Metal contamination and methane oxidising bacteria around a formerly industrialised suburban river

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Dedication

This thesis is dedicated to my family

Abstract

Heavy metal pollution is a very prominent problem persisting globally at the present time. Many technologies have been put forward to remediate such pollution. Bioremediation is a promising and eco-friendly tool to resolve environmental pollution. Microbes belonging to diverse phylogenetic groups have been investigated previously for remediation and immobilisation of pollutants. One among such groups are methanotrophs, which grow on methane as their sole source of carbon and energy and are able to remediate diverse hydrophobic organic pollutants due to the wide range of substrates utilised by their oxygenase enzymes. Recently, it was shown that the methanotroph *Methylococcus capsulatus* (Bath) was also able to remediate hexavalent chromium contamination. In the current study sediment samples were enriched from an urban river in Sheffield in Northern England that has previously suffered serious heavy metal pollution due to previous activity of the steel industry (17th to 19th century).

The site selected for the present study was the River Sheaf located on south west of Sheffield in Northern England and approximately 3 miles from city centre, and which flows from Totley through Millhouses Park. Sediment samples were collected and characterised, according to various size fractions and then heavy metal analysis was carried out in the various fractions of the sediments. The most abundant heavy metals found at site were lead, chromium, nickel, arsenic and cobalt. The maximum concentrations of the heavy metals Pb, Cr, Ni, As and Co in the site were 412.80 mg/kg, 25.232 mg/kg, 25.196 mg/kg, 8.123 mg/kg and 7.66 mg/kg, respectively.

Methanotrophs were enriched and isolated from the Sheaf sediments and then the isolated methanotrophs were investigated to determine their ability to reduce the hexavalent chromium. A strain of *Methylomonas koyamae*, which was given the strain designation SHU1, was isolated and found to remove hexavalent chromium across a range of concentrations in the range of 10-1000ppm after cultivation on methane as growth substrate. The Cr (VI) may be reduced to Cr (III) but the production of Cr (III) was not experimentally investigated and so there is the possibility that some or all of the chromium removal could be due to biosorption and uptake into the cells e.g. via the sulphate transport pathway.

It was also found that the removal of Cr (VI) was inhibited by the addition of the metabolic inhibitor sodium azide, thus indicating that removal of chromium is largely a metabolic reaction mediated by enzymes rather than a passive biosorption process.

It was speculated that methane monooxygenase (MMO) provides electrons from the oxidation of methane which may be used by other enzymes for removal (e.g. chemical reduction) of hexavalent chromium. Phenyl acetylene is a strong inhibitor of soluble MMO (sMMO) but inhibits particulate MMO (pMMO) less effectively. When cells expressing sMMO and pMMO were inhibited by phenyl acetylene the chromium removal reaction was completely inhibited compared in cells expressing sMMO.

The above isolated organism produces a sMMO when there is copper deficiency in the media which is another distinguishing characteristic to potentially apply the organism in bioremediation of hydrophobic organic compounds, because sMMO generally has a wider substrate range than pMMO.

The proteins encoded by available genome sequences of *Methylomonas* strains were compared with proteins from other microbes that are involved in chromium reduction, efflux systems and chromium uptake. Highly significantly similar proteins were found in the *Methylomonas* strains which resembled the proteins known to be involved in chromium removal, uptake and reduction. A number of strains of the *Methylomonas* genus are known to possess a gene for the sulphate transporter systems which could also play a major role in transportation of chromium (VI) into the cells.

To the author's knowledge this is the first description of a strain of the widely environmentally distributed genus *Methylomonas* that is capable of remediating hexavalent chromium.

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CHAPTER 1

LITERATURE REVIEW

Chapter 1

1.1 Introduction

Rapid industrialisation and population growth are the two factors which most affect the global environment and are a general risk to health. The release of pollutants into the environment accidentally or through anthropogenic activities distributes pollutants in soil, water and air, affecting the environment (Das and Dash 2014).

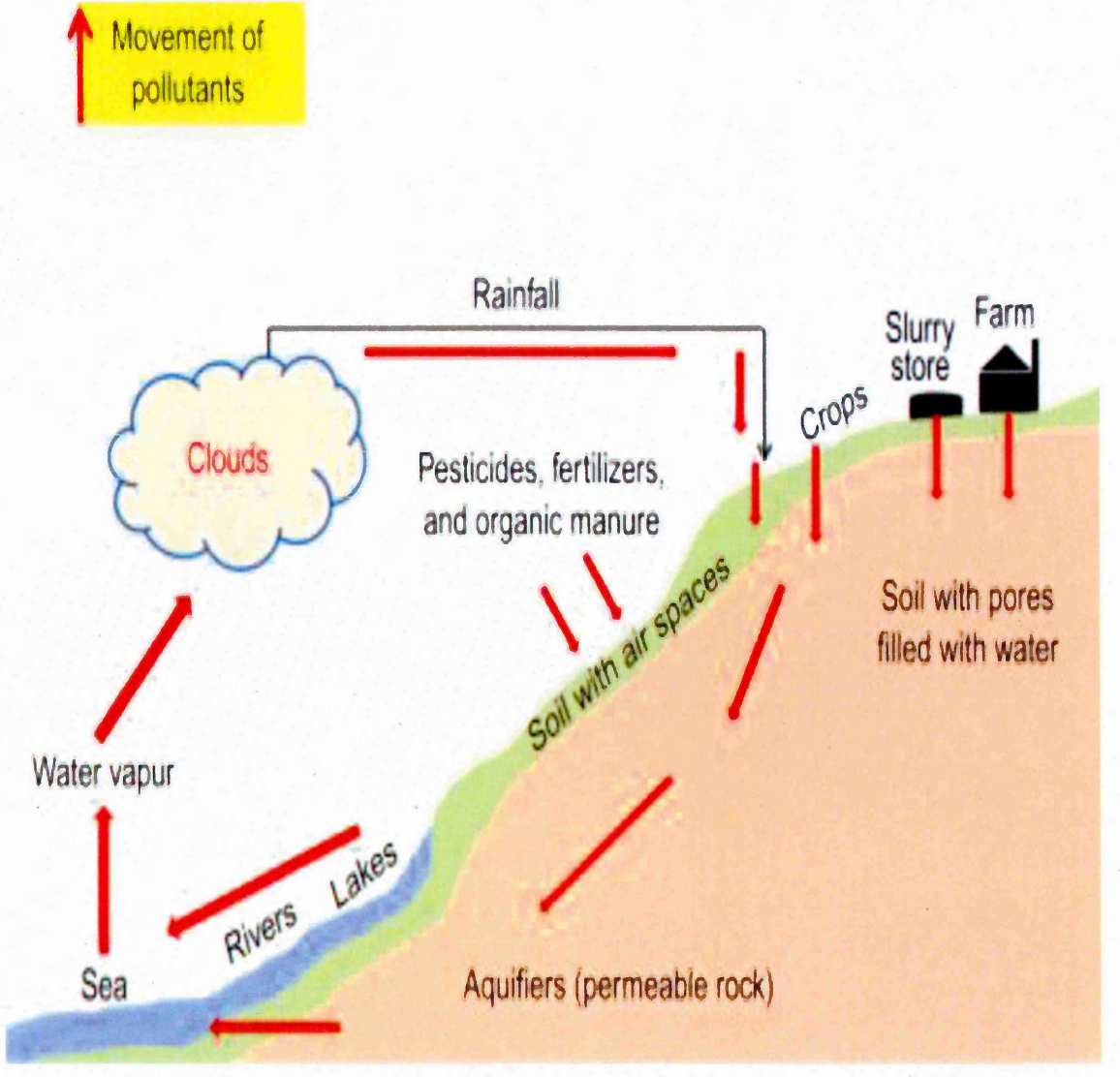
Highly industrial areas throughout the globe pose a high risk of heavy metal pollution to living beings. Natural processes also release some amount of heavy metals into the environment but their concentration is negligible in most parts of the world but serious in some locations. Volcanic eruptions, weathering and erosion are the principal natural processes that release heavy metals into the environment (Abdullahi 2015).

The pollutants once they enter into the environment can pass through the food chain and flow from lower constituents to higher trophic levels and risk the ecosystem by bioaccumulation and transferring from one food chain to other (Das and Dash 2014). The pollutants from air water and soil can move and be transported via various biogeochemical cycles and thus affect every component of environment as shown in figure 1.1.

Heavy metals and poly cyclic aromatic hydrocarbons are among the major pollutants in present day. Globally the order of the heavy metals found at polluted sites in terms of their concentrations is as follows: Pb>Cr>As>Zn>Cd>Cu & Hg (USEPA 1996). Their non-biodegradable nature and bioaccumulation in the environment makes heavy metal pollution a long term serious issue.

The most efficient technique to remediate the heavy metal pollution is treatment with microbes. Microbial remediation is gaining significant interest due to the interaction of microorganisms with metals which can alter the physical and chemical state by making changes in metal speciation thus increasing or decreasing their mobility. Thus by transformation or immobilisation by microbes remediation of metal contaminated sites can be achieved (Ali et al 2015).

Figure 1.1 Movement of pollutants in various parts of environment



Source: Adapted from Das et al 2014

1.2 Chromium

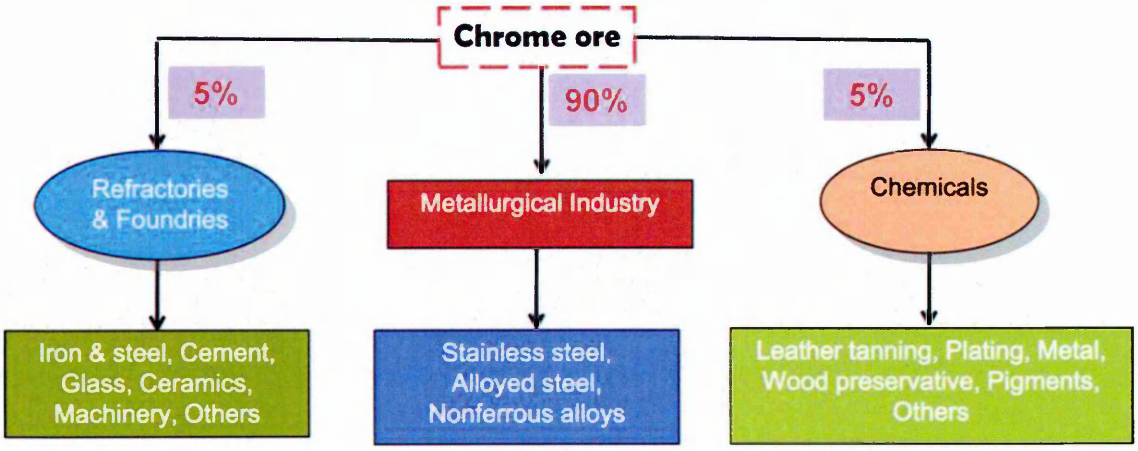
1.2.1 Definition, Sources & Uses

Chromium was first identified in Siberian red lead ore (Crocoite) in 1798 by the French chemist Vacqueline. It belongs to group VI-B (transition element) of the periodic table. Chromium is a steel-grey, radiant, hard and malleable metal occurring in nature in various chemical forms, which constitutes 0.1-0.3 mg kg^{-1} of the earth's crust (Fendorf 1995). Chromium has numerous ores of which the mineral chromite is the major economically extractable form. The stable forms of Cr are the trivalent (III) and the hexavalent Cr (VI) species; although there are various other valence states between Cr^{-2} and Cr^{+5} which are unstable and short lived in biological systems.

Cr (III) and Cr (VI) are the usual common species in soil. These two forms exhibit quite different chemical properties and affect organisms in different ways (Fendorf 1995). Cr (VI) is mediated as the most toxic form which usually occurs associated with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) oxy anions. Cr (III) is less harmful and less mobile than Cr (VI).

The various sources of chromium can be found in different industries such as electroplating, steel and automobile manufacturing, mining, leather tanning, cement, wood preservation, metal processing, textile manufacture and production of paints, pigments and dyes. The extensive usage of chromium in various industrial applications is tabulated in figure 1.2 below.

Figure 1.2 Usage of chromium in different sectors

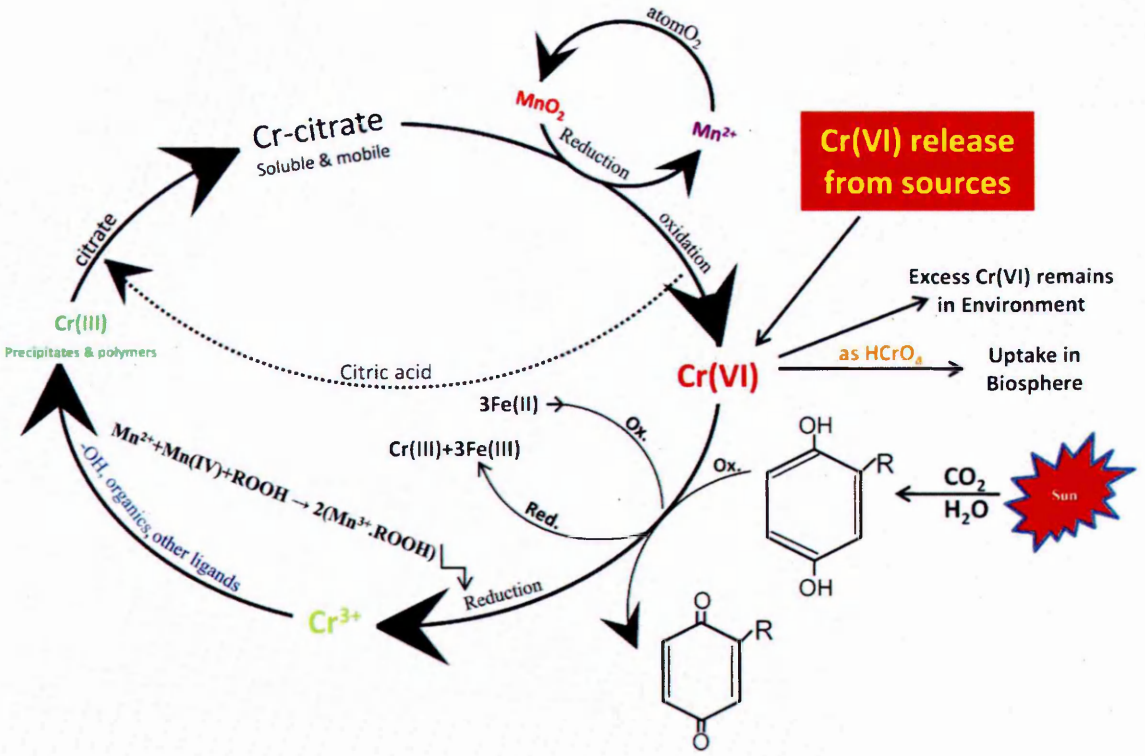


Source Adapted from Dhal et al (2013)

1.2.2 Properties and forms of chromium

As described above the two forms of chromium (III) and (VI) commonly exist in soil. Cr (VI) is water soluble across a wide range of pH, while trivalent chromium tends to be adsorbed on the surface of soil particles and precipitate as chromium hydroxide to some extent in acidic and alkaline conditions thus posing Cr (III) a high possibility for persistence in the environmental for long periods. Figure 1.3 shows how the various forms of chromium exist in a natural cycle.

Figure 1.3 General phases of chromium in natural environment



Source: Adapted from Dhal et al., (2013)

Chromium (VI) is the most oxidised, free, active and noxious form of chromium and exists in the environment in equilibrium with other chromium species (Figure 1.3). The existence of Cr (VI) in nature is a consequence of the oxidation of natural Cr III in smaller proportions while larger concentrations are released anthropogenically as pollutants from oxidation of Cr III (Dhal et al 2013). Manganese oxides present in soil favour the oxidation of Cr III to Cr VI (Dhal et al 2013) while soil carbon compounds aid in reduction of Cr (VI) to Cr (III) (James 2001). Hence the oxidation and reduction reaction takes place simultaneously there is balance of different chromium containing ions in the soil environment

Under neutral pH the oxidation of Cr (III) to Cr (VI) facilitated by manganese oxides via surface oxidation and proceeds as

$$Cr^{3+} + 1.5\ MnO_2 + H_2O \longrightarrow HCrO_4^- + 1.5\ Mn^{2+} + H^+ \qquad (1)$$

Reduction by organic compounds, e.g. hydroquinone (with formation of quinine) proceeds as

$C_6H_6O_2 + CrO_4^{2-} + 2H_2O \longrightarrow 0.5\ Cr_2O_3 + 1.5\ C_6H_6O_2 + 2.5\ H_2O + 2OH^- \ \Delta G^0_{298} = -427 kJ\ mol^{-1}$ (2)

Cr (III) is less bioavailable and less mobile in water and soils compared to Cr (VI) which has higher solubility and bioavailability thus making its toxicity a greater environmental problem. In soils the oxidation reaction of Cr (III) to Cr (VI) occurs via surface oxidation facilitated by manganese oxides under neutral pH conditions. However reduction reactions of Cr (VI) to Cr (III) are favoured by organic compounds like hydroquinone, moreover under most natural conditions in soil, reduction of Cr (VI) to Cr (III) is favoured over the oxidation of Cr (III) to Cr (VI) by oxidising agents. Barlett and James (1979) who conducted several experiments on the oxidation of Cr (III) to Cr (VI) concluded that soils containing manganese oxides were more extensively oxidised than the soils without manganese oxides.

Although pH plays a very important role on the oxidation and reduction reactions of chromium in soils it is a complicated process, but typically high pH values intensifies the oxidative power while low pH values increase reduction reactions. In the laboratory at optimum conditions such as neutral pH and at elevated levels of Mn oxides and appropriate aeration conditions, it was observed that soluble and freshly precipitated forms of Cr (III), such as $CrCl_3$ and $Cr(OH)_3$ supplemented to soil may be oxidised up to 15% (Dhal et al 2013).

1.2.3 Speciation of Cr (VI)

Toxicological studies have shown that the extent of toxicity of a metal depends on its chemical form and oxidation state hence there is growing interest to study the speciation of chemicals in the environment (Hintsa 2013)

Cr (VI) can form distinct species, such as CrO_4^{2-} (chromate), $HCrO_4^-$ (hydrogen chromate) or $Cr_2O_7^{2-}$ (dichromate) according to the pH and total Cr (VI) proportion in the medium.

$H_2CrO_4 \longrightarrow H^+ + HCrO_4^-$, $K_1 = 10^{-0.75}\ mol\ dm^{-3}$

$HCrO_4 \longrightarrow H^+ + CrO_4^{2-}$, $K_2 = 10^{-6.75}\ mol\ dm^{-3}$

$2HCrO_4^- \longrightarrow Cr_2O_7$, $K_3 = 10^{-2.2}\ dm^3\ mol^{-1}$

H_2CrO_4 (chromic acid) is a strong acid and at higher than pH 1 a deprotonated form of Cr (VI) exists, while at pH more than 7 only CrO_4^{2-} ions prevail in solution throughout the concentration range. At pH ranging from 1-6 $HCrO_4^-$ is the prevailing species.

1.2.4 Chromium toxicity

Chromium is a fundamental micronutrient in humans and animals; it is crucial for normal sugar, lipid and protein metabolism in mammals (Dhal et al. 2013). Deficiency of chromium leads to amendment to lipid and glucose metabolism in humans and animals, while no positive effects have been observed for chromium in plants.

Cr (VI) toxicity can be attributed to its easy dispersion across the cell membrane in prokaryotes and eukaryotes with consecutive reduction of Cr (VI) giving free radicals that cause DNA transformations and other toxic effects (Dhal et al 2013 & Xia et al 2014). Cr (III) has been predicted at 10-100 times less toxic than Cr (VI) (Flora et al 1990) because of the impervious nature to cellular membranes to almost all Cr (III) complexes.

1.3 Bioremediation

1.3.1 Introduction

Industrialization has produced many pollutants in the environment, where their removal becomes essential for environment to produce sustainability for living beings. A range of physical and chemical methods have been investigated for eradication of toxic metals but these are not sustainable to the environment (Das 2014).

The conventional techniques that can be employed in the remediation process include chemical precipitation, coagulation, adsorption by activated carbon, adsorption by natural minerals, ion exchange and reverse osmosis. In chemical precipitation coagulants such as aluminium sulphate, iron salts, and lime are employed to remove the noxious elements from the higher concentration of pollutants present in waste materials. A desired level of precipitate is generally not obtained to remove pollutants by this technique (Das 2014).

Adsorption is a surface based phenomenon in which molecules, atoms, and ions from gas, liquid or dissolved solid adsorb to the surface of adsorbent. Adsorption by activated carbon is an extensively used technique that was first employed in United States in 1930 to eliminate taste and odour from contaminated water. Activated carbon is a raw form of graphite with a random/irregular structure which is highly penetrable, demonstrating an immense range of pore sizes, from visible cracks and crevices to slits of molecular dimensions. The large surface area, micro porous structure and high degree of surface reactivity contribute to the adsorption by activated carbon (Mohan & Pittman Jr. 2006)

Various components such as coconut shells, woodchar, lignin, petroleum coke, bone char, peat, sawdust, carbon black, rice hulls, sugar peach pits, fish fertilizer wastes, waste rubber tyres etc., aid in preparation of activated carbons. The adsorption by activated carbon has advantages since it is highly effective process, it has great scope to adsorb a broad range of pollutants, due to the porous nature and high surface area of the adsorbent (for example activated carbon) it is an efficient remediation technique (Mohan & Pittman Jr. 2006).

The adsorption by activated carbon also has several disadvantages. It is an expensive technique and the costs for reactivation of the adsorbents are high. The performance of the technique can be improved by complexing agents. Activated carbons are not applicable to

removal of hydrophilic substances and rejuvenation results in loss of carbon (Mohan & Pittman Jr. 2006).

Ion exchange is a versatile chemical reaction wherein ions from water or waste water are exchanged with similarly charged ions attached to an immobile solid particle. Naturally occurring inorganic zeolites or artificial resins serves as immobile solid particles. Ion exchange technique mainly employs hydrocarbon derived polymeric resins (Akpor & Muchie 2010). The technique has been proposed to eliminate several heavy metals from waste water. For example the naturally occurring mineral clinoptilolite has been used to remove chromium, lead and cadmium (Vaca et al 2001).

Disadvantages of ion-exchange technique are lack of selectivity against specific target ions, susceptibility to fouling by organic substances and microorganisms present in water and generation of wastes as a result of ion exchanger regeneration (Metcalf & Eddy 1991).

Reverse osmosis removes over 99% of dissolved minerals via a membrane process which acts as a molecular filter. When cellulose acetate or aromatic polyamide membranes are applied at high pressures the solvent is forced out through this membrane to dilute solution. The clean water passes through the membrane leaving dissolved and particulate matter. This technique is efficient to remove ionic species from solution (Akpor & Muchie 2010).

The concentrations of ionic and dissolved organic compounds can be reduced to great extent by reverse osmosis (Volesky et al 2003, Pawlak et al 2005). This technique has gained utmost importance in removal of heavy metals on large and small scales. The disadvantages of these techniques are the high cost of membranes used, very expensive operational costs and usage of high pressure, all of which make the technique expensive and sensitive to operating conditions.

Chemical precipitation is a widely used technology to remove metals from industrial waste waters. The process involves the alteration of dissolved contaminants to insoluble solids and then removal of contaminants from the liquid phase using physical methods such as clarification and filtration (Nomanbhay & Palaniswamy 2005). In a precipitation process the chemical precipitants are added to intensify the particle size through aggregation. The concentration of chemical is dependent on pH and alkalinity of water. Heavy metals are removed from waste water with sodium hydroxide and lime during the neutralisation process (Akpor & Muchie 2010).

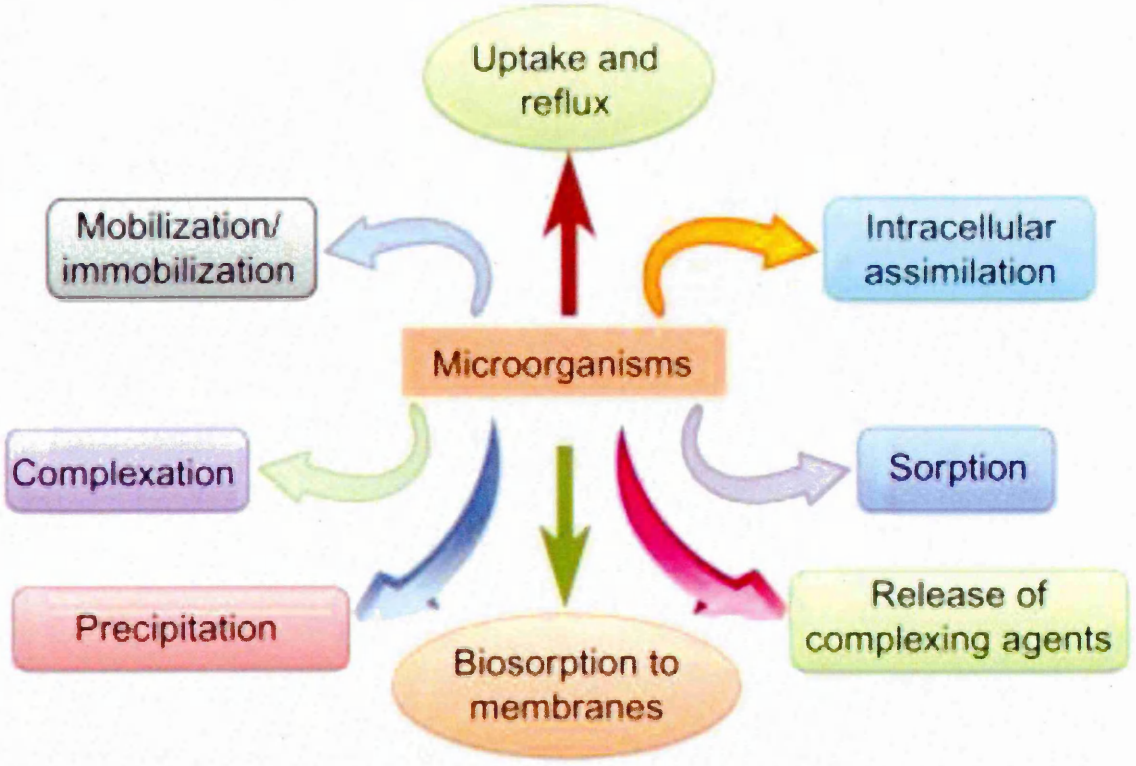
The disadvantage of this technique is that light small flocs are generated which require extra coagulation and flocculation procedures. The method generates a large volume of sludge whose disposal incurs additional costs. This technique does not meet regulatory requirements with hydroxide and carbonate precipitations. The method is also not considered to be safe due to working with corrosive chemicals (Akpor & Muchie 2010).

Because of the disadvantages described above relating to conventional techniques for remediation of metals, use of bacteria to bioremediate various natural and man-made compounds and the resulting reduction of their toxicity on ecosystems draws an increasing significance. The enzymatic transformation of metals generally does not generate any toxic products therefore posing less risk to ecosystems. Bioremediation and biotransformation can be applied to facilitate the naturally occurring microbes to enhance their metabolic activity by degrading, transforming, or accumulating toxic compounds like hydrocarbons, radionuclides, heterocyclic compounds, pharmaceutical substances and toxic metals (Das 2014).

1.3.2 Characteristics of microorganisms suitable for bioremediation

The applications of microorganisms to detoxify metals have increasingly gained recognition as a significant approach for remediation of metals, in spite of some limitations. A substantial amount of work in the literature suggests that a microbe to be employed for bioremediation should possess a resistant genotype for a particular pollutant or have the following features as described below in figure 1.4.

Figure 1.4 Features of microbes to be used in bioremediation



Source: Adapted from Das (2014)

1.3.3 Various methods of chromium reduction

Microbes can produce several enzymes that can transform toxic metal ions to less/ nontoxic forms under various environmental conditions. Traditionally enzymes such as mono or di oxygenases, reductases, dehalogenases, cytochrome p450 monooxygenases, bacterial phosphotriesterases, hydrolases, transferases and oxidoreductases obtained from bacteria, fungi, plants and microbe plant associations can be employed in bioremediation. Enzymes participating in lignin metabolism such as laccases, lignin and manganese peroxidases isolated from white rot fungi and bacterial phosphotriesterases are all employed in the bioremediation process (Thatoi et al 2014).

Hexavalent chromium usually enters the cell via the sulphate transport pathway and gets reduced to Cr (III) by various enzymatic and non-enzymatic processes. During this process, reactive oxygen species (ROS) are formed, which exert deleterious effects on cells by interacting with proteins and nucleic acids. While trivalent chromium is less toxic and less

bioavailable and it readily forms insoluble hydroxides/oxides above pH 5.5 and also Cr (III) are impermeable to biological cell membranes. Detoxification of Cr (VI) to Cr (III) by reduction of hexavalent chromium species is gaining utmost importance in the environment (Thatoi et al 2014).

The chromium reduction can be carried out by bacteria, fungi and algae. Bacterial chromium reduction can be of two types

1. Enzymatic or direct chromium reduction

2. Non-enzymatic or indirect chromium reduction

1.3.4 Direct reduction of chromium by bacteria

Microorganisms that have the ability to reduce Cr (VI) are usually referred to as chromium reducing bacteria (CRB). Among CRB, the Gram positive bacteria are shown to have significant tolerance to Cr (VI) toxicity at relatively high concentrations, whereas Gram-negative bacteria are more sensitive to Cr (VI) (Thatoi et al 2014).

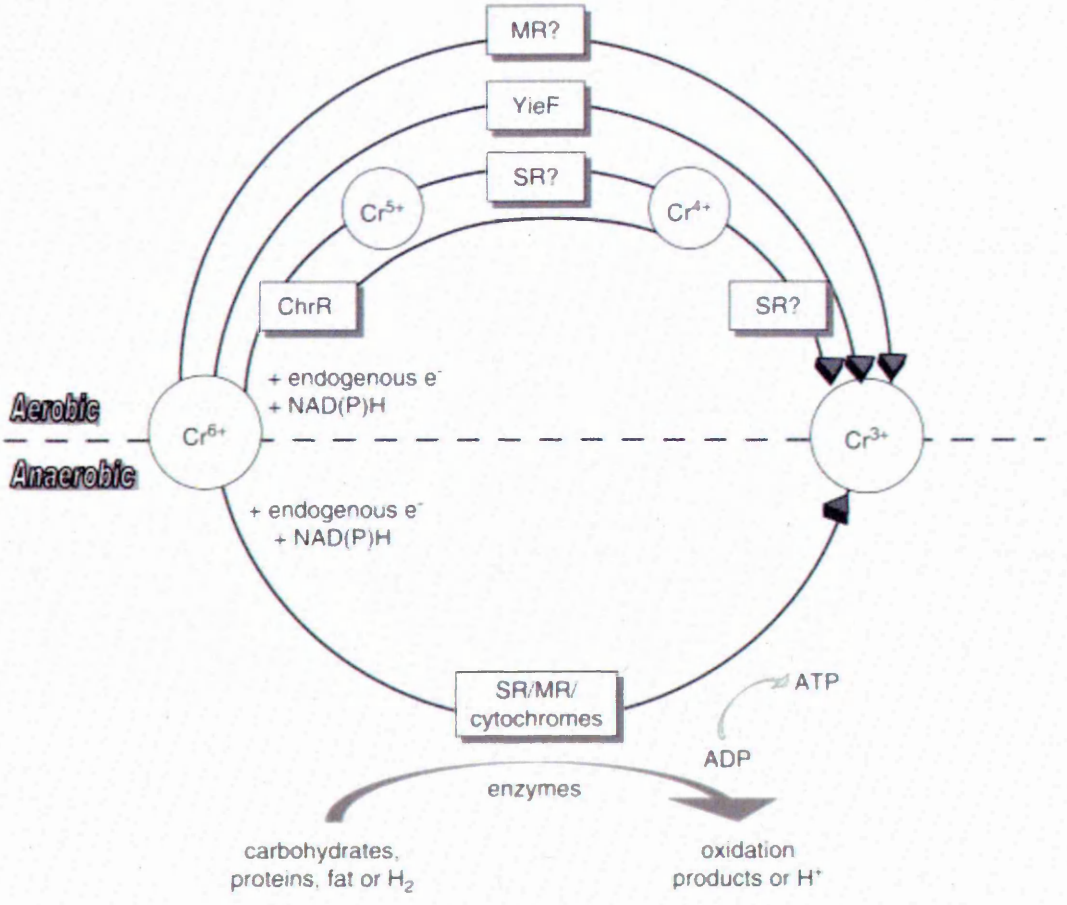
The enzymatic reduction of chromium takes place in two ways.

1. Aerobic process

2. Anaerobic process

The enzymatic reduction of Cr (VI) is shown below in Figure 1.5

Figure 1.5 Diagrammatic representation of Cr (VI) reduction via enzymatic process



Source: Adapted from Cheung and Gu (2007) MR- Membrane associated reductases and SR-soluble reductases

1.3.4.1 Aerobic reduction of Cr (VI)

Under aerobic process the bacterial Cr (VI) reduction occurs via two or three steps. Initially Cr (VI) is reduced to Cr (V) and/or Cr (IV) which are usually short lived intermediates preceded by further reduction to the Cr (III) which is a thermodynamically stable product. The reactions are shown below in the following equations.

$$Cr^{6+} + e^- \longrightarrow Cr^{5+}$$

$$Cr^{5+} + 2e^- \longrightarrow Cr^{3+}$$

Electrons from internal sources, NADH, and NADPH act as electron donors in the Cr (VI) reduction reaction. The Chr R Cr^{6+} reductase immediately reduces Cr^{6+} via one step with an electron to form Cr^{5+}, followed by a two electron transfer to generate Cr^{3+}. Although some amount of Cr^{5+} intermediate is automatically re-oxidised to generate ROS, the probability of harmful radicals are lessened with the reduction via two electron transfer by Chr R. The enzyme Yie F is exclusive enzyme which catalyses the straight forward reduction of Cr^{6+} to Cr^{3+} through a four-electron transfer, in which three electrons aid in reducing Cr^{6+} and the other is transferred to oxygen. The amount of ROS produced by Yie F in Cr^{6+} reduction is minimal, and so it is considered as a less potent reductase than Chr R for Cr^{6+} reduction (Cheng, Gu 2007 & Barrera-Diaz et al 2012).

In *Pseudomonas aeruginosa*, Cr^{6+} resistance is attributed to the decreased uptake and/ or increased efflux of Cr^{6+} by the cell membrane. An identical mechanism of resistance is reported for *Alcaligenes eutrophus* CH34 (recently classified as *Waustersia eutropha*). A number of bacteria in other genera such as *Bacillus* spp., *E.coli* ATCC 33456, *Shewanella alga* BrY-MT and a few unidentified strains have been shown to reduce Cr^{6+} and utilise x soluble reductases in the cytosol (Cheung & Gu 2007). In *Ps. maltophila* O-2 and *Bacillus megaterium* TKW3 Cr^{6+} reduction was facilitated with the membrane cell fractions indicating that the reductase enzyme is membrane associated (Cheung et al 2006).

A lot of research has been carried out regarding the Cr^{6+} reductases and their purification from pseudomonads. A partially soluble Cr^{6+} reductase from *Ps. putida* PRS2000 was reported by Ishibashi et al (1990). A 38 kDa soluble Cr^{6+} reductase from *Ps. ambigua* G-1 was reported by Suzuki et al., (1992). A 600 kDa soluble Cr^{6+} reductase, ChrR, from *Ps. putida* MK1 was reported by Park et al., (2000). Ackerley et al (2004) described ChrR as a dimeric flavoprotein activating the reduction of Cr^{6+} optimally at 70^0C. The membrane associated Cr^{6+} reductase was reported from the proteome of *B. megaterium* TKW3 which was identified on a two dimensional electrophoresis gel (Cheung et al 2006).

1.3.4.2 Anaerobic reduction of Cr (VI)

In the anaerobic process, Cr (VI) can act as a terminal electron acceptor in the respiration process utilising a large range of electron donors, including carbohydrates, proteins, fats, hydrogen, NAD (P) H and endogenous electron reserves. Both soluble and membrane-associated enzymes have been found to enhance Cr^{6+} reduction. Sulphate and iron reducing bacteria (SRB and IRB) are important members of anaerobic microbial communities.

Chromium (VI) reduction by biogenic iron (II) and sulphides generated by IRB and SRB are estimated as being 100 times greater than that due to CRB alone. SRB produce H_2S, which serves as a Cr (VI) reductant via a process that involves three stages

1. Reduction of sulphates

2. Reduction of chromate by sulphides and

3. Precipitation of Cr (VI) by sulphide.

The reduction of Cr (VI) by Fe (II) occurs when IRB reduce Fe (III) to Fe (II) which in turn reduces Cr (VI) to Cr (III). Research into Cr^{6+} reduction has also been performed in the facultative anaerobe *Enterobacter cloacae* HO1 (Barrera-Diaz et al., 2012).

A consortium of bacteria has been described with the capability to reduce Cr^{6+} and also to degrade benzoate. This consortium contained *Microbacterium* sp. MP30, *Geobacter metallireducens*, *Shewanella putrefaciens* MR-1, *Pantoea agglomerans* SP1, *Agrobacterium radiobacter* EPS-916 (Cheung & Gu 2007).

1.3.5 Indirect reduction of chromium by bacteria

The non-enzymatic Cr (VI) reduction to Cr (III) can be attributed to different chemical compounds, produced during the bacterial metabolic process. The most potential non-enzymatic chromate reductants could be ascorbic acid, glutathione (GSH), cysteine or hydrogen peroxide in microbial cells and ascorbate in higher organisms.

Reduction of Cr (VI) may also occur via chemical reactions that are present in intra/extra cellular locations such as amino acids, nucleotides, sugars, vitamins, organic acids or glutathione associated compounds (Thatoi et al 2014).

1.3.6 Reduction of chromium by Algae

Interaction between chromium and algae has been researched less intensively compared to interaction between chromium and bacteria, or chromium and fungi. Reports on tolerance and resistance of algae to chromium are limited. The exact mechanism of chromium resistance and uptake of chromium by algae is not clearly understood (Kamuledeen et al 2003).

Hexavalent chromium reduction in Algae can be mainly done by the accumulation of chromium by algal cells, followed by the removal with the help of chelating agents. Possible uses of immobilized algae for chromium bioremediation suggest a feasible technique.

Filamentous alga *Cladophora* accumulated several heavy metals but Cr uptake rate was higher and faster (72% after 15 minutes). It is reported from tannery effluent samples of post-anaerobic digestor *Selenastrum* could remove 395ppm of Cr in solutions (Cervantes et al 2001). Using immobilised cells in columns with kappa carragenan (fluidized bed) or polyurethane foam (packed bed), *C. vulgaris* removed 48 and 34% of Cr respectively, whereas *S. acutus* removed 36 and 31%. (Cervantes et al 2001).

1.3.7 Reduction of chromium by Fungi

Yeasts and filamentous fungi offer a possible alternative for bioremediation of waters and soils polluted by Cr (VI). However, no practical use of fungal cells has been reported. The fungal cell wall constitutes chitin, a heteropolymer of N-acetylglucosamine and glucosamine. The deacetylated amino groups of glucosamine act as an essential binding site for metals. In *Mucorales*, the siderophore rhizoferrin shows increased Cr (III) biosorption (Cervantes et al 2001).

A polycarboxylate siderophore rhizoferrin is able to bind Fe (III), Cr (III) and Al (III). Mainly in yeasts and fungi the remediation of hexavalent chromium is by biosorption rather than reduction from the cells. Biomasses obtained from *Rhizomucor arrhizu, Candida tropicalis* and *Penicillum chrysogenum* are excellent biosorbents of chromium. *S. cerevisiae* and *Candida utilis* have the ability to sorb Cr (VI) and the sorption capacity of dehydrated cells is considerably higher than that of intact cells (Cervantes et al 2001).

Aspergillus sp. N2 and *Penicillum* sp. N3 are chromate resistant filamentous fungi and when tested in 50ppm of Cr (VI) aqueous solution at about neutral pH *Aspergillus* sp. N2 reduced the Cr (VI) concentration by 75% whereas *Penicillum* sp. N3 reduced it to 35%. The mechanism of Cr (VI) reduction in both the species were enzymatic and sorption to mycelia (Cervantes et al 2001).

1.4 Methanotrophs

1.4.1 Introduction

Methanotrophic bacteria can utilise methane as sole source of carbon and energy. Methanotrophs are omnipresent in the environment and show an important role in the universal oxidation of methane. Their unique microbiological and metabolic features have led to investigation of methanotrophs for a number of biotechnological applications (Jiang et al 2010). Methanotrophs play a major role in universal cycling of carbon, nitrogen and oxygen as well as in deterioration of hazardous organic materials (Semrau et al., 2010).

1. 4. 2 What are methanotrophs?

Methanotrophs are a group of the methylotrophic bacteria. Other methylotrophic bacteria can use different one-carbon compounds, including methanol, methylated amines, and halomethanes and methylated compounds containing sulphur. The key enzyme of methanotrophic microorganisms, methane monooxygenase (MMO) performs the oxidation of methane to methanol is the chief defining metabolic feature of methanotrophs.

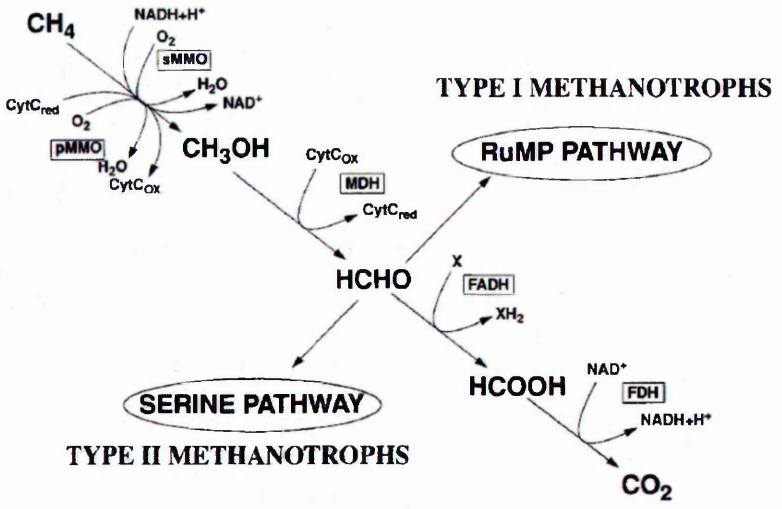
Methanotrophs are pervasive in environment, including many extreme environments, and can grow at temperatures as low as 40^0C or as high as 72^0C (Jiang et al 2010).

Most known methanotrophs survive at steady pH (5-8) and at wide temperature ranging from (20-35^0C), with psychrophilic (growth <15^0C), thermophilic (growth> 55^0C), alkaliphilic (growth at pH > 9.0), and acidophilic (growth at pH <5) (Semrau et al 2010).

The first methanotroph *Bacillus methanicum* was isolated in 1906, but it was in 1970 when Whittenbury and his colleagues isolated and characterized over 100 new methane utilising bacteria which form basis for current classification of bacteria (Jiang et al 2010).

Methane monooxygenases are the inherent enzymes of methanotrophs that catalyse methane to methanol, as shown in the figure 1.6 illustrating metabolism of substrates by methanotrophs with formaldehyde as intermediate.

Figure 1.6 Pathway for oxidation of methane and assimilation of formaldehyde

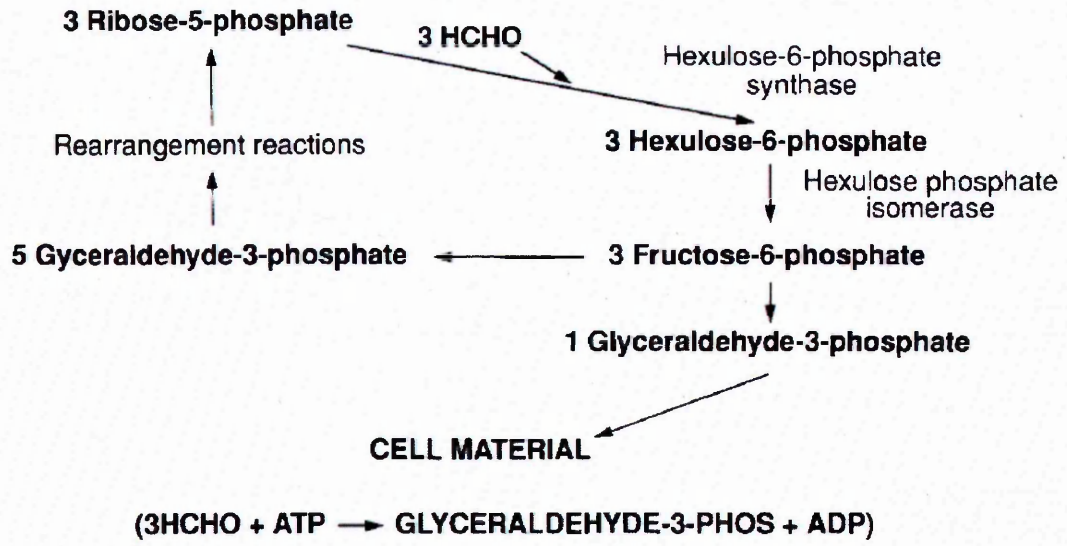


Source: Adapted from Hanson &Hanson 1996

Abbreviations- CytC- Cytochrome C; FADH-formal dehydrogenase; FDH-formate dehydrogenase.

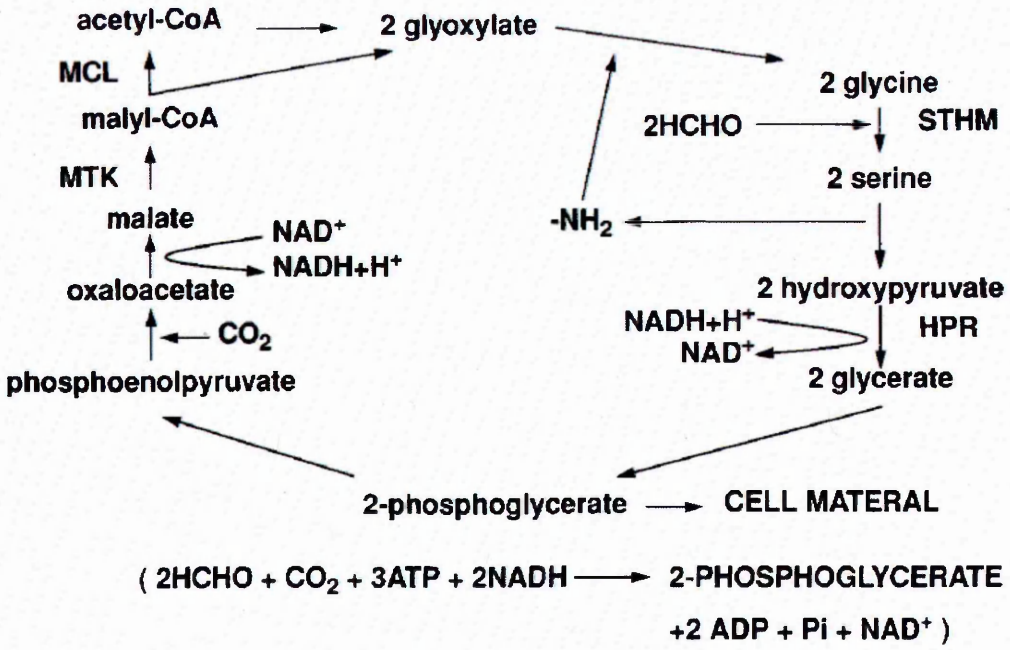
The two different pathways of formaldehyde assimilation in methanotrophic bacteria are RuMP pathway and Serine pathway are shown in the figure 1.7 and 1.8 respectively.

Figure 1.7 RuMP pathways for formaldehyde fixation



Source: Adapted from Hanson &Hanson 1996

Figure 1.8 Serine pathways for formaldehyde assimilation



Source: Adapted from Hanson &Hanson 1996

1. 4. 3 Forms of MMO

Methanotrophs oxidise methane to methanol with the key enzyme methane monooxygenase (MMO). This MMO is present in two forms 1. Membrane associated or particulate methane monooxygenase (pMMO) present in most known methanotrophs and is located in cytoplasmic membrane. 2. Soluble methane monooxygenase (sMMO), which is present in some methanotrophs, is located in the cytoplasm.

pMMO is a copper containing enzyme. It consists of three polypeptides with molecular masses of approximately 45000 Da (α-subunit, PmoB), 26000 Da (β-subunit, pmoA), and 23000 Da (γ-subunit, pmoC) with a $(\alpha\beta\gamma)_3$ subunit structure (Semrau et al 2010). pMMO is present in all tested methanotrophs except *Methylocella* sp., which possess sMMO alone (Semrau et al 1999).

The sMMO is a three component enzyme comprising of a hydroxylase, a reductase and a regulatory protein. The hydroxylase component is composed of three subunits with molecular masses of approximately 54000 Da (α-subunit), 42000 Da (β-subunit), and 22000 Da (γ-subunit) with a subunit molecular structure of $(\alpha\beta\gamma)_2$ (Semrau et al 2010). To date the sMMO gene is found in *Methylococcus, Methylosinus, Methylomonas, Methylomicrobium, Methylocella* and *Methylocystis* (Semrau et al 1999).

In Type I methanotrophs the SMMO gene is found in the *Methylomonas* sp. Strain KSWIII and *Methylomonas* sp. strain KSPIII. The sMMO genes from these organisms were cloned and sequenced and the genealogical analysis of sMMO amino acid sequences showed that the strains are closer to the *Methylococcus capsulatus* (Bath) of type X methanotrophs. (Shigematsu et al., 1999).

1. 4.4 Classification of methanotrophs

The methanotrophic bacteria have been grouped into three types, Type I, Type II and Type X based on the morphological differences, types of resting stages, fine structure of intracytoplasmic membrane and some physiological characteristics as shown in table 1.1 below (Hanson & Hanson 1996). The characteristics of the various types of methanotroph are described.

Table 1.1 Characteristics of type I, type II, and type X methanotrophs

Characteristic	Type I	Type II	Type X
Cell morphology	Short rods, usually occur single; some cocci or elipsoids	Cresent- shaped rods, rods, pear-shaped cells, sometimes occur in rosettes	Cocci, often found in pairs
Growth at 45^0C	No	No	Yes
G+C content of DNA (mol%)	49-60	62-67	59-65
Membrane arrangement	Yes	No	Yes
Bundles of vesicular disks paired membranes aligned to periphery of cells	No	Yes	No
Nitrogen fixation	No	Yes	Yes
Resting stages formed Exospores Cysts	No Some strains	Some strains Some strains	No Some strains
RuMP pathway present	Yes	No	Yes
Serine pathway present	No	Yes	Sometimes
Ribulose-1,5-biphosphate carboxylase present	No	No	Yes
Major PLFAs	14.0, 16:1ω 7c, 16:1ω5t	18:1 ω8c	16:0, 16:1ω7c
Proteobacterial subdivision	Gamma	Alpha	Gamma
Phylogenetic signature probe (S)	1041 (5'-CTCCGCTATCTCTAACAGATT-3'), 1035 (5'-GATTCTCTGGATGTCAAGGG-3'), MM650 (5'-CCTCTACTCAACTCTAGT-3'), MM850 (5'-TACGTTAGCTCCACCACTAA-3')	1034 (5'-CCATACCGGACATGTCCAAAGC-3')	No specific probe has been tested.

1.4.5 Environmental application of methanotrophs

Methanotrophs have important and major applications in applied microbiology and biochemical engineering along with bioremediation.

1. Pollutant degradation- possession of pMMO or sMMO by methanotrophs is significant feature to be applied in various pollutants degradation. sMMO will bind and oxidise alkanes up to C-8, as well as ethers, cyclic alkanes, and aromatic hydrocarbons. pMMO can oxidise alkanes up to C-5 but cannot oxidise cyclic alkanes or aromatic compounds (Seramu et al 2010). Methanotrophs can be used in bioremediation of halogenated hydrocarbons via co-metabolism with MMO's, biotransformation of organic substrates like propylene to epoxy propane and production of chiral alcohols (Jiang et al 2010). Methanotrophic enrichments from various habitats such as aquifers, landfills, wastewaters and waste disposal sites can degrade pollutants such as chlorinated hydrocarbons.

2. Greenhouse gas removal- with increased global warming concern, efforts have been made to reduce the man-made emissions of various greenhouse gases particularly CH_4 from landfills and agricultural soils. CH_4 is almost 25 times more powerful than carbon dioxide at absorbing infrared radiations (IPCC, 2007) and atmospheric CH_4 proportions have dramatically increased since the industrial revolution. Landfills are potential source of atmospheric CH_4 emissions and various strategies have been proposed to reduce the CH_4 emissions, engineered systems like 'biocovers' or 'biofilters' to reduce CH_4 emissions (Semrau et al 2010).

Biocovers constitutes porous materials like organic matter (eg. compost, sewage sludge and wood chips) above the surface of landfill, which can effectively transport gas with sufficient water retention capacity to increase methanotrophic activity. Various column studies of biocover material in laboratories reported the removal rates of CH_4 ranging from 22 to 242 g CH_4 $m^{-2}day^{-1}$ (Scheutz et al 2009). It was also reported that methanotrophs can remove convincing amounts of CH_4 from the atmosphere, i.e., at concentrations on the order of 1.7 p.p.m.v., orders of magnitude lower than what is found in engineering environments such as landfills and factory farms (Semrau et al., 2010).

In western Michigan 2 ½ year field experiment was conducted at a closed landfill where methanotrophs, were enhanced by nutrient addition to soil without increasing biogenic nitrous oxide (N_2O) production. The methane flow were reduced drastically about more than

half with addition of KNO_3 and NH_4Cl into the soil in comparison to control plots, while N_2O flow increased considerably during spring and summer. With the addition of phenylacetylene an inhibitor of sMMO to the soil fields it decreased peak N_2O proportions by half and methane oxidation by one third. The results indicate such addition in connection with soil moisture management provides viable method to reduce greenhouse gas emissions from landfills (Lizik et al 2013).

3. Production of single cell protein- an alternative mode of protein production for humans and animal consumption has increased since World War I, and importance has risen among developing nations to provide adequate protein diets to their populations (Kuhad et al., 1997). Yeasts, fungi, algae, and bacteria, including methanotrophs can be used as source of microbial protein. The Norferm Denmark A/s in Norway can produce 8000 tons $year^{-1}$ of protein obtained from *M. capsulatus* Bath, named as Bioprotein and was estimated that production could be increased to 40000 tons $year^{-1}$ (Winder 2004).

1.4.6 Copper regulation in methanotrophs

Copper is the key factor that controls methanotrophic activity and plays a major role in their physiology. Copper is essential for expression of pMMO, while at low concentrations of copper few methanotrophs express sMMO that contains diiron active site.

MMO activity in *M. capsulatus* (Bath) is primarily dependent on concentration of copper. In excess of copper the MMO activity was observed in particulate fractions but switched to soluble fractions in response to copper stress i.e., low copper to biomass ratio (Stanley et al 1983). The copper switch mechanism was demonstrated in *Methylosinus trichosporium* OB3b but some methanotrophs do not have ability to defend copper stress in this way (Stanley et al 1983).

In strains which express both pMMO and sMMO copper in the growth medium inhibits expression of sMMO genes leading to elucidation of pMMO and formation of considerable intracytoplasmic membranes that express pMMO (Balasubramaniam and Rozenweig 2008). The switch is associated with the secretion of a chalkophore known as methanobactin and expression of another polypeptide MmoD, which plays an important role in the copper switch by increasing copper bioavailability that controls expression of MMOs, the MmoD with methanobactin amplifies the bacterial response to copper (Semrau et al 2010, Semrau et al 2013). Lack of copper in the medium, facilitates methanobactin to adhere to a variety of

transition and near transition metals that may have potential effects on metals mobility metals in soils and aquatic systems (Semrau et al 2013).

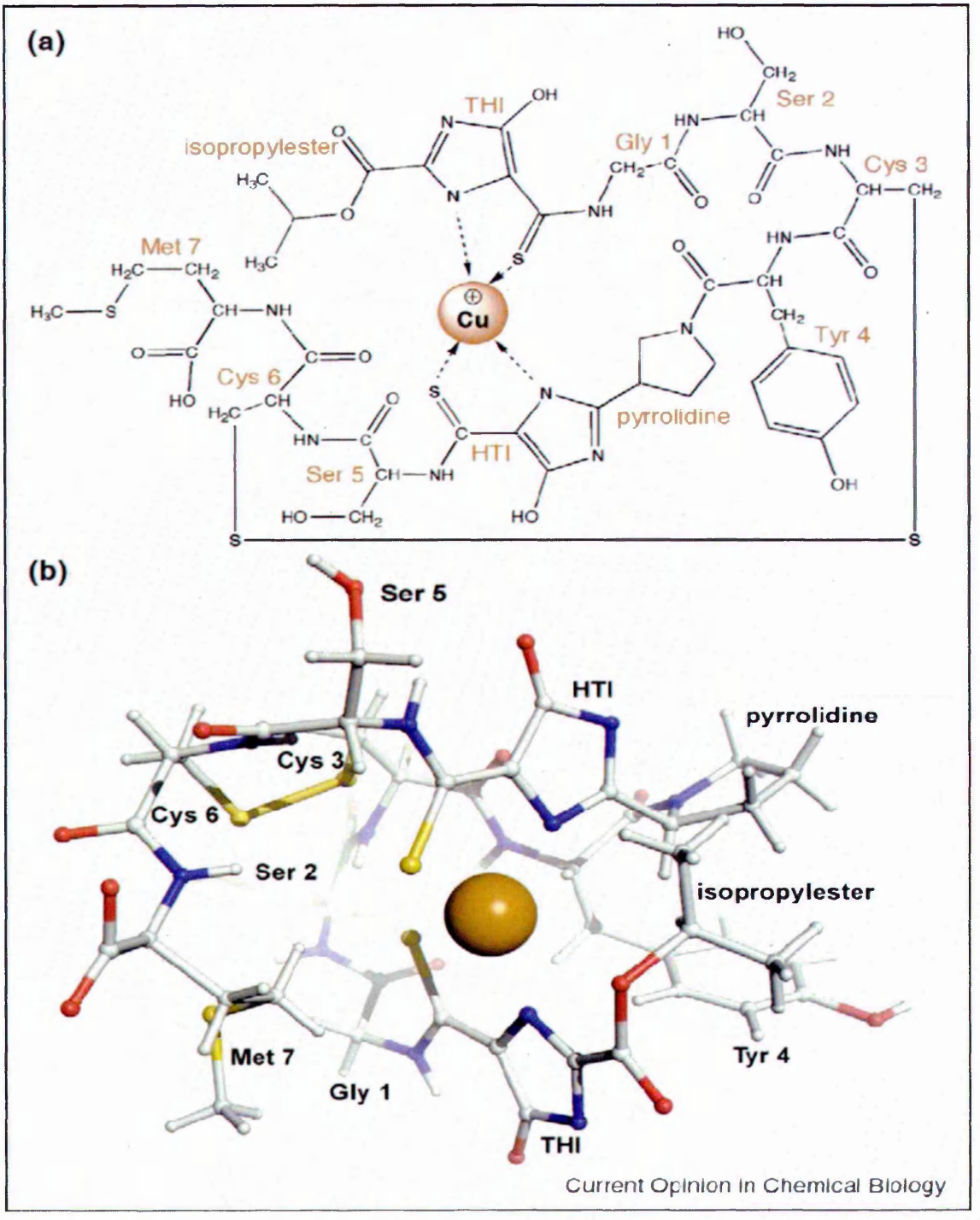
Copper plays a vital role in metabolism, and co-ordinating expression of two methane monooxygenases. Copper also influence the elucidation of two out of four formaldehyde dehydrogenases, development of internal membranes, and expression of other polypeptides related to copper regulation or transport (Kim et al., 2004). Methanobactin fulfils the role of copper trafficking molecule by passaging higher amounts of copper while protecting cellular components from its toxic effects (Kim et al 2004).

Methanobactin is a small (<1200 Dalton) modified polypeptide with two five or six- member rings (imidazole, oxazolone or pyrazinedione rings) (Figure 1.9) with associated enethiol groups that binds copper with affinities of greater than 1021 M-1. (Semrau et al 2013). Methanobactin was first identified from *M. trichosporium* OB3b is a modified polypeptide with two oxazolone rings that are responsible for the high affinity binding of copper (Semrau et al 2013).

Methanobactin can be described as a compact pyramid like structure with a metal complexation site located at the base of the pyramid. The isopropylester group (Figure 1.9) folds underneath the surface creating a tail like projection and a cleft, and it appears to obscure the metal site to some extent. The metal co-ordination environment is composed of dual N- and S- donating systems that are derived from two 4-thionyl-5-hydroxy imidazolate moieties. The N atom of each imidazole and the S atom of the two thionyl substituents coordinate the copper in distorted tetrahedron geometry (Kim et al 2004).

The cells secrete methanobactin continuously and it accumulates in the copper deficient conditions in culture media. If copper is provided in culture media, methanobactin binds the copper and the methanobactin copper complex is internalised to the cell possibly to be associated with pMMO activity (Kim et al 2004).

Figure 1.9 (a) Diagrammatic illustration of the copper-methanobactin complex (Cu-mb) from *M. trichosporium* OB3b (b) Ball-and-stick representation of crystal structure (Cambridge Crystallographic Data Centre deposition number CCDC 241254)



Source: adapted from Balasubramaniam and Rozenweig (2008)

1.4.7 Bioremediation by methanotrophs

Potential use of methanotrophs in biotechnology and bioremediation process is mainly due to their unique physiology and the capability of the bacteria to be cultivated on large scale (Jiang et al., 2010).

The possession of MMO enzyme is the distinguishing characteristics of methanotrophs to allow them to degrade diverse organic compounds. Methanotrophs can also perform the remediation of inorganic pollutants. The capability of MMO to detoxify or transform toxic elements to less toxic forms by redox reactions with the enzyme is the basis for their potential remediation of metal contaminated environment. These organisms influence the speciation and bioavailability of metals in environment which is another characteristic feature to be employed in bioremediation (Pandey et al., 2014).

Bioremediation of hexavalent chromium is well characterised by methanotroph *M. capsulatus* (Bath) over wide range of concentrations 1.4-1000 mg L^{-1}. These organisms' genome sequences demonstrate that five potential chromium reductases genes may be responsible for the Cr (VI) reduction and making the organism potential for bioremediation of this pollutant (Hasin et al., 2010)

Another interesting aspect of *M. capsulatus* (Bath) is its ability to detoxify Hg (II) to the less toxic Hg (0) via a reaction catalysed by MerR-derived polypeptides. This NADH dependent mercuric reductase is active in *M. capsulatus* (Bath) with NADH provided at expense of methane oxidation (Boden & Murrell 2011).

Methanotrophs are known to remediate many organic pollutants because of their enzyme MMO which has affinity to a wide range of organic substrates. TCE (trichloro ethylene) which is used in many industrial solvents is a known carcinogen and can be degraded by several methanotrophic bacteria such as *M. trichosporium* OB3b, *M. capsulatus* (Bath), *Methylocystis* sp., *Methylsinus sporium* strain 5, *Methylocystis daltona* SB2, *Methylocystis* strain SB2 and unidentified strains of methanotrophs (MP18, MP20, P14) (Pandey et al 2014).

Some strains of the *Methylomonas* genus were found to exhibit the sMMO enzyme which will help to degrade trichloroethylene. For instance *Methylomonas methanica* (KSWIII, KSPII & KSPIII) can enhance trichloroethylene degradation (Shigematsu et al., 1998).

Methylomicrobium album BG8 which can utilise methane and methanol as sole carbon and energy source can degrade several halogenated hydrocarbons: dibromomethane, dichloromethane, vinyl chloride, 1,1 dichloroethylene, cis-dichloroethylene, trans-dichloroethylene and trichloroethylene (Semrau et al 1999).

A new strain *Methylocystis* strain JTA1 which was isolated from Loagong municipal solid waste landfills Shangai, China. The 16S rRNA & PmoA gene shows that the organism can tolerate chloroform (50 mg L^{-1}) and can be employed in the soils and landfills bioremediation and waste water decontamination (Zhao et al 2013).

Methanobactin, a chalkophore which binds copper, was found to bind other metals including mercury when added as $HgCl_2$ and reduced Hg (0) thus is helping in the detoxification process. Methanobactin from *M. trichosporium* OB3b ΔmbnA (a mutant defective in methanobactin production), *Methylocystis* sp. strain SB2 and *Methylomicrobium album* BG8 showed that methanobactin can adhere to mercury and leads to reduction of Hg (II) to Hg (0) by formation of grey precipitate and was associated with cell biomass (Vorobev et al 2013).

A facultative methanotroph *Methylocystis* strain SB2 when grows on methane can degrade vinyl chloride, trans-dichloroethylene, trichloroethylene, 1,1,1 trichloroethane and chloroform. The organism grows on methane and co-oxidises these hydrocarbons and chlorinated hydrocarbons. When the strain grew on ethanol it can degrade vinyl chloride, trans-dichloroethylene, trichloroethylene, 1,1,1 trichloroethane and except few 1,1,1 trichloroethane the growth on ethanol was not affected by chlorinated hydrocarbons. No reduction of chlorinated hydrocarbons was observed when cells were inhibited by growth on ethanol indicating pMMO activity for the degradation of pollutants. When mixtures of chlorinated alkanes/alkenes were added to cultures growing on methane/ethanol chlorinated alkene degradation occurred but not chlorinated alkanes and the growth significantly reduced on methane and ethanol (Semrau & Im 2011).

A facultative methanotroph *Methylocystis* strain SB2 which also grows on multi carbon compounds can degrade 1,2 dichloroethane, 1,1,2-trichloroethane, 1,1-dichloroethylene and cis-dichloroethylene when grown on methane or ethanol. This organism can use ethanol as growth substrate to enhance mobility of chlorinated hydrocarbons in situ and pollutant transport and biodegradation (Jagadevan & Semrau 2013).

Methanotrophs can also be employed for metal recovery as biosorbents from waste water. Such methanotrophs were isolated from pristine and metal impacted (acid mine drainage) environments (Xie et al 1996).

De Marco et al (2004) studied the capability of 31 methylotrophic strains isolated from range of soil, sediments and contaminated areas to study heavy metal tolerance among these strains. The isolates exhibited some interesting features such as resistance to heavy metals, arsenate and organic pollutants. Four of the strains were regarded as "super bugs" because of their ability to withstand high concentration of heavy metals. The strain *Methylophilus methylotrophus* EHg7 resistant to cadmium and *M. methylotrophus* ECr4 resistant to chromium.

The organism *Methylacidiphilum fumariolicum* (SoIV) strain isolated from volcanic environment survive at low methane and oxygen concentrations and pH as low as 1. The genome sequence reveals that the organism has pMMO and assimilates C_2 compounds and thus is a facultative methanotroph. This organism helps the study of methane cycling in the volcanic environment (Khadem et al 2012).

The ubiquitous nature of methanotrophs, adaptability to copper stress environment, wide range of substrate specificity by MMO enzyme, the detoxification process of Cr (VI) and Hg (II) by *M. capsulatus* (Bath) and degradation of several halogenated compounds by various methanotrophs is an interesting aspect to isolate and characterize methanotrophs from River Sheaf and study the detoxification process of Cr (VI) by the isolated organisms.

1.4.8 Aims and objectives

The main aims and objectives in the present study are

1. To collect the sediment samples from the River Sheaf

2. To characterize the physical and chemical properties of sediments

3. To enrich and isolate the methanotrophic bacteria from the sediments of the River Sheaf

4. To characterize and study the isolate enriched from the River Sheaf sediments

5. To determine the remediation properties of hexavalent chromium by the isolated methanotrophic bacteria.

Chapter 2

MATERIALS AND METHODS

2. Materials and Methods

2.1 Materials

All chemicals were supplied by Sigma, Fisher or BDH and were of analytical grade. The water used was glass distilled or deionised.

2.1.1 Bacterial strains and growth conditions

The following bacterial strains were used in the study:

- *Methylomonas koyamae* (SHU 1) isolated from River Sheaf sediments
- *Methylosinus trichosporium* OB3b was obtained from the culture collections of Howard Dalton and Colin Murrell (University of Warwick)

The methanotrophs *M. trichosporium* and *Mm. koyamae* (SHU1) were grown and propagated aerobically in nitrate minimal salts (NMS) medium or NMS agar using methane (1:4 v/v in air) as sole source of carbon and energy. The methanotrophs *M. trichosporium* and *Mm. koyamae* (SHU1) were cultivated on NMS agar plates and incubated inside airtight jars in the presence of methane at 30^0C for 8-10 days using methane as growth substrate. The recipe for NMS medium is given in section 2.1.5 below.

2.1.2 Enrichment and isolation of new methanotrophs

0.5 g of sediment samples collected from the River Sheaf were enriched with 50 ml of NMS media (Nitrate minimal salts) in 200 ml Erlenmeyer flasks. Methane gas was introduced at 1:4 v/v with air into the culture flasks at regular intervals using hypodermic syringes and the flasks were sealed with subaseals (Fisher) to prevent methane loss. The flasks were incubated at 30^0C on a rotary incubator for 1 week to 10 days for the growth of methanotrophs in the flask. The growth of methanotrophs was observed by monitoring the turbidity of the enrichments in flasks.

The cultures in the flasks were sub cultured into fresh NMS medium and incubated at 30^0C on a rotary incubator for 1 week to 10 days of growth. After 1 week to 10 days of growth in fresh flasks, a loopful of culture was streaked on fresh NMS plates and incubated at 30^0C in a methane air atmosphere until single colonies of the isolate were obtained. The plates were

then put into a gas tight jar and methane gas was introduced into the air in the jar as the sole source of carbon and energy and incubated at 30^0C for 1-3 weeks. Depending on the physical appearance and morphological features of colonies, colonies of the same appearance were streaked on fresh NMS plates and incubated at 30^0C for 1-3 weeks for appropriate growth of methanotrophs on the plates.

The isolated organisms were streaked several times on fresh NMS plates and, prior to sequencing of the 16S rRNA genes, were also examined under the microscope to observe features like shape and motility.

After obtaining pure cultures the methanotrophs *M. trichosporium* and *Mm. koyamae* (SHU1) were grown and propagated aerobically in nitrate minimal salts (NMS) medium or NMS agar using methane (1:4 v/v in air) as sole source of carbon and energy.

The growth of the organism was determined by plotting a graph of absorbance at A600 nm vs time.

2.1.3. Growth of methanotrophs for chromium (VI) removal experiments

All growth cultures and chromate reduction experiments were carried out at 30^0C using an orbital shaker incubator at 180 to 200 rpm. Bacterial cultures were prepared in 250 mL Erlenmeyer flasks containing 50 mL of medium as the working volume in triplicate. The flasks were inoculated with pure cultures of *M. trichosporium* OB3b, *Mm. koyamae* (SHU1) at 30^0C. Flasks were fitted with subaseals to prevent the loss of methane. 50 cm^3 of air was removed by using a plastic syringe fitted with a hypodermic needle and then the air was replaced aseptically with 60 cm^3 of methane, whilst allowing the addition of liquids or taking the samples for spectrophotometric analysis.

Cultures were allowed to grow to an OD_{600} of 0.3-0.8 before addition of hexavalent chromium in the form of potassium dichromate solution. The cells were cultured in the presence of hexavalent chromium using the same culture methods described above, with addition of the same concentration of hexavalent chromium to control flasks, containing medium plus dead cells or just medium as stated for each experiment.

The resistance of the organism to chromium was studied by cultivating the organism in fresh NMS medium and then by addition of hexavalent chromium and taking absorbance readings at A600 nm at regular intervals. The resistance of *Mm. koyamae* SHU1was also observed by

preparing fresh NMS plates with the different chromium(VI) concentrations of 5, 10, 25, 50, 75 and 100 ppm and the resistance of the organism was identified by growth of organism on these NMS plates.

2.1.4 Equipment

Orbital incubators were used from Stuart Scientific S150 and Gallenkamp, UK

Inductively coupled plasma-mass spectrometer (ICP-MS) model Hewlett Packard (HP) 4500, Yokogawa Corporation, Japan was used

Spectrophotometer: 6715 UV/Vis Jenway

Centrifuge: Heraeus Pico 17, Thermoscientific

Thermal cycler: Primus 86 plus, MWG-Biotech

Microwave Oven MDS2000 CEM Corporation USA

2.1.5 Media

Nitrate minimal salts (NMS) liquid medium and agar medium containing 15 g of bacteriological agar (Oxoid) per litre were used for cultivation of methanotrophs. Phosphate buffer was added after the medium had cooled to about 60^{0}C, just before pouring the agar media in sterile plates. The recipe of NMS is as follows:

NMS Medium

This recipe is for NMS with 0.1 mg per litre $CuSO_4.5H_2O$. To vary the concentration of copper, copper free NMS trace elements (recipe below) were used and copper sulphate was added to bulk medium as a separate solution before autoclaving.

The following components were mixed in the following given proportion

10 X NMS salts - 100ml

Na molybdate solution - 1ml

NMS trace elements - 1ml

Fe EDTA solution - 0.1ml

Water to 1 litre

Just before using 10 ml of sterile NMS phosphate buffer solution was added per litre of medium (when cooled to lower than 60^0 C)

10 X NMS salts

KNO_3 - 10 g

$MgSO_4.7H_2O$ - 10 g

$CaCl_2.2H_2O$ -2 g

Water to 1 litre

Stored at 4^0C

Na molybdate solution

$NaMoO_4.2H_2O$ - 0.5 g

Water to 1 litre

Stored at 4^0C

FeEDTA solution

FeEDTA -3.8 g

Water to 100 ml

Stored in the dark at 4^0C.

NMS trace elements

$CuSO_4.5H_2O$ - 100 mg (for 0.1 mg per litre in the final medium)

$FeSO_4.7H_2O$ - 500 mg

$ZnSO_4.7H_2O$ - 400 mg

H_3BO_3 - 15 mg

$CoCl_3.6H_2O$ - 50 mg

Na_2EDTA - 250 mg

$MnCl_2.4H_2O$ - 20 mg

$NiCl_2.6H_2O$ - 10 mg

Water to 1 litre

Stored in the dark at 4^0C.

NMS phosphate buffer solution

$Na_2HPO_4.12H_2O$ - 107.4 g

(OR Na_2HPO_4 - 49.7 g)

KH_2PO_4 - 39 g

Water to 1 litre

pH was 6.8 without adjustment. Sterilised by autoclaving in 100 ml aliquots and stored at room temperature.

2.2. Analytical measurements

2.2.1 Optical density measurements

During the whole experimentation process, the OD (for growth curve experiments) was measured at a wavelength 600 nm using a spectrophotometer.

2.2.2 Preparation of chromium (VI) stock solution

A stock solution containing 1000 ppm of chromium was prepared by adding 1.130 g of $K_2Cr_2O_7$ in 500 ml of deionised water.

2.2.3 Preparation of chromium (VI) standard curve

A 1, 2.5, 5.0, 7.5 and 10 ppm range of Cr (VI) solutions were prepared including a blank. 50 µl of diphenyl carbazide (0.5 g in 200 ml of acetone) and 30 µl of 10 % sulphuric acid were added to 1 ml of each of the standard Cr (VI) solutions and allowed to stand for 5 minutes in order to develop colour (Al Hasin et al.,2010). A calibration curve with the standards of

hexavalent chromium was obtained, from which the unknown values are interpolated with the help of prism 6 Graph pad software.

After the colour development the absorbance is measured at 540 nm and the graph was plotted against absorbance vs concentration to get standard curve.

2.2.4. Determination of reduction assay in microbial cultures

The reduction of hexavalent chromium in microbial cultures was determined by measuring growth, inhibiting metabolic enzyme and also MMO enzyme described below.

The removal of the hexavalent chromium was determined by culturing the organism *Mm. koyamae* SHU1 in fresh NMS medium to optical density (0.1/0.3 OD) and then addition of hexavalent chromium (10 ppm) in the form of potassium di-chromate solution and then determining the removal of hexavalent chromium via the diphenyl carbazide (DPC) assay and recording the absorbance readings at $A_{540\ nm}$. A 1 ml aliquot of each bacterial culture and control was taken in an Eppendorf tubes and it was centrifuged for 5 minutes at maximum speed and the supernatant was collected and the diphenyl carbazide assay was performed as described for the standard curve above. The unknown concentration in the samples was determined by using the standard curve.

An experiment was designed to determine whether the removal of hexavalent chromium was carried out by the organism enzymatically or whether the chromium removal was a non enzymatic process such as biosorption. The organism was cultivated as described above. 0.5% sodium azide was also added which is a metabolic inhibitor. Removal of Cr (VI) is determined by the DPC assay. In a separate flask containing NMS medium the organism was cultivated and optical density measured 0.1/0.3 at OD600 nm and the cells were autoclaved, in order to kill them and inactivate enzymes. After cooling hexavalent chromium was added and chromium (VI) concentration was monitored by the DPC assay at A540 nm.

In another set of experiments, the organism *Mm. koyamae* was again cultivated in fresh flasks with optical density of 0.3 and then addition of 10 ppm hexavalent chromium. Phenyl acetylene (0.05%v/v) was added to the culture flasks to inhibit the enzyme methane monooxygenase. The inhibition of the enzyme is assessed by monitoring the removal of hexavalent chromium by DPC assay.

Fresh NMS medium was prepared with 0.1 mg of copper per litre and the organism was cultivated until it reached 0.3 OD_{600nm}. Then phenyl acetylene (0.05% v/v) and 10 ppm of hexavalent chromium were added to determine whether the sMMO enzyme was inhibited and its possible role in reduction of chromium (VI). The removal of hexavalent chromium was monitored by DPC assay.

2.2.5 Determination of Napthalene oxidation assay

The expression of sMMO and pMMO in the methanotrophs is controlled by the concentration of the copper in the medium. pMMO is expressed at high copper to biomass ratio and sMMO is expressed at low copper to biomass ratio. In order to investigate the effect of a copper dependent MMO and possibly other gene expression on the effect of phenyl acetylene on chromium (VI) removal, the experiments were performed at a range of copper concentrations. The fresh NMS medium with various concentrations of copper 100 mg L^{-1}, 1 mg L^{-1}, and 0.1 mgL^{-1} were prepared and the organism was cultivated and the expression of pMMO or sMMO was determined by the naphthalene oxidation assay (Graham et al., 1992).

In order to determine whether methanotroph cells on plates were expressing sMMO, the naphthalene oxidation test was performed and the plates were compared with the plates of known sMMO producing *Methylosinus trichosporium* OB3b, at a low copper to biomass ratio as a positive control.

The naphthalene oxidation assay was mainly used to assess whether the sMMO gene is active in methanotrophs because napthalene is substrate of sMMO but not pMMO. The test was performed by adding a few crystals of naphthalene (~ 25-30 mg) across the petri dish lid and the agar plate was turned upside down on top of it. The plate was kept in an air tight jar and incubated at 30^0C for 60 minutes. The plates were removed from the incubator and a freshly prepared solution of tetrazotised-o-dianisidine (5 mg mL^{-1}) was added drop wise onto the colonies. A purple or pink colour that developed instantly or within a few minutes indicated the oxidation of napthalene to 1, 2- napthol by the sMMO enzyme.

In the case of liquid cultures 1 mL of liquid cultures were taken into Eppendorf tubes and a few crystals of naphthalene were added and the tubes were incubated on a rotary shaker at 30^0C for 60 minutes and sMMO activity was assessed by adding tetrazotised-o-dianisidine as above.

2.2.6 pH measurement in sediment samples

After transporting the sediment samples to the laboratory, their pH was measured using the pH meter (a suspension was made by adding 0.1 g to 10 ml distilled water) and the results indicated that the pH was near neutral (6.7-6.9) for all samples.

2.3 Template DNA extractions for polymerase chain reaction (PCR)

This is the method of extracting DNA from a culture of bacterial cells that was used to prepare the template for PCR amplification.

1. Liquid culture (1 ml) / or a loopful of bacterial colonies from the plate were taken into an Eppendorf tube.

2. In the case of liquid cultures, centrifugation for 1 minute at maximum speed in a bench top centrifuge was carried out to pellet the cells and then the supernatant was discarded

3. The cells were resuspended in 50 µl of sterile distilled (SD) ultrapure water by vortexing, and centrifuging for 1 minute.

4. The supernatant was discarded

5. The cells were again resuspended in 30 µl of SD H_2O

6. The cell suspension was boiled in a heat block at 100^0C for 10 minutes

7. Centrifugation was carried out for 10 minutes at maximum speed.

8. 5 µl of the resulting supernatant was used as the DNA containing template for PCR.

PCR Reagents

10X PCR buffer -5 µl

dNTPs (25 mM each dNTP) - 0.5 µl

50 mM $MgCl_2$ - 2 µl

Primer 1 [16S1 AGAGTTTGATCMTGGCTCAG ((100-200ng)] - 1 µl

Primer 2 [16S2 TACGGYTACCTTGTTACGACTT (100-200 ng)] - 1 µl

DNA template - 5 µl

BSA (bovine serum albumin) solution - 1 µl

Taq Polymerase - 1 µl

SD H_2O - 33.5 µl

After addition of all reagents the tubes were placed in the thermal cycler and the program selected for amplification is shown in table 2.1

The first phase is denaturation where the double helical strands of DNA are separated from one another in the template DNA at 94^0C - 95^0C. In the second phase or annealing step the primers, which are short sequences of single stranded DNA added to the sample DNA, anneal with complementary similar sequences on the template DNA at a specific temperature. In the final phase or extension phase the polymerase enzyme makes primers extend along the length of DNA to make a new strand of DNA. Successive cycle of these steps result in an exponential amplification of the target DNA sequences.

Table 2.1 Thermal cycling conditions

Step	Temperature	Time (Minutes)	No. of cycles
Initial denaturation	95^0C	1-3	1
Denaturation	95^0C	0.5	25-40
Annealing	Tm-5	0.5	
Extension	72^0C	1 min/kb	
Final extension	72^0C	5-15	1

Source: Fisher scientific protocol

After completion of the thermal cycler program (about 2 h) the tubes were kept on ice for 30 minutes and 5 µl of the solution was used for gel electrophoresis.

2.4 Gel Electrophoresis

1. The agarose gel was prepared by adding 1g of agarose in 100 ml TAE buffer. The suspension was heated in the microwave oven to get clear solution.

2. After cooling to about 50^0C 40 ml of the solution was poured into the gel plates and ethidium bromide (5 µl of a 5 mg/ml solution) was added to permit subsequent visualisation of the DNA.

3. The comb was inserted into the gel.

4. After the gel had solidified the comb was removed and the gel was placed into the gel tank filled with the TAE buffer.

5. A piece of parafilm was taken and 1 µl of 6 X DNA loading dye (Thermal Scientific) was added on to the parafilm.

6. 5 µl of water (which serve as negative sample) was added to a separate 1µl of loading dye and pipette it out and released to mix it well.

7. The procedure was repeated with the given number of samples.

8. 6 µl of the sample plus loading dye was loaded into the second wells in the gel.

9. In the same way other samples were filled in the next well one after the other.

10. 1 µl of loading dye and 3 µl DNA ladder were mixed well and the resulting 4 µl of solution were loaded into the first well of the gel to quantify the approximate size and amount of DNA in the experimental samples.

11. In the same way another 4 µl of DNA ladder plus loading dye was placed in the last well after all the samples were loaded.

12. The lid was placed onto the gel tank and connected to the power supply and the voltage was adjusted to 90 V and checked after 10 min

13. The gel was allowed to run for 40 minutes after which the power was switched off.

14. The lid was removed and the agarose gel was placed on to the uvp trans- illuminator.

15 The uv light was turned on and the ethidium bromide which is a DNA-intercalating agent caused the bands of DNA to fluoresce with orange colour.

16. An image of the gel electrophoresis was captured via a digital camera and the connected computer.

2.5 PCR product clean up

The PCR product which was obtained had to be cleaned up prior to sending for sequencing, which was performed by using the Qiagen PCR clean up kit.

1. Depending on the number of samples the Eppendorf tubes were taken in duplicate and labelled accordingly.

2. 200 µl of buffer PB (binding buffer) from the kit was added to each tube.

3. 50 µl of sample was added to each tube and was mixed by hand.

4. 250 µl of the resulting solution was poured into DNA-binding column tubes from the kit.

5. The tubes were centrifuged at 13,000 rpm in the micro centrifuge for 1 minute.

6. The liquid that ran through the columns was discarded.

7. 750 µl of buffer PE (wash buffer) was added to each of the column tubes.

8. The centrifugation was carried out as in the above step and the flow through was discarded and the process was repeated again until no further buffer flowed out of the columns.

9. The flow through was discarded and the column tubes were placed onto new other tubes to collect the DNA.

10. 30 µl of buffer EB (elution buffer) was added to the column tubes and centrifuged again as above.

11. The column tubes were discarded and the purified PCR product was stored in the freezer until sequencing.

2.6 Sequencing of 16S rRNA genes

After the PCR amplification of the 16S rRNA gene from the purified bacterial DNA from the isolated organisms from the River Sheaf sediments, the amplified DNA was visualised by gel electrophoresis and the samples were cleaned up with the Qiagen PCR kit, as detailed below. The samples were then sent to Eurofins MWG for sequencing (Carson et al., 2012).

2.7 Bioinformatics analysis of sequences

Bioinformatics is a discipline that combines the computer technology and biology for processing biological data.

BLAST is the Basic Local Alignment Search Tool and is an algorithm for comparing biological sequences such as amino acid sequences and nucleotide sequences of different proteins or DNA. A BLAST search compares the query sequences with the library of sequences databases to identify sequence that resemble the query sequence above certain threshold limit.

The result that was obtained from the sequencing was compared with database of sequences using the NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and identified the organism with nearest similarity in terms of the sequence of its 16S rRNA gene.

2.8. Acid digestion for heavy metal analysis

A known amount of each sample (0.1 g) was taken into a digestion tube and 2 ml of 50% HNO_3 was added to the tube in fume hood and was left for 15 minutes for pre-digestion. The tubes were then sealed and digested in a microwave digestion unit (10 minutes). After the samples had cooled a further 2 ml of 50% HNO_3 was added and the mixture was filtered through Whatman filter paper. The filtered supernatant was stored in capped bottles in the cold room until further analysis on ICP-MS. The tubes were washed in soap solution after filtration and then soaked in 10% HNO_3 overnight and washed with distilled water. The tubes were air dried and re-used (EPA method 3050 B).

2.9 Heavy metal analysis by ICP-MS

ICP-MS (Inductive coupled plasma mass spectrometry) is a mass spectrometry technique which can measure several metals and non-metals at a concentrations down to the parts per trillion range of non-interfered low background isotopes. A schematic representation of ICP-MS is shown below in the figure 2.1.

The ICP-MS consists of following parts:

1. Sample introduction system which composes nebuliser and spray chamber to introduce the sample into the system.

2. ICP torch and RF coil which generates argon plasma and also serve as ion source

3. Interface which links the atmospheric pressure to high vacuum mass spectrometer

4. Vacuum system which provides high vacuum for ion optics, quadrapole and detector

5. Collision or reaction cell which remove interfaces

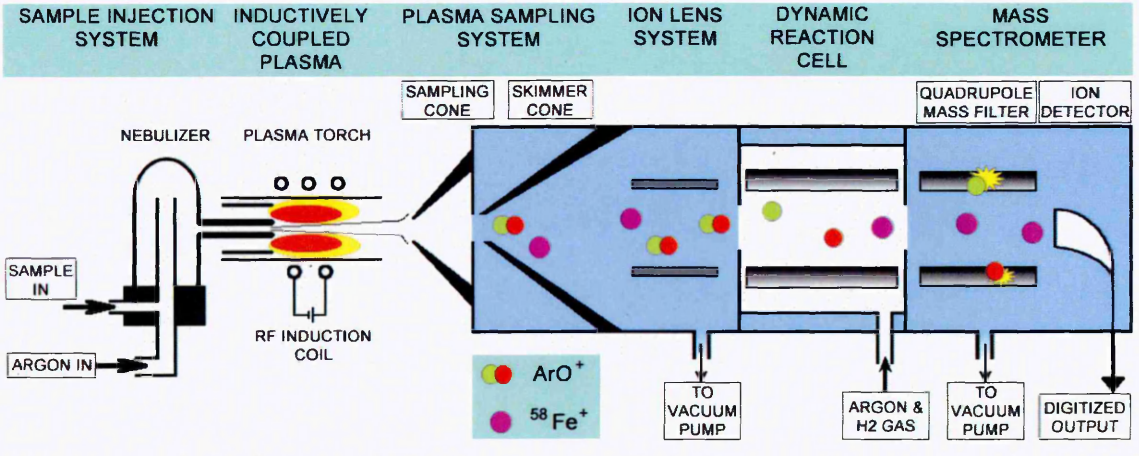
6. Ion optics which guide the desired ions into quadrupole

7. Mass spectrometer which sorts ions by mass-to-charge ratio (m/z)

8. Detector which counts individual ions

9. Data handling and system controller which controls instrument and handles data to obtain final output

Figure 2.1 Schematic diagram of ICP-MS



Source: Adapted from www.ualberta.ca

The principle behind the ICP-MS is when the samples are introduced into the sample injection system the fine droplets created in nebulizer will be passed through spray chamber and finally into argon plasma. The plasma dries the aerosols dissociates the molecules there by removing electron from components thus forming single charged ions which are directed into mass spectrometer. Most ICP-MS use quadrupole mass spectrometer which rapidly scans mass-to-charge-ratio at one time.

Once the ions exit from the spectrometer, ions strike the dynode of an electron multiplier, which acts as detector. At this stage the ions releases a cascade of electrons which are

amplified until a measurable pulse. The software program installed on the computer compares intensities of measurable pulses to those of standards, which make up calibration curve, to determine the concentration of the element.

The ICP-MS equipment was calibrated by running with blank solutions, followed by a standard solution, ICP-MS Calibration standard (XXI) from Sigma.

After calibration, the samples in which the heavy metal analysis has to be carried are injected via nebuliser and the processing of sample takes place in the ICP-MS and the final output is generated in the report.

2.10 Grain size distribution in sediment samples

After collecting sediment samples the samples were air dried in the oven and then separated into different sizes using a series of sieves as per the mesh size (75 μm, 150 μm, 300 μm, 600 μm, 1.18 mm, 2.36 mm and 5 mm). The weight of each sieve was determined first and then the sieves were stacked one on top of another with the 75 μm sieve at the bottom and the 5.0 mm sieve on top. A known amount of soil sample was placed on the uppermost sieve and sieving was carried out for 15 minutes. After sieving the weight of soil plus sieve was determined for each individual sieve to allow the weight of each particle size fraction to be calculated. Finally the sieves were cleaned with a brush before use with further samples.

The sampling protocol for the River Sheaf sediments is described in the sampling chapter.

3. Sampling of River Sheaf Sediments

The sampling protocol used the steps described below to obtain the samples from suitable locations in appropriate quantities in order to determine the physical and chemical characteristics of the sediments and also enrich and isolate microorganisms to employ in bioremediation.

3.1 Introduction

Sheffield is known for large scale metal manufacturing and processing resulting in the introduction of heavy metals into top soils. A wide range of pollutants such as Cr, Ni, and Pb have been found in the top soils of Sheffield and these have resulted from point and diffuse pollution. Dispersal of trace elements due to mining and exploitation of coal has further enhanced the heavy metal concentration in the top soils of Sheffield. Deposition of heavy metals in the top soils of Sheffield has resulted from the metal working industries of Sheffield along the Rivers Don, Sheaf and Porter during the last century and establishment of 150 firms for steel manufacturing in the city during the mid-18th century. The long history of the steel manufacture in Sheffield, including the large steel works of British Steel at Tinsley in the 1960s have led to elevated levels of Ni and Cr in top soils of the Sheffield area (Rawlins et al., 2005).

Knowledge of the history of the steel industry and historical heavy metal contamination of the Sheffield area serves as an important base in analysing the type and concentration of pollutants in the sediments of the River Sheaf and employing the River Sheaf as a study area for further research.

3.2 Sampling Purpose: -

Health and safety procedures have to be taken into consideration during sampling. A risk assessment has to be prepared considering all risks involved during sampling to minimise the chance of accidents during field trips.

The aim of the sampling procedure was to designate an appropriate area where the samples were to be collected, and take photographs when the samples were collected at appropriate locations along the section of the River Sheaf which passes through Millhouses Park. The device used to collect the samples was a plastic scoop.

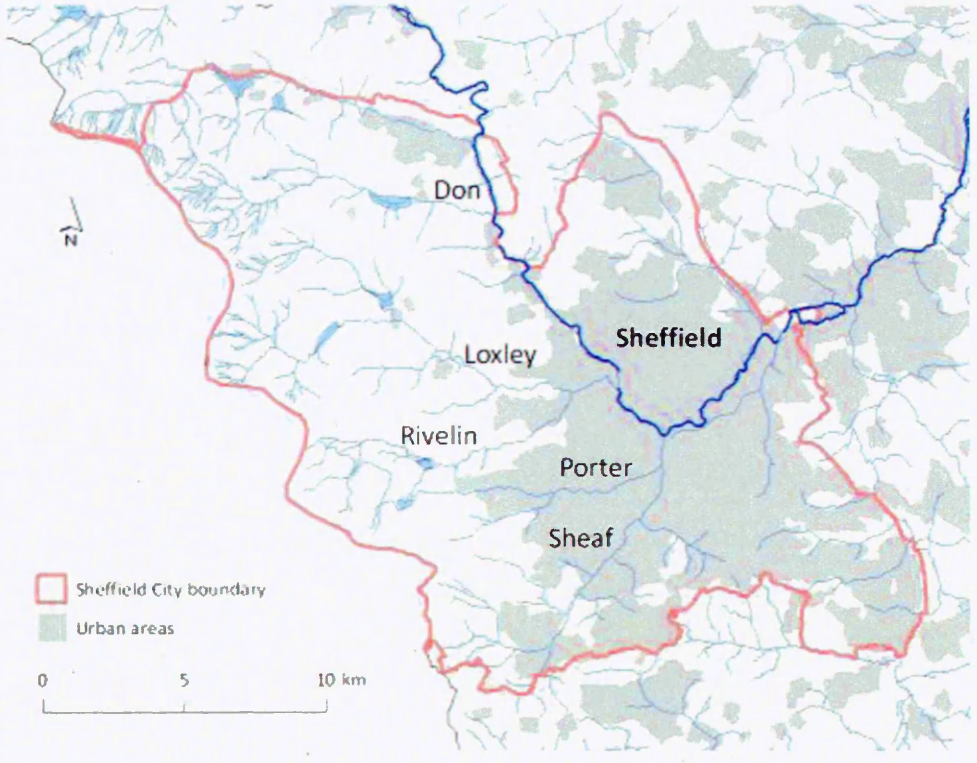
The main rationale for identification of this particular location of the River Sheaf as the study site is due to its historical contamination and also some former industrial pollution sources present along the River Sheaf upstream from the chosen sampling site. The heavy metals were expected to persist in the environment for a longer time than other organic pollutants, due to their non-biodegradable nature in the sediments; as a result the section of the River Sheaf described below was selected as the sampling site.

3.3 Description of site: -

The present site employed for the study is the River Sheaf which is a river in Sheffield 53° 23′ N 1° 28′ W53.383° N 1.467° W, South Yorkshire, England. Situated in the Pennine foothills in the extreme south west corner of Yorkshire, Sheffield city is built on seven hills and watered by five rivers. The Rivers Sheaf, Porter, Loxley and Rivelin finally join into the River Don as shown in figure 3.1. There are continuous ridges which rise from the Don Valley and connect with the high ground to the north and west. With cross valleys and minor ridges the impression is gained that Sheffield is all hills, except for one corner of flat terrain containing Attercliffe and Carbrook on the lower Don valley. The source of the River Sheaf is in the union of the Totley Brook and the Old Hay Brook in Totley, which was formerly a separate settlement and is now a suburb of Sheffield (total 56 suburbs). The main tributaries of the River Sheaf are Porter Brook and Meers Brook. It flows northwards, past the suburb of Dore through the valley called Abbeydale and north of suburb of Heeley. The River Sheaf joins the River Don near Blonk Street Bridge in Sheffield city centre.

Millhouses is a public urban park located in Millhouses neighbourhood in the south of Sheffield. It is a 12.87 hectare park stretching 1.2 km along the floor of the River Sheaf valley sandwiched between Abbeydale Road South (A 621) and the railway tracks of the Midland Mainline. Prior to the construction of the park it was used for farmland and industrial purposes due to various power mills located on the river. (Sheffield libraries archives & information 2006)

Figure 3.1 Rivers of Sheffield



Source: Adapted from Crown Copyright/database right 2011. An Ordnance Survey/EDINA supplied service. River layer kindly licensed from the Environment Agency.

The soils of Sheffield city have developed over carboniferous Lower and Middle coal measure formations, although the soils of part of the west of the city have been developed by older Millstone Grit Formation. The Lower and Middle coal measures in the Sheffield region consist of cyclothems, including mudstones, shales and inter-bedded sandstones. Soils derived from the coal measures in this region were shown to have naturally elevated concentrations of several trace elements including Pb, Cr and Ni in comparison to their average contents throughout England and Wales. There are no extensive quaternary deposits found in these soils ensuring that all the soils are formed from two parent materials (Rawlins et al. 2002).

The history of metal manufacturing and processing, has contributed to significant amounts of trace metal pollution in the urban areas of Sheffield top soils. The historic coal usage, metal

working industries on the rivers Don, Sheaf and Porter along with the Steel Manufacturing by British Steel in 1960s have led to elevated levels of Ni and Cr in Sheffield soils. Historically Pb was known in Sheffield for manufacturing of special alloys and more recently as an additive (alkyl-lead) in petrol. Although the adding of lead compounds to petrol ceased in the UK in 2000, high road density and heavy traffic in industrialized areas was evident throughout Sheffield and has contributed to significant point and diffuse metal pollution (Rawlins et al., 2002).

3.4 Sample collection: -

The sediment sample collection protocol was designed to collect the samples according to the size of site and physical and chemical parameters to be analysed. A stratified random sampling method was employed to collect the samples at the River Sheaf site passing along Millhouses Park. The total site was broken into two areas and samples were collected randomly from each area depending on the accessibility to sediment samples at the location. Depending on the length of River Sheaf that is passing through Millhouses Park the nine samples were collected at approximately equal spacing along the river from where the River enters Millhouses Park with grid reference SK 332835 to the point at which the River leaves the Park, considering the accessibility to the site in view with health and safety. The nine different sampling sites at which samples were collected are shown in figure 3.2

Figure 3.2 Map showing different sampling locations along River Sheaf

Scale 1:5000

Red dots denote sampling locations

3.5 Sampling locations: -

The sediments of the River Sheaf passing along Millhouses Park were collected for the present study. The samples were collected during the month of October in 2011. A total of nine samples were collected for physico chemical and microbiological analysis. The photographs of the site at the individual locations of sample collected are shown below and marked with arrows in Figure 3.3-3.11.

Figure 3.3 (Sample MH-1)

Figure 3.4 (Sample MH-2)

Figure 3.5 (Sample MH-3)

Figure 3.6 (Sample MH-4)

Figure 3.7 (Sample MH-5)

Figure 3.8 (Sample MH-6)

Figure 3.9 (Sample MH-7)

Figure 3.10 (Sample MH-8)

Figure 3.11 (Sample MH-9)

3.6 Sample preparation and handling: -

The sediments samples of 1.5-2.0 kg each were collected in zip lock bags by using a plastic scoop at each designated location as shown in the figure 3.1. After collection at each site the scoop was cleaned in the flowing water of the river to minimise cross contamination and the samples were transported to laboratory for further analysis and were stored in the cold room prior to the analysis. The litter, mostly domestic waste such as tins, paper and plastic bags, was observed along with some dog faeces and debris at some places which could result in pollution of sediments to some extent in and around the site, along with leaves and twigs around the site; these were separated prior to placing the soils samples in zip lock bags.

The weather on the day during mid October 2011 when the samples were collected was cloudy with 7^0C temperature. All the samples were collected on the same day. The river was flowing very slowly throughout its course in Millhouses Park. The Park is used by public for social and recreational activities and can contribute to some amount of waste which can alter the biological properties of sediments.

The samples were stored in the cold room and then the physical and chemical characteristics were analysed by separating the sediments according to the fraction size and also heavy metal analysis were determined in these sediments samples which are described in the next chapter "physical and chemical characterisation of River Sheaf sediments" in this thesis.

3.7 Conclusion and Discussions: -

The site is used by general public for recreational activities. Waste due to recreational activities such as tins, paper and plastic bags have been found around the site which can alter the biological properties of sediment and water. The water looks clean to naked eye in all the areas except at one junction where the pipe of 20 cm diameter from unknown origin joined into river which could also have an effect on the sediments and water.

Chapter 4

PHYSICAL AND CHEMICAL CHARACTERISATION OF SEDIMENTS

4. Physical and chemical characteristics of sediment samples

4.1 Introduction

The sediment samples from the River Sheaf were collected and were transported to the laboratory for further physical and chemical characterization. Trace elements or heavy metals are the primary concern in environmental pollution. The main sources of pollution include weathering of soils and rocks, anthropogenic activities such as drainage of land and land use alterations and industrial activities. The hydrological cycle which maintains the water in the environment dissolves less than 1% of pollutants in water and 99% are stored in sediments, thus making sediments major carriers of pollutants. Analysis of sediments indicates the nature of pollutants and the type of pollution associated with the water body (Cited by Filgueiras et al., 2004).

Human activities like tanning, smelting, electricity production, mining and domestic and industrial waste waters release several aquatic toxic heavy metals into the terrestrial environment and through natural processes enter into the aquatic environment and become deposited in the sediments.

The particle size distribution was measured in the sediment samples by the sieving method as described in the section 2.9 of Materials and Methods chapter. The concentration of the pollutants stored in sediments is affected by the sediment mineralogy and dimensions and distribution of the particles. Trace elements are adsorbed by organic substances such as carbohydrates and minerals such as Fe and Mn oxides. Heavy metals of anthropogenic origin are generally introduced into the environment as inorganic complexes or hydrated ions which can easily bind to the surface of sediment particles by relatively weak physical and chemical bonds (Bartoli et al., 2012). The concentration of chemicals in sediments tends to increase with the decreasing particle size, because the surface area per unit mass increases rapidly with decreasing particle size, as suggested by Fontaine et al (2000).

After particle size fractionation of the samples the heavy metals in each selected fraction were determined by Nitric acid digestion followed by analysis on ICP-MS.

4.2 Results

The sediment samples were divided into various fragments according to size of particles and were categorised on the Wentworth scale as shown in table 4.1. Grain size is the fundamental aspect of sediments. According to geological criteria sediments can be classified into four fractions that constitute gravel, sand, silt and clay and sediments can be characterised based on the ratios of the various proportions of these fractions. The fractions have long been defined according to the grade scale described by Wentworth (1922). According to Wentworth's scale the grain size is a logarithmic scale in which each grade limit is twice as large as the next smaller grade limit. The gravel sized particles have a diameter of 2 mm or greater; sand-sized particles diameters range from <2 mm to >62.5 μm; silt-sized particles have diameters ranging from <62.5 μm to >4 μm; and clay is less than < 4 μm. The table 4.1 shows the relationship between the Wentworth scale and the ϕ (phi) scale devised by Krumbein which has been extensively used in recent work (Krumbein & Aberdeen 1937).

Table 4.1 Size classification of sample fractions

Fractions	Wentworth Grade	Phi(ϕ) scale
Fraction 1- ≥5 millimeters	Pebble	-2
Fraction 2 ≤5.0 mm≥2.36 mm	Granule	-1
Fraction 3 ≤2.36 mm≥1.18 mm	very coarse sand	0
Fraction 4 ≤1.18 mm≥600 μm	coarse sand	1
Fraction 5 ≤600 μm ≥300 μm	medium sand	2
Fraction 6 ≤300 μm ≥150 μm	fine sand	3
Fraction 7 ≤150 μm≥75 μm	very fine sand	4
Fraction 8 ≤75 μm	Silt	5-8

The sample fractions which are larger than sand (granules, pebbles, cobbles and boulders) are collectively known as gravel and the fractions smaller than the sand (silt and clay) are known as mud. A total of 9 samples were collected from the River Sheaf that was passing through the Millhouses Park and was designated as MH-1 to MH-9. The sediment samples are

categorised into various fractions according to sizes and are described below from table 4.2-4.10.

Table 4.2 Particle size distribution at sample location MH-1

MH-1	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8
Weight of sample fraction (g)	630.6	283.0	200.1	118.2	67.1	26.0	10.7	11.8
Percent retained	46.87	21.03	14.88	8.79	4.99	1.93	0.79	0.88

see Table 4.1 for definitions of fractions

Weight of soil sample = 1345.5g

% retained= weight of sample fraction/weight of soil sample *100

Table 4.3 Particle size distribution at sample location MH-2

MH-2	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8
Weight of sample fraction (g)	368.5	203	216.6	251.4	179.1	63.8	19.3	17.0
Percent retained	27.92	15.38	16.41	19.05	13.57	4.83	1.46	1.28

see Table 4.1 for definitions of fractions

Weight of soil sample= 1319.4g

Table 4.4 Particle size distribution at sample location MH-3

MH-3	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8
Weight of sample fraction (g)	213.6	105	124.7	168.0	138.5	63.9	19.9	15.1
Percent retained	25.13	12.35	14.67	19.76	16.29	7.51	2.34	1.77

see Table 4.1 for definitions of fractions

Weight of soil sample=849.8g

Table 4.5 Particle size distribution at sample location MH-4

MH-4	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8
Weight of sample fraction (g)	291.9	150.3	153.3	217	145.2	41.5	11.7	10.4
Percent retained	28.57	14.71	15.00	21.24	14.21	4.06	1.14	1.01

see Table 4.1 for definitions of fractions

Weight of soil sample=1021.5g

Table 4.6 Particle size distribution at sample location MH-5

MH-5	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8
Weight of sample fraction (g)	584.7	205.5	170.2	251.5	297.7	106.2	31.0	23.6
Percent retained	34.86	12.25	10.1	15.35	17.15	6.33	1.84	1.40

see Table 4.1 for definitions of fractions

Weight of soil sample=1677.0g

Table 4.7 Particle size distribution at sample location MH-6

MH-6	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8
Weight of sample fraction (g)	399.2	189	193	198.2	131.7	53.8	23.1	27.1
Percent retained	32.82	15.54	15.86	16.29	10.82	4.42	1.89	2.22

see Table 4.1 for definitions of fractions

Weight of soil sample = 1216.2 g

Table 4.8 Particle size distribution at sample location MH-7

MH-7	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8
Weight of sample fraction (g)	694.3	316.9	208.3	111.0	80.3	41.4	16.6	21.4
Percent retained	46.58	21.27	13.98	7.45	5.39	2.78	1.11	1.43

see Table 4.1 for definitions of fractions

Weight of soil sample = 1490.4 g

Table 4.9 Particle size distribution at sample location MH-8

MH-8	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8
Weight of sample fraction (g)	612.8	257.9	240.7	226.8	273.1	161.2	38.8	21.7
Percent retained	33.41	14.06	13.12	12.37	14.89	8.79	2.11	1.18

see Table 4.1 for definitions of fractions

Weight of soil sample = 1834.1 g

Table 4.10 Particle size distribution at sample location MH-9

MH-9	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7	Fraction 8
Weight of sample fraction (g)	234.2	134.6	110.1	125.9	255.5	292.6	97.6	51.8
Percent retained	17.94	10.31	8.43	9.64	19.57	22.41	7.48	3.97

see Table 4.1 for definitions of fractions

Weight of soil sample = 1305.3 g

The abundance of each of the particle size fractions in each sample was in the following rank order pebble≥ granules≥ coarse sand ≥ very coarse sand≥ medium sand≥ fine sand≥ very fine sand≥ silt. The percent of silt is low at all sample locations with an average of 1.68% and pebbles being the highest contributing to average of 32.67% in each fractions.

After fractionating into the various particle sizes heavy metal analysis was carried out on fractions 5-8 i.e., the four finest fractions with the ICP-MS as described in the Materials and Methods section 2.8 and the results are tabulated below (table 4.11 to 4.16).

Table 4.11 Heavy metal concentration at sample locations MH-1, MH-2 &MH-3 fractions

Sample Location	Mg (mg/kg)	Ca (mg/kg)	Cr (mg/kg)	Mn (mg/kg)	Fe (mg/kg)	Co (mg/kg)	Ni (mg/kg)	Cu (mg/kg)
MH-1 Fraction 5	-	52.08	-	12.084	575.2	-	-	-
MH-1 Fraction 6	-	1051.2	-	45.8	1902	-	-	-
MH-1 Fraction 7	-	1776	7.808	212.2	9596	-	0.1502	1.136
MH-1 Fraction 8	-	1965	13.86	-	-	0.236	0.538	3.238
MH-2 Fraction 5		47.68	-	10.56	676	-	-	-
MH-2 Fraction 6	-	1156.8	-	35.63	2083	-	-	-
MH-2 Fraction 7	-	1665.8	3.568	200.96	9659	-	0.328	0.985
MH-2 Fraction 8	-	1896	12.685	-	-	0.486	0.785	2.56
MH-3 Fraction 5	10.36	3865	-	3.86	478	-	-	-
MH-3 Fraction 6	-	986.3	-	25.65	1896	-	-	-
MH-3 Fraction 7	-	1336	2.86	125.36	8758	-	0.236	1.686
MH-3 Fraction 8	-	1683.2	6.538	-	-	1.214	0.685	3.825

'-' denotes below detection limit

Table 4.12 Heavy metal concentration at sample locations MH-1, MH-2 &MH-3 fractions

Sample Location	As (mg/kg)	Se (mg/kg)	Cd (mg/kg)	Pb (mg/kg)	Zn (mg/kg)
MH-1 Fraction 5	5.244	-	-	5.076	5.12
MH-1 Fraction 6	5.368	-	-	50.76	8.2
MH-1 Fraction 7	6.063	-	-	238.18	21.772
MH-1 Fraction 8	6.126	-	-	336.88	35.68
MH-2 Fraction 5	4.832	-	-	6.072	3.26
MH-2 Fraction 6	4.954	-	-	49.96	5.78
MH-2 Fraction 7	5.032	-	-	210.36	18.76
MH-2 Fraction 8	5.123	-	-	280.68	29.36
MH-3 Fraction 5	4.768	-	-	4.068	4.86
MH-3 Fraction 6	4.855	-	-	48.86	6.85
MH-3 Fraction 7	4.980	-	-	196.98	18.75
MH-3 Fraction 8	5.023	-	-	212.36	27.36

'-' denotes below detection limit

Table 4.13 Heavy metal concentration at sample locations MH-4, MH-5 &MH-6 fractions

Sample Location	Mg (mg/kg)	Ca (mg/kg)	Cr (mg/kg)	Mn (mg/kg)	Fe (mg/kg)	Co (mg/kg)	Ni (mg/kg)	Cu (mg/kg)
MH-4 Fraction 5	10.36	1230	-	3.368	65.8	-	-	-
MH-4 Fraction 6	-	1236.7	-	-	2136	-	0.896	-
MH-4 Fraction 7	-	3658	1.123	-	8588	1.536	9.856	6.368
MH-4 Fraction 8	-	71.36	9.865	-	-	5.658	22.121	40.386
MH-5 Fraction 5	11.388	1460.8	-	1.422	83.6	-	-	-
MH-5 Fraction 6	-	1464.8	-	-	4392	-	1.2912	-
MH-5 Fraction 7	-	4688	4.744	-	10744	3.9676	11.892	9.668
MH-5 Fraction 8	-	8592	25.232	-	-	7.66	25.196	59.08
MH-6 Fraction 5	9.568	1336	-	4.656	96.5	-	-	-
MH-6 Fraction 6	-	1385	-	-	3136	-	0.435	-
MH-6 Fraction 7	-	4025	2.265	-	9759	2.368	6.785	3.856
MH-6 Fraction 8	-	65.96	13.68	-	-	6.865	19.578	22.516

'-' denotes below detection limit

Table 4.14 Heavy metal concentration at sample locations MH-4, MH-5 &MH-6 fractions cont...

Sample Location	As (mg/kg)	Se (mg/kg)	Cd (mg/kg)	Pb (mg/kg)	Zn (mg/kg)
MH-4 Fraction 5	4.186	-	-	70.36	2.868
MH-4 Fraction 6	4.583	-	-	100.58	22.85
MH-4 Fraction 7	4.765	-	-	175.86	47.13
MH-4 Fraction 8	4.950	-	-	200.58	96.58
MH-5 Fraction 5	5.184	-	-	100.56	4.164
MH-5 Fraction 6	5.596	-	-	113.96	24.88
MH-5 Fraction 7	6.24	-	-	228.02	58.56
MH-5 Fraction 8	6.6	-	-	271.44	121.48
MH-6 Fraction 5	3.131	-	-	81.36	3.868
MH-6 Fraction 6	3.468	-	-	121.85	36.580
MH-6 Fraction 7	3.935	-	-	157.96	49.235
MH-6 Fraction 8	4.123	-	-	210.98	101.53

'-' denotes below detection limit

Table 4.15 Heavy metal concentration at sample locations MH-7, MH-8 &MH-9 fractions

Sample Location	Mg (mg/kg)	Ca (mg/kg)	Cr (mg/kg)	Mn (mg/kg)	Fe (mg/kg)	Co (mg/kg)	Ni (mg/kg)	Cu (mg/kg)
MH-7 Fraction 5	21.05	4356.8	-	63.8	7136	-	-	-
MH-7 Fraction 6	-	4878	-	27.6	3856	-	-	-
MH-7 Fraction 7	-	2136.5	4.868	-	4538	0.438	-	1.365
MH-7 Fraction 8	-	3985	18.965	-	-	2.136	9.675	53.785
MH-8 Fraction 5	-	2500.2	-	175.18	9250	-	-	-
MH-8 Fraction 6	-	2342.5	-	136.0	6758	0.734	-	-
MH-8 Fraction 7	-	1876.5	2.5685	-	8136	0.865	-	0.9765
MH-8 Fraction 8	-	2895	7.132	-	-	3.135	12.185	29.365
MH-9 Fraction 5	-	2635.6	-	195.12	8084	-	-	-
MH-9 Fraction 6	-	2395.2	-	129	4880	0.8472	-	-
MH-9 Fraction 7	-	1374.4	3.6764	-	7360	0.9208	-	1.1008
MH-9 Fraction 8	-	3238	9.644	-	-	5.036	13.416	37.508

'-' denotes below detection limit

Table 4.16 Heavy metal concentration at sample locations MH-7, MH-8 &MH-9 fractions cont...

Sample Location	As (mg/kg)	Se (mg/kg)	Cd (mg/kg)	Pb (mg/kg)	Zn (mg/kg)
MH-7 Fraction 5	7.125	-	-	116.53	45.468
MH-7 Fraction 6	7.368	-	-	140.38	31.38
MH-7 Fraction 7	7.835	-	-	200.65	48.75
MH-7 Fraction 8	8.123	-	-	335.65	95.68
MH-8 Fraction 5	5.928	-	-	101.56	29.368
MH-8 Fraction 6	5.735	-	-	116.86	11.756
MH-8 Fraction 7	6.013	-	-	216.53	33.652
MH-8 Fraction 8	6.236	-	-	412.80	80.568
MH-9 Fraction 5	6.028	-	-	108.96	34.632
MH-9 Fraction 6	5.672	-	-	124.48	13.78
MH-9 Fraction 7	5.944	-	-	218.64	38.756
MH-9 Fraction 8	6.656	-	-	439.20	85.96

'-' denotes below detection limit

The analysis of heavy metals in the River Sheaf sediments indicate that sediments have been polluted with toxic heavy metals, of which the major representatives were lead (Pb), chromium (Cr), cobalt (Co), nickel (Ni) and arsenic (As). The magnesium was found only in the medium sand fractions at sample locations 3, 4, 5 and 6. The minimum concentration of calcium, copper, zinc, manganese, and iron at various locations was 47.68 mg/kg, 0.9765 mg/kg, 3.26 mg/kg, 1.422 mg/kg, and 65.8 mg/kg while maximum concentration of calcium,

copper, zinc, manganese and iron was 8592 mg/kg, 59.08 mg/kg, 121.48 mg/kg, 212.2 mg/kg and 10744 mg/kg. The heavy metals found at site are chromium, cobalt, nickel, arsenic and lead. The concentration of lead was very high compared to the other toxic metals. The minimum concentration of chromium, cobalt, nickel, arsenic and lead are 1.123 mg/kg, 0.236 mg/kg, 0.1502 mg/kg, 3.131 mg/kg and 4.068 mg/kg while maximum concentrations of these heavy metals chromium, cobalt, nickel, arsenic and lead at various sample locations are 25.232 mg/kg, 7.66 mg/kg, 25.196 mg/kg 8.123 mg/kg and 412.80 mg/kg.

4.3 Conclusions and discussions:-

Size is a fundamental property of sediment particles. It provides important clues about the nature and provenance of sediments (Pye & Blott 2004). Most heavy metals are found in greatest abundance in the fractions of fine sand, very fine sand and silt as it would be easier for the metals to get adsorbed to the smaller particles (Duyusen et al, 2013).

The mobility and biological effectiveness of soil heavy metals have a strong correlation with the size and composition of heavy metal fractions (Cited by Zhang et al., 2013). The various size fractions have different composition and properties which affected the behaviour of pollutants. Fine particles have a higher ability to carry heavy metals because of their increasing specific surface area and presence of clay minerals, organic matter and Fe/Mn/Al oxides in micro aggregates (Gong et al., 2013).

Sheffield is known for extensive iron and steel working since the industrial revolution along the upstream region of the River Sheaf, which could contribute to the heavy toxic metals such as chromium, nickel, cobalt, arsenic and lead. The main source of the iron and steel industry is usage of chromium, nickel and cobalt with steel alloys in manufacturing and thus resulted in the chromium pollution with sediments. The lead in the sediments comes from industries and urban pollution and automobiles which have been predominant in Sheffield (Gilbertson et al., 1997).

Sheffield has a wide history of pollution from the period of Roman occupation which caused small scale geo chemical contaminants. In the 17^{th} and 18^{th} centuries a lot of pressure was put on woodlands for charcoal production for domestic and industrial use and burning of charcoal intensified the pollution. During mid18^{th} and 19^{th} centuries coal burning followed by coke utilisation for industrial and domestic purposes has led to atmospheric pollution. From 1897-

1937 activities of the Rother Vale mine in what is now a Tinsley Park led to widespread pollutants of the atmosphere, water and soil. From 1913 to 1980 introduction of alloying agents to stainless steels, special steels and high speed steels led to the introduction of organic contaminants, heavy metals, charcoal and soot. After 1980 intensive use of lead additives in automobiles has led to increasing lead concentration and pollution in city of Sheffield.

Sheffield's historical development has been chartered by Hey (1998), Crossley et al. (1989), and Hutchinson and Rothwell (2008). Hutchinson and Rothwell (2008) surveyed 115 sites of water powered industry on the city's rivers with 34 sites documented along the River Sheaf and concluded that Pb was the major industrial pollutant along this river. According to Crossley et al. (1989) the former lead working site operated along the Upper Sheaf Valley located along side water courses provided early sources of power between the 17^{th} and 19^{th} centuries. A large proportion of the heavy metal pollution resulted from smelting of metal ores and has continued to cause problems even though the industrial activities that produced the pollution have ceased (Blake et al 2003, Hutchinson and Rothwell 2008).

In 2008 Hutchinson & Rothwell surveyed 12 sample sites located along the upper River Sheaf to the south west of Sheffield, a span of approximately 5 km. The samples were analysed to investigate spatial and temporal distribution of heavy metal mobilisation along the former water powered and Pb working sites. The results showed high concentration of Pb at all sampling locations. Chromium was not analysed during this previous study. During summer the concentrations of lead observed by Hutchinson and Rothwell (2008) were approximately twice as compared to the winter. The concentration of organic matter in the samples also increased (from 20-40%) from winter to summer at the uppermost sample sites on the very edge of the urban area and the concentration of organic matter generally decreased going downstream.

Hutchinson and Rothwell (2008) performed heavy metal analysis for two sampling periods and considerable spatial and temporal variation was observed in suspended sediment metal concentrations. The sediment associated Pb concentrations were relatively high during the winter at sampling sites 7, 8, 9, 10 and 12. The highest concentration of lead recorded in samples was 2132 mg/kg which was very high compared with the Pb concentrations in the present study sampling sites (maximum Pb concentration of 412.80 mg/kg). The high Pb levels at the site can be contributed due to particular industrial history of valley. Comparison of the results in the current study with those of Hutchinson and Rothwell (2008) indicates a

difference in the lead concentrations between the flood plains of the upper Sheaf (sampled by the previous study in 2008) and the lower Sheaf (sampled in the current study in 2011). The substantial difference in the concentrations of lead found in the two studies are probably the result of a number of factors, including likely continued release of lead into the environment due to acid mine drainage from historic mine workings and an expected general decline in lead concentrations at these sites since the industrial activity that caused the problem ceased in around 1970.

During the sampling the suspended sediment Cu concentrations observed by Hutchinson and Rothwell (2008) were also high at sampling site 3 during summer (maximum Cu concentration 107 mg/kg) compared with winter (maximum Cu concentration 102 mg/kg) sampling, although this seasonal difference was less than that observed for Pb (which was also at higher concentrations in winter as detailed above). The maximum concentation of Cu found in the present study was lower than these values (maximum copper concentration 59.08 mg/kg. The other metals found by Hutchinson and Rothwell (2008) were Zn, Fe and Mn at sampling site 5, which was located on tributary of the River Sheaf. These three elements showed a strong temporal variation in their concentrations. The Zn, Fe and Mn at the present sampling study site are low (see Tables 4.11-4.16) as compared to the upper Sheaf sampled by Hutchinson and Rothwell (2008). The reason could be attributed due to some remediation works carried out that can have impact on the low concentration of these metals, in addition to the expected decline in heavy metal concentration with time since mining activities ceased.

Another study was performed by collecting 5 samples from the soils near the Sheba Leather Industry Wukro, Ethiopia and also 2 samples from 2 km away from main industry and effluent stream. The samples were collected at a depth of 20 cm across a 5 m radius. The samples near the industrial area showed higher concentration of Chromium (VI) which could be possibly due to release of untreated sewage. The maximum concentration of Cr (VI) found at the site was 9.99 mg kg^{-1} which falls just within the WHO limits of 10 mg kg^{-1} (Gitet et al., 2013). This result is comparable to the maximum total chromium concentration of 7.66 mg kg^{-1} in the present study, though it should be borne in mind that the Gitet et al. data are for hexavalent chromium alone and the data from the current study are total chromium, which is likely to comprise both trivalent and hexavalent chromium.

A further study which was carried out at a slag heap associated with the steel industry in China analysed a total of 45 samples were collected from this site. Of these, 3 samples were

from location A (Steel alloy factory), 9 samples from location B (adjacent to slag heap), 23 samples from location C (in the vicinity of factory) and 10 control samples from far away from chromate slag site.

The highest concentration of Cr (VI) recorded at Location A was 2239.5 mg/kg which crossed the threshold of secondary environmental quality standard for soil in china by 540% and is 21 times higher than local background value. At location B it is 1589.3 mg/kg which is 15 and 4 times higher than standard limit and local background value. However the chromium concentrations in the vicinity of factory at location C are all low, close to local background value. Even outside the factory the chromium concentration was 959.3 mg/kg which is 9 times higher than local background value (Huang et al., 2009). These concentrations are more than two orders of magnitude higher than in the current study, consistent with a very high degree of contamination from the site in China where steel manufacture and deposition of contaminated waste continues.

Jajmau & Unnao the two industrial areas and parts of Kanpur & Unnao districts of Uttar Pradesh in India located at 80^0 15^1- 80^0 34^1 E longitude and 26^0 24^1 - 26^0 35^1 N latitude is a chronic polluted area and one of the biggest exporting centers of tanned leather. The industry is located on the banks of River Ganga along with its tributary Pandu River. A total of 53 samples were collected from site and 11 elements were analysed (As, Ba, Co, Cr, Cu, Ni, Mo, Pb, Sr, V and Zn) out of which As, Co, Ni and Mo were below the detection limit. The concentration of the metals exceeded the international threshold values.

The average values of these heavy metals were Cr (2652.3 mg/kg) Ba (295.7 mg/kg) Cu (42.9 mg/kg) Pb (38.3 mg/kg) Sr (105.3 mg/kg) V (54.4 mg/kg) and Zn (159.9 mg/kg) and these metals showed positive correlation due to industrial contamination and sinks of the soil in the study site. The presence of Sr in the study site showed the adsorption and enrichment of Pb, V and Cu (Srinivasa Gowd et al., 2010). These results indicate a site highly contaminated with chromium, presumably due to the on-going tanning industry in this area

Across the world, the average chromium concentration in pristine natural waters ranges from 0.2-1 g/ltr. Natural chromium concentrations in sea water are found to be 0.04-0.5 µg/ltr. The natural total chromium content in natural surface waters ranges from 0.5-2 µg/ltr approximately, while the dissolved total chromium content is 0.02-0.3 µg/ltr (WHO guidelines 2003). In light of these data, it is evident that if the chromium contained in the sediment samples from the River Sheaf were released into the surrounding river water, with a

substantial proportion of the chromium in the hexavalent oxidation state, a substantial pollution problem could result.

CHAPTER 5

ENRICHMENT AND ISOLATION OF *Methylomonas. koyamae* (SHU1)

from sediment samples

5. Enrichment and Isolation of *Methylomonas koyamae* (SHU1) from sediment samples

5.1 Introduction:-

Environmental pollution can be attributed to several anthropogenic activities. When various toxic metals are introduced into the environment they can remain persistent, thereby affecting human health by entry through food chain and produce toxic effects. The metals which remain in the environment are held there by sorption, precipitation and complexation reactions. These metals can be removed from the soil by uptake with plants, leaching and volatilization process. The fate of the metals in the soil environment depends on the soil properties and environmental factors (Das et al 2014).

Metals are cycled in the various segments of the environment by biological, geological and chemical process that transport and mobilise the elements simultaneously between living systems and non-living systems. Several physical and chemical techniques have been studied to remediate these metals but these have proven to be non-economical, so transformation by microbes offers a promising alternative to physical and chemical remediation (Das et al 2014).

Methanotrophs or methane oxidising bacteria are capable of oxidising methane which is a very potent greenhouse gas. These organisms can remediate a range of pollutants as well as oxidising methane and using it as sole source of energy, thus adding a beneficial effect to the environment.

Methanotrophs have been used in the present study because of their potential and promising results with the metal remediation (as detailed in the literature review chapter) and also these organisms which are widespread in the environment has not been extensively explored in terms of metal remediation compared to the other microbes. In the present study methanotrophic bacteria were enriched from the sediments of the River Sheaf to study the characteristics of methanotrophs from this environment in respect to reduction of heavy metals.

5.2 Methods

Enrichment of microbes from the study site was carried out to ensure isolation of various pure strains of methanotrophs to be cultivated to employ in the experiment. The sediment samples collected from the River Sheaf were used for enrichment and isolation of methanotrophs. Sampling of sediments is described in detail in chapter 3 of the thesis. Figure 3.2 from chapter 3 shows the sample collection site used for the above experiment.

A pure colony of the same type that was obtained from the plates described in materials and methods chapter was grown on a fresh NMS plate to produce cells for the DNA preparation, and then the 16S rRNA gene was amplified by PCR (the methods for DNA purification and PCR are described in detail in chapter 2).

The PCR product of the 16S rRNA gene from the cultivated methanotrophs was subjected to gel electrophoresis (method is described in chapter 2 section 2.5) to identify the presence of a product of the expected size. Later the gel electrophoresis product was cleaned with the Qiagen kit and then subjected to sequencing.

The sequencing results obtained from the PCR product were subjected to BLAST which is an online tool to identify the similar sequences which can be used to classify 16S rRNA sequences to the species level.

5.3 Results

5.3.1 Enrichment Results

When sediments samples were enriched into fresh NMS medium in Erlenmeyer flasks, the cultures showed high turbidity within 3-4 days indicating the growth of methanotrophs in the media, with methane as sole added carbon and energy source. The flasks were then sub cultured 2-3 times until several single isolated colonies were obtained depending on colour and texture on fresh NMS plates. Based on the colour the colonies isolated are tabulated in table 5.1 below.

Table 5.1 . Different colours of isolated colonies from NMS plates

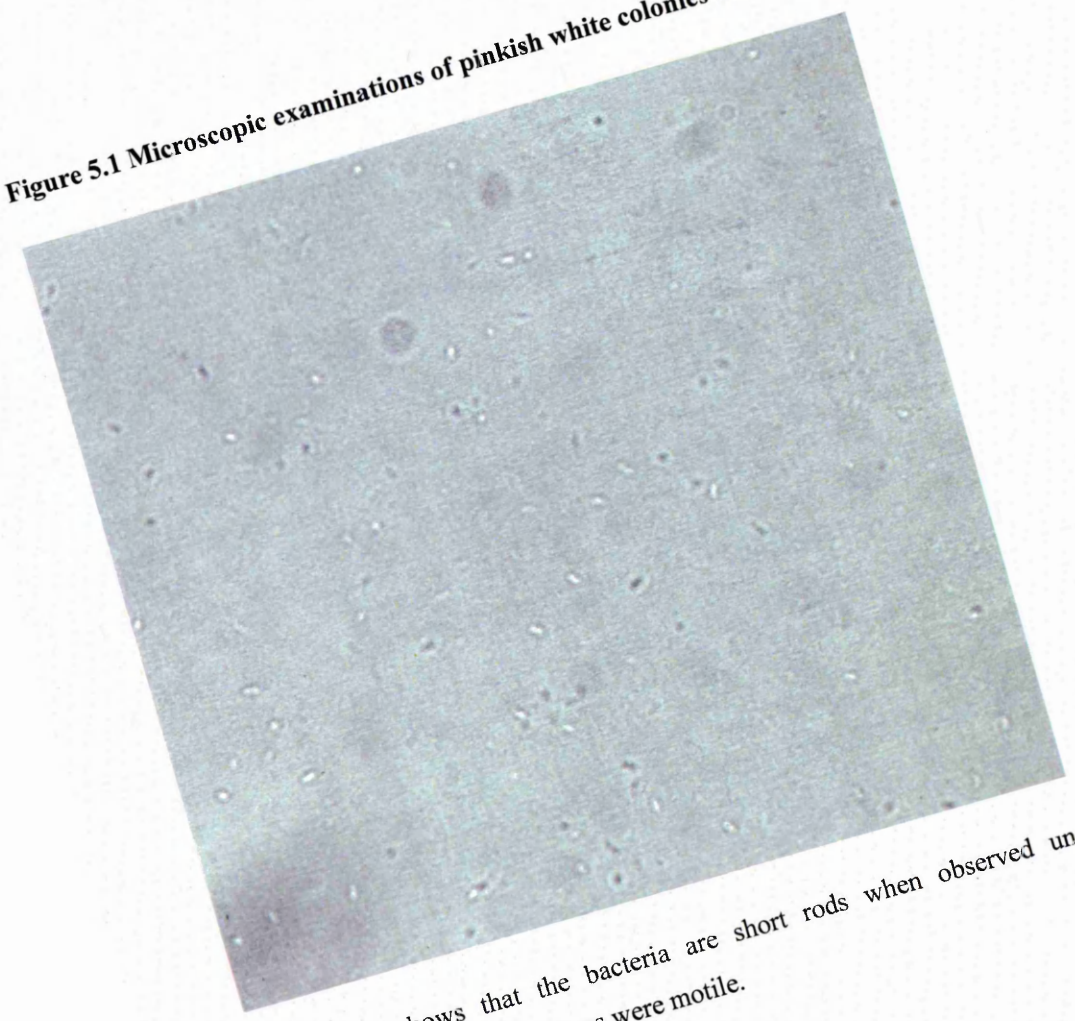
Isolate number	Colony colour
1	pinkish white from 25/9/12 plate 1
2	white colonies from 17/9/12
3	orange colonies from 25/9/12 plate 1
4	cream colonies from 25/9/12 plate 1
5	creamish orange colonies from 25/9/12 plate 1
6	pink colonies from 25/9/12 plate 1
7	cream colonies from 25/9/12 plate 2
8	creamish orange colonies from 25/9/12 plate 2
9	pink colonies from 25/9/12 plate 2
10	orange colonies from 25/9/12 plate 2
11	creamish orange colonies from 25/9/12 plate 3
12	orange colonies from 25/9/12 plate 3

Among all of the samples analysed six different colours and morphology of colonies were obtained. Representatives of these organisms were cultivated in fresh NMS plates and used for further study.

5.3.2 Microscopic examination of isolated colonies

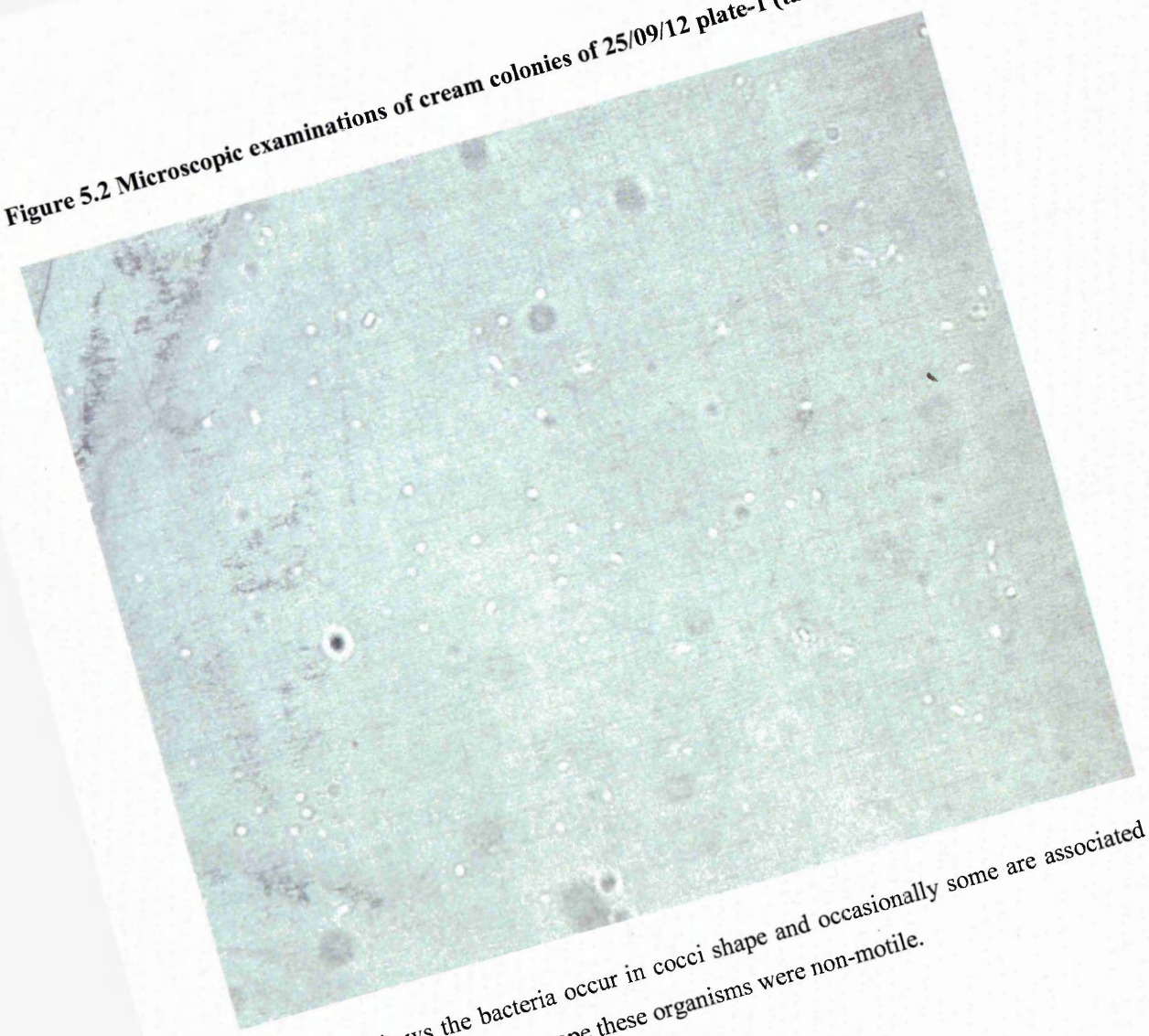
The enriched isolated colonies were prepared and observed under the microscope to view the shape and motility of the organisms (Figures 5.1 and 5.2; Table 5.2).

Figure 5.1 Microscopic examinations of pinkish white colonies of 25/9/12 plate-1 (table 5.1)



The image shows that the bacteria are short rods when observed under microscope (1000X magnification) these organisms were motile.

Figure 5.2 Microscopic examinations of cream colonies of 25/09/12 plate-1 (table 5.1)



This image shows the bacteria occur in cocci shape and occasionally some are associated in chains, when observed under microscope these organisms were non-motile.

Table 5.2 Characteristics of the isolated organisms

Isolate number	Colony colour	Colony shape and Motility
Isolate number 1 from table 5.1	pinkish white colonies	rods and motile
Isolate number 2 from table 5.1	white colonies	cocci and non-motile
Isolate number 4 from table 5.1	cream colonies	cocci and motile
Isolate number 5 from table 5.1	Creamish orange colonies	rods and motile
Isolate number 6 from table 5.1	pink colonies	rods and motile
Isolate number 10 from table 5.1	orange colonies	rods and non-motile

5.3.3 Identification of non methanotrophic contamination in isolated samples

The isolated colonies were streaked on the fresh nutrient agar plates and incubated at 37^0C for 24 hrs to check for the growth of non methanotrophic bacteria in the samples. No growth on three out of six cultured nutrient agar plates indicated absence of any contamination by other types of cultivable bacteria from these cultures. The results of the growth on the nutrient agar are shown in the table 5.3.

Table 5.3 Purity of isolated samples

Isolate number	Type of colony	Growth on Nutrient Agar plates
Isolate number 1 from table 5.1	Pinkish white colonies	-
Isolate number 2 from table 5.1	White colonies	+
Isolate number 4 from table 5.1	Cream colonies	+
Isolate number 5 from table 5.1	Creamish orange colonies	-
Isolate number 6 from table 5.1	Pink colonies	-
Isolate number 10 from table 5.1	Orange colonies	+

5.3.4 Molecular identification of microorganisms

The isolated colonies which are detailed in table 5.1 were used for sequencing. After amplification of 16S rRNA genes from single isolated colonies by PCR, the PCR products were sequenced and the sequences were subjected to analysis via BLAST, which is an online tool to compare the sequences with the database. The results of the BLAST searches are interpreted in table 5.4.

Table 5.4 Molecular identification of organisms

Isolate	Serial No (table 5.1)	Length (bp)	Closest match	Genbank accession no.	Similarity	E_0 Values
Pinkish white P1	No.1	973	*Methylomonas koyamae*	NR113033.1	99%	0.0
Pinkish white P2	No.1	914	*Methylomonas koyamae*	NR 113033.1	99%	0.0
White P1	No.2	35	N/A	-		
White P2	No.2	22	N/A	-		
Creamish orange (plate-1) P1	No.5	236	N/A	-		
Creamish orange (plate-1) P2	No.5	315	*Methylophilus leisingeri* *Methylophilus methylotrophus* *Methylophilus rhizosphararae*	AB 193725.1 NF 911346.1 AB 698737.1	99% 99% 99%	2e-157 1e-155 1e-155
Creamish Orange (plate-2) P1	No.5	366	*Methylomonas koyamae*	NR 113033.1	99%	0.0
Creamish Orange (plate-2) P2	No.5	277	*Methylomonas koyamae*	NR 113033.1	99%	2e-117
Pink P1	No.6	-	N/A	-		
Pink P2	No.6	467	*Methylomonas koyamae*	NR 113033.1	99%	0.0
Creamish orange (plate 1) P1	No.8	792	*Methylomonas koyamae*	NR 113033.1	99%	0.0
Creamish orange (plate 1) P2	No.8	819	*Methylomonas koyamae*	NR 113033.1	99%	0.0
Orange P1	No.10	980	*Acidovorax facilus*	JQ236816.1	99%	0.0
Orange P2	No.10	973	*Acidovorax facilus*	JQ236816.1	100%	0.0

The results in table 5.4 show that there were three types of organisms that were been isolated from the sediments of the River Sheaf and were identified as

1. *Methylophilus* spp.

2. Methylomonas koyamae

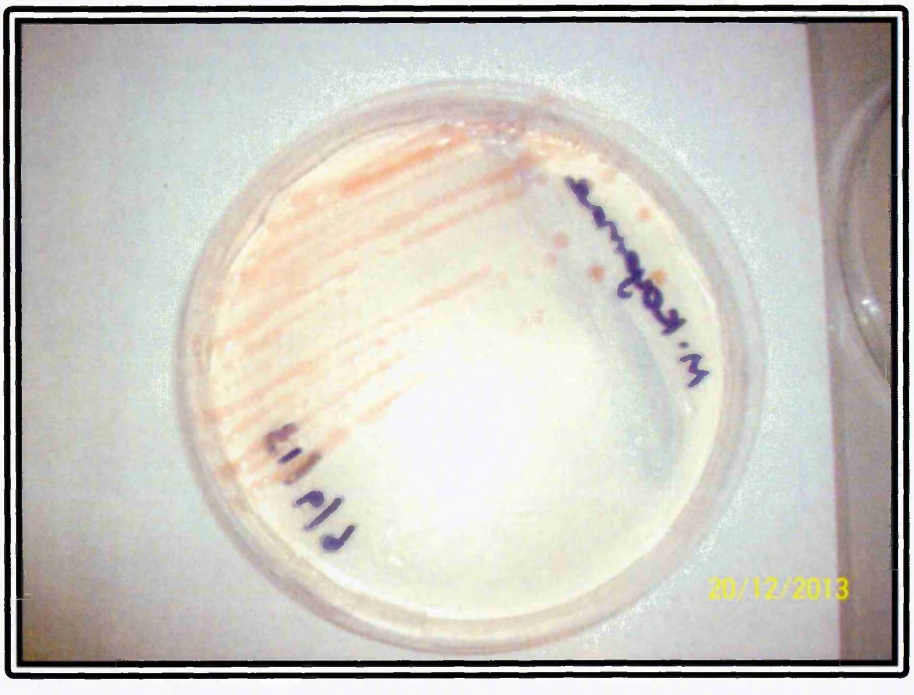
3. Acidovorax facilus

Among the isolates obtained from this study only *Mm. koyamae* belongs to the genus or species previously known to grow on methane. The isolated strain *Mm. koyamae* from table 5.4 with serial number 6 was used for the further study and this strain was given the name *Mm. koyamae* (SHU1).

5.3.5 Growth of *Mm. koyamae* (SHU1)

After identification of *Mm. koyamae* (SHU1) it was cultivated and examined under the microscope prior to making glycerol stocks of the organism. The colonies appeared pink in colour on fresh NMS agar plates and changed further to pinkish orange at longer incubation time. The isolated colonies of *Mm. koyamae* (SHU1) are shown in the figure 5.3. The colonies appeared as motile rods under the microscope.

Figure 5.3 *Mm. koyamae* on NMS plate



5.3.6 Growth of *Mm. koyamae* (SHU1) on Methanol

The ability of *Mm. koyamae* (SHU1) to grow using methanol (0.5%) as sole carbon and energy source was tested in liquid culture at 30^0 C on rotary incubator. Within 3-4 weeks of innoculation the cultures developed turbidity the colour of the culture was pinkish orange. This indicates growth of the *Mm. koyamae* (SHU1) on methanol.

5.4 Discussion and conclusions

In the present study when the samples of the River Sheaf were enriched with methane as sole carbon and energy source *Mm. koyamae* (SHU1) was isolated. These particular organisms belong to methanotrophic bacteria which are Gram negative bacteria that utilize CH_4 as the sole carbon and energy source. *Mm. koyamae* (SHU1) belongs to type I methanotrophs of *Methylomonas* family which are ubiquitous in the environment. As detailed in chapter 2 the sediments samples from the River Sheaf had near neutral pH and so isolation of a *Methylomonas* strain from these samples is consistent with the neutrophilic phenotype previously reported for members of the genus *Methylomonas*. Similarly the isolation of the strain of *Methylomonas* in enrichments at 30^0C is consistent with the known mesophilic growth temperature range of this genus (Marco et al., 2004).

Methane oxidising bacteria of the genus *Methylomonas* have previously been found in fresh water lakes and rivers, wetland muds, activated sludge and waste water, and coal mine drainage water. The reason for the growth of type I methanotrophs in the present study can possibly be attributed to their faster growth compared with the type II methanotrophs. Type I methanotrophs growth is significantly influenced by the O_2 concentration compared to type II methanotrophs and not only that the former grow faster than latter and former bacteria are more sensitive to environment such as, O_2 concentration and nitrogen. Higher concentrations of methane CH_4 favour the growth of type II methanotrophs. (Zifang et al.,2012).

Mm. koyamae (SHU1) the species which was found in the present study, was first isolated by Japanese bio geochemist Tadashiro Koyama and is named after him. The strain was isolated from floodwater of rice paddy fields in Japan (Ogiso et al., 2012). *Methylogaea oryzae* and *Mm. koyamae* are the only two organisms belonging to Type I of methane oxidising bacteria that have been isolated from paddy fields so far (Dianou et al., 2012).

Methanotrophs have been extensively studied in rice field cultivation because rice fields are water logged and anaerobic. The methanogenesis activity under these anaerobic conditions makes rice fields one of the major global sources of methane and has led to interest in studying the methane oxidising bacteria in rice fields, which may be important in reducing the amount of the greenhouse gas methane released into the atmosphere. In a year-round experiment conducted in Japan on methanotroph communities with DGGE (denaturing gradient gel electrophoresis) and DNA sequencing analysis of key functional genes (*pmoA, amoA*). 38 DGGE bands were observed in this study. The investigation of these DGGE bands showed that methanotrophs, particularly type I, dominated during rice cultivation and during the winter fallow season type I and type II were dominant in sheath segments on soil surface and in the plough layer, while ammonia oxidisers dominated blade segments placed in the plough layer (Jia et al 2007).

Methanotrophic bacteria that are associated with roots of submerged rice plants have been assessed by cultivation independent techniques such as T-RFLP (terminal restriction fragment length polymorphism) technique, to study *pmoA, mmoX, mxaF*, and 16S rRNA genes. The results obtained from *pmoA* based (T-RFLP) from rice roots and bulk soils of hooded rice microcosms indicated that there was a greater abundance of type I methanotrophs in rice roots than in bulk soil. The organisms belonged to *Methylomonas, Methylobacter* and *Methylococcus* (Horz et al., 2001).

Rice and paddy fields are the major source of anthropogenic emissions of atmospheric methane which can contribute to 15-20% of global anthropogenic methane emissions. The rice fields have optimum conditions like temperature ranging from 25^0C - 30^0C and pH 6-8 which is most appropriate for the methane oxidation by methanotrophs (Fazli et al., 2013).

Fresh water sediments also contribute 40-50% of annual atmospheric methane flux thus giving rise to the growth of methanotrophic organisms by providing methane as sole carbon and energy source. In fresh water sediments the zone of methane oxidation is restricted to the top 0.8 cm where the oxidation of methane takes place and 65% of methane is oxidised to cell materials and other metabolites. The high oxygen concentration and low methane concentration in this zone of soil provide a suitable environment for various type I strains of methanotrophs (Auman et al 2000).

Previous isolates of *M. koyamae* have been shown to be Gram-negative motile rods with a single polar flagellum and type I intracytoplasmic arrangement. These organisms grow on

methane and methanol as sole carbon and energy source. The cells of this organism do not form chains or surface pellicle. The optimum conditions for this organism are 30^0C at pH 6.5 and with 0-0.1% (w/w) NaCl; cells are sensitive to NaCl above 0.5%. The major quinone is MQ-8 and $C_{16:1}$ and $C_{14:0}$ are predominant fatty acids. The DNA G+C content of the type strain is 57.1 mol%. Consistent with this, the isolated organism *Mm. koyamae* SHU1 exists as motile rods and can grow on methane and methanol as source of energy at optimum temperature 30^0C (Ogiso et al 2012).

The organism *Mm. koyamae* SHU1 is pink or pinkish orange in colour which may indicate the presence of carotenoid pigments. Possession of carotenoids is a distinctive feature of species in the genus *Methylomonas* which is rarely found in other species of methanotrophs (Shigematsu et al, 1999). Carotenoids are naturally occurring lipid soluble pigments, the majority being C_{40} terpenoids which act as antioxidants scavenging O_2 and peroxy radicals; their anti-oxidant property can be attributed to their structure. Carotenoids are found in all higher plants, algae, phototrophic bacteria and some non-phototrophic bacteria. The non-phototrophic bacteria and fungi rely on carotenoids for protection when they grow in abundant light and air (Mata Gomez et al., 2014).

Species of *Methylomonas* have been applied in bioremediation of organic pollutants such as trichloroethylene and thus this particular genus can be employed in industrial applications of remediation of various pollutants.

In the study a *Methylophilus* sp. strain is identified these are obligate methylotrophs that can use reduced one carbon compounds such as methane or methanol as carbon source for their growth (Anthony 1982). *Methylophilus* sp. has earlier been studied in terms of its metal tolerance, plant growth promotion, plant disease resistance and soil fertility enhancement. This genus has earlier been explored for the industrial production of amino acids, polyhydroxy alkanoates and carotenoids as well as for bioremediation (Giri et al., 2013). $NADP^+$ specific isocitrate dehydrogenase and glutamate dehydrogenase activities have not been observed in strains of this genus (Doronina et al., 2012). Isocitrate dehydrogenase is an enzyme from citric acid cycle which catalyses oxidative decarboxylation of isocitrate to α-ketoglutarate and CO_2. Glutamate dehydrogenase is present in most microbes and is used convert glutamate to α-ketoglutarate and in urea synthesis.

Acidovorax facilis was also enriched when isolating methanotrophs in this study. *A facilus* is a chemo organotrophic Gram negative bacterium which as shown in table 5.3 in this chapter

is able to grow on nutrient agar plates. The organism is mainly associated with soil and agriculture as a soil inoculant and derives its energy and biomass from the end products of methanotrophs which can result in growth during enrichment of methanotrophs. The genus *Acidovorax* belongs to class Betaproteobacteria and the family Comomonadaceae. (Li et al., 2011) The term *facilis* indicates quick and easy cultivation of this organism. *A. facilis* was first isolated from lawn soil in the United States. The mean G+C contents of DNAs range from 64-65 mol%. *A.facilis* grow at 30^0C (found in the above experiment) in the presence of 0.5% NaCl and grow on the following substrates D-glucose, glycerol, butyrate, succinate, suberate, azelate, sebacate, DL-lactate, D-malate, DL-3 hydroxybutyrate, L-proline and L-glutamate. (Williams et al., 1990). *A. facilis* is likely to use DL-3 hydroxybutyrate and butyrate from methanotrophs and so it may have been isolated in the current study because of its ability to grow alongside methanotrophs when methane was provided as the sole source of carbon and energy.

The bioremediation potential of the newly isolated *Mm. koyamae* (SHU1) is explored in the next chapter.

CHAPTER 6

CHARACTERISATION OF

Methylomonas koyamae SHU1

6. Characterisation of *Methylomonas koyamae* SHU1

6.1 Introduction

Heavy metal pollution associated with concomitant industrial development is a great concern with regard to sustainability of the environment. Heavy metal pollution is complex as compared to organic pollution because the former is non-biodegradable in nature and can persist in environment for many years. Microbes play a prominent role in remediation of the environment, by biosorption and transformation of heavy metals as microorganisms from contaminated environment tend to be resistant to these metals because of natural selection and evolution in polluted environments. They have detoxification as well as resistance mechanisms. Intracellular bioaccumulation or biotransformations are two processes that are associated with Cr (VI) detoxification which can be attributed to enzymatic reactions or chemical reaction with metabolites and other cellular components (Guria et al., 2014).

Methane, which is a potent greenhouse gas and causes global warming 23 times more than CO_2, has only one biological methane sink which are methanotrophs. These organisms growing on methane can co-metabolise many organic and toxic compounds. Methanotrophs are of great interest in industrial applications due to their unique microbiological and metabolic features (Jiang et al., 2010).

Methanotrophs are ubiquitous in nature and can grow at temperatures as low as 4^0C and as high as 72^0C and can adapt themselves to various environmental conditions thus increasing the potentiality of the organisms for biotechnological and industrial applications. Methane monooxygenase (MMO) is a distinguishing enzyme in methanotrophs which facilitates oxidation of many hydrocarbons and other hydrophobic compounds (Jiang et al., 2010).

6.2 Methods

The organism *Mm. koyamae* SHU1 isolated as described in isolation and enrichment chapter *was* cultivated in fresh NMS medium and incubated at 30^0 C to study the growth characteristics of the organism.

The growth curve, resistance to hexavalent chromium experiment, removal of hexavalent chromium by adding metabolic inhibitor and inhibiting enzyme MMO are all described in the materials and Methods chapter.

The results of all the experiments are discussed below.

6.3 Results

6.3.1 Growth of *Mm. Koyamae* SHU1

The *Mm. koyamae* SHU1 organism was grown in fresh NMS medium with methane as carbon and energy source to determine its growth. The optical density at OD_{600nm} of the culture was recorded and a graph was plotted of optical density vs time and the readings are shown in the growth curve for the organism as depicted below in Figure 6.1. A parallel experiment was conducted with the well characterised methanotroph *M. trichosporium* OB3b in order to compare its growth curve (Figure 6.2) with that of *Mm. Koyamae* SHU1.

Figure 6.1 Growth curve of *Mm. koyamae* SHU1

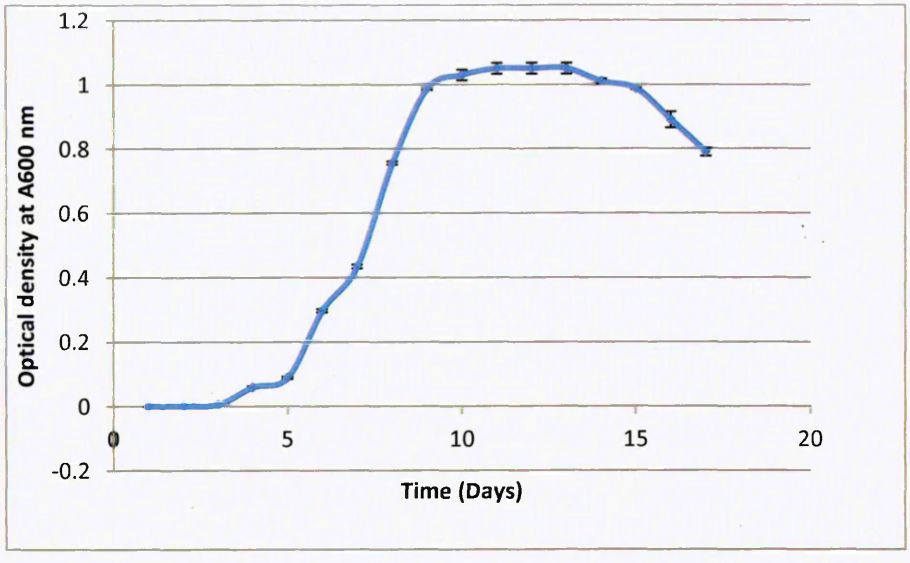
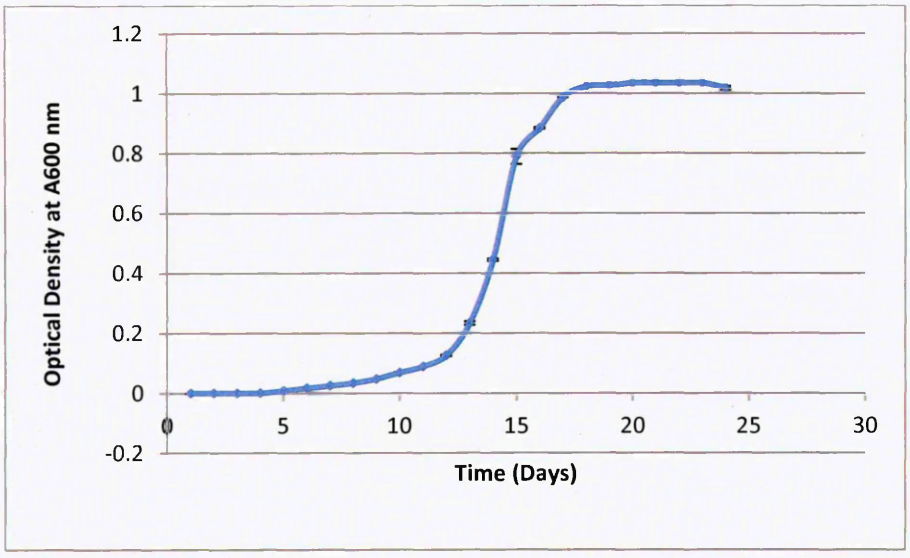


Figure 6.2 Growth curve of *M.trichosporium* (OB3b)

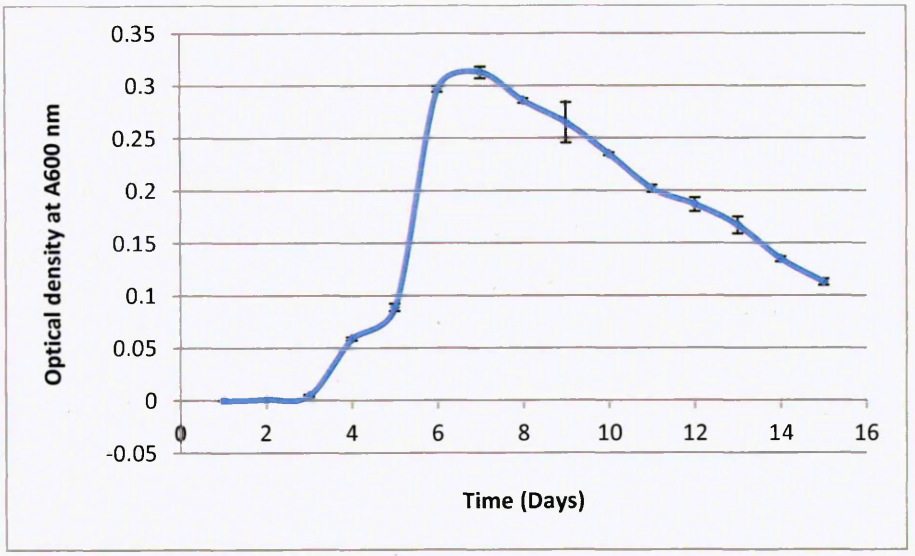


Both the organisms grew to a maximum optical density of approximately 1.0 and the growth in terms of time was compared for both the organisms. The lag phase of *Mm.koyamae* SHU1is less than that compared with the *M. trichosporium* (OB3b). The time taken for *Mm. koyamae* to reach the maximum OD was about 9 days when compared with *M. trichosporium* OB3b which took approximately 17 days. The shorter lag phase of *Mm. koyamae* could be due to its faster adaptability to the environment and thus reaching its fastest growth rate earlier than compared with *M. trichosporium* OB3b.

6.3.2 Resistance of the *Mm. koyamae* SHU1 with hexavalent chromium

After the addition of hexavalent chromium to 10 ppm to a liquid culture (0.3 OD) of *Mm. koyamae* SHU1 the optical density readings decreased indicating the cells are not tolerant or resistant to the hexavalent chromium (Figure 6.3). Growth of *Mm. koyamae* SHU1 was not recorded in any of the plate cultures with chromium (VI) with varying concentrations of 5, 10, 25, 50, 75 and 100 ppm indicating the organism is non-resistant. The resistance to hexavalent chromium was compared with *M. trichosporium* (OB3b) and this organism also proved to be non-resistant and non-tolerant to hexavalent chromium. The decrease in the optical density of the culture after the addition of hexavalent chromium suggests the autolysis of cells.

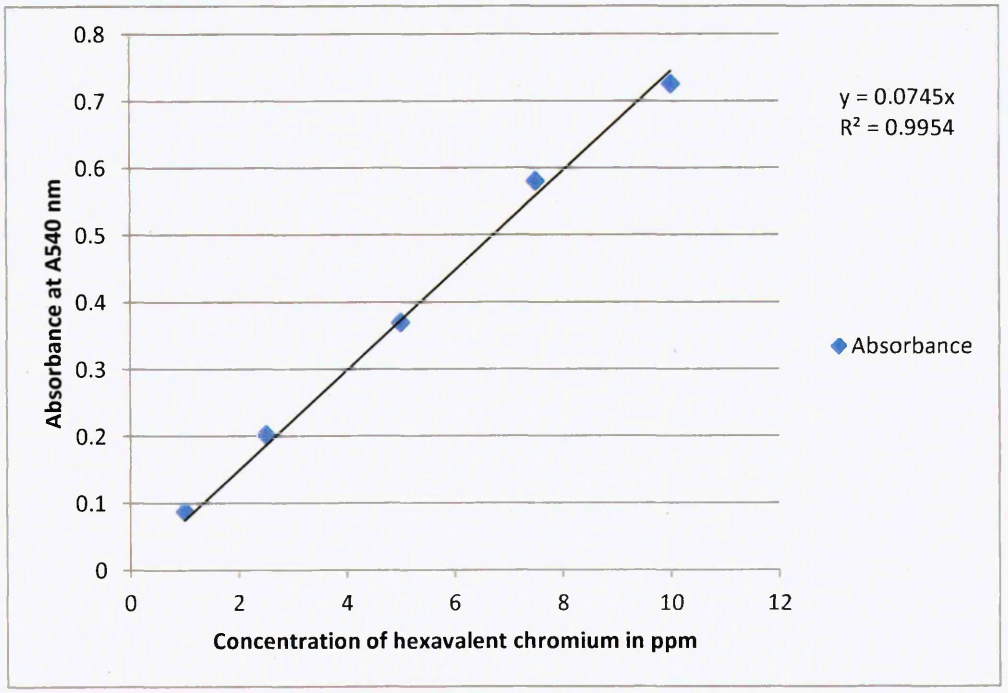
Figure 6.3 Growth curve of *Mm. koyamae* SHU1 after addition of 10 ppm of Cr (VI)



6.3.3 Removal of hexavalent chromium by *Mm. Koyamae* SHU1

The standard curve (Figure 6.4) with different concentrations of chromium (VI) vs absorbance at A_{540} nm was obtained and this graph was used as a basis to calculate the unknown concentrations.

Figure 6.4 Standard curve of chromium Cr (VI) by DPC Assay

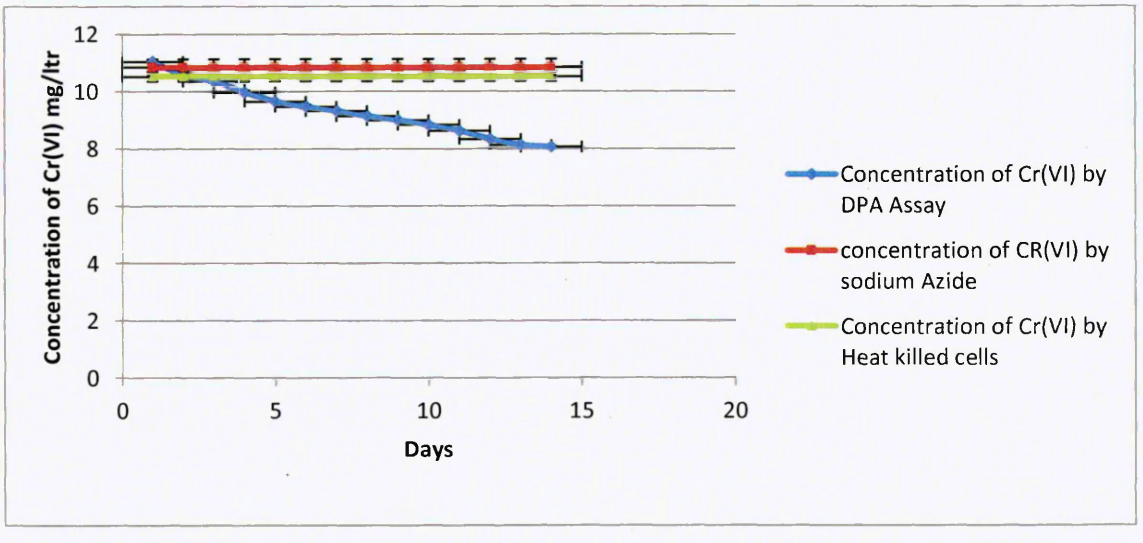


Removal of hexavalent chromium by *Mm. koyamae* SHU1 in the presence of sodium azide, blank and heat killed cells was also recorded and reported. Figure 6.5 shows the removal of hexavalent chromium by *Mm. koyamae* SHU1 in the presence and absence of inhibitors, blank and heat killed cells. There was no removal of hexavalent chromium observed by *Mm. koyamae* SHU1 in the flasks with added sodium azide, which is a metabolic inhibitor. Removal of hexavalent chromium was not observed with the blank and in heat killed cells indicating that the live cells of *Mm. koyamae* SHU1 are solely responsible for the removal of the chromium.

Reduction of hexavalent chromium was observed in cultures of *Mm. koyamae* SHU1 despite the data described earlier in this chapter that indicate that the strain was not resistant to hexavalent chromium. Together these data show that even though the hexavalent chromium inhibits the growth of *Mm koyamae* SHU1 these cells remain to some extent metabolically intact and metabolically remove the hexavalent chromium.

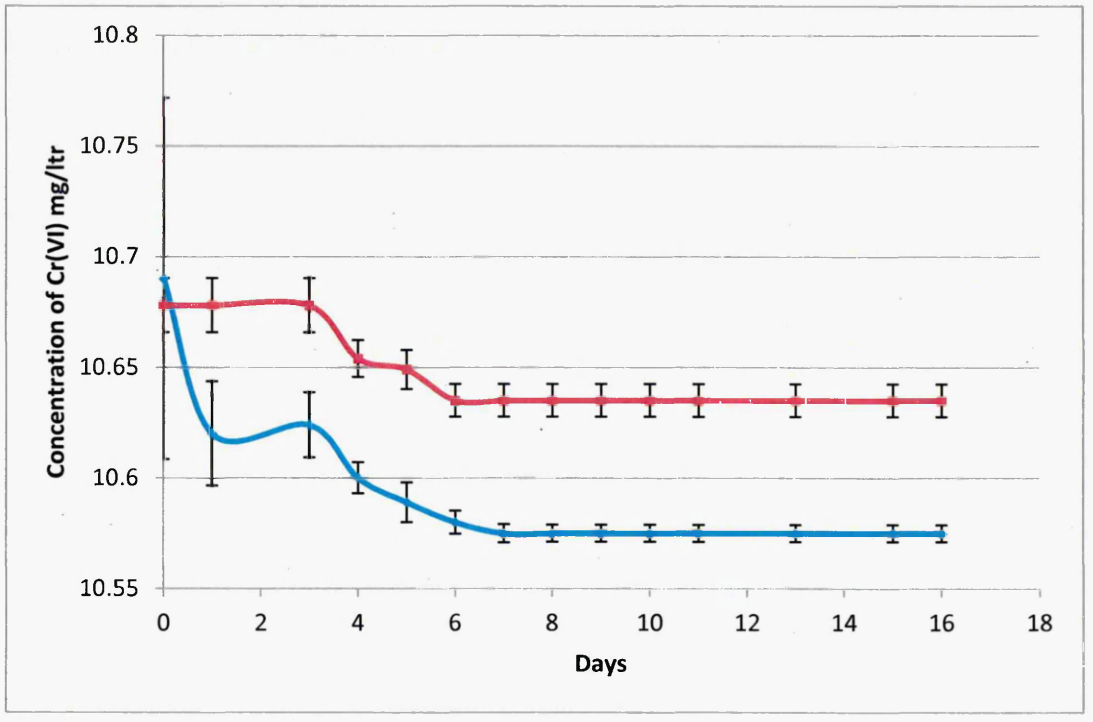
The heat killed cells did not show any removal of hexavalent chromium indicating that these could be an enzymatic process since that heat killing makes all the enzymes inactive. This provides additional information that removal is enzymatic process compared to the results with sodium azide.

Figure 6.5 Removal of hexavalent chromium by *Mm. koyamae* (SHU1) in various conditions



In order to investigate the role of MMO enzyme in the chromium removal from cells by *Mm. koyamae* SHU1 the organism was grown in high copper medium and low copper medium and the enzyme was inhibited by phenyl acetylene as phenyl acetylene is a well known inhibitor of the sMMO enzyme. When the cells were grown in low copper medium with phenyl acetylene the chromium removal activity was completely inhibited, indicating that the phenyl acetylene completely inactivated the sMMO enzyme and the chromium removal reaction was completely inhibited as a consequence (Figure 6.6). When the cells were grown in high copper medium in the presence of phenyl acetylene, the cells could perform the removal of chromium for the period of experiment, consistent with continued activity of pMMO in the presence of phenyl acetylene and hence continue with general metabolism including chromium (VI) removal.

Figure 6.6 Removal of hexavalent chromium by *Mm. koyamae* (SHU1) by inhibiting two enzymes pMMO and sMMO

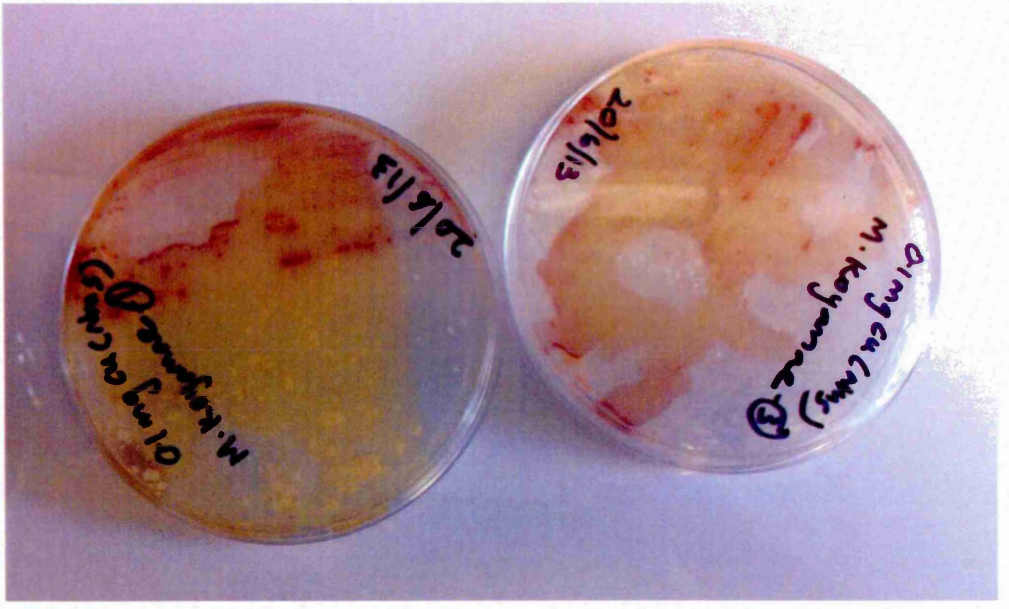


The organisms were cultivated in normal NMS medium and 0.1 mg L^{-1} cu NMS medium and were grown to OD 0.3 and then phenyl acetylene (0.05% v/v) and 10 ppm of hexavalent chromium were added.

6.3.4 Effect of copper concentrations on *Mm. koyamae* SHU1

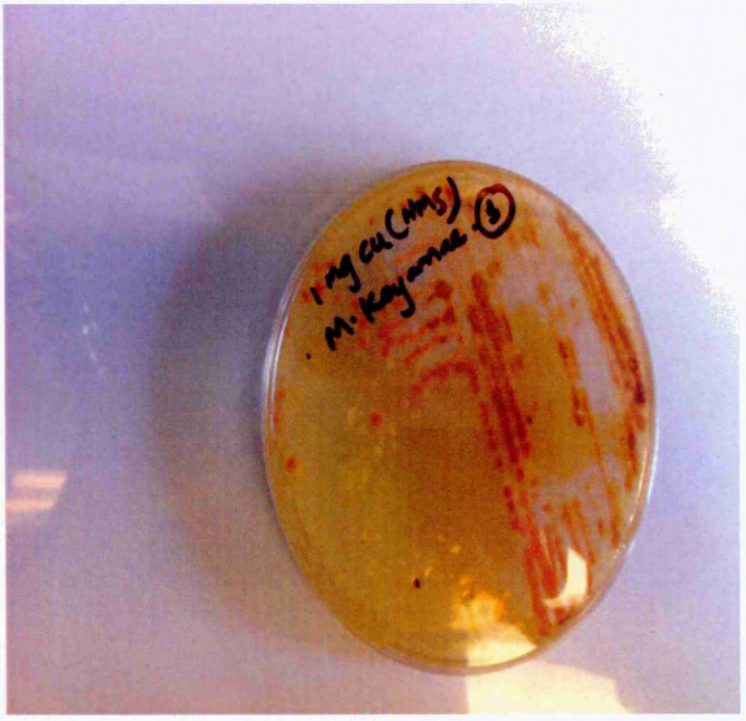
The *Mm. koyame* SHU1 was cultivated on fresh NMS plates with the various copper concentrations of 100 mg L^{-1}, 1mg L^{-1} and 0.1 mg L^{-1} and after the growth of organism on the plates the naphthalene oxidation assay was carried out to determine the possible expression of sMMO. The plates of 100 mg L^{-1} and 1 mg L^{-1} copper showed negative results for the naphthalene assay while the plates with 0.1 mg L^{-1} copper showed a positive result with purple colouration to the reagent as shown in the figures below (Figures 6.7, 6.8 and 6.9)

Figure 6.7 Naphthalene assay for 0.1 mg L^{-1} concentration of copper for *Mm. koyamae* (SHU1)



The image (figure 6.7) shows a positive result for sMMO expression by *Mm. koyamae* SHU1 at low copper concentration.

Figure 6.8 Naphthalene assay for 1 mg/L^{-} concentration of copper for *Mm. koyamae* (SHU 1)



The image (figure 6.8) shows negative result for sMMO expression by *Mm. koyamae* SHU1 at high copper concentration.

Together these two results suggest that in this organism *Mm. koyamae* SHU1 the expression of MMO's is strongly controlled by the copper concentration.

Figure 6.9 Naphthalene assay for 0.1 mg/L^{-} concentration of copper for *M. trichosporium* (OB3b)

This image (figure 6.9) confirms sMMO expression by the positive control culture *M. trichosporium* (OB3b) at low copper concentration.

6.3.5 Genome sequences of chromate reductase genes in *Methylomonas* genera

At the time of writing of this thesis the genome sequence of the *Methylomonas koyamae* JCM16701 is underway at the University of Tokyo but it is currently incomplete. In order to investigate the genes and proteins that are possibly involved in the chromium removal by *Methylomonas* strains, BLAST searches were performed to compare the sequences known to be involved in chromium removal in other microbes with available *Methylomonas* sequences, including the completed genome sequence of *Methylomonas methanica* and a number of draft genome sequences of other *Methylomonas* strains.

The literature survey has been done to identify various genes and protein sequences involved in the chromium reduction and based on the sequences BLAST searches were done to identify these proteins in *Methylomonas*. The query sequences which were used for the BLAST searches were identified from the bacteria which are known to be involved in either uptake of Cr (VI) or enzymatic reduction of Cr (VI). Based on the hits E values less than or equal to 1 have been reported in this thesis.

A BLAST search of *Methylomonas* sequences was performed with the chromate transport and sulphate uptake protein SrpC gene of *Synechococcus elongatus* (Accession number Q55027 and locus SRPC_SYNE7) which is known to be involved in the transport of Cr (VI) oxy anions into cells. The results are represented as follows (Table 6.1)

Table 6.1 *Methylomonas* proteins similar to the chromate transport and sulphate uptake protein Srp C

Type of Protein	E-value	% Identity	Accession number	% Similarity
Chromate transporter *Methylomonas* sp FJG1	1e-41	29%	WP 036278748.1	46%
Chromate transporter *Methylomonas* sp 11b	2e-41	30%	WP026603374.1	47%
Chromate transporter *Methylomonas* sp MK1	6e-41	30%	WP 020483265.1	46%
Chromate transporter *Methylomonas* sp MK1	2e-37	29%	WP 020485852.1	45%
Chromate transporter *Methylomonas* sp LW 13	6e-29	40%	WP 033158674.1	59%

A number of sequences were identified from *Methylomonas* strains with the highly significant similarity to the chromate transporter and sulphate uptake protein SrpC from *S. elongatus*. This indicates genes with potential to enable Cr (VI) uptake are widespread within the genus *Methylomonas*.

A BLAST search of *Methylomonas* sequences was performed using an example of the flavo reductases namely the flavin/Fe_2S_2 oxido reductase protein Fre of *Escherichia coli* (Accession number M74448 and locus AAA91058) which is known to be involved in Cr (VI) reduction. The results are represented as follows (Table 6.2).

Table 6.2 *Methylomonas* proteins similar to the Flavin oxido reductase Fre of *E. coli*

Type of Protein	E-value	% Identity	Accession number	Similarity
Oxido reductase *Methylomonas methanica*	8e-13	25%	WP013819134.1	39%
Hypothetical protein *Methylomonas* sp MK1	6e-11	23%	WP 020483906.1	45%
Methane monooxygenase *Methylomonas* sp LW13	5e-10	23%	WP 033157500.1	45%
Methane monooxygenase Methylomonas sp 11b	5e-10	23%	WP 026602862.1	44%
Methane monooxygenase *Methylomonas methanica*	4e-09	23%	WP 013818326.1	44%
Soluble methane monooxygenase reductase (MMOR) *Methylomonas* sp KSPIII	5e-09	23%	BAA84756.1	45%
Oxygenase *Methylomonas* sp MK1	3e-07	23%	WP 020485016.1	43%

Type of Protein	E-value	% Identity	Accession number	Similarity
Na (+)-translocating NADH quinine reductase subunit F *Methylomonas methanica*	5e-07	25%	WP013819247.1	47%
Oxygenase *Methylomonas* sp FJG1	6e-07	22%	WP036279265.1	43%
Na (+)-translocating NADH quinine reductase subunit F *Methylomonas* sp FJG1	4e-06	24%	WP 036277821.1	47%
Multispecies Na (+)- translocating NADH quinine reductase subunit F *Methylomonas*	7e-06	24%	WP 020481961.1	46%
Oxygenase *Methylomonas* sp LW 13	8e-06	23%	WP 033155708.1	43%
Na (+)-translocating NADH quinine reductase subunit F *Methylomonas* sp LW13	9E-06	24%	WP 033159347.1	46%
Oxygenase	1e-05	21%	WP 013819792.1	40%

Type of Protein	E-value	% Identity	Accession number	Similarity
Methylomonas methanica				
Pyridoxamine 5' phosphate oxidase *Methylomonas* sp LW 13	5e-04	25%	WP 033159429.1	46%
Hypothetical protein Methylomonas sp MK 1	0.066	32%	WP 020482799.1	54%
Pyridoxamine 5' phosphate oxidase *Methylomonas* sp FJG1	0.41	31%	WP 036272407.1	52%

A number of sequences were identified from *Methylomonas* strains with the highly significant similarity to the flavin reductase protein Fre from *E. coli*. This indicates genes encoding protein with potential to enable Cr (VI) reduction are widespread within the genus *Methylomonas*.

A BLAST search of *Methylomonas* sequences was performed with the nitro reductase protein NfsA gene of *E coli* (Accession number YP_ 007556383) which is also known to be involved in Cr (VI) reduction. The results are represented as follows (Table 6.3)

Table 6.3 *Methylomonas* proteins similar to the nitro reductase NfsA which catalyses chromate reduction

Type of Protein	E-Value	%Identity	Accession number	Similarity
cob(II)yrinic acid a,c diamide reductase Methylomonas spFJG1	5e-08	24%	WP036275280.1	41%
cob(II)yrinic acid a,c diamide reductase Methylomonas methanica	1e-07	26%	WP013816958.1	41%
Cob(II)yrinic acid a,c diamide reductase Methylomonas sp LW13	2e-07	24%	WP 033157530.1	41%
Cob(II)yrinic acid a,c diamide reductase Methylomonas sp 11b	1e-06	24%	WP 036277672.1	41%
Cob(II)yrinic acid a,c diamide reductase Methylomonas sp MK1	1e-05	24%	WP 026147064.1	40%
drg A Methylomonas methanica	1e-04	23%	WP 01381799.1	43%

A number of sequences were identified from *Methylomonas* strains with the highly significant similarity to the nitro reductase protein NfsA from *E. coli*. This indicates genes encoding protein similar to NfsA with potential to enable Cr (VI) reduction are widespread within the genus *Methylomonas*.

A BLAST search of *Methylomonas* sequences was performed with the old yellow enzyme chromate reductase from *Thermus scotoductus* (Accession number CAP 16804) which represents another class of enzymes known to be involved in Cr (VI) reduction. The results are represented as follows (Table 6.4)

Table 6.4 *Methylomonas* proteins similar to the old yellow enzyme of chromate reductases

Type of Protein	E value	%Identity	Accession number	Similarity
Oxido reductase *Methylomonas* sp LW13	2e-119	51%	WP 033157034.1	65%
hypothetical protein *Methylomonas* sp MK1	1e-118	53%	WP 020483038.1	66%
Oxido reductase *Methylomonas* sp FJG1	2e-117	51%	WP 036275863.1	65%
Oxido reductase *Methylomonas* sp 11b	5e-114	50%	WP 026601964.1	65%
NADH flavin oxido reductase *Methylomonas* sp FJG1	5e-30	35%	WP 036276325.1	52%
NADH flavin oxido reductase *Methylomonas* sp	1e-29	35%	WP 020484195.1	52%

Type of Protein	E value	%Identity	Accession number	Similarity
MK1				
NADH flavin oxido reductase *Methylomonas* sp 11b	3e-29	35%	WP 026602636.1	53%
NADH flavin oxido reductase *Methylomonas* sp LW13	5e-29	35%	WP 033156900.1	52%
NADH flavin oxido reductase *Methylomonas methanica*	5e-29	29%	WP 013816803.1	44%
N-ethylmalemide reductases *Methylomonas methanica*	8e-28	33%	WP 013818191.1	52%
NADH flavin oxido reductase *Methylomonas* sp MK1	2e-26	28%	WP 033193894.1	44%
NADH flavin oxido reductase *Methylomonas methanica*	8e-26	33%	WP 013818206.1	45%
Hypothetical protein *Methylomonas* sp LW 13	0.18	28%	WP 033155511.1	42%
Hypothetical protein *Methylomonas* sp FJG1	1.0	32%	WP 036274026.1	40%

A number of sequences were identified from *Methylomonas* strains with the highly significant similarity to the old yellow enzyme type chromate reductases protein from *T. scotoductus*. This indicates genes with potential to enable Cr (VI) reduction are widespread within the genus *Methylomonas*. The BLAST search also identified two *Methylomonas* proteins which are annotated as hypothetical proteins in the data base with E values which are greater than 0.1 and may have similar function and structure to the nitro reductase protein, but the relatively high E values indicate that this conclusion can only be made tentatively in these cases.

A BLAST search of *Methylomonas* sequences was performed with the chromate reductase from *Pseudomonas putida* ChrR (Accession number AAK56852) which is known to be involved in Cr (VI) reduction. The results are represented as follows (Table 6.5)

Table 6.5 *Methylomonas* proteins similar to chromate reductase ChrR

Type of Protein	E-value	%Identity	Accession number	Similarity
FMN reductase *Methylomonas* sp FJG1	7e-19	35%	WP 036273967.1	56%
FMN reductase *Methylomonas* sp LW 13	2e-18	36%	WP 033158735.1	54%
FMN reductase *Methylomonas* sp 11b	8e-13	37%	WP 036276266.1	59%
Hypothetical protein *Methylomonas* sp MK1	0.25	30%	WP 020481640.1	42%
NAD (P)H quinone oxido reductase *Methylomonas methanica*	0.58	23%	WP 013816938.1	42%
NAD (P)H quinone oxido reductase *Methylomonas* SP 11b	0.97	19%	WP 026602733.1	39%
Hypothetical protein *Methylomonas* sp FJG1	0.97	29%	WP 036272568.1	42%

Three sequences were identified from *Methylomonas* strains with the highly significant similarity to the chromate reductase protein from *P. putida.* This indicates genes with potential to enable Cr (VI) reduction are widespread within the genus *Methylomonas*. The BLAST search also identified four *Methylomonas* proteins in the data base with E values which are greater than 0.1 and may have similar function and structure to the Chr R protein, but the relatively high E values indicate that this conclusion can only be made tentatively in these cases. It is also interesting that two of these are annotated as reductase proteins in the data base.

A BLAST search of *Methylomonas* sequences was performed with the chromate efflux system from *Pseudomonas aeruginosa* ChrA (Accession number AAA88432) which is known to be involved in Cr (VI) reduction. The results are represented as follows (Table 6.6)

Table 6.6 *Methylomonas* proteins similar to chromate reductase ChrA

Type of Protein	E-value	%Identity	Accession number	Similarity
Chromate transporter *Methylomonas* sp FJG1	1e-43	33%	WP 036278748.1	47%
Chromate transporter *Methylomonas* sp MK1	3e-42	33%	WP 020485852.1	47%
Chromate transporter *Methylomonas* sp 11b	3e-25	37%	WP 026603374.1	58%
Chromate transporter *Methylomonas* sp MK1	1e-24	37%	WP 020483265.1	58%
Chromate transporter *Methylomonas* sp LW13	2e-24	34%	WP 033158674.1	57%

A number of sequences were identified from *Methylomonas* strains with the highly significant similarity to the chromate efflux systems protein from *P. aeruginosa.* This indicates genes with potential to enable Cr (VI) resistance due to chromium (VI) efflux are widespread within the genus *Methylomonas*. The BLAST search indicates the species of *Methylomonas* have shown the presence of potential chromate efflux systems which would be

expected to confer the resistance to Cr (VI). Nonetheless the strain used in the study did not show resistance to Cr (VI) but facilitated removal of Cr (VI).

6.4 Discussion and conclusions

Methanotrophic bacteria are considered as suitable organisms for bioremediation of organic pollutants because of their co-metabolic transformation of such compounds, possibility of complete compound degradation without the formation of toxic metabolites, broad spectrum of compounds availability and widely available and inexpensive growth substrate (Sullvian et al., 1998). The methane monooxygenase enzyme (MMO) also plays a major role in the bioremediation of several organic compounds making methanotrophic bacteria as suitable organisms for bioremediation of several compounds (Marco et al., 2004).

The possibly shorter lag phase of *Methylomonas koyamae* SHU1 compared with *M. trichosporium* OB3b could be another distinguishing characteristic to employ them in bioremediation. These organisms could serve two purposes 1. Use of methane as sole source of energy and carbon, thereby able to reduce methane gas emissions in the natural environment 2. Removal of hexavalent chromium for bioremediation purposes.

Metals play an important role in the life processes of microorganisms. Many metals like Ca, Co, Cr, Cu, K, Mg, Zn and Na are required nutrients and play essential role in metabolic activities. Typically presence of these metals and other nutrients at polluted sites select microbes resistant to these pollutants and such strains may be applicable in the bioremediation of pollutants (Srivastava et al., 2008).

Microbes when encountered with heavy metals will utilise them in one or more of the following ways 1. Utilise trace amounts of metals for metabolic activities 2. Tolerate the metal ions to threshold limit 3. Detoxify the metal spp. 4. Offer resistance to toxic levels.

The uptake of heavy metals in the environment by microorganisms is mainly by the following mechanisms, biosorption, bioaccumulation, and efflux and chemical transformation such as reduction or precipitation. Chromium reduction has been reported in several species of bacteria, fungi, yeasts and some actinomycetes to date (Sandana Mala et al., 2014). The different population of microbes present in different habitats have different capabilities to reduce the hexavalent chromium and transform other heavy metal pollutants.

The reduction of hexavalent chromium to trivalent chromium is of renewed interest in research field. The discovery of chromium reducing microbe in 1970's (Romaneko & Korenkov 1977) was followed by Several Cr (VI) resistant species belonging to *Pseudomonas, Ochrobactrum, Bacillus, Shewenella, Arthobacter, Lysinibacillus* and *Cupriavidus* have been isolated in recent years. (Pei et al., 2009). These microbes have demonstrated their ability to survive in the presence of hexavalent chromium by acquiring resistance to chromium (VI) by exposure to chromium (VI) or by participating in the detoxification process. Cr (VI) reduction can be co-metabolic or could be predominantly disimilatory or respiratory under anaerobic conditions (Pei et al., 2009). Cr (VI) reduction can either be plasmid borne as in *Pseudomonas* species or located on chromosomal DNA as in *Bacilli* and *Enterobacteriaceae* (Pei et al., 2009).

Microorganisms that have the ability to reduce Cr (VI) are usually called chromium reducing bacteria (CRB), Among CRB, Gram positive bacteria have high tolerance to Cr (VI) concentrations compared to Gram negative bacteria. (Coleman 1988). Bacteria employ different resistance mechanisms to overcome chromium toxicity in the environment which include the reduced uptake of Cr(VI), extracellular Cr (VI) reduction, detoxification of reactive oxygen species (ROS), detoxifying enzymes/intracellular Cr(VI) reduction, DNA repair enzymes and efflux of hexavalent chromium from the cell (Thatoi et al., 2014).

The *chrR* gene located on chromosome of *P. aeuginosa* conferred resistance to chromate (Aguilar-Barajaset al 2008). Morais et al. 2011 reported *Ochrobactrum tritici* contains *chrB, chrA, chrC and chrF* in chromosomal DNA of which *chrB* and *chrA* are essential for chromium resistance in sensitive bacteria. Genetic studies show *ruvR* gene of *O. tritici* is related to chromium resistance. Genetic analyses of chromate resistant *P. aeruginosa* (Cervantes et al 1990) and *Alcaligenes eutrophus* (Nies et al 1990) has shown that the Cr reduction is mediated by plasmid. Genes for hydrophobic polypeptide, *chrA,* were identified in chromate resistance plasmids of both *P. aeruginosa* and *A. eutrophus*.

Aerobic Cr (VI) reduction is usually associated with soluble proteins and require NAD (P) H as an electron donor (Shen and Wang 1993). Cr (VI) reducing activity in aerobes like *Pseudomonas ambigua*, *P. putida, Escherichia coli* and *Bacillus coagulans* have been found in the soluble fraction of cells. In *B. subtilis* the reduction is mediated by cell free extracts of bacteria. (Thatoi et al. 2014)

NADH was electron donor in the reduction of chromate by soluble enzyme present in cytoplasm of *Pseudomonas* sp. CRB5. NADH/NADPH served as electron donors for Cr (VI) reduction by soluble enzymes present in *P. ambigua*, *P. putida* and *Vigribacillus* sp. *P. maltophila* O-2 and *Bacillus megaterium* TKW3 were found to utilise membrane associated reductases for Cr (VI) reduction in spite of being aerobes (Thatoi et al 2014).

Anaerobic chromium reduction is associated with membrane bound reductases such as flavin reductases, cytochromes and hydrogenases that are part of electron transport systems and use chromate as electron acceptor (Thatoi et al 2014). *Pseudomonas dechromaticans* isolated from sewage sludge under anaerobic chromium reduction use Cr (VI) as electron acceptor. *Enterobacter cloacae* isolated from industrial waste water in periplasmic space uses membrane bound hydrogenase or reduced cytochrome (Wang et al. 1991).

In *Shewanella putrefaciens* MR-1 chromate reduction was associated with cytoplasmic membrane where formate and NADH served as electron donors. (Park et al 2000). Membrane associated reductase have been reported in Cr(VI) reduction in some bacteria where H_2 was an electron donor and Cr(VI) was an electron acceptor in electron transport chain (Thatoi et al 2014). The reduction of Cr (VI) in *Desulfovibrio vulgaris* has been shown by cytochrome c3 which is anaerobic bacterium.

In the present study it has been shown that the removal of hexavalent chromium by *Mm. koyamae SHU1* is likely to be enzyme mediated as when a metabolic inhibitor was added to the cultures the reduction was not carried out and when cells were heat killed the reduction was inhibited, strongly suggesting reduction is carried out enzymatically and it is an active cellular reaction rather than the reaction between cellular constituents and chromate. The chromium reduction carried out by *Mc. capsulatus* Bath showed that it can reduce hexavalent chromium from 1.4 to 1000 mg L^{-1} and inhibition of organism by sodium azide caused loss of 57% of chromium removal. It can be shown that the inhibition by metabolic indicator effects the organism as cytochrome oxidizers are affected and there by affecting the metabolism of organism. The effect of azide is presumably an indirect one since inhibition by azide causes reduction of dioxygen to water and thus prevent channelling of reducing equivalents into reduction of chromate (Hasin et al 2010). In the case of *Mm. koyamae* (SHU 1) studied in this thesis the sodium azide may be functioning in the same way as proposed for *Mc. capsulatus* Bath to remove the electrons supply from a reductase enzyme. Since trivalent chromium has not yet been detected as the product of hexavalent chromium transformation in *Mm. koyamae*

(SHU1), it is also possible that sodium azide exerts its effect by removing the source of the energy for active uptake of hexavalent chromium.

Cr (VI) transport across the cell membranes can occur via the sulphate uptake pathway because sulphate and chromate are two oxy anions with similar structure (Ramirez-Diaz et al., 2008). The Cr (III) ion transport into cells is quite slow because of its insolubility and slow rate of ligand exchange. However the complexes of Cr (III) can be easily transported with siderophores, which are iron uptake mediators. Siderophores are structurally diverse group of biogenic chelating agents associated with uptake of iron and other metals. These siderophores bind to a wide range of metal ions, solubilize metal complexes and enhance mobility of metal ions in the environment, including toxic heavy metals (Duckworth et al., 2014).

Chromate accumulation has been reported in *P. aeruginosa* and *A. eutrophus* via the sulphate transport pathway. Microbes such as *E. coli* and *Salmonella typhimurium* possess the ATP binding cassette (ABC) type sulphate- thiosulphate transport systems which is regulated in parallel with cysteine biosynthetic enzymes and is part of the cysteine regulon. The components of sulphate-thiosulphate permease from *E.coli* and *Salmonella typhimurium* are largely encoded by a cluster of genes. The sulphate transport pathway in *E. coli* is encoded by the cluster of genes *cysP, cysT, cysW* and *cysA* and the unlinked gene *sbp*. The products of *cysT* and *cysW* span the membrane and form a channel for the passage of sulphate and related ions; *cysA* encodes an associated hydrophilic membrane binding ATP protein, while *sbp* and *cysP* encode the sulphate and thiosulphate periplasmic binding proteins respectively (Marcia & Diego 2000).

Heat killing is one of the most efficient methods for inactivating the enzymes in bacteria. Optimum temperature is one of the criteria and plays an important role in the growth of bacteria and reduction of Cr (VI). Variations in temperature affect viability of cells and ultimately leading to death. At low temperatures the fluidity of membranes decreases and thereby affecting the functioning of transport systems thus as a result the substrates do not enter the cell to support the low growth rate of cells. The increase in temperatures leads to thermal denaturation of proteins which is irreversible. As the removal of chromium is almost certainly enzyme mediated loss of denatured protein will result in loss of chromium removal function, altering membrane function, inactivation of protein synthesizing mechanism due to alteration in ribosome conformation (Narayani & Shetty 2013). As discussed earlier in this

section the key protein in chromium removal by *Mm. koyamae* (SHU1) that is inactivated by heating could be a reductase or a transmembrane pump that actively imports chromium into the cells.

The optimum temperature 30^0C for chromium reduction is found in all the following species *Pseudomonas fluorescens* LB300, *Microbacterium* Sp. NCIMB 13776, *Streptomyces* sp. MC1, *Brevibacterium casei*, *Arthobacter crystallopoites, Thiobacillus thioparus, Bacillus* sp. and *Pseudomonas plecoglossicida* (30^0-37^0C) (Naryani & Shetty 2013).

In all methanotrophic organisms' pMMO or sMMO requires reducing equivalents for its activity. Methanotrophs are known to generate NADH from oxidation of formaldehyde and formate which provides the reducing equivalents for pMMO and sMMO. The fact that the phenyl acetylene inhibits chromium removal by *Mm. koyamae* SHU1 expressing either form of MMO may indicate a direct involvement of the MMO enzyme in Chromium (VI) removal but more likely is due to general inhibition of energy requiring processes within the cell when it can no longer metabolise its carbon and energy sources.

Oxidation of methane to methanol is the carried out by the enzyme methane monooxygenase enzyme (MMO). Two distinct forms of MMO have been characterised, cytoplasmic and soluble forms of methane monooxygenase (sMMO) and membrane bound or particulate methane monooxygenase (pMMO). These two distinct MMOs have different kinetics for methane oxidation with sMMO having higher turnover but lower affinity than pMMO. (Lee et al 2006). Several strains of methanotrophs including *Methylococcus capsulatus* (Bath) are known to express two forms of enzymes pMMO or sMMO. At higher copper levels (4 μM) pMMO is expressed whereas at low copper levels (< 0.8 μM) sMMO is expressed. The α and γ proteobacteria have been found to synthesize and excrete a chalkophore (a siderophore like molecule) known as methanobactin which can increase the bioavailability for copper and plays an important role in copper switch that controls expression of two forms of MMO (Semrau et al 2013).

The sMMO activity has been observed in small number of strains of type I methanotrophs including *Methylomonas*. The two isolates from the genus *Methylomonas* that have been shown to express the two forms of MMO are *M. methanica* 68-1 and *Methylomonas* sp. strain GYJ3 (Fru 2011).

Mm. koyamae SHU1 is likely to be useful in bioremediation of both hydrocarbons and Cr (VI) due to its possession of sMMO and a chromium(VI) removal activity.

Since the genome sequence for *Methylomonas koyamae* was not available, BLAST searches were done to search the other available genome sequences of *Methylomonas* species. The BLAST search for proteins potentially involved in chromium reduction, Cr (VI) uptake and chromium efflux systems were performed with all available *Methylomonas* sequences. The results demonstrate the potential presence of all these functions associated with Cr (VI) removal and resistance in the genus *Methylomonas*. This is consistent with the ability of *Methylomonas koyamae* SHU1 to remove Cr (VI). Also the presence of Cr (VI) resistance genes suggests that other *Methylomonas* strains, unlike *Mm. koyamae* SHU1, may be resistant to hexavalent chromium. The laboratory results and the BLAST search results suggest that the chromium resistance and chromium reduction genes are widespread among the genus *Methylomonas* in the environment. It could be possible that a diversity of strains from this genus can be used in the bioremediation of chromium (VI).

CHAPTER 7

DISCUSSION AND CONCLUSIONS

7. Discussions and Conclusions

7.1 General conclusions

In the present study methanotrophs have been isolated from the sediment samples of the River Sheaf passing along Millhouses Park in Sheffield, UK. The site selected for the study has a past industrial history alongside the steel manufacturing plants, and water power mills which contributed to a significant pollution and as the pollutants are non-biodegradable in nature they have been persistent in the environment for many years. Thus it may be advantageous to remediate the pollutants by using microorganisms rather than any other physical and chemical technique. Microbes can help in the detoxification process, where heavy metals are converted to a less toxic state and microbiological methods may be more economical than chemical alternatives.

The Sheaf sediments were collected and the sediment samples were fractionated on the basis of particle sizes to analyse the concentration of pollutants in each particle size fraction of the sediments. The concentration of lead was higher in the samples than any other heavy metals probably because of the past industrial history. The other heavy metals found in the study area are chromium, cobalt and nickel. These heavy metals can be attributed due to development of steel industries in the Sheffield city.

The sediment samples collected from the River Sheaf were enriched for the methanotrophs and the methanotroph obtained was identified based on the sequencing of 16S rRNA gene as *Methylomonas koyamae* SHU1 and the organism was employed in the removal of chromium. It was found that the organism can reduce the concentration of chromium in the range of 2-10 ppm.

It was found that *Mm koyamae* SHU1 could remove hexavalent chromium although the organism was not resistant at the concentrations tested. The removal of hexavalent chromium was carried out in the presence of sodium azide a metabolic inhibitor and in the presence of heat killed cells to ensure that the reaction is metabolically carried by the enzymes within the cells.

The removal of chromium by *Mm. koyamae* SHU1 was not observed in the heat killed cells and also in the cells where sodium azide was added indicating that the removal of Cr (VI) is

an active metabolic process and depends on the cellular constituents rather than an environmental mediated reaction.

The enzyme methane monooxygenase was inhibited with phenyl acetylene and there was no removal observed probably indicating that the organism requires reducing equivalents for reduction reaction and the methane oxidation pathway provides the reducing equivalents necessary for the reduction of chromium. Alternatively *Mm. koyamae* SHU1 may remove chromium (VI) in another way such as active Cr (VI) uptake rather than the chemical reduction.

The organism *Mm. koyamae* SHU1 produces a sMMO during the deficiency of copper in the medium indicating that under the low copper conditions the organism can be potentially applied for the bioremediation of several hydrocarbons and chromium (VI) reduction because of the greater substrate range of sMMO.

BLAST searches were performed to identify the chromium (VI) reduction, Cr (VI) efflux system and Cr (VI) uptake genes with the available *Methylomonas* sequences to identify the potential presence of these genes.

7.2 Discussion

The isolated organism *Mm. koyamae* SHU1 can reduce hexavalent chromium (which was seen in the previous chapter) but as the trivalent chromium has not been characterised in this study there could be the possibility that the organism can accumulate or sorb the hexavalent chromium ions from the solution rather than the complete enzymatic mediated reduction process.

7.2.1 Mechanism of chromium (VI) bioremediation

In the natural environment when the microbes interact with metals or metalloids some of these elements could be beneficial to the microbes and others harmful. Interaction of metals with microbes can lead to four mechanisms which can be employed to remediate metal contaminated environment by microbes.

The various levels of interaction of metals/metalloids with the organisms include

1. Metabolic/enzymatic- uptake of metals or metalloids in the metabolic activities of microbes. Indeed enzymes such as nitrogenase and cytochrome oxidases contain metals (Cu, Mo and Fe) that under certain circumstances can be toxic.
2. Some microbes can use certain metals or metalloids as electron donors or electron acceptors in energy metabolism. A number of Eubacteria and archaea have been researched eg. The archeon *Sulfolobus* sp. reduces MoO_4^{2-} to a lower oxidation state (Brierley and Brierley 1982) and reduction of CrO_4^{2-} to Cr (III) by the bacterium *Pseudomonas fluorescenes* LB300 form part of respiration (Wang and Shen 1995).
3. Enzymatic microbial detoxification- where the toxic metal species is converted to a less toxic or non-toxic species. The oxidation of AsO^{2-} to AsO_4^{3-} by *Alcaligenes faecalis* (Ehrlich 1997) and reduction of hexavalent chromium to trivalent chromium by *Methylococcus capsulatus* (Bath)
4. Non enzymatic processes such as accumulating metal ions on the cell surfaces either by living or dead biomass. This process can also be referred to as biosorption. The process of biosorption is detailed below.

   Biosorption is a physico-chemical process where the substances can be removed from the solution with the help of biological materials (Gadd 2009). Biosorption can be passive or metabolically independent process. The biosorbents used for the process can be dead biomass or fragments of cells and tissues. The biosorption can be performed by the live cells where the metal ions get adsorbed on to the surface of the cell walls or the outer layers (Fomina & Gadd 2014). Biosorption is an important natural phenomenon like sorption of metals and microbes in soil.

   The biosorption process involves a solid phase (biosorbent) and liquid phase (solvent: water) or some dissolved or suspended ions to be sorbed (sorbate). A wide range of biosorbates can be removed from the solutions. These include metals, particulates, and colloids, inorganic and organic compounds like dyes, flouride, pthalates and pharmaceuticals (Fomina & Gadd 2014).

The mechanisms involved in the biosorption process include adsorption, ion exchange and complexation/coordination. In the case of biological materials the functional groups present in cell wall structure interact with metal species like carboxyl, phosphate, hydroxyl, amino, thiol etc., the process of biosorption depends on system and given conditions. Precipitation and crystallisation are also possible mechanisms that can occur in the biosorption process (Gadd 2009).

The process of biosorption is influenced by various factors like pH, ionic strength of the solution, initial pollutant concentration, biosorbent nature and the availability of binding sites, agitation and temperature (Gadd 2009). Several analytical techniques have been employed to study the efficiency of the biosorption process such as atomic absorption spectrophotometry (AAS), UV-Vis spectrophotometry, Scanning or transmission electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM/TEM-EDX), X-ray diffraction (XRD) analysis, Nuclear magnetic resonance (NMR) and X-ray photoelectron spectroscopy (XPS) (Gadd 2009). In the case of xenobiotics which are extremely resistant to biodegradation these can be removed by biosorption. The dyes, phenolic compounds and pesticides have gained increasing attention to remove from waste stream by biosorption (Fomina & Gadd 2014).

A material which has affinity for absorbing various types of substrates can be used as a biosorbent. There are a wide range of biosorbents available which includes biological materials, industrial wastes and natural residues. The various biosorbents used are microbial biomass like bacteria, archaea, cyanobacteria, filamentous fungi and yeasts, and seaweeds. Industrial wastes like fermentation and food wastes, anaerobic and activated sludges are also employed as the biosorbents. Agricultural wastes such as rice straw, wheat bran, sugar beet pulp, soyabean hulls, fruit and vegetable wastes are also employed. Natural residues such as tree barks, weeds, and sphagnum peat moss sawdust and plant residues have also been employed as biosorbents (Park et al., 2010; Dhankar and Hooda, 2011). Biosorbents have been prepared from commercial products to remove various types of pollutants from solution (Gadd 2009).

Peptidoglycan carboxyl groups are the main site for binding of metal ions in Gram positive bacterial cells with phosphate groups contributing in Gram negative bacteria (Gadd 2009). Many microbes have the ability to produce extracellular polymeric substances (EPS), which constitutes polysacchrides, capsules, slimes and sheaths which is an important biosorptive component in living cell systems especially biofilms depending on associated components and polysaccharide (Comte 2008). EPS are involved in biosorption of cadmium in activated sludges (Comte 2008). EPS also adsorb or trap particulate matter such as precipitated metal sulphides and oxides (Gadd 2009).

Previously, an attempt was made to test the efficiency of metal ions removal from waste water using the biosorbents from the isolates of metal contaminated environments. Several heterotrophic, methanotrophic, sulphate reducers and algae were isolated from water and

sediment samples collected at abandoned coal mining site. One culture IGTM17 which is mixed methanotrophic culture has a binding capacity of 56 mg/g biomass making it superior to ion exchange resins. The biomass was found to exhibit good metal binding capacities at low pH.(Xie et al., 1996).

It is possible that biosorption plays a role in chromium removal by *Mm. koyamae* SHU1 it may be possible that the metal binding peptide methanobactin that methane oxidising bacteria produce is involved.

Methanobactin (mb) is a small copper binding compound or chalkophore produced in response to copper limitation, which strongly bind to copper and are found in almost all methanotrophs. These chalkophores promote copper internalisation into cells and protect the cells from copper toxicity. These chalkophores are known to display redox reactions and weak antibiotic properties and are possibly associated with pMMO (Kim & Graham 2004).

Methanobactins is a chalkophore which is similar to the siderophores which are utilised by several other organisms for collecting iron. Although mb is known to be produced in several methane oxidisers, it has not been investigated in *Mm. koyamae* SHU1. A simple plate assay helps in the identification of methanobactin production, as detailed below. Such a test could be used as a first step to determine whether *Mm. koyamae* SHU1 produces mb.

Semrau and colleagues have developed a method to detect mb on plates by modifying the previous chrome azurol S (CAS) for determining siderophores on plates. In the previous method a blue complex forms with iron and CAS in the presence of detergent hexadecyltrimethylammonium bromide (HDTMA) and the removal of iron by siderophores can be determined by colour change in medium from blue to orange as the siderophore strips iron from the CAS. In the modified method of Semrau, determination of chalkophore iron is replaced by copper; CAS has high affinity for copper as well as iron. The change in the colour from blue to yellow shows the production of chalkophore in the plates, as the chalkophore removes copper from the CAS. Four methanotrophs have been tested for chalkophore production in this way and *M. trichosporium* OB3b, *M. capsulatus* (Bath), and *Methylomicrobium album* BG8 produced chalkophores, while *Methylocystis parvus* OBBP did not produce detectable chalkophore in this plate assay (Yoon et al., 2011).

Methanobactins are known to bind to variety of the metals and play an important role in solubilisation or immobilisation of metals making methanotrophic activity a potential role in remediation of metal contaminated environment. Research findings by Choi et al 2006 using UV-visible absorption spectroscopy have demonstrated that the methanobactin from *Methylosinus trichosporium* OB3b in the absence of Cu (I) or Cu (II) binds several metals such as Ag(I), Au (III), Cd (II), Co (III), Fe (III), Hg (II), Mn (II), Ni (II), Pb (II), U(VI) and Zn (II) but not Ba (II), Cr(VI), La (III), Mg (II) and Sr (II). If the mb from *Mm. koyamae* SHU1 matches or is almost similar to mb of other methanotrophs it is unlikely that it will bind to Cr (VI).

Nonetheless, the possibility of methanobactin being involved in chromium reduction could be evaluated since the methanobactins have the redox capacity and also have been found to bind to mercury and detoxify it as shown in *Methylocystis* strain SB (Baral et al., 2014). If in future it is possible to isolate methanobactin from *Mm. koyamae* SHU1 its potential role in detoxification of heavy metals, including chromium (VI), could be investigated.

The ability of methanobactin to bind the heavy metals as shown by Choi et al 2006 could be used in bioremediation by methanotrophs and also to evaluate the role of methanobactin from methanotrophs in the biosorption of heavy metals.

The possibility that chromium (III) is produced from Cr (VI) by *Mm. koyamae* SHU1 needs to be experimentally investigated. The techniques are discussed below to measure both Cr (III) and Cr (VI). The chromium speciation in the solution can be detected with the ICP-MS (Inductively coupled plasma emission mass spectrometry) coupled with HPLC (High pressure liquid chromatography). Because these techniques provide high resolution and ease in separation and detection of ions, they are gaining importance. In the HPLC-ICP-MS technique the consistent mobile phase of HPLC carries the chromium species and they are separated according to their affinity for the mobile and stationary phases. The outflow from the HPLC is coupled to the nebuliser of the ICP-MS to quantify the chromium in the fractions as they elute (McSheehy and Martin 2006).

It is likely that the removal of hexavalent chromium by *Mm. koyamae* SHU1 is enzymatic because the addition of the sodium azide to the reaction completely inhibited the removal of Cr (VI) indicating the likely role of enzymatic activity.

The NAD (P) H reductase could play a role in chromium removal as when the enzyme methane monooxygenase is inhibited by the phenyl acetylene chromium removal was completely abolished. Alternatively, the reducing equivalents supplied from the reduction of methane to methanol by MMO enzyme may enable the reduction of hexavalent chromium by the organism using other enzyme(s).

M. capsulatus (Bath) can also play an important role in reduction of toxic mercury (II) ions to elemental mercury with the help of NADPH dependent mercuric reductase (Boden 2011). The reduction of hexavalent chromium by *M. capsulatus* Bath as shown by Ismael (2014) by NADPH reductase could be leading to possibility to *Mm. koyamae* SHU 1 could also reduce hexavalent chromium with an NADPH reductase and also be employed and tested for the detoxification of mercury (II) ions.

Many basic steps in the chemical transformation of compounds in microbes involve oxidation and reduction reactions. These usually involve important co-enzymes whose substrate metabolism and energy metabolism have been studied (Feng, Shi et al 2009) and the co-enzymes function to allow oxidation or reduction of the substrate by accepting or donating electrons.

Such redox co-enzymes include NAD and its phosphorylated derivative NADP, which can be oxidised or reduced by loss and gain of two electrons. ATP-NAD kinase utilizes ATP as sole phosphoryl donor for phosphorylation of NAD (H), While NADH kinase phosphorylates both NAD^+ and NADH to form $NADP^+$ and NADPH. The NADP and NAD (P) H are thought to be involved in the chromium reduction in methane oxidising bacteria and the determination of NAD (P) H activity in solutions can give significant background information on reduction of hexavalent chromium in the organisms and its vital role in the oxidation reduction reactions.

The possible involvement of an NAD (P) H reductase in reduction of chromium (VI) by *Mm. koyamae* SHU 1 could be investigated as follows. The cells would be grown to mid exponential phase and then the cells harvested by centrifugation, followed by the resuspension of cells in 10mM phosphate buffer and then breakage of the cells through sonication, followed by centrifugation. The cell free extracts would be stored to allow subsequent analysis of the chromium (VI)-stimulated dehydrogenase activity in the cells, via spectrophotometer assays at A_{340} nm. The reduced flavin nucleotides (NADH and $NADPH^+$)

have strong absorbance at this wavelength compared to oxidised nucleotides (NAD^+ and $NADP^+$) (Ray et al 1989).

The chromium removal observed in the present study could also be attributed to the involvement of the sulphate transport pathway. The similarities of the two oxy anions may allow chromium (VI) to permeate inside the cell membrane of the microbes. The pathway thus could take up the chromate as well as sulphate into the cells.

Bioinformatics is a range of tools which uses computer to study the biological data and process it. Various BLAST searches were performed with the available genome sequences of the *Methylomonas* genus with the chromium reductases enzymes and other proteins that could possibly be involved with chromate uptake, efflux systems and chromate reduction. Various highly significant proteins likely to be involved in the process were found in a number of *Methylomonas* species which have been clearly discussed with the results in chapter 6 under heading 6.3.5 section. The presence of these proteins in *Mm. koyamae* SHU 1 could possibly enable the application of *Methylomonas* in the bioremediation of Cr (VI).

7.2.2 Distribution of *M. koyamae* across the globe

Methanotrophs are present in many natural ecosystems consuming methane which is formed biogenically by methanogenesis or abiotically generated by seeping from ocean beds natural gas mines and coal fields. The type I methanotrophs are termed as efficient oxidisers and also used as biofilters in industrial applications. These organisms have been used in several remediation applications (Rosenberg et. al., 2014).

The genus *Methylomonas* is ubiquitous in nature, although this is only the second report of the isolation of *Mm. koyamae*. The first time this species was isolated, it was associated with the nitrogenous fertilisation in the rice field soils. The fact that *M. koyamae* has been isolated from two contrasting environments is consistent with its presence in wide range of habitats. The organism *M. koyamae* was first isolated from flood water of rice paddy field and the strain was classified as FW 12E- Y^T (NCIMB=14606). Next, in the current study, the strain was isolated from sediments of the River Sheaf, Sheffield, UK, and so it is present in at least different habitats and two different continents. Also, the strain *M. koyamae* along with four other strains R-45378, R-45383, R-49799, and R-49807 (97.9-100% 16S RNA sequence similarity) were detected at the molecular level in a culture-independent study of nitrogen metabolism in methane oxidising bacteria in Belgium (Hoefman et al., 2014).

Since *M. koyamae* has been isolated from two contrasting environments across two environments across two continents and also identified in a culture independent molecular study, it is possible that this species is widely distributed across the globe. It is also reasonable to draw the conclusion that *Methylomonas* are widespread and their ability to use in the bioremediation and other studies along with several other methanotrophs contribute a great significance in the upcoming research field which is described in detail in 1.4.5 of the literature review

7.2.3 Significance of results

Researchers have tried experimenting with many bacteria, fungal and algal species to bioremediate hexavalent chromium and found several organisms which are able to effectively remediate hexavalent chromium to the less toxic trivalent state in the environment by employing techniques such as enzymatic reduction, biosorption, bioaccumulation by sulphate transport pathway with low operational and chemical costs.

In a similar way researchers have been exploiting methanotrophs and studying the application of the organisms in remediation of hexavalent chromium, which is a growing trend in the field of biotechnology. If we can cultivate such organisms as *Mm. koyamae* SHU1 or other Cr (VI) reducers in bioreactors and employ them on large scale for cleaning up the contaminants it would be a considerable success in biotechnological industry.

7.3 Recommendations for future work

- The above the removal of hexavalent chromium by *Mm. koyamae* SHU1 was observed by diphenylcarbazide assay. These reduction reactions can also be characterised by applying TEM transmission electron microscopy) and EXFAS (Extended X ray absorption fine structure). The speciation of chromium during microbial reduction can also be employed with these techniques.
- The organism could be employed for reduction of other potential heavy metal pollutants or detoxification of lead, mercury and arsenic. Biosorption by methanotrophs could also be employed to remove the toxic metals from the environment.

- Organisms which can reduce hexavalent chromium also have potentiality to remediate several organic compounds. The organisms can be employed in the bioremediation of the organic pollutants with or without simultaneous chromium (VI) contamination.
- The methanobactin from the isolated organism *Mm. koyamae* SHU1 should be tested for binding of other metal ions which may be one efficient way to detoxify the heavy metals by this compound.

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