate or Nitrite	Distillation	Decompose to form cyanide.
Misc. Organics + Nitrate or Nitrite	Distillation	Decompose to form cyanide.
Carbonate	Distillation, Gas diffusion	Excessive foaming and possible violent release of carbon dioxide. Passes through the diffusion membrane.

Ghosh, Dzombak, Drop, et al. (2006) provide a concise overview of widely employed standardized techniques for total cyanide determination after distillation:

1. The titrimetric method is based on the titration of the cyanide absorber solution with silver nitrate. The reaction between silver nitrate and free cyanide yields $\text{Ag}(\text{CN})_2^-$. Excess silver ions remain in the solution as Ag^+ and are detected utilizing a rhodanine indicator, which undergoes a colour change from yellow to salmon red. Cyanide concentration is then calculated based on the normality and volume of the titrant employed. The typical calibration range for this method is between 0.1 and 10 mg/L. Despite its approval by USEPA (Standard Methods 4500-CN-D) and ASTM (D2036-98), this method has limitations such as the high-concentration calibration range, susceptibility to disturbance from various

interferences, operational complexity, use of hazardous solvents and time-intensive sample preparation. These limitations diminish the method's applicability for cyanide determination in complex environmental matrices, where the desired detection limits are often on the $\mu\text{g/L}$ level.

2. The colourimetric method is the most commonly applied. The method involves the conversion of free cyanide in the absorber solution to CNCl using chloramine-T under pH conditions below 8. Subsequent addition of pyridine–barbituric acid yields a stable red-blue colouration, detectable at wavelengths between 578 and 587 nm via spectrophotometry. A standard calibration curve is essential for determining cyanide concentration, typically within the range of 0.02 to 0.2 mg/L. Common drawbacks associated with this method include high-level calibration range, susceptibility to oxidizing agents, sulfuric compounds, and sample alkalinity, alongside operational complexity, use of hazardous solvents, and time-consuming procedures. Despite the drawbacks, the method has been standardized by USEPA (Standard Methods 4500-CN-E) and ASTM (D2036-98). When coupled with UV irradiation and either distillation or gas diffusion, the colourimetric method adheres to ISO Standard 14403-2:2012.
3. Cyanide Ion-Selective Electrode (ISE) measures the CN^- concentration in the absorber solution potentiometrically, utilizing a standard calibration curve to derive concentration from the potential (mV). A typical calibration range for this method is between 0.05 and 10 mg/L. The ISE method is hindered by numerous interferences like halides, pseudohalides and heavy metals, rendering it highly susceptible to matrix effects. Despite its approval by USEPA (Standard Methods 4500-CN-F) and ASTM (D2036-98), its susceptibility to interferences and lower sensitivity compared to the colourimetric method limit its usage.
4. Flow Injection Amperometry (FIA) employs a gas diffusion membrane selective for HCN during distillation. In this method, CN^- is released from cyanide complexes by UV irradiation. The generated HCN diffuses across the selective membrane, which makes it possible to employ a nonselective electrical conductivity measurement to determine the cyanide concentration in the absorber solution. FIA is susceptible to interference from sulfides, but it has been standardized as ASTM D7511-09 and recognized as a valid alternative to colourimetry in the ISO 14403-2:2012 standard.

OI Analytical (2009) has compiled a summary of interferents during the final measurement phase (Table 10), noting that these interferences often remain undetected, particularly when employing automated methods.

Table 10: Interferents in the determination of cyanide ions during sample measurement (OI Analytical, 2009)

Interferent	Measurement technique	Description of Interference
Sulphide	Titration, ISE, Colorimetry, FIA	Detected as cyanide, Detected as cyanide, Detected as cyanide (at > 10 mg S/L), Detected as cyanide (at > 50 mg S/L)
Thiocyanate	Colorimetry	Detected as cyanide
Fatty acids	Titration	Mask endpoint
Sulphur Dioxide	Colorimetry	Increase chloramine-T demand resulting in a negative bias. The increased demand may not be noticed with automated methods.
Carbonates	FIA	Negative bias at > 1,500–mg CO ₃ /L

2.5.3. Direct free cyanide

When analysing water samples, the concentration of free cyanide is often the primary interest due to its high toxicity. There have been continuous efforts over decades to develop reliable, rapid, and cost-effective techniques for measuring cyanide at concentrations as low as micrograms per litre in water samples of various compositions. Three methods have been standardized for free cyanide measurement in environmental water samples: ASTM D 4282, ASTM D 7237, and ISO 14403-2:2012 (Ghosh, Dzombak, Drop, et al., 2006; OI Analytical, 2009).

The first method entails microdiffusion of hydrogen cyanide (HCN) from an acidified sample, targeting the cyanide dissociation from simple cyanides and weaker weak acid dissociable (WAD) species at room temperature, within a solution of pH 6 (Ghosh, Dzombak, Drop, et al., 2006). Employing a Conway microdiffusion cell comprising two nested chambers, the sample is introduced into the outer chamber, buffered to pH 6, and allowed to diffuse in a darkened environment for 6 hours. Subsequently, the liberated HCN gas, comprising free, simple, and weaker WAD cyanide, is absorbed as CN⁻ into an inner chamber containing a sodium hydroxide (NaOH) absorber solution. The resultant absorber solution is analyzed using the standard colourimetric procedure outlined in the preceding Chapter(2.5.2), with a typical calibration range spanning from 0 to 150 µg/l.

The second method, ASTM D 7237, employs a flow injection technique (FIA) into a reagent solution at a pH range of 6-8 (Stanley & Antonio, 2012). Initially, the sample is introduced into the FIA system's carrier solution via an injection valve, subsequently merging downstream with a phosphate buffer solution. The neutralized sample then traverses a gas-diffusion cell, wherein released hydrogen cyanide (HCN) diffuses through a hydrophobic gas diffusion membrane into an alkaline acceptor stream. This stream passes through a debubbler before entering the injector sample loop. Cyanide ions are detected using an amperometric flow cell detector featuring a silver-

working electrode. In the presence of cyanide, oxidation of silver occurs at the applied potential within the working electrode, with the resultant anodic current being proportional to the cyanide concentration in the injected standard or sample.

The ISO 14403-2:2012 method incorporates the addition of a zinc sulfate solution to the distilling sample at pH 3.8, aimed at precipitating any iron cyanides present as zinc-cyanoferrate. Additionally, a tetraethylenepentamine solution is introduced to liberate cyanide from nickel complexes before analysis when using the gas diffusion method. Unlike the total cyanide measurement outlined in Chapter 2.5.2, UV light is not utilized in this method. Hydrogen cyanide at pH 3.8 is separated via online distillation at 125°C or through gas diffusion at temperatures ranging from 30°C to 40°C across a hydrophobic membrane. With gas diffusion, hydrogen cyanide is absorbed into a sodium hydroxide solution. Subsequently, the absorber solution is subject to measurement either by colourimetry or by amperometry when employing gas diffusion.

2.5.4. Non-standardized methods

Scientists are constantly seeking to improve existing methods and innovate new methods to suit their specific requirements. Therefore, several methods outside of the standardized methods to measure cyanides exist, covering almost all aspects of modern instrumental analysis. For example, in their paper introduction Themelis et al. (2009) list and cite research utilizing capillary electrophoresis after single-drop microextraction, chemiluminescence, indirect flame atomic absorption spectrometry, FI fluorimetry, gas chromatography with electron capture or mass spectrometric detection, ion chromatography, piezoelectric quartz crystal sensors, potentiometry using modified carbon paste electrodes, surface modified CdSe quantum dots as luminescent probes, amperometric and optical sensors, indirect adsorptive stripping voltammetry at a mercury electrode, batch spectrophotometry based on the Berthelot reaction or a revised phenolphthalein method, FI spectrophotometry using aquacyanocobyrinic acid heptamethyl ester and emometrics based on partial least squares regression. Ma & Dasgupta (2010) and Randviir & Banks (2015) assess several of these methods in their review papers, however, these evaluations are primarily focused on human samples such as blood. In a more recent review paper by Jaszczak et al. (2017), a comprehensive array of analysis methods for environmental samples spanning water, soil, air, food, and human biological samples is enumerated. Specifically focusing on water samples, including drinking water, mineral water, petrochemical wastewater, electroplating wastewater, and groundwater, the authors compiled research utilizing Ion Chromatography with pulsed amperometric detection (IC-PAD), Gas

Chromatography, and the spectrophotometric method. Notably, the spectrophotometric method is highlighted as the most prevalent due to its standardization within the field.

In this project Ion Chromatography (IC) paired with an amperometric detector was selected as the preferred method. IC has emerged as a widely employed and continually refined technique for detecting cyanide in liquid samples, as evidenced by studies such as those by Cheng et al. (2007), Christison & Rohrer (2007), Giuriati et al. (2004), Jackson (2000), Kumar Meher et al. (2018), Wu et al. (2015). The IC-PAD offers several advantages: streamlined operation due to fewer sample preparation steps compared to standardized methods, chromatographic separation of the cyanide anion from other interfering ions effectively reduces common interferences, capability to detect microgram per litre ($\mu\text{g/L}$) level concentrations with high precision and sensitivity, rapid sample analysis (typically 5-10 minutes per sample), and minimal sample volume requirement. However, it is important to note that certain limitations have been identified in the literature. These include manual preparation of eluents (although options for online eluent preparation and ready-made solutions are available), time-consuming operation (typically referring to the overall duration of the analytical process), baseline instability, high noise levels, low reproducibility, and fouling of the working electrode. While silver working electrodes were previously commonplace, recent studies, such as those by Kumar Meher et al. (2018) and industry sources like Metrohm (2018), have underscored the distinct advantages of gold working electrodes. Consequently, this thesis has opted to employ a gold working electrode in accordance with these findings.

3. Materials and Methods

3.1. Study sites

3.1.1. *Siebenhirten*

The first study site is a 32 ha industrial area located in Liesing, the 23rd district of Vienna. The site is listed as W8 on the atlas of legacy contaminated sites maintained by the Environment Agency Austria (Dörrie, 2011). Since 1828, the site has been used for diverse chemical production, at first mainly sulphuric acid. In 1858, production expanded to illuminating gas and processing of gasworks. At the turn of the century, sulphuric acid production expanded to artificial fertilizer, among other types of acid production. Production residues were not processed but piled in various-sized pits across the site, resulting in acid mountains up to 8 m high with pH values as low as 2.

The primary contaminants detected at the site are cyanides, hydrocarbons, phenols, ammonium, nitrite and sulphate. During the monitoring period spanning from 2005 to 2010, the highest recorded cyanide concentration in the groundwater reached 10.72 mg/L. This surpasses the actionable threshold value of 0.05 mg/L (ÖNORM S 2088-1) by 215-fold.

The uppermost soil layer is characterized by anthropomorphic fills of various types, origins and thicknesses. The natural subsoil layers consist of the fluvial fills of the Liesing River, characterized by alternating layers of water-bearing sand-gravel and impermeable silt-clay. The groundwater table is between 3 and 7 metres deep, its thickness varies from nothing to 2 meters, and its gradient varies greatly, up to max. 2 %. The general groundwater flow direction is towards the northeast and east.

In 2002 and 2003 the site was enclosed with a 7-13 m deep sealing wall, reinforced with a sealing foil, in the north, south and west. A 12 m deep seepage wall with an internal drainage system, one pump shaft and three inspection shafts were built on the east side. The pumps maintain a low point of groundwater, hydraulically preventing any outflow of seepage water into the surrounding area. The acidic excavated material was deposited on the eastern side of the site into two piles, which have been sealed, covered and planted with vegetation. See Figure 4 for further details.

Groundwater samples for this study were collected from 5 sampling points at different depths, marked blue in the map. The sampling points are marked with running numbering that is later used in the sample coding in the laboratory.

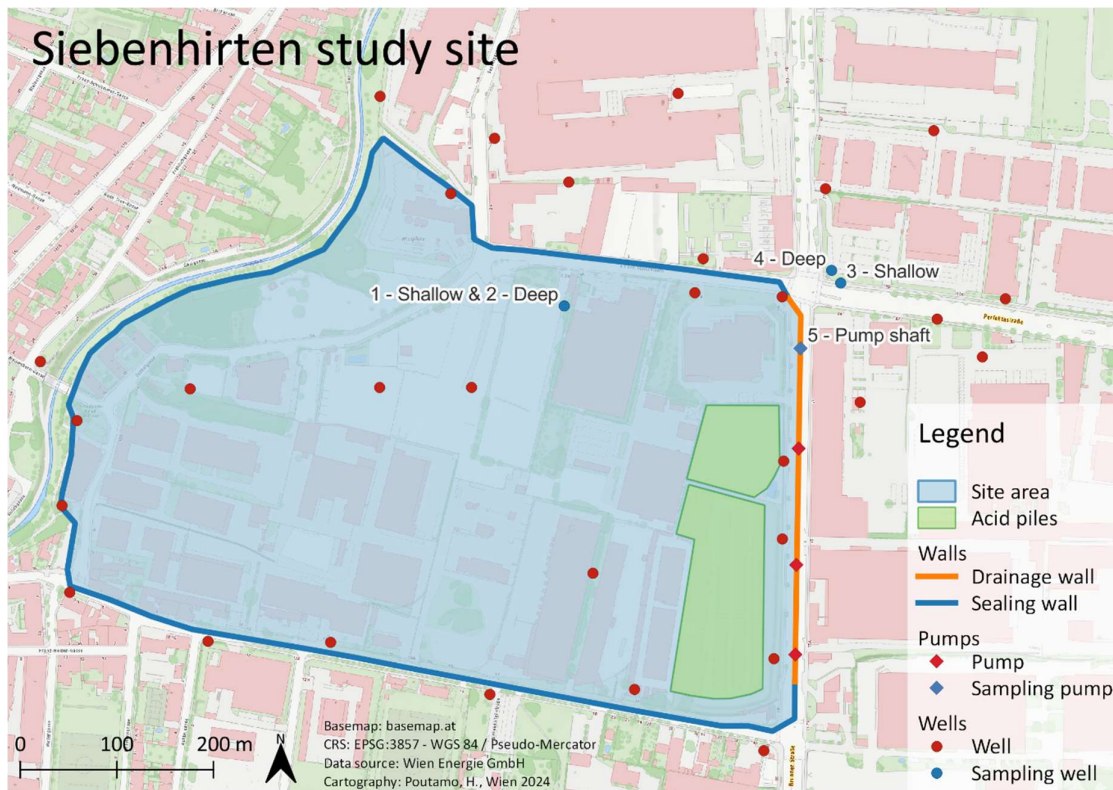


Figure 4: Map of the Siebenhirten study area

3.1.2. *Simmering*

The second contaminated study site is listed as W18 on the atlas of legacy contaminated sites (Moser, 1999). The former Gaswerk Simmering covers an area of around 32.5 hectares and is located in Vienna's 11th district. The gasworks was operated from 1900 to 1975 and was one of the largest gas production plants in Austria. In the period from 1900 to 1960, around 8.6 billion cubic metres of town gas were produced. The main by-products of town gas production are tar, gas purifier mass and aqueous ammonia.

Typical pollutants from these by-products are polycyclic aromatic hydrocarbons (PAHs), phenols, aromatic hydrocarbons, cyanides, sulphur and sulphides. During 1997-1998, contaminant concentrations were measured from eluates of drill cores. In the most contaminated soils, total cyanide concentration reached 1,235 mg/kg (dry mass). This exceeds the actionable threshold value of 250 mg/kg dm (ÖNORM S 2088-1) by a factor of 5.

The site lies on the Vienna Basin, at the western end of the Schwechat trough. Its uppermost soil layer, spanning 1-3 meters, primarily comprises anthropogenic fills. Below this layer, the natural subsoil consists of floodplain fills (1-3 m), quaternary Danube gravel (3-8 m), tertiary fine sands (0-5

m), and tertiary clay marls and silts starting from 10-17 m below ground level. Notably, the Danube gravel is water-bearing, with permeability of approximately $1 \cdot 10^{-4}$ m/s. The groundwater table is 5-7 m deep and fluctuates by ~ 1.5 m throughout the year. The groundwater flows towards the east and southeast, but it is significantly influenced by the nearby Danube Canal, situated only 200 m northeast of the site, as well as industrial and agricultural activities in the surrounding area.

Between October 2010 and April 2012, some remediation measures took place (PORR Umwelttechnik GmbH, 2012). Hot spots with the highest concentration of pollutants or most potentially mobile pollutants were excavated, including the former tar and ammonia cisterns. In total, around 100,000 tonnes of material were extracted from the site. Additionally, two rows of barrier wells were installed to hydraulically secure the site.

Groundwater samples for this study were collected from 5 sampling points, marked blue in Figure 5. The running numbering of the sampling points is later used in the sample coding in the laboratory.

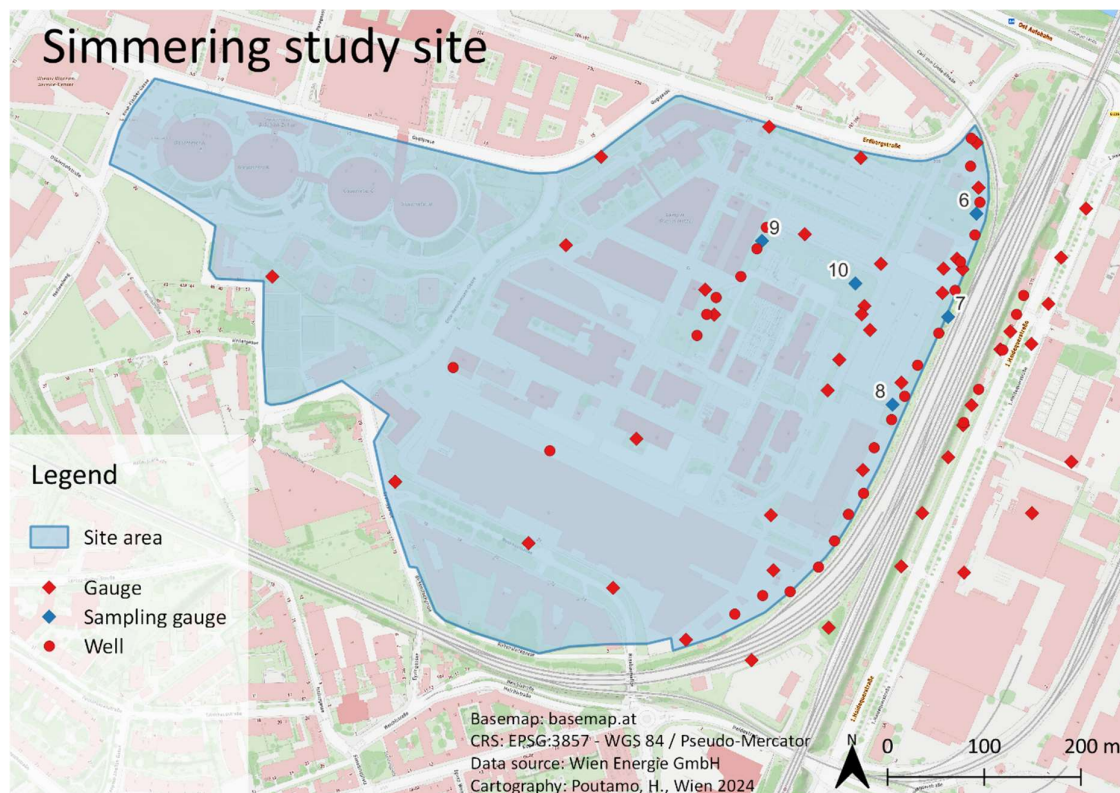


Figure 5: Map of the Simmering study area

3.2. Enrichment tests of liquid samples

6 L of groundwater was collected from 5 wells in Siebenhirten: the water was pumped from well 1-4 and scooped from well 5. The samples were transported in cooling boxes to the laboratory, where they were stored at 4°C in the dark. DNA from samples was extracted for other experiments. Experiments were conducted at room temperature inside the laminar hood to avoid airborne contaminants from the laboratory. To concentrate the sample microorganisms, 1.4 litres of groundwater from each sampling point was equally divided into 50 mL Falcon tubes, which were then centrifuged at 4,800 rpm for 15 minutes in the Heraeus Megafuge 40R Centrifuge (Thermo Scientific™). Following centrifugation, the supernatants were carefully extracted, and the resulting concentrated groundwater fractions were combined into single Falcon tubes. Since no pellets were formed, approximately 1.5 – 2 mL was collected as the concentrated fraction.

The concentrated groundwater samples were subsequently cultured in a Mineral Salt Medium (MSM) adapted from Kandasamy et al. (2015) (see Table 11 for the recipe): 10 mL of concentrated sample was added into a sterile, 100 mL, foil-wrapped Schott bottle containing 50 mL of MSM. Bottle sterilisation was done at 121°C for 30 minutes in one of two autoclaves (HMC Europe Hiclave HG-133 or the CertoClav MultiControl). The culture medium was supplemented with 20 mg/L cycloheximide (CAS: 66-81-9, Carl Roth 8682, purity ≥ 96 %) to prevent fungal contamination, 65 mg/L potassium cyanide (KCN, CAS: 151-50-8, Sigma Aldrich 60478, BioUltra ≥ 98.0 %) as the sole nitrogen source to selectively encourage the growth of cyanide-utilizing bacteria, and 1 g/L glucose (CAS: 50-99-7, Carl Roth HN06, CELLPURE® ≥ 98 %) to provide a carbon- and energy source for bacterial growth. All supplements were filter-sterilized, and stocks were stored in autoclaved bottles at 4°C. The same supplements were used in all later tests. Two cultures were made from each concentrated groundwater well sample, totalling 10 cultures. One control sample was also created, with ultrapure water (Sartorius Arium® Pro, resistivity ≥ 18.2 MΩ x cm, ASTM Type I) instead of groundwater.

Table 11: MSM recipe adapted from Kandasamy et al. (2015) for the cultivation of enriched bacterial cultures in liquid samples. Concentrations are final concentrations in the cultures. pH was adjusted with NaOH and HCl.

pH	7 ± 0.05
Ingredient	(g/L)
K ₂ HPO ₄	1
MgSO ₄ x 7 H ₂ O	0.2
MnSO ₄ x H ₂ O	0.2
CuSO ₄ x 5 H ₂ O	0.2
ZnSO ₄ x 7 H ₂ O	0.2
CaCl ₂ x 2 H ₂ O	0.01
NaCl	0.01

To assess the growth performance of the bacterial cultures, periodic measurements of optical density (OD) were conducted with a Spectrophotometer/Fluorometer (DeNovix DS-11 FX+) at 600 nm wavelength. Due to a notable visual lack of increase in turbidity, confirmed by the measured OD values, it was determined that the MSM did not perform adequately. Consequently, a decision was made to explore alternative MSM recipes, a process detailed in the subsequent chapter.

3.3. MSM trials for best performance

A literature review was conducted to identify different MSM used in the enrichment of cyanide-degrading bacteria. A diverse set of MSM was chosen for trials to find the best performing MSM for samples collected from the study sites. Media with pH values exceeding 10 were excluded. Eventually, six distinct media recipes were adapted from Coleman et al. (2002), Kao et al. (2003), Mirzadeh et al. (2014), Potivichayanon & Kitleartpornpairat (2010), Sharma et al. (2012), and Watanabe et al. (1998). Refer to Table 12 for detailed recipes. To ensure comparability across the performance tests, only the mineral salts were included from each recipe, with glucose and cyanide concentrations fixed at 1 g/L and 65 mg/L, respectively. Cycloheximide, following the original liquid enrichment test, was also added at a concentration of 20 mg/L to each recipe.

Table 12: Six MSM recipes tested for best performance in culturing groundwater samples. Concentrations are final concentrations in the cultures. pH was adjusted with NaOH and HCl. The first row shows the source literature, and the second row is the abbreviation used in the text and result graphs of this thesis.

(Kao et al., 2003)		(Mirizadeh et al., 2014)		(Potivichayanon & Kitleartpornpairat, 2010)	
KAO		MIRI		POTI	
pH	7 ± 0.05	pH	9.5-10	pH	7.2 ± 0.05
Bulk medium	(g/L)	Bulk medium	(g/L)	Bulk medium	(g/L)
Na ₂ HPO ₄	7.098	KH ₂ HPO ₄	4.35	KH ₂ PO ₄	2.7
KH ₂ HPO ₄	13.709	Trace salts solution	10 mL	K ₂ HPO ₄	3.5
MgSO ₄	0.120	Trace salts solution	(mg/L)	Trace salts solution	10 mL
CaCl ₂ x 2 H ₂ O	0.015	FeSO ₄ x 7 H ₂ O	300	Trace salts solution	(mg/L)
		MgSO ₄ x 7 H ₂ O	140	FeSO ₄ x 7 H ₂ O	300
		CoCl ₂ x 6 H ₂ O	240	MgCl ₂ x 6 H ₂ O	180
		CaCl ₂ x 2 H ₂ O	50	Co(NO ₃) ₂ x 6 H ₂ O	130
		MnCl ₂ x 4 H ₂ O	40	CaCl ₂ x 2 H ₂ O	50
		MoO ₃ x H ₂ O	20	ZnSO ₄ x 7 H ₂ O	70
				MoO ₃ x H ₂ O	20

(Coleman et al., 2002), further adapted from (Hartmans et al., 1992)	
PK	
pH	7 ± 0.05
Bulk medium	(g/L)
K ₂ HPO ₄	2.27
KH ₂ PO ₄	0.95

(Sharma et al., 2012)	
SHARMA	
pH	9.5-10
Bulk medium	(g/L)
K ₂ HPO ₄	0.5
KH ₂ PO ₄	0.25
(NH ₄) ₂ SO ₄	0.23
MnSO ₄ x H ₂ O	0.1
MgSO ₄ x 7 H ₂ O	0.1
CaCl ₂ x 2 H ₂ O	0.01
FeCl ₃ x 6 H ₂ O	0.002

(Watanabe et al., 1998)	
WATA	
pH	7.2 ± 0.05
Bulk medium	(g/L)
K ₂ HPO ₄	0.829
MgSO ₄ x 7 H ₂ O	0.2
CaCl ₂ x 2 H ₂ O	0.01
NaCl	0.01
Trace salts solution	10 mL
Trace salts solution	(mg/L)
MnSO ₄ x H ₂ O	15.3
CuSO ₄ x 5 H ₂ O	20.2
ZnSO ₄ x 7 H ₂ O	20.2

Groundwater samples from wells 1-3 were cultured following the liquid enrichment test protocol (see Chapter **Error! Reference source not found.**). Due to the limited amount of centrifugally concentrated groundwater, most of which was used in the original liquid test, 10 mL of unprocessed groundwater was cultured instead. One culture was created from each of the three wells, for each six MSM. Additionally, a control sample with ultrapure water instead of groundwater was made for each MSM, to be used as a baseline reference in the OD measurements and to track possible laboratory cross-contamination. A total of 24 samples were created.

After the initial culturing of the groundwater samples, a baseline for OD was measured with the control samples. OD measurements were conducted twice during the first week of incubation, later reduced to once per week, performed right before and after subculturing. To subculture, 10 mL of the cultures were moved to fresh MSM mixtures with identical ingredients to the original cultures, which were discarded. Subcultures were made once per week. Just before each subculturing, 100 μ L of the cultures were plated, undiluted, on 10 % Tryptic Soy Broth (TSA) plates to monitor the diversity and fitness of the bacterial microcosm. The duration of the experiment spanned one month, encompassing 7 OD measurements, 4 plates, and 3 subculturing events, the timeline outlined in Table 13:

Table 13: Timeline of MSM trials.

Date	OD taken	Plating, days until colony count	Subculturing
29/06/2023	x		
03/07/2023	x		
05/07/2023		x, 7 days	x
12/07/2023	x2	x, 8 days	x
20/07/2023	x2	x, 6 days	x
26/07/2023	x	x, 9 days	

To further assess the general bacterial activity in the cultures, an oxygen consumption test was executed. Fifteen brown 150 ml bottles were sterilized and prepared with PK-MSM and groundwater from well 3. Three bottles were opened daily, and O₂ levels were measured to quantify bacterial metabolic activity.

A corresponding test with multiple MSM was conducted in a collaborative laboratory in Germany. Upon combining results from both laboratories, two well-performing MSM recipes were chosen for the enrichment of bac-trap material, a process detailed in the subsequent chapter.

3.4. Enrichment tests of bac-trap samples

Bac-traps containing sterilized quartz sand (grain size <2 mm) were placed in all 10 groundwater wells in depths ranging from 6-17 meters below well head in Siebenhirten and 9-10 meters below well head in Simmering. After 8 weeks of exposure to the local microcosm, the bac-traps were removed, immediately cooled to 4°C and transported to the laboratory storage in 100 mL brown sterile glass bottles. In the laboratory, the bac-trap material was cultured similarly to the liquid groundwater samples: approximately 10 g of material was weighed into 100 mL sterile, foil-wrapped Schott bottles, 50 mL of MSM (see Table 14 for recipes) was added together with glucose, KCN and cycloheximide in final concentrations of 1 g/L, 20 mg/L and 250 µg/L, respectively. In the first culture, 60 µL of a garden NPK fertilizer (IMPOS Blumendünger Universal, Art-Nr.: 866854) was added as an extra nitrogen source. The only nitrogen source in the following subcultures was the KCN.

Table 14: MSM recipes used to enrich bacteria from the bac-trap material. Concentrations are stock concentrations. pH was adjusted with NaOH and HCl.

Medium 1		Medium 2	
pH	7.2 ± 0.05	pH	7.2 ± 0.05
Ingredient	(g/L)	Ingredient	(g/L)
K ₂ HPO ₄	1	KH ₂ PO ₄	2.7
KH ₂ PO ₄	0.5	K ₂ HPO ₄	3.5
NaCl	0.5	Trace salts solution B	10 mL
MgSO ₄ x 7 H ₂ O	0.25	Trace salts solution B	(mg/L)
CaCl ₂ x 2 H ₂ O	0.01	CaCl ₂ x 2 H ₂ O	50
FeSO ₄ x 7 H ₂ O	0.01	CoCl ₂ x 6 H ₂ O	100
Trace salts solution A	10 mL	CuSO ₄ x 5 H ₂ O	2.6
Trace salts solution A	(mg/L)	FeSO ₄ x 7 H ₂ O	300
CoCl ₂ x 6 H ₂ O	100	MgSO ₄ x 7 H ₂ O	150
CuSO ₄ x 5 H ₂ O	2.6	MnSO ₄ x H ₂ O	50
MnSO ₄ x H ₂ O	50	Na ₂ MoO ₄ x 2 H ₂ O	10
Na ₂ MoO ₄ x 2 H ₂ O	10	ZnSO ₄ x 7 H ₂ O	40
ZnSO ₄ x 7 H ₂ O	40		

The timeline of the enrichment tests conducted on the bac-trap material is outlined in Table 15. The first culture was incubated for 2 days, the first subculture for 1 week, and subsequent subcultures for 3 weeks. To subculture, 2.5 mL of the previous liquid culture was added to 50 mL of MSM and equal concentrations of glucose, KCN and cycloheximide as previously. On weeks when samples were not subcultured, they were fed with the same amount of KCN as during subculturing.

Samples were incubated in a fume hood at room temperature on a shaker set to 100 rpm. To track the performance of the incubation settings, optical densities of the samples were measured weekly, except from the first and second cultures where remaining suspended quartz sand clouded the sample. Simultaneously, samples were plated on 10% TSA plates; the first two cultures as undiluted plates and later as dilution series. The dilution series were incubated overnight at 27°C and counted on the next day.

Table 15: Timeline of the enrichment cultures from the bac-trap material. Darker horizontal lines mark changing weeks.

Date	OD taken	Plating	(Sub)culturing	KCN feeding
07/08/2023	arrival of samples			
09/08/2023		x	x	
11/08/2023			x	
18/08/2023	x	x	x	
24/08/2023	x	x		
25/08/2023				x
31/08/2023	x	x		
01/09/2023				x
07/09/2023	x	x		
08/09/2023			x	
12/09/2023		x ¹⁾		
14/09/2023	x	x		
15/09/2023				x
21/09/2023		x		
22/09/2023	x			x
28/09/2023	x	x		
29/09/2023			x	

1) Plating on 4 different agar plates with dilution of 10^{-4}

Additionally, on the 5th week of the enrichment test, a 10^{-4} dilution of the samples were plated on 4 different types of agar plates (see Table 16 for recipes) to see if the results of the colony counts could be improved.

Table 16: Agar plate recipes. *) CN was percolated on an already poured and solidified plate.

LB-Medium (LB)	g/L
Tryptone (Peptone)	10
Yeast extract	5
NaCl	5
Agar	15
Nutrient Agar (NA)	
Nutrient Broth	8
Agar	15
10% Tryptic Soy Broth (TSA)	
Tryptic Soy Broth	3
Agar	15
Water Agar (WA)	
Agar	15
CN*	4

3.5. CN degradation in different O₂ environments

A preliminary cyanide degradation test was conducted to produce a baseline for ensuing microbial degradation assessments. 1 mM of KCN, corresponding to approximately 0.065 g/L, was dissolved into 50 mL of ultrapure water in nine sterile, foil-wrapped Schott bottles. The experiment was divided into three distinct treatment groups, each with 3 bottles: open lids, partially open lids, and closed lids with daily openings when feasible. Before measurements in the IC, the samples were diluted by a factor of 1000, done in 2 steps, to avoid column overload.

The instrument set-up for measuring the cyanide solution consisted of Metrohm 930 Compact IC Flex, equipped with a column oven, chemical suppression system, peristaltic pump for suppressor regeneration, and built-in degasser. Samples were automatically introduced by the Metrohm 863 Compact IC Autosampler. The separation column employed was Metrosep A Supp 10 – 100/4.0. Sample signals were measured with an amperometric detector featuring an Au working electrode and a Pd reference electrode. A solution of 100 mM NaOH and 0.01 mM EDTA (NaOH CAS: 1310-73-2, Merck 72064, 49-51 % in water and EDTA CAS: 6381-92-6, Merck 324503, purity ≥ 98.5 %) in ultrapure water was used as eluent. To derive cyanide concentrations from the signal peak areas, a 12-step cyanide standard curve was produced, ranging from 0.25 ppb to 80 ppb ($R^2 = 0.998$). A check standard of 2 ppb or 10 ppb was used on every measurement session to determine the reliability of the measurements. The standard curve and check standard solutions were prepared from cyanide standard solution (Merck 1.19533, 1000 mg/l CN in H₂O Certipur®, traceable to SRM from NIST K₂[Zn(CN)₄]).

3.6. Microdistillation test

The Ion Chromatograph is limited to measuring liquid samples, and more specifically, the free cyanide in the liquid phase. However, cyanide found in polluted areas is often complexed by trace metals in both solid and liquid phases (Kjeldsen, 1999). To measure the total cyanide found in the groundwater samples, it was necessary to find a way to free the bound cyanide for the IC measurements. Trials were made with a microdistillation setup sourced from SKAD Analysing Systems and Services. The setup consisted of a thermostat (Aufschlussblock LT200-2, LTV08251) and a 2-tube digestion system (Aufschlussröhrchen, KS173). The lower glass tube accommodated the sample alongside an acidic digestion solution, while the upper glass tube housed the basic collection solution. Between the tubes, a coupling system with a permeable membrane (PTFE, pore size 0.2 μm , PT020) connected the tubes.

In order to ensure the precise quantification of the freed cyanide, predetermined quantities of iron-complexed cyanides were prepared for the test. 2 different iron-complexed cyanide stock solutions were prepared: the first solution contained 100 ppm $\text{C}_6\text{FeK}_4\text{N}_6 \times 3\text{H}_2\text{O}$ (abbreviated Fe^{II} , CAS:14459-95-1, Sigma-Aldrich P9387, ReagentPlus®, $\geq 98.5\%$) and the second 100 ppm $\text{C}_6\text{FeK}_3\text{N}_6$ (abbreviated Fe^{III} , CAS: 13746-66-2, Sigma-Aldrich P8131, ReagentPlus®, $\sim 99\%$). Both stock solutions were diluted to a series of 7 sample concentrations: 5, 2.5, 1, 0.5, 0.25, 0.1 and 0.01 ppm. Both series were made in duplicate, one series to be distilled for 30 minutes and the second for 60 minutes. The acidic digestion solution was prepared with 7.11 M H_2SO_4 and 0.79 M MgCl_2 and the collector solution with 1 M NaOH.

For the distillation, the thermostat was set to heat up to 120°C. 6 mL of sample solution was measured into the lower tube together with 0.75 mL digestion solution. The coupling was screwed onto the tube and the permeable membrane was inserted into it. The upper tube was then fastened to the coupling, and 1.5 mL collector solution was added to it. On top, the system was closed with a membrane cap to release excess pressure but keep volatile cyanide compounds inside. The system was then set onto the preheated thermostat for the predetermined time. After the heating, the tube system was removed from the thermostat, the lower digestion tube uncoupled from the system and the upper collector tube with the distilled cyanide was set to cool down for 10 minutes. The cooled solution was then collected by pipette to a small glass vial and topped up to 6 mL with ultrapure water.

Distilled samples were stored in the dark at 4°C for 4-5 days before measurements in the IC could take place. Before measuring the distillates in the IC, they were diluted to be within the detection range of the IC (0-80 ppb). The same IC setup was used as described in Chapter 3.5.

4. Results

4.1. Enrichment tests of liquid samples

The growth curves of the cultured microorganisms were measured six times over a 4-week period by optical density (OD). As can be seen in Figure 6 below, the microorganisms from any sampling point did not perform well. None of the samples reached optical density above 0.2, when well growing bacterial communities usually reach an OD of around 1. All samples had a similar growth behaviour – none of the samples behaved exceptionally differently.

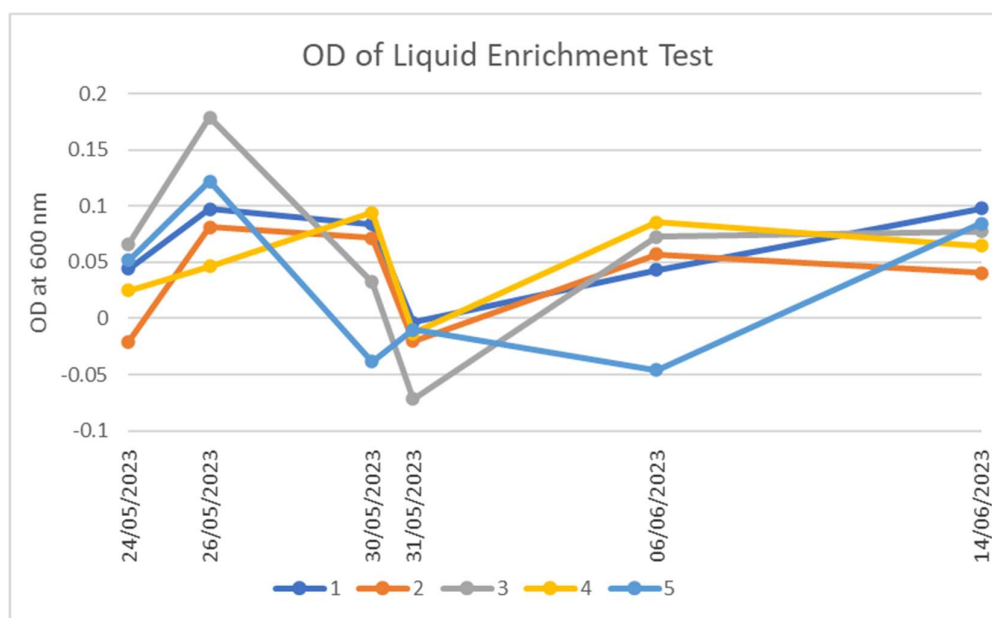


Figure 6: ODs of the liquid enrichment test. Numbers 1-5 are sampling points, i.e. groundwater well numbers from the Siebenhirten study area (see Figure 4 for map). The values are averages of two cultures. MSM with added distilled water instead of groundwater was used as baseline reference value.

Due to the lack of growth in the medium, confirmed by the measured OD values, it was determined that the MSM did not perform adequately. Alternative MSM recipes were explored to find a better performing medium before arrival of the bac-trap samples.

4.2. MSM trials for best performance

6 different MSM were trialled for best performance for cyanide-degrading bacteria growth. The outcomes derived from the OD measurements failed to provide valuable information. In a manner similar to the liquid enrichment test, the growth curves did not exhibit an exponential growth phase

during the 4-week trial. Instead, the OD values ranged from -0.14 to 0.18, without any discernible trendlines that were consistently increasing or decreasing. The result graphs can be viewed in Annex 1. Notably, one measurement point reached a value of 0.75, which was the third measurement taken with the SHARMA MSM from well number 3, 14 days after the initial culturing, prior to the first subculturing. However, this growth was not reproduced in subsequent measurements of the subcultures.

More informative than the OD values were the data acquired from the plated samples. These samples were plated undiluted before the subculturing events and counted a week later. As depicted in Figure 7, the various MSM employed produced significantly different results.

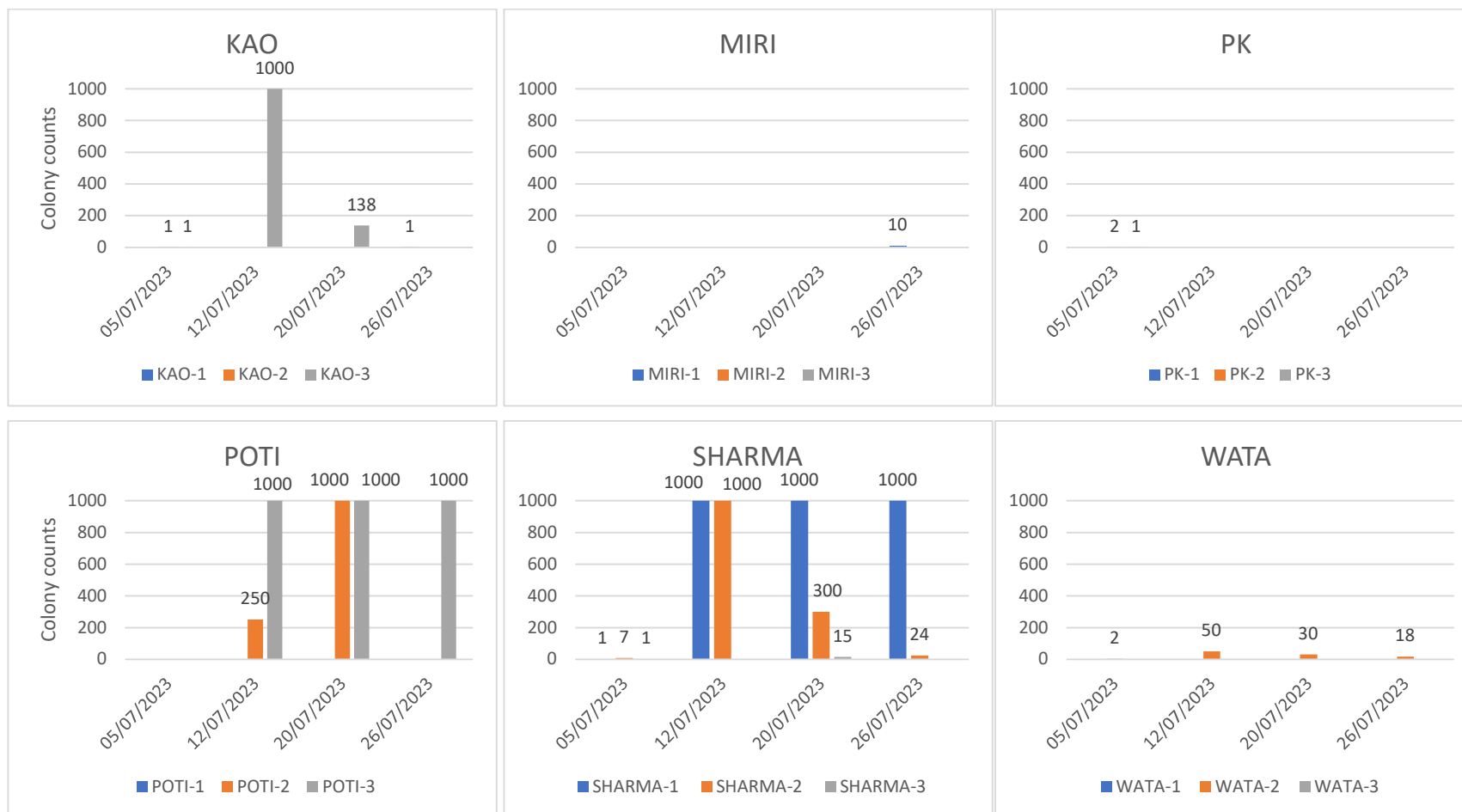


Figure 7: Colony counts of groundwater samples from wells 1-3, enriched in six different MSM. Among the recipes, POTI and SHARMA included extra nitrogen alongside KCN. Cultures were plated undiluted. Colonies were counted approximately one week after the plating date marked on the graph. A count of 1000 indicates a fully grown plate.

The poorest results were obtained with the simple PK-recipe, which yielded fewer than three colonies from the initial plating event, with no further colonies evident. This is likely due to the recipe's simplicity, which included only two potassium phosphate sources and no other mineral salts, resulting in a lack of nutrients for the microorganisms.

The MIRI recipe also performed poorly, with countable colonies only observed from the last plating event from sampling well 1. However, these colonies were agglomerated at the edge of the plate, suggesting a possible contamination source from the laboratory. Moreover, the recipe's pH was set to 9.5-10, much higher than the pH of approximately 7 in the original sampled groundwaters. It is likely that the microorganisms from the groundwater did not survive the higher pH in the MSM, and the observed colonies were from contamination.

The MSM WATA recipe also yielded poor results, although a small number of colonies consistently appeared from samples taken from groundwater well 2. However, other sampling points did not yield any colonies. While the results were consistent, the number of colonies was small compared to other trialed recipes.

The KAO recipe was also subpar in terms of performance. Although two plating events from samples cultured from well 3 yielded a full plate and a plate with over 100 colonies, these events did not replicate in further subcultures. It is unlikely that the colonies originated from laboratory contamination because they were evenly distributed on the plate and had a similar appearance to colonies on other plates.

MSM recipes for POTI and SHARMA both contained an extra source of nitrogen in addition to potassium cyanide, which significantly enhanced the growth of microorganisms in these media, resulting in more consistent colony count outcomes. Remarkably, samples from different groundwater wells showed optimal growth in different media: cultures from wells 1 and 2 achieved full growth or at least 20 large colonies after the first subculturing in MSM SHARMA, while cultures from wells 2 and 3 similarly flourished in MSM POTI.

The microcosms' activity was also assessed through an oxygen consumption test. As detailed in Chapters 2.4.1 and 2.4.2, many cyanide degradation pathways consume oxygen. The greater the activity of the bacteria in the samples, the more oxygen they consume in closed test bottles. However, no oxygen consumption was observed during the test; instead, oxygen levels increased from the first day and remained steady throughout week and over the weekend (Table 17).

Table 17: Results of O₂ consumption test.

day	Average O ₂ (mg/L)	std
0	7.08	0.11
1	8.08	0.04
2	8.23	0.02
3	8.15	0.02
4	8.32	0.06
7	8.12	0.08

4.3. Enrichment tests of bac-trap samples

The growth curves presented in Figure 8 do not reveal a clear exponential growth phase. However, the OD values generally exceeded those observed in previous liquid sample tests, indicating relatively robust growth. It is apparent that microorganisms were growing in numbers within the culture media between subculturing events, marked by a general increase in optical density after the red lines on the graph. It is plausible that more frequent measurements could have helped identify an exponential growth curve, which now looks more linear.

Control samples, mirroring cultured samples but substituting groundwater with ultrapure water in the medium, were employed as OD baseline references whenever feasible. However, upon examination of the plated control samples, it was evident that they had been contaminated. As the plating of the samples occurred after the OD measurements, the contamination of the controls was identified only post-measurement. Consequently, due to using contaminated controls as baselines, the OD values for MSM-1 might be somewhat elevated compared to results obtained on the dates 31/08/2023, 07/09/2023, and 22/09/2023. Similarly, for MSM-2, this discrepancy is noted on the dates 24/08/2023 and 22/09/2023, as highlighted by red dates on Figure 8.

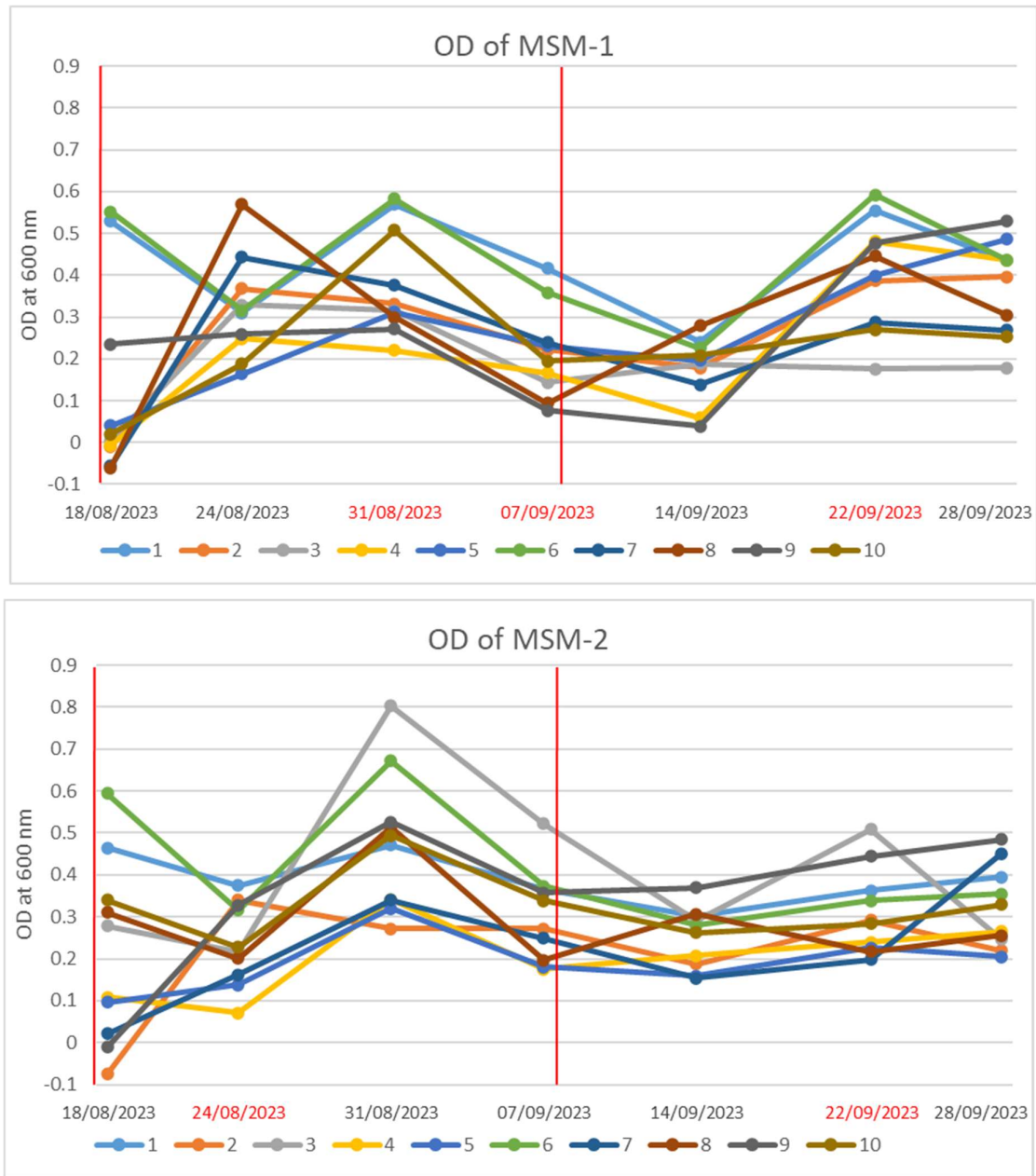


Figure 8: ODs of enrichment cultures derived from bac-trap material in MSM-1 (above) and MSM-2 (below). Numbers 1-10 are sampling points (see Figure 4 and Figure 5 for maps). The red lines mark the second and third subculturing events. The red dates mark the possibility of too low values due to a contaminated control sample used as a reference baseline.

Although statistical validation is precluded by the limited dataset, it can be noted that all sampling points have similar trends in optical density, with no sample displaying consistently significantly higher or lower optical density than others. The variation in the order of sample density over time further supports the similarity of the samples. However, discernible differences in microbial growth were observed between the two different MSM recipes. Before the third

subculturing, MSM-2 consistently yielded better results compared to MSM-1, as evidenced by higher OD values and colony counts on agar plates. Moreover, the diversity of colonies obtained from samples plated on different agar plates was more pronounced for MSM-2 than MSM-1 (Table 18). This discrepancy diminished following the third subculturing.

The first agar plates were plated directly before the second subculturing, but the samples were plated undiluted, following the precedent set by the undiluted liquid samples tested previously. The plates were fully grown, rendering accurate counting of colonies impossible. Six days after the second subculturing event, samples were plated as dilution series. Dilution 10^{-5} consistently provided counts above zero but not reaching a fully grown plate, therefore this dilution was used to graph the development of the counts, as depicted in Figure 9. The results parallel the trends observed in the OD values, albeit with more pronounced growth before subculturing and decline after subculturing. This enhanced contrast may be attributed to the use of contaminated baseline references in the OD measurements, which likely influenced the results. Similarly to the OD values, the plated cultures were alike, with no sample demonstrating significantly higher vitality than another that would have persisted over time.

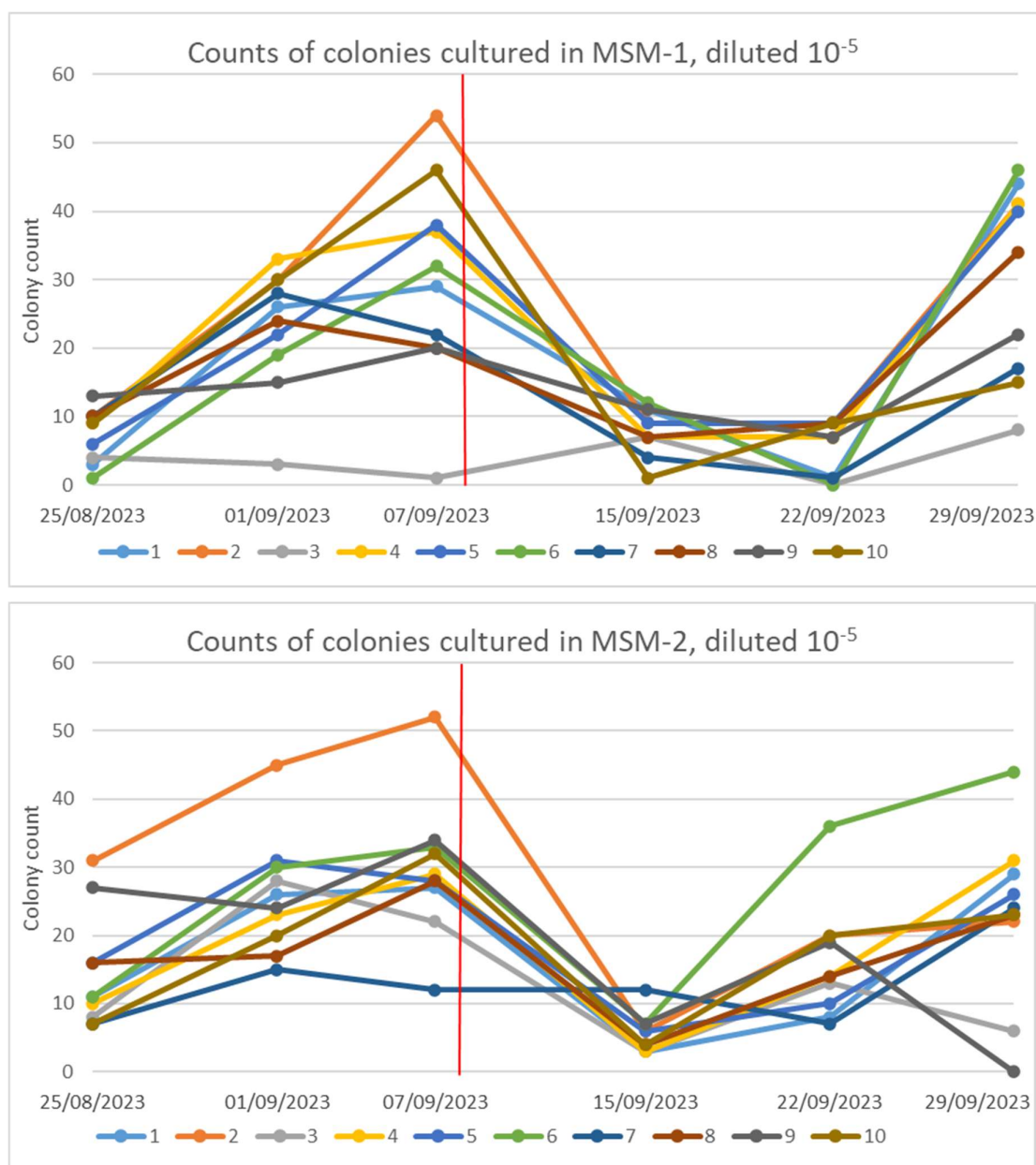


Figure 9: Colony counts of enrichment cultures derived from bac-trap material cultured in MSM-1 (above) and MSM-2 (below). The red line marks the third subculturing event.

Samples were plated on four different agar plates four days after the third subculturing to assess if any improvement in colony count results could be achieved. Plating occurred two days earlier than the dilution series were normally plated, prompting the selection of a dilution of 10^{-4} in hopes of obtaining countable colonies. Unfortunately, many plates were fully grown within two days at room temperature, making colony counting impractical. Instead, the number of distinct colonies per plate was noted. Table 18 reveals that nutrient agar (NA) supported the growth of a wider variety

of colonies compared to other agar recipes. Moreover, LB agar performed better than the previously used TSA recipe. An experiment was also conducted to evaluate microbial growth when cyanide was the sole nutrient source together with agar. While no colonies were observed on the cyanide plates during the initial counting event, colonies became visible nine days later. MSM-2 had a higher abundance of colonies capable of surviving in the cyanide environment compared to MSM-1.

Table 18: Averages of number of different colonies plated with different agar recipes.

Agar	LB	NA	TSA	WA
MSM1	2.25	2.25	2.00	-
MSM2	2.42	2.75	2.08	-

Table 19: Colony counts of WA plates after a longer incubation time.

Average colony counts of WA plates	
MSM1	64.5
MSM2	167.8

4.4. CN degradation in different O₂ environments

The results of the CN degradation test reveal significant challenges. Only the outcomes from the open-lid experiment showcased reasonable trends, displaying a consistent and rapid decline in cyanide concentration over time, with a near-complete evaporation observed within a three-day period. The findings from the other test conditions displayed inconsistencies, with occasional instances of cyanide concentrations higher than previously recorded, even though no cyanide was added to samples during the test. These discrepancies will be further explored in Chapter 5.4.

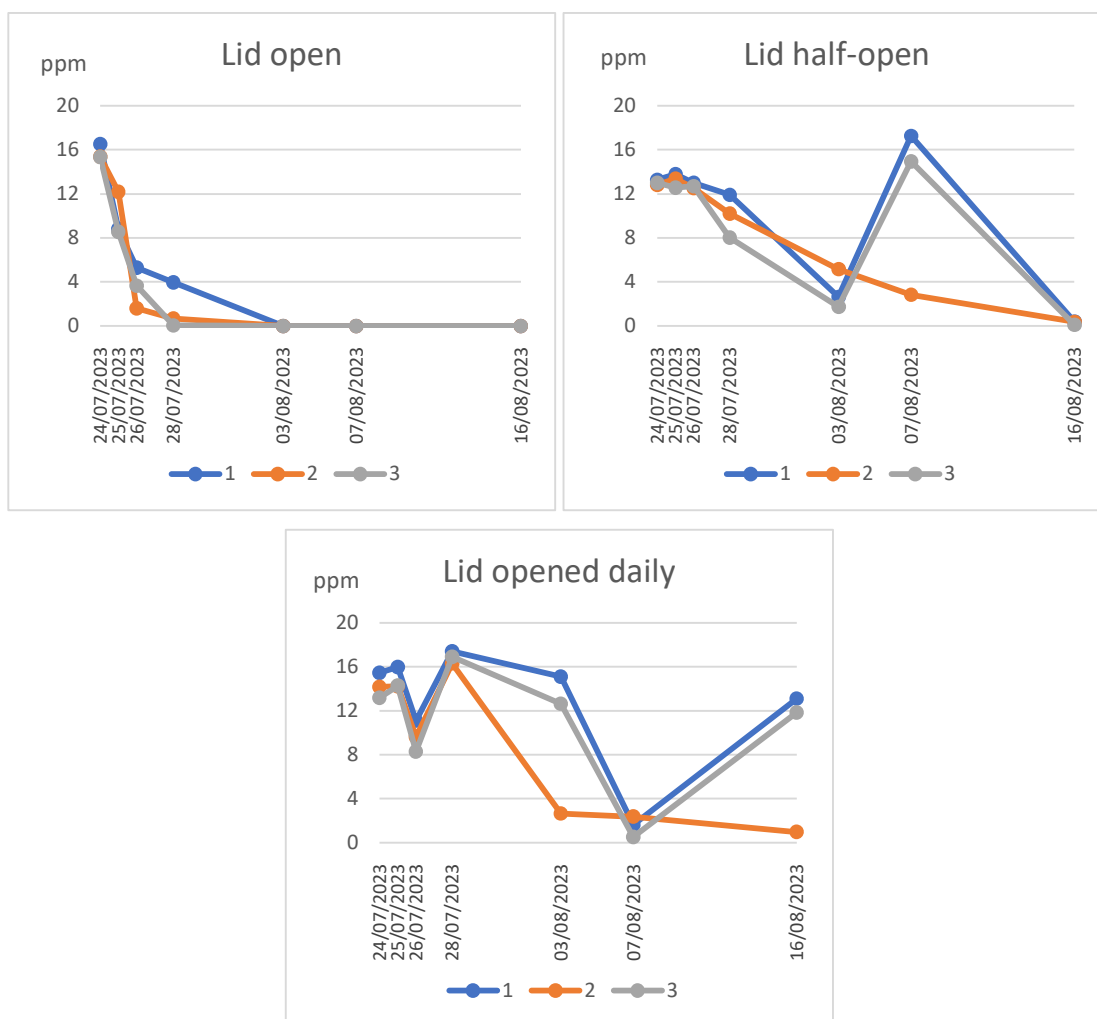


Figure 10: Degradation of 1 mM potassium cyanide in varying oxygen environments: bottles with lid open, bottles with lid screwed half open and bottles with lid closed but opened once daily.

Throughout each measurement cycle, a check standard was employed to assess the reliability of the results. Typically, measured check standard concentrations should align within a 5 % margin of the prepared concentration to ensure reliability. However, during this test, check standards consistently deviated significantly beyond this threshold, with one instance recording a discrepancy of 135.8 % from the prepared concentration (refer to Table 20). Consequently, the validity of all results is called into question, prompting a need for thorough investigation and potential recalibration of the experiment procedures, to be discussed in Chapter 5.4. At present, it suffices to say that the CN degradation results are substantially compromised. While individual data values may be deemed unreliable, their relative positioning to other data points may still offer insights worth considering.

Table 20: Check standards' prepared concentrations, measured values and their differences. Measured concentrations should be within 5% of the created checks.

	Check std (ppb)	measured (ppb)	difference
24/07/2023	2	1.33	66.5 %
25/07/2023	2	1.274	63.7 %
26/07/2023	10	13.575	135.8 %
28/07/2023	10	8.663	86.6 %
03/08/2023	2	0.319	16.0 %
07/08/2023	10	9.337	93.4 %
16/08/2023	10	5.191	51.9 %

4.5. Microdistillation

The observed recovery rates of microdistilled cyanide samples behaved unexpectedly: the measured concentrations of cyanide significantly exceeded the prepared concentration in the sample, resulting in recovery rates surpassing 100% (see Figure 11). When the anticipated cyanide concentration exceeded 250 ppb, the recovery rates stabilized around 100%. Conversely, for expected cyanide concentrations below 500 ppb, the recovery rate increased inversely with the expected concentration, approaching 800%. This observed escalation in recovery rate appears to follow an exponential trend (as seen in Figure 11).

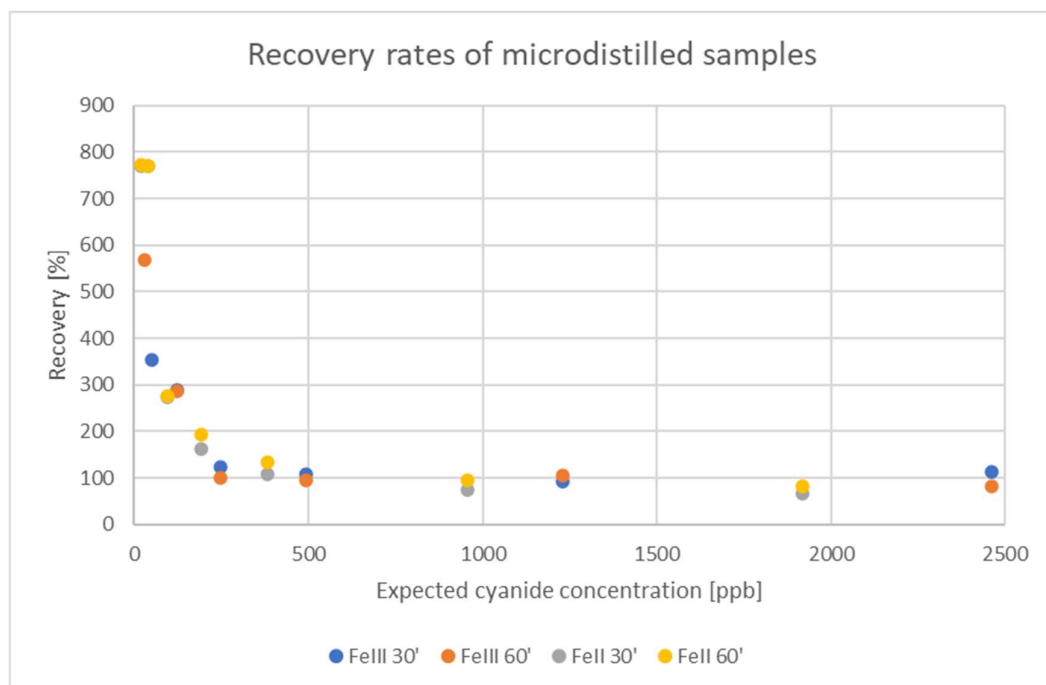


Figure 11: Recovery rates of microdistilled samples. FeIII denotes $\text{C}_6\text{FeK}_3\text{N}_6$ (potassium hexacyanoferrate III) and FeII denotes $\text{C}_6\text{FeK}_3\text{N}_6 \times 3\text{H}_2\text{O}$ (potassium hexacyanoferrate II trihydrate)

Both potassium hexacyanoferrate II and III behaved similarly, which was anticipated given their similar solubility products, as outlined in Table 2. The utilized potassium hexacyanoferrate II ($\text{K}_4\text{Fe}(\text{CN})_6$, labeled as FeII) was a trihydrate, in contrast to the unhydrated potassium hexacyanoferrate III ($\text{K}_3\text{Fe}(\text{CN})_6$, marked as FeIII), resulting in a smaller relative cyanide content. Therefore, the expected concentrations of FeII were lower than FeIII in Figure 11.

Considering the erratic behavior of the samples, it is challenging to ascertain whether a 30-minute microdistillation period yielded comparable results to a longer distillation duration. However, it appears that for FeII, the prolonged distillation period yielded marginally higher concentrations, whereas the impact on FeIII remains inconclusive.

5. Discussion

5.1. Enrichment tests of liquid samples

Typically, optical density measurements capture a clear lag-phase followed by an exponential growth phase before reaching the stationary and cell death phases, forming an S-curve on a graph. However, during the liquid enrichment test, despite 4 weeks of growth time, the exponential phase failed to appear. The lack of growth was assumed to stem from the composition of the culture medium, adapted from Kandasamy et al. (2015), the ingredients specified in Table 11. The recipe included high concentrations of metal salts such as magnesium, manganese, copper and zinc sulphates, usually added in trace amounts. Particularly, the high concentration of the copper sulphate imparted a strong sky-blue colour to the medium, rendering it opaque and potentially disrupting the optical density measurements. It was later discovered that the recipe used by Kandasamy et al. (2015) was identical to that used by Watanabe et al. (1998), except for thousandfold higher concentrations of manganese, copper and zinc sulphates. These elevated metal salt concentrations likely impaired the bacterial growth in the MSM. Due to the lack of growth, subculturing was not performed, and instead, alternative growth media were explored to find conditions more conducive to microbial growth.

5.2. MSM trials for best performance

Although the various MSM formulations produced varying outcomes on the agar plates, no medium was performing satisfactorily. None of the media provided consistently well growing plates across all sampling points. The most effective media, POTI and SHARMA, were those containing an additional nitrogen source, possibly promoting the development of bacteria resistant to cyanide but unable to degrade it. These recipes provided fully grown plates from certain sampling wells, but not from all wells.

The measurements conducted to support the findings derived from colony counts, OD and oxygen consumption tests, were not informative. The OD values remained low for all MSM recipes, without any discernible growth phases appearing. Oxygen was not consumed in the closed environments, instead, the oxygen levels rose on the first day and remained high until the end of the test. The lack of growth and oxygen consumption in the media might be explained by the general inactivity or small number of microorganisms. Unconcentrated groundwater was used for this test, meaning that from the start, the number of microorganisms in the samples was very low. However,

in well performing, sterile mineral salt media the low count of introduced microorganisms should not hinder their growth, only prolong the lag phase before the exponential growth phase. Fewer microorganisms should have more space and nutrients to grow and thrive in the medium, without having to compete.

Ultimately, two effective MSM formulations, which were employed in parallel trials at a laboratory collaborating on the same project in Germany, were selected for the enrichment of the bac-trap material.

5.3. Enrichment tests of bac-trap samples

From the outset of testing the enrichment cultures derived from the bac-trap material, it was evident that these cultures outperformed cultures enriched from pure groundwater samples in the testing methods, namely the OD measurements and plating series. The improved results can be attributed primarily to the bac-trap material's ability to capture a greater quantity of bacteria. Bacteria tend to live on or nearby surfaces, which the quartz sand of the bac-trap provides.

However, the microcosm experiments did not exhibit the expected level of activity. Although some growth was observed in OD measurements and colony counts, it proceeded at a relatively slow pace. Following the third subculturing, a lag phase lasting two weeks was observed. Unfortunately, only the initial stages of what looks like the exponential growth phase after the third subculturing were captured, precluding any assessment of its speed or steepness. The preceding growth phase, initiated after the second subculturing, displayed a seemingly linear progression rather than exponential, both on the OD-derived growth curve and the colony count curve (Figure 8 and Figure 9). To verify any growth, be it linear or exponential, more frequent measurement of OD and colony counts would have been necessary. Without more data, the growth can only be described as very weak or even non-existent due to the low cell density.

The contamination of control samples likely resulted from cross-contamination from other samples. It is improbable that the controls were contaminated during storage in the laboratory, as the Schott bottles were kept 1.5 rotations open with minimal exposure to airborne contaminants, but enough for the samples to have an oxygen supply. However, during sample feeding with KCN or during subculturing events, when bottles were left open side by side, cross-contamination may have occurred, despite rigorous sterilization of bottle caps, bottle necks, and all equipment before operating in the laminar hood. Furthermore, the persistence of contamination in the control samples for weeks, despite being devoid of any nitrogen source other than KCN, suggests that cross-contamination from other samples is more plausible than contamination from elsewhere in the

laboratory. Since the controls were contaminated, it is likely that the other samples were similarly affected. The similarity of results across sampling points may, in part, be attributed to cross-contamination. More importantly, cross-contamination likely influenced the OD measurements by elevating the baseline when measuring with contaminated controls, potentially yielding results lower in value than they should have been.

Encouragingly, samples plated on agar plates containing only agar and potassium cyanide showed growth. Although degradation tests could not be conducted within the timeframe of this thesis, the ability of colonies to grow not only in liquid media but also on cyanide-agar plates is a promising indication for the success of the degradation tests.

5.4. CN degradation test

The results of the CN degradation test were met with disappointment due to the significant discrepancy observed between the prepared concentrations and measured values from the check standards. Due to the discrepancy, the test results are compromised. However, while individual data values maybe unreliable, their relative positioning to other data points may still provide valuable insights worth considering.

The measurements conducted with the lid fully open show rapid disappearance of cyanide in the water within a matter of days, as expected. No pH correction was applied to prevent cyanide from volatilizing. Given the high volatility of cyanide, continuous exposure to air facilitated its rapid evaporation from the solution.

In contrast, measurements conducted under different oxygen conditions were inconsistent, with some results having values higher than previously recorded. When lids were kept closed or only partially open, direct and continuous cyanide volatilization during storage was prevented. Instead, cyanide evaporated during the measurement process. Samples needed to be prepared in open air for measurement in the Ion Chromatograph, which exposed the cyanide for volatilization. Samples were prepared into IC vials, which could not be closed, as the provided vial lids were unsuitable for their intended purpose: the IC needle frequently got stuck in the lid, disrupting the measurement cycle and potentially harming the machine. Instead, samples were placed on the sample wheel uncovered during the cycle, exposing the cyanide to volatilization. Consequently, samples measured more promptly after preparation or placed earlier in the order on the sample wheel had less cyanide volatilization, whereas samples measured later had longer exposure to air. When measured CN⁻ concentrations are plotted against their measuring time, this effect becomes visible (see Figure 12). The different exposure times could have led to discrepancies in the values and enabled cyanide

concentrations higher than previously measured. Additionally, as is always the case in laboratory work, human error during sample preparation or dilution may have contributed to discrepancies in the results.

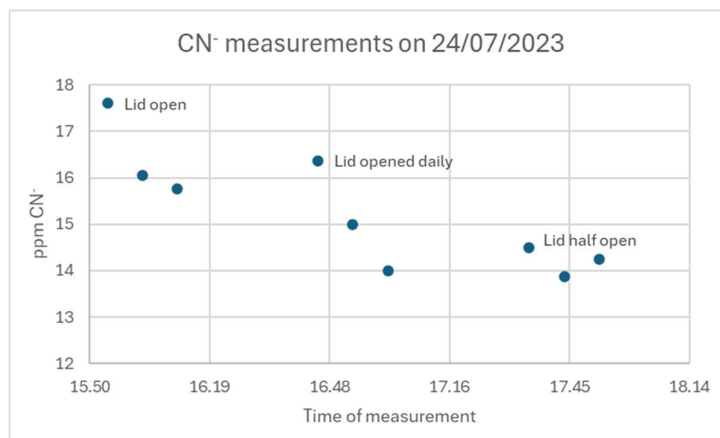


Figure 12: Impact of measurement timing on CN⁻ concentration: At the start of the CN⁻ degradation test on 24/07/2023, all samples were prepared with 1 mM KCN. Due to air exposure during IC sample preparation and on the IC sample wheel, CN⁻ volatilized over time, resulting in lower concentrations in samples measured later.

It's worth noting that each sample was prepared with 1 mM of KCN, which should have resulted in approximately 26 ppm CN per sample. However, the highest measured concentrations only reached around 16 ppm. It is unlikely that the missing 39% of CN remained chemically bonded with the potassium, as KCN has a very low solubility product constant. A more plausible explanation is that there were issues with the volatilization of cyanide. Issues were also encountered with the Ion Chromatograph setup that led to unreliable results, as indicated by the disparity between the expected and measured check standard values.

Many corrective measures were implemented in attempts to correct the check standard issue. The working electrode and reference electrode of the IC were regularly cleaned according to the official instructions provided by the manufacturer. Fresh eluent was always used, and inline filters were periodically replaced. The system was flushed, and other IC vial lids were tested to prevent cyanide volatilization. Despite these efforts, the problem persisted unabated. Addressing the technical issues encountered during the experiment will be essential for conducting any future experiments.

5.5. Microdistillation

Explaining the manyfold recovery rates for low concentrations of expected cyanide presents a formidable challenge. Common sources of error in laboratory procedures include calculation mistakes, errors in sample preparation and dilution, issues during microdistillation or in the ion chromatography setup, or errors occurring during the measuring cycle. However, meticulous care was taken at every stage of the process, with calculations and preparations often subjected to double-checking. While the possibility of human error can never be dismissed, it is intriguing to note that the false results were consistent: all batches had recovery rates that appeared to follow an inversely exponential pattern, with higher recovery rates towards lower expected concentrations. If the errors were solely attributable to human factors, one might expect a more random distribution of results. On the other hand, at low expected concentrations, minor interferences can have a larger proportional effect, causing disproportionately high recovery rates. Even small amounts of contamination during stock preparation, sample dilution, microdistillation, or IC measurement can result in disproportionately high readings. However, the consistency of the erroneous results does not support this hypothesis.

If the error did not stem from human factors, potential issues within the test setup must be considered. It is possible that a chemical used in the microdistillation process acted as an interferent, increasing the measured values as hexacyanoferrate concentrations decreased. However, the chemicals utilized in the microdistillation—ultrapure water, H_2SO_4 , MgCl_2 , and NaOH —are not known to interfere with cyanide measurements via ion chromatography. These reagents are routinely employed in microdistillation procedures. Cyanide carryover from previous sample to the next during measurement in the IC was considered, but this theory was dismissed as it would imply that, on occasion, more than double the preceding sample's concentration was carried over to the subsequent sample, which is highly improbable. Moreover, all samples were diluted to the lower end of the detection range, reducing the likelihood of carryover impacting the results.

Nevertheless, as with the problems encountered during the CN-in-water degradation test, they must be solved before enriched samples can be used to begin any microbial CN degradation experiments.

6. Conclusions

The main purpose of this study was to establish active enrichment cultures of indigenous bacteria capable of degrading cyanide collected from cyanide-polluted legacy contamination sites. This thesis documents the meticulous efforts and numerous experiments required to successfully establish active bacterial enrichment cultures and ensure their degradation capabilities.

The results indicate limited yet promising success in cultivating viable bacterial cultures. In the enrichment tests derived from bac-trap material, the lag phases of the growth curves extended up to two weeks, and the initial growth stages were very weak due to low cell density. Nevertheless, the ability of these cultures to grow on plates containing only agar and potassium cyanide as nutrient sources is a positive sign. This growth indicates potential degradation capabilities of the bacteria, despite the initial slow start and low density.

In the future, this project will proceed with degradation tests to further evaluate the performance of the bacterial cultures. The ultimate aim remains to apply the enriched cultures in field-scale remediation efforts. The groundwork laid by this study provides a foundation for continued research and development in cyanide bioremediation, moving closer to practical and effective environmental restoration solutions.

7. References

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Annexes

Annex 1

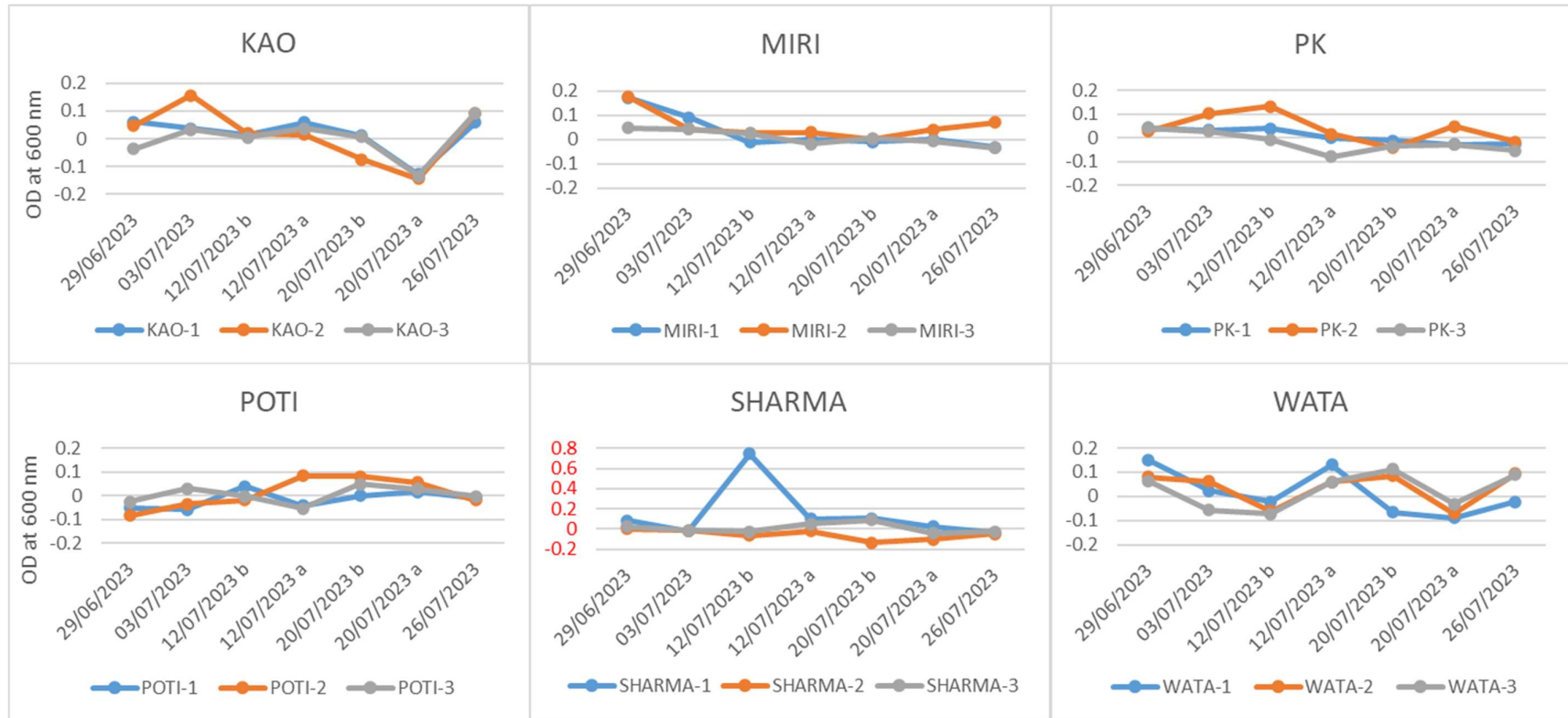


Figure A - 1: Results of OD-values taken during the MSM trials for best performance. MSM codenames can be referred to in Table 12 on page 34. On the days when subcultures were made, OD was measured both before and after the act, marked next to the date as b and a, respectively. The range of the vertical axes are fixed between -0.2 and 0.2, except for SHARMA (axis marked in red), where one data point reached the value of 0.75.

