CHAPTER 1

Literature Review

Introduction

Bioremediation is an increasingly favoured method of treating industrial wastes as microorganisms present a unique and safer method for remediation of hazardous materials in the environment compared to chemical means. Bioremediation processes are also often cost effective as these strategies utilise innate metabolic functions of microorganisms. In addition, microorganisms are able to degrade multiple hazardous and complex pollutants much more safely than treatment by chemical means (Hanson, 1999). For example, certain microorganisms are able to utilise hazardous pollutants such as methane for the generation of ATP and energy compounds in their respiratory pathways (Kightley *et al.* 1995 and Hanson, 1999).

Methane in the natural environment

Methane (CH_4) is the most abundant organic gas in the atmosphere (Hanson and Hanson, 1996 and Hilger *et al.* 2000) commonly released from a variety of environments such as soils, wetlands and rice paddies and to a lesser extent from oceans, lakes and deserts (Hanson and Hanson, 1996). CH_4 is an important greenhouse gas as it absorbs infrared radiation more actively than the other greenhouse gas, carbon dioxide (CO_2). Due to this characteristic, CH_4 reportedly contributes to global warming at a rate of at least 26 times more than CO_2, once it re-emits radiation back into the atmosphere (Bowman *et al.* 1997 and Hanson and Hanson, 1996). CH_4 is classified as a 'long-lived' gas as it has a long residence time in the atmosphere enabling it to efficiently release the absorbed radiation into the atmosphere over a longer period of time (Reinhart *et al.* 1996). Its contribution to

global warming is estimated to be at 12% of the enhanced rate of global warming, with a rate of 500 – 700 Tg (1 Tg = 1 million tons) per year globally (De Visscher *et al.* 1999).

Landfills and bioremediation

A landfill is a disposal area for domestic waste / municipal solid waste (MSW) consisting of a basal liner, the waste body and cover layers above this waste layer (Chapter1, Figures 1.1 and 1.2). MSW's are rich in materials such as cellulose, lignin, hemicellulose, protein, starch and sugars (Crecchio *et al*. 2001; Liwarska-Bizukojc *et al.* 2001 and Rohrs *et al*. 2002)**.** Once degraded, MSW produces compost rich in organic compounds as well as organic and inorganic nitrogen (Crecchio *et al.* 2001 and Liwarska-Bizukojc *et al*. 2001). Atmospheric CH_4 generated from landfills is due to anaerobic degradation of the organic wastes by anaerobic methanogenic bacteria (Lay and Noike, 1998) with other by-products being CO_2, H_2, O_2 and water. Anaerobic degradation processes occur under optimum conditions of neutral pH and mesophilic (30 °C – 40 °C) temperatures (Yuen *et al.* 2001; Yu and Fang, 2003). Other important factors for efficient biodegradation are the use of shredded waste, moist conditions as well as nutrient rich wastes that are compacted maximally while still allowing the efficient flow of water through the voids present in the waste (Yuen *et al*. 2001). The use of shredded domestic waste increases the surface area for maximum biodegradation to occur.

The gas composition of a landfill is reportedly 60 %: 40 % (vol./vol.) of CH_4 and CO_2 (Cummings and Stewart, 1995). CH_4 concentrations in the atmosphere are reportedly increasing at a rate of 1 % per year and landfill emissions are thought to contribute 20 to

70 Tg of CH_4 globally (De Visscher *et al.* 1999) and to constitute the fourth largest contributor (Kightley *et al.* 1995). CH_4 emission from soil is the net result of CH_4 production and subsequent oxidation and is formed under low redox potential conditions by methanogens via CO_2 reduction or transmethylation conditions. It has been suggested that landfill methanotrophic bacteria can efficiently consume 80 % - 90 % of this generated CH_4 (Dunfield and Knowles, 1995; Murrell *et al*. 2000).

i) The five phases of waste degradation in landfills

There are five phases in the process of landfill waste degradation (Figure 1.3). Biodegradation processes in each phase are carried out by naturally occurring bacteria in the wastes and the environment. Phase I involves the hydrolysis of organic compounds into simple sugars by aerobic microorganisms (Swarbrick, 2001). This degradation process requires water and oxygen with glucose as the primary product. Initially, an adequate oxygen concentration in the MSW fraction facilitates chemical oxidation reactions for aerobic microorganisms initiating the MSW degradation process (Cummings and Stewart, 1995). However, potentially toxic compounds are reportedly also formed from the oxidation of nitrogen and sulphur, such as, nitrates, sulphates and chlorinated organic compounds that can be dissolved and may percolate in the leachate to contaminate South African groundwater bodies, possibly eventually resulting in eutrophication of surface waters (Hanson and Hanson, 1996). This phase is also responsible for the rise in temperature of the landfill, primarily due to the high levels of energy generation (Dunfield and Knowles, 1995; Kightley *et al.* 1995; Murrell *et al.* 2000).

Phase II is characterised by acidogenesis. Acid producing bacteria ferment glucose and form long chain organic acids that decrease the pH to acidic levels (pH <6) (Lay and Noike, 1998) with propionate, butyrate, caproate, valerate, heptoate, complex alcohols and acids being the end products (Swarbrick, 2001). The formation of these long chain organic acids increases the concentration of the organic compounds leading to higher levels of chemical oxygen demand (COD) and biochemical oxygen demand (BOD) in the leachate (Swarbrick, 2001). Shorter chain organic acids are rendered volatile and form odorous compounds at the landfill surface. As the oxidation reactions occur, the oxygen supply is depleted, leading to an anaerobic environment. There is an overall increase in CO_2 levels emitted from the landfill and hydrogen formation is initiated by acetogens (Swarbrick, 2001).

Acetogenesis occurs in phase III where acetogenic bacteria form acetate from the degradation of organic acids e.g. from butyrate. The pH decreases further to acidic levels (minimum pH 5) (Lay and Noike, 1998) and more hydrogen is produced. However, this hydrogen has a negative impact on the acetogens as high concentrations inhibit acetogenesis. The hydrogen formed here also serves as a substrate for methanogenesis in the fourth phase (Swarbrick, 2001).

In phase IV, methanogenic degradation dominates. CH_4 is produced from the MSW fraction by methanogens, either by using acetate as the substrate or by the combination of hydrogen and CO_2 (Cummings and Stewart, 1995). Methanogens utilising acetate as the substrate have a slower growth rate due to lower energy levels reportedly produced

during this pathway. By contrast, those methanogens that utilize hydrogen for methanogenesis have a faster growth rate than the acetate utilizing methanogens (Cummings and Stewart, 1995 and Gurijala *et al*. 1997). CH_4 generation from the MSW fraction into the atmosphere increases as the organic acids are utilised. Hereafter, the pH rises to neutrality due to the increased utilisation of hydrogen, and levels of COD and BOD gradually decrease (Swarbrick, 2001). Phase III is then repeated as a close symbiotic relationship exists between the methanogens and acetogens. In addition, the production of CH_4 is usually equal to the formation of hydrogen and acetate (Cummings and Stewart, 1995 and Lay and Noike, 1998).

The final stage is phase V. Anaerobic degradation is complete and CH_4 or CO_2 production ceases and the landfill is regarded as being stabilised. Air is thought to diffuse back into the landfill, as there is no more CH_4 emissions to cause its displacement (Farquhar and Rovers, 1973 and Swarbrick, 2001). Stabilised South African landfills are usually later used for parks and recreational areas.

ii) Waste settlement in landfills

An important factor in municipal landfills is that of refuse settlement (Wall and Zeiss, 1995) where three compression phases occur. The first phase involves initial compression where an external load is mechanically applied to the MSW by a roller so as to decrease the height of the deposited wastes, causing an immediate compaction of void spaces in the MSW fraction (Ueberbach, 2002). Primary compression is the second phase and occurs when compaction results in the removal of water and gas in these void spaces.

This removal inhibits methanogenesis as water and gases are prevented from reaching the methanogens. Primary compression generally occurs within the first 30 days of waste deposition in a landfill after which secondary compression occurs, the third phase of compression. Secondary compression occurs due to biological decay and 'creep' of the degraded refuses. "Creep" occurs when degraded refuse falls back on itself and further reduces voids in the decomposed layer. This phase generally takes years to complete (Wall and Zeiss, 1995) and is important during methanogenesis, which has an onset of a few months in a warm moist environment (Wall and Zeiss, 1995). Methanogenesis may be inhibited, as substrates are restricted from reaching bacterial cells due to creep.

iii) Environmental factors affecting methanogenesis

Biodegradation of organic components of wastes is a highly controlled and sensitive process involving acidogenic and methanogenic bacteria requiring a co-ordinated procedure for efficient degradation. Any upset in the metabolic rates of these two bacterial types results in a disturbance of anaerobic degradation. If an imbalance in acidogenesis occurs, an accumulation of acidic products follows which inhibits methanogenesis (Zain *et al.* 2002). Temperature changes from the optimal for methanogenesis can also drastically reduce the methanogenic biodegradation potential, with optimum temperature being between 37 ˚C – 55 ˚C (Sanchez *et al.* 2000; Zain *et al.* 2002). However, 37˚C works best due to there being a greater stability of the processes of acidification and methanogenesis at mesophilic temperatures, as higher temperatures are only for 'hot' industrial wastewaters (Sanchez *et al.* 2000). Reportedly in South African landfills, the anaerobic phase can reach temperatures as high as 70 ˚C.

Temperatures especially from 45 ˚C to 50 ˚C are reported to negatively impact on the rate and efficiency of degradation (Yuen, 2001; Zain *et al.* 2002; Yu and Fang, 2003). If the temperature is below the optimal, then substrate utilisation in methanogenesis does not occur, decreasing the maximum specific growth rate of the methanogens**.** Changes from the pH optimum (an increase or decrease) can affect the growth rate of the methanogens where metabolic processes involving the use of carbon and energy sources, metabolites release from cells and cell synthesis are hindered thereby decreasing the substrate degradation efficiency. Morphology and cell structures of bacterial cells are also affected leading to a change in cell capabilities and adhesion properties. The optimum pH for acidogenesis is postulated at pH 5.9 or 6.0 (Sanchez *et al.* 2000; Yu and Fang, 2003).

Sulphate reducing bacteria reportedly compete with methanogens for acetate and hydrogen leading to an inhibition of methanogenesis (Yuen, 2001; Minimum Requirements, 1998). An increased rate of acetogenesis that exceeds the methanogenesis rate decreases the pH resulting in souring of environmental conditions in the MSW fraction (Yuen, 2001). This pH decrease inhibits methanogenic activity.

Landfills in South Africa

The procedure used for landfilling in South Africa is based on that designed for wetter, European climates. In South Africa compaction is performed on deposited waste to reduce voids present in the waste. South African landfill covers are composed of low permeability soil so as to facilitate reduced levels of gas and pollutant emissions as well as minimising water ingress to the stored wastes (Minimum Requirements, 1998).

Waste volume dictates the physical size of the landfill. The required cover to waste ratio is 1:4 to 1:6 by volume specifying the total air space to be used by the site. For every m^3 of cover, 4 to 6 m^3 of compacted waste is disposed of requiring adequate soil cover material (Mimimum Requirements, 1998; Du Preez, 2002; Novella *et al.* 2002). The soil cover layer is important as it forms the physical separation of the landfill waste from the atmosphere, thereby providing protection from the long-term effects of wind and water erosion. This soil cover layer also limits and controls precipitation entering the waste (Du Preez, 2002). Another specified requirement for landfills in South Africa is the addition of a clay layer between the waste deposition layer and the topsoil layer (Figure 1.3) to contain the waste body so as prevent the migration of pollutants into the atmosphere (Minimum Requirements, 1998). However, due to the dry South African climate, especially inland, this clay layer tends to dry out and crack where gaseous emissions are released.

Future designs are considering the elimination of this clay layer from the landfill design and the addition of a higher permeability soil cover (sandy soil) to facilitate the process of CH_4 oxidation (methanotrophs) and air permeability (Fourie, 2002). In the Johannesburg area, evaporation (1550mm per annum) exceeds precipitation (750mm per annum) thereby accelerating the drying effect on landfills leading to a decrease in CH_4 oxidation as water is evaporated from the vital topsoil layer.

Methanotrophs and CH_4 oxidation

Methanotrophic bacteria are common inhabitants of the oxic – anoxic zones in aquatic and soil environments and, on plant surfaces (Mancinelli, 1995; Hanson and Hanson, 1996; Hanson, 1999; Murrell *et al*. 2000). Methylotrophic bacteria can use a variety of one-carbon compounds as their carbon and energy source (Hanson and Hanson, 1996; Murrell *et al.* 2000). Methanotrophs are a subgroup of the methylotrophs and are gram-negative bacteria that utilise one-carbon compounds for the generation of ATP (Hanson and Hanson, 1996; Murrell *et al.* 1998; Hanson, 1999; Murrell *et al.* 2000; Wise *et al.* 2001). Methanotrophs oxidise CH_4, as their sole carbon and energy source, to water and CO_2 thereby reducing CH_4 levels in the atmosphere (Mancinelli, 1995; Hanson and Hanson, 1996; Bowman *et al*. 1997; Murrell *et al*. 1998; Hanson, 1999). Certain methanotrophs are also capable of oxidising toxic groundwater pollutants like trichloroethylene (Mancinelli, 1995; Wise *et al.* 2001), dichloroethylene, dichloroethane, chloroform, chloride and dichloromethane (Hanson and Hanson, 1996; Hilger *et al.* 2000; Vorholt, 2002), rendering methanotrophs useful for bioremediation purposes. Colony morphology and colour vary widely between the methylotrophic bacteria, which include pink, orange, white, tan, yellow, transparent and deep red to purple or, colourless colonies. Cell morphologies include cocci, rods, pear shaped, rosettes, single cells or, vibroids arranged as diplococci. Growth is reported to occur at pH 4.0 – 6.6 (Hanson and Hanson, 1996; Hanson, 1999; Hilger *et al.* 1999). The formation of exospores or cysts makes methanotrophs heat and desiccation resistant. These bacteria also possess cytochromes a, b and c and are also catalase and oxidase positive. Organic nitrogen

compounds, ammonia, nitrate and nitrite serve as nitrogen sources (Hanson, 1999; Murrell *et al*. 2000).

i) Methanotroph types

Type I methanotrophs assimilate formaldehyde by using the ribulose monophosphate pathway (RuMP) (Figure 1.4) and belong to the family *Methylococcaceae* found in three clusters in the gamma-subdivision of the Proteobacteria (Hanson and Hanson, 1996; Hanson, 1999). The three genera in this family are *Methylococcaceae* (or *Methylomonas)*, *Methylobacter* and an unnamed group. They have disc-shaped bundles of intracytoplasmic membranes and have a G + C content of 51 - 59 mol %. Type I can be rods or cocci (Hanson and Hanson, 1996; Hanson, 1999) with a growth optimum between 25 °C and 35 °C – growth is not reported to occur above 40 °C (Murrell *et a*l. 1998; Hanson, 1999; Heyer *et al.* 2002; Vorholt, 2002).

Moderately thermophilic methanotrophs fall into the genus *Methylococcus* (RuMP pathway), generally referred to as type X (Hanson and Hanson, 1996; Hanson, 1999). These cocci shaped bacteria belong to the gamma subdivision of the Proteobacteria. The mole G + C content is 59 – 66 % with an optimum temperature growth range of 37 ° C – 50 °C. They can also fix atmospheric nitrogen and possess the soluble methane monooxygenase (sMMO) enzyme (Hanson and Hanson, 1996; Murrell *et al.* 1998; Hanson, 1999; Heyer *et al.* 2002; Vorholt, 2002).

Type II methanotrophs fix formaldehyde using the serine pathway (Figure 1.5). The DNA content is 62-67 mol % and most members are also capable of nitrogen fixation. Type II's belong to the alpha subdivision of the Proteobacteria and exist as rods, which include the *Methylosinus* and *Methylocystis* genera (Hanson and Hanson, 1996; Hanson, 1999; Murrell *et al*. 2000). These methanotrophs differ from the type I and X methanotrophs by the types of resting forms, phospholipid fatty acid profiles, nitrogen assimilation pathways and the morphology of the intracytoplasmic membranes (Hanson and Hanson, 1996; Murrell *et al.* 1998; Hanson, 1999; Vorholt, 2002).

ii) Biochemistry of methane oxidation

Oxidation of the one-carbon compounds occurs by the action of the enzyme MMO. Two types of methane monooxygenases exist - particulate methane monooxygenase (pMMO) and sMMO (Semrau *et al.* 1995; Hanson and Hanson, 1996; Jensen *et al.* 1998; Hanson, 1999; Murrell *et al.* 2000). The pMMO exists in all methanotrophic species and is a membrane bound form that is active in growth medium with a copper content of more than 0.9 grams per gram of dry weight of bacterial cells (Hanson and Hanson, 1996). The sMMO is less common and only occurs in certain methanotrophs under conditions of copper limitation where oxidation of carbon compounds occurs at high rates only under these conditions (Hanson and Hanson, 1996).

MMO enzymes work by cleaving the O-O bonds of O_2 using a reducing agent (Semrau *et al*. 1995; Hanson and Hanson, 1996; Jensen *et al.* 1998; Hanson, 1999). SMMO uses NADH + H^+ as the electron donor and still remains soluble after cell extracts have been

centrifuged, giving the sMMO its name. Type II and type X methanotrophs possess sMMO where members of the *Methylosinus* and *Methylococcus* genera mostly encode for sMMO (Hanson and Hanson, 1996 and Hanson, 1999). However, sMMO occurrence is not restricted to these groups. All sMMO's have three components; a hydroxylase component (245 kDa) that possesses a nonheme iron and is an oligomer of beta, gamma and alpha subunits; a B component (15.8 kDa) which is a colourless protein with no cofactors; and a reductase component (38.4kDa), which has a flavin adenine dinucleotide and a Fe_2S_2 cluster (Semrau *et al.* 1995; Hanson and Hanson, 1996 and Murrell *et al.* 2000). This B component is proposed to form a specific complex with the hydroxylase component which modulates the MMO reaction (Murrell *et al.* 2000). The reductase component shuttles electrons from NADH to the hydroxylase component (Niellsen *et al.* 1996).

The genes that encode for the sMMO components are *mmoX*, *mmoY*, *mmoB*, *mmoZ*, *orfY* and *mmoC* occurring in this order in the genome (Niellsen *et al.* 1996). These gene sequences are highly conserved and are generally used as probes for detecting sMMO presence and activity. The pMMO requires copper for efficient functioning as it is thought that copper exists in the active site of the pMMO (Murrell *et al.* 2000). The pMMO contains three subunits of 45, 27 and 23 kDa, respectively. It is thought that the active site for the pMMO occurs in the 45 and 27 kDa subunits (Semrau *et al.* 1995; Murrell *et al.* 2000). Copper binding compounds, possessing a high affinity for copper, bind any copper in the environment. However, the sMMO has been shown to have broader substrate specificity than the pMMO where sMMO is able to oxidise typically

'non-growth' substrates such as alkanes, alkenes and aromatic compounds (Hanson, 1999; Murrell *et al.* 2000). In this way sMMO is able to simultaneously carry out CH_4 oxidation reactions and bioremediation strategies (Hanson and Hanson, 1996; Hanson, 1999; Murrell *et al.* 2000).

The reaction for CH_4 oxidation is as follows:

$CH_4 + 2O_2 \rightarrow CO_2 + 2H_20$.

The MMO enzyme is active in the first step of the reaction with oxygen being the most important factor (Roslev and King, 1994; Semrau *et al.* 1995; Hanson and Hanson, 1996; Hilger and Humer, 2003). MMO catalyses the addition of one oxygen atom into the C-H bond of CH_4 to form methanol and the second oxygen atom is reduced to water (Hanson and Hanson, 1996; Hanson, 1999 and Murrell *et al.* 2000). In sMMO this occurs in the active site of the hydroxylase component (Niellsen *et al.* 1996). The action of the methanol dehydrogenase enzyme on methanol results in the formation of formaldehyde (Hanson and Hason, 1996).

Formaldehyde serves to form the intermediates for metabolic pathways that result in the formation of cell material synthesis (Semrau *et al.* 1995; Hanson and Hanson, 1996; Hanson, 1999; Murrell *et al.* 2000). In the ribulose monophosphate pathway (RuMP) (Figure 1.4), formaldehyde reacts with ribulose-5-phosphate forming hexulose-6-phosphate, this reaction being catalysed by the enzyme hexulose-6-phosphate synthase (Hanson and Hanson, 1996). Isomerization of hexulose-6-phosphate by the hexulose phosphate isomerase enzyme yields 3 molecules of fructose-6-phosphate (Mancinelli,

1995 and Hanson and Hanson, 1996). A single molecule of fructose-6-phosphate undergoes conversion to 2-keto-3-deoxy-6-phosphogluconate and cleavage of this molecule generates pyruvate and glyceraldehyde-3-phosphate. A rearrangement reaction also occurs involving the other 2 molecules of fructose-6-phosphate (now with glyceraldehyde-3-phosphate), which forms more ribulose-5-phosphate for further reactions with formaldehyde (Mancinelli, 1995 and Hanson and Hanson, 1996). The pyruvate is incorporated into cell biomass (Mancinelli, 1995). NAD^+ or $NADP^+$ serves as electron acceptors in the RuMP cycle (Hanson and Hanson, 1996; Hanson, 1999). Formaldehyde oxidation yields the reducing power required for the initial CH_4 oxidation reaction (Hanson and Hanson, 1996; Murrell *et al*. 2000) where at this point, the carbon component of the entire bacterial cell is assimilated (Hanson and Hanson, 1996).

The serine pathway for formaldehyde assimilation (Figure 1.5) is common in the type II methanotrophs (Hanson and Hanson, 1996). Once again, the cell's carbon is assimilated during formaldehyde oxidation. Serine transhydroxymethylase is responsible for the assimilation of formaldehyde in this pathway whereby it catalyses the addition of formaldehyde to glycine, which in turn results in the formation of serine (Mancinelli, 1995 and Hanson and Hanson, 1996). Serine is then converted to 2-phosphoglycerate (two molecules) where 1 molecule forms 3-phosphoglycerate, which eventually is incorporated into cell biomass. The other molecule of 2-phosphoglycerate is converted to phosphoenolpyruvate (Mancinelli, 1995; Hanson and Hanson, 1996 and Murrell *et al*. 2000). This then combines with CO_2 to form oxaloacetate, which in turn is regenerated to

glycine for further reactions with formaldehyde. Cellular components are then synthesized from precursors that are obtained in this pathway (Murrell *et al*. 2000).

iii) In vivo and in vitro studies of methanotroph ecology

No methanotrophs grow on multicarbon compounds (Semrau *et al*. 1995; Hanson and Hanson, 1996; Nesheim and Lipscomb, 1996; Jensen *et al*. 1998; Hanson, 1999). The potential uses for methanotrophs include bulk chemical production e.g. single cell protein or propylene oxide and degradation of trichloroethylene (Nesheim and Lipscomb, 1996), a groundwater pollutant. Soil methanotrophs are exposed to reduced CH_4 levels in soils as compared to rice paddies and marine sediments. CH_4 oxidation is dependent on its concentrations i.e. methanotrophs are more likely to exhibit higher CH_4 oxidation rates in CH_4 concentrations similar to those found in soil compared to laboratory conditions of 10 to 20 % CH_4 (Whalen *et al.* 1990; Jensen *et al.* 1998; Dunfield *et al.* 1999). This reiterates the fact that environmental microorganisms are thought to be 'overfed' under laboratory conditions and so do not grow optimally. Kightley *et al.* (1995) reported that laboratory conditions use too much CH_4 when isolating landfill bacteria. This selects for methanotrophic communities that have a low affinity for CH_4 and are not able to survive under conditions of low CH_4 concentrations, such as in soil under winter conditions where rainfall is sporadic such as in the Johannesburg area. Reportedly, field methanotrophs may as yet be uncultured and are more distinct than cultured methanotrophs, due to them being overfed under laboratory conditions (Dunfield and Knowles, 1995; Ren *et al.* 1997; De Visscher *et al.* 1999). Therefore, there exists a

possibility that field methanotrophs are able to undergo a wider range of environmental stresses compared to their laboratory counterparts.

CH_4 oxidation in oxic microsites (e.g. anoxic-oxic interfaces) reportedly results in more than 80 – 90 % of the CH_4 being oxidised (Kightley *et al*. 1995; Murrell *et al.* 2000). In upland soils, methanotrophs show high affinity for CH_4 oxidation of atmospheric CH_4. Rice paddy soils undergo a flooding period and then a drainage period. This affects the CH_4 oxidation potentials of the methanotrophs whereby an onset of desiccation conditions results in an eventual decrease in the CH_4 oxidation potential, as bacteria experience CH_4 starvation conditions and water stress (Kightley *et al.* 1995). High moisture levels (saturation conditions) favour methanotophic bacteria in paddy soils but not in landfills, where paddy soil methanotrophs have a low affinity for CH_4 oxidation. Cai and Mosier (2002) showed that CH_4 oxidation capabilities were restored after a desiccated paddy soil was re-watered. This is important during winter periods when rainfall is minimal and desiccation is most possible. This also implies that the re-initiation of CH_4 oxidation was performed by methanotrophs capable of surviving stressful environmental conditions. It is these bacteria that are the most useful in dry, arid climates (Roszak and Colwell, 1987; Roslev and King, 1994).

iv) The importance of molecular techniques in the study of methanotroph ecology

DNA analysis of bacterial communities is advantageous in situations where bacterial communities cannot be cultured on laboratory growth media as only 1 % of environmental samples are cultivable in the laboratory since their metabolic requirements

are unknown (Amann *et al*. 1995). Phylogenetic analysis of DNA therefore reveals a greater diversity of microorganisms in these sample types (Nesheim and Lipscomb, 1996 Murrell and Radajewski, 2000; Bussman *et al*. 2004) being complementary to laboratory culture methods. DNA analysis also provides information on the phylogenetic relationships between bacterial groups, being an efficient indicator of evolution in various environments.

Phylogenetic probes have been designed for methanotrophs based on 16s rRNA gene sequences of existing methanotrophs (Brusseau *et al.* 1994; Murrell *et al.* 1998; Wise *et al.* 1999; Murrell and Radajewski, 2000; Wise *et al*. 2001). These are used in 16s rRNA gene libraries constructed from DNA directly extracted from the environment (soil, peat bogs, etc.) or in flourescent *in situ* hybridisation (FISH). Oligonucleotide probes with a broad specificity for methanotrophs were one of the first to be made (Henckel *et al*. 1999; Wise *et al.* 1999; Heyer, *et al.* 2002; Bussman *et al*. 2004). Oligonucleotide probe 10-γ is specific for γ-proteobacterial and RuMP pathway methanotrophs and probe 9-α is specific for α-proteobacterial, serine pathway methanotrophs (Murrell and Radajewski, 2000). Both are able to detect all methanotrophic species in soil samples enriched with a CH_4/air atmosphere. Narrow specificity probes target different genera of only methanotrophs and have been used successfully for isolates (*Methylococcus capsulatus*) from peat bogs (Murrell and Radajewski, 2000; Heyer *et al.* 2002). The accuracy of 16s rRNA gene sequences is crucial for the design of probes as it dictates probe specificity. Accurately designed probes have been shown to yield a wider diversity of methanotrophs in environmental samples (Hanson and Hanson, 1996; Murrell and Radajewski, 2000).

Functional gene probes for methanotrophs involve those genes that specify key functional enzymes in methane metabolism and it is these probes that are used as the primer sets (Murrell and Radajewski, 2000). Polymerase chain reaction (PCR) primers are designed by using conserved regions of genes, which amplify MMO and methanol dehydrogenase (MDH) gene products. MDH, pMMO and sMMO are highly conserved genes and so are preferable when developing probes for methanotrophs allowing the detection of all the methanotrophic bacterial species in a given sample (Hanson and Hanson 1996, Murrell and Radajewski, 2000; Heyer *et al.* 2002). Probes for the five sMMO structural genes *mmo X*, *Y*, *Z*, *mmoB* and *mmoC* were part of one of the first primer sets (Heyer *et al.* 2002). The most widely used primer set is that for the *mmoX* gene which encodes for the active site of the MMO polypeptide (Murrell *et al*. 2000). It is used to detect methanotrophs in peat soil and more importantly, the monitoring of methanotroph population in bioreactors (Wise *et al.* 1999). However, since not all methanotrophs have sMMO, another functional gene primer set is for the *pmoA* gene that is involved in the universally possessed pMMO. This probe is based on known *pmoA* and *amoA* sequences for *M. capsulatus* and *Nitrosomonas europaea* respectively, amplifying a 525 base pair (bp) DNA fragment for all methane oxidisers tested (Murrell and Radajewski, 2000; Heyer *et al*. 2002). These probes also reveal greater methanotroph diversity than those methanotrophs grown in culture. Another functional gene probe is that for the *mxaF* gene used for environmental samples (soils, peat, and marine sources) (Murrell and Radajewski, 2000).

Although phylogenetic methods have proved to be indispensable for bacterial identification purposes, they do not reveal the physiological traits of bacteria. These traits can only be revealed by careful manipulation of standard laboratory culture media for isolation of methanotrophs for bioremediative purposes. Phylogenetic analysis thereafter, is able to identify the specific methanotrophic strain that was isolated. Phylogenetic identification is important as it has been suggested that certain heterotrophic bacteria may also show growth using CH_4, and can be mistaken for methanotrophic species (Hanson, 1999).

Factors affecting CH_4 oxidation

i) Chemical inhibitors of CH_4 oxidation

Ammonium, primarily from fertilisers, inhibits CH_4 oxidation in soils (King and Schnell, 1994; Schnell and King, 1994; Dunfield and Knowles, 1995; Begonja and Hrsak, 2001; Bodelier and Laanbroek, 2004) especially if ammonium, nitrite and nitrate are present in high concentrations in the soil. This inhibition is due to competitive inhibition by ammonia for the active site of the methane monooxygenase (MMO) enzyme that is capable of oxidising NH_3 to nitrite (Hanson, 1999; Bodelier and Laanbroek, 2004). This NH_3 oxidation reduces CH_4 consumption by methanotrophs thereby reducing the methane oxidation rate (Steudler *et al.* 1989; Yu *et al.* 2001; Bodelier and Laanbroek, 2004). Since ammonia is therefore toxic to methanotrophs, these bacteria prefer a nitrogen source in the form of nitrate. Toxicity results as methanotrophs can oxidise ammonia, but cannot use it as a sole carbon source and hence do not grow. This

inhibitory effect has been shown in landfill cover soils, forest soils and agricultural soils (Schnell and King, 1994; Seghers *et al*. 2003; Bodelier and Laanbroek, 2004). Nitrogen containing fertilisers convey a long-term inhibitory effect on CH_4 oxidation where ammonium ions can persist for several years even once ammonium levels return to their original concentrations (Schnell and King, 1994; Begonja and Hrsak, 2001; Seghers *et al.* 2003; Bodelier and Laanbroek, 2004). If high levels of methane are present (8%), ammonia and its derivatives have no effect on the rate of CH_4 oxidation. Rather, the high levels of CH_4 then inhibit ammonium oxidation. However, this ammonium oxidation inhibition is important in the zones where the highest concentration of methanotrophs exists, as here there will be low levels of CH_4 so leading to possible CH_4 oxidation inhibition if increased levels of nitrogen and its derivatives are present (Schnell and King, 1994; Bodelier and Laanbroek, 2004). On the other hand, if nitrogen sources are depleted then CH_4 oxidation is also inhibited as the methanotrophs lack a source of nitrogen (Schnell and King, 1994). Nitrogen depletion is common in landfill cover soils (Schnell and King, 1994; Begonja and Hrsak, 2001) where there are no renewable nutrient sources. Ammonium was found to stimulate CH_4 oxidation in ricefield soils (Schnell and King, 1994). However, it is not known whether this increased oxidation rate was carried out by nitrifying bacteria present in the same environment as the methanotrophs (Schnell and King, 1994). It therefore depends on the soil type where nitrogen concentrations need to be first evaluated for that particular environment, where possible nitrogen addition must not be at inhibitory concentrations for CH_4 oxidation.

ii) Soil and moisture characteristics with respect to CH_4 oxidation

Approximately half of the anaerobically degraded organic carbon in the waste body results in CH_4 formation (Whalen *et al*. 1990). CH_4 formation from landfills varies widely, with values between 0.0002 and 4000 grams per m^{-2} d^{-1} (Dunfield and Knowles, 1995; De Visscher *et al.* 1999; Murrell *et al.* 2000) having being reported. Soil moisture has been shown to be an important factor in specifying the rate of CH_4 oxidation (Rosqvist, 2000; Hilger and Humer, 2003). Water acts by redistributing nutrients and biodegradative substrates in solid state areas of landfills. Water mobility and presence are not uniform in landfills due to spatial and temporal variations, dictated by preferential flow in voids (Fourie, 2000), resulting in certain areas where no biodegradation occurs at all as these may be devoid of adequate moisture. A reduced rainfall rate can have a negative feedback on gas consumption in soil whereby this changes to gas emission. Net CO_2, CH_4, N_2O, and NO emissions rely on the soil water content. At the end of the wetter seasons, soil layers start to dry out, with this drying effect reaching a maximum towards the end of the winter period, resulting in decreased microbial respiration (Cattanio *et al.* 2002; Jiminez *et al.* 2002). The resulting increased net emission rate is possibly due to reduced levels of CH_4 oxidation occurring in the soil cover (Hilger *et al.* 1999; Hilger and Humer, 2003).

The spatial location of microorganisms is facilitated in landfill regions with optimum moisture content. Changes in soil water content affect microbial activity (Czepiel, 1996) where decreases in the soil moisture content decreases CH_4 oxidation levels. However, too much hydration also results in decreased CH_4 oxidation rates as water present in air

spaces prevents gaseous entry to methanotrophs. Optimal moisture allows for gas phase molecular diffusion of CH_4 and O_2 whereby there is enough moisture in air spaces to allow gaseous entry to regions of bacterial cells. Desiccation of the bacterial cell is also prevented. Hence, the soils must not be waterlogged so as to ensure efficient CH_4 oxidation (Cai and Mosier, 2002).

Surface soils periodically experience prolonged drying periods and in-between these periods rapid rewetting of soils occurs, being most common in areas with a warm, dry climate where rainfall occurs infrequently e.g. winter months in Johannesburg. Soil bacteria experience frequent environmental stresses where rewetting is seen as a disturbance as it sends bacterial cells into a state of osmotic shock that can lead to cell lysis and release of cellular solutes that can be 30 – 60 % of the biomass carbon (C) (Fierer *et al.* 2003). In optimal growth conditions, bacterial cells undergo cell growth resulting in the formation of biomass. However, under stress conditions cells will perform functions that are involved in survival of the cell so as to maintain the cell physiologically (Fierer *et al.* 2003). Any drying-rewetting cycles affect the community structure of a soil community and the negative impact of this cycle is that cell respiration decreases. This is due to the bacterial cell adapting to differing conditions that spontaneously occur. The surviving bacteria will generally have slow growth cycles if they are to cope efficiently with the periodic changes in the environmental stresses. Numerous drying-rewetting cycles can thus select for those microorganisms that are capable of surviving environmental stresses. Fierer, *et al.* (2003) found that soil microbial communities that

were not adapted to stress conditions were not able to cope with induced stress conditions.

Air permeability and gas diffusivity are both determined by soil water content. Too much water has a rapid flow that prevents gases from diffusing (and remaining) in the soil for extended time periods. If there are low water levels in the soil the possibility of air displacement by the upward biogas flow is greater. Certain studies have shown that a soil moisture content of between 5 – 15 % is generally optimal (Ball *et al*. 1997), this is typical of semi-arid areas of South Africa. The methanotroph population will also increase as the CH_4 concentration increases, which will stop once the flow of CH_4 stabilises.

The importance of cover soil characteristics in landfill bioprocesses

i) Soil quality and leachate percolation

The prevention of landfill leachate percolation into groundwater is a function of the basal liner that separates refuse from the land below it (Figure 1.2) (Minimum Requirements, 1998 and Fourie, 2000). However, the landfill cover soil should also not allow sufficiently large amounts of rainfall water to percolate through the waste body to the groundwater beneath it. Sandy soils are regarded as having relatively high water holding capacities, so being potentially efficient soil cover layers. Coarse soils allow for a greater degree of percolation compared to finer grained soils. Coarse soils also promote CH_4 oxidation, as there is a greater flow of oxygen in these soils compared to finer grained soils.

The clay layer between the topsoil and waste, required by current guidelines (Minimum Requirements, 1998) is prone to desiccation in dry climates (Figures 1.1 A and 1.2). Erosion of the topsoil layer by nature e.g. wind, exposes the clay layer to air and clay desiccation occurs, especially as the topsoil layer is only about 20–30 cm thick. The waste layer below the clay layer also usually settles unevenly resulting in a non-uniform clay layer leading to a greater incidence of crack development and the release of methane into the atmosphere (Fourie, 2000).

ii) Soil depth and CH_4 oxidation

CH_4 oxidation has been shown to vary with soil depth as the transport properties and porosity of soil differs as depth increases (Ball *et al.* 1997). The uppermost horizons of the soil cover (0-3 cm) are more susceptible to reduced moisture levels due to the drying effects of air above. CH_4 oxidation rates have been shown to be higher in the soil layers below the upper-most horizons (3 – 10 cm) (Whalen *et al.* 1990). De Visscher *et al.* (1999) found that optimum CH_4 oxidation rates occurred in the uppermost 20 cm of soil. The CH_4 oxidation potential for landfill topsoil has been estimated at 45 g CH_4 per oxidised m^2 of soil. For methanotroph sampling purposes it is suggested that soil samples be taken from the 3 - 10 cm soil horizons.

Compaction decreases both the soil diffusivity rate and the CH_4 oxidation rate by filling voids with soil particles and decreasing diffusion properties. The soil layer closest to the surface has been shown to have the greatest permeability. Air permeability is reportedly

dependent rather on pore size between soil particles than on soil diffusivity (Ball *et al.* 1997). Airflow is laminar because its downward migration into the soil is dependent on the upward pressure of the gases being emitted from the cover soil. If the soil pore size is large, then air diffuses more easily into soil thereby increasing the oxygen levels of the soil. If the soil pore is small, the upward pressure of the emitted gas will be greater as more gas is 'squeezed' through tiny pores preventing air entry into the soil as this air is displaced. Large soil pores will allow more gas to escape at a lower pressure and increases the chances of downward air migration into the soil. 'Wide' pores or high permeability soils favour methanotrophic bacteria and the potential for CH_4 oxidation with efficient water and gas diffusivity in the soil.

iii) Soil fertility

Soil fertility is a measure of its biological productivity to maintain a high environmental quality and henceforth promote the health of plants and animals. An important indicator of soil quality is its organic content (Roling *et al*. 2000), an important nutrient source for microorganisms where it promotes their biological activity. Organic matter content is thought to change very slowly with time (Roling *et al.* 2000). However, microbial processes that are more sensitive to environmental changes, such as population structure and metabolic capabilities, occur faster. A decrease in organic matter in soil leads to a decrease in microbiological activity in this soil and subsequently microbial biomass (Roling *et al*. 2000). Any deviation from the optimum soil pH inhibits microbial activity

and the bacterial cell's ability to perform biochemical reactions efficiently (De Visscher *et al*. 1999).

Compost is a soil-conditioning agent as it improves the water holding capabilities of soil, air movement in soil and cation exchange ability thereby optimising CH_4 oxidation capabilities of methanotrophs. It is defined as "a homogenic, completely fermented material of animal or plant origin to which no plant nutrients have been added" (van Heerden, 1996). Since the application of raw waste material directly to soil can confer toxicity to plant and animal species, processed waste material is used, as it is a more stable form. Compost is a stable form of wastes as it has been naturally attenuated by microbial action (fungi and bacteria) (Crecchio *et al.* 2001). South African landfill cover soil is rendered productive for bacterial reactions by the addition of compost, which increases the organic component of this soil. Dissolved organic components also present in compost are much more easily absorbed into the soil. Although this proportion may be low compared to the bulk organic matter, it aids in the transport of nutrients and so enhances methanotroph activity in the soil (Chae *et al.* 2000) thereby ensuring efficient development of the bacterial soil community (Kightley *et al*. 1995).

Leachate recycling in landfills

To increase the hydration of South African landfills, leachate recycling is being considered to overcome the problem of drying out in landfill cover soils thereby facilitating the activity of methanotrophs. Due to the high evaporation rates in drier inland areas, South African landfills produce little or no leachate, lowering the risk for

groundwater contamination. As a result, the Minimum Requirements for Waste Disposal by Landfill are based on continuous monitoring of gas emissions rather than leachate quality (Minimum Requirements, 1998). Leachate recycling occurs when leachate that is formed at the bottom of the landfill is re-circulated and applied to the top of the landfill where it percolates down through the soil cover and waste (Novella *et al*. 1996). Leachate recirculation allows the landfill to be continually hydrated optimizing phase I of biodegradation (acidogenesis). If microbial metabolism is enhanced then the rate of energy production is increased thereby speeding up this degradation process as compared to non–recirculating systems, leading to the onset of methanogenesis in a shorter time period (Ball *et al*. 1997; Reinhart, 1996; Yuen *et al*. 1997; Yuen, 2001; Yuen *et al*. 2001).

The other important advantage of leachate recycling is that pH decreases are readily remedied by the natural buffering capacity of the MSW. Ash is also added to the MSW as it has proven to be an efficient buffering agent with a high water holding capacity to retain moisture in the landfill waste body. Gas and leachate quality depends on the availability of moisture, nutrients and substrates in the MSW where microbial degradation is optimal if these three requirements are fulfilled, as leachate is rendered less volatile by continual cycles of biodegradation by the microorganisms (Reinhart, 1996; Chae *et al.* 2000, Yuen *et al.* 2001). This could be because as leachate recycles through the landfill, it goes through further decomposition processes thereby reducing its strength.

Leachate recycling is also thought to decrease the time period in which a landfill stabilises (Figure 1.3, phase V) by 5-fold (Reinhart, 1996) e.g. from 50 years to 10 years, which inevitably leads to reduced operating costs of the landfill. Increased degradation also means increased gas formation rates where high CH_4 emission rates causes the methanotroph population to increase considerably to facilitate oxidation. An enhanced biodegradation state of waste also allows for rapid settlement, rapid volume reduction, early landfill closure and subsequent land-usage of a landfill (Nedwell and Reynolds, 1996; Novella *et al.* 1996; Pohland and Kim, 1999). Rapid volume reduction also indicates that more waste can be disposed of in a landfill. Moisture that is added to the refuse contributes to the amount of water that will be available in the leachate recycling operation.

Sewage sludge can be co-disposed of with solid waste in landfills, which is effective and efficient at sludge attenuation. Any disposal method must be highly controlled to protect the environment and human health. Land application is the "spraying or spreading of sewage sludge onto the land surface," (Zain *et al*. 2002) or by surface injection by incorporating sewage sludge into soil where it serves as a conditioning agent for crop fertilisation (Pohland and Kim, 1999; Strachan *et al.* 2000). Land application also relies upon chemical, physical and biological soil properties. Heavy metal concentrations in the sludge are important here (Zn, Cu, Pb, Ni, Cd and Cr) and are proposed to be immobilised in soil with a pH of 6.5 – 7.0. Heavy metals in the elemental form cannot be transformed into a more attenuated form leading to environmental problems. If large amounts of sewage sludge are applied to the land surface then large amounts of heavy

metals accumulate. Excessive concentrations may enter the plant and animal food chain, which subsequently reaches humans (Strachan *et al*. 2000; Zain *et al*. 2002). The metal mobility ranking in soil is Cd, Cu, Cr, Zn, Ni and Pb. However mobility depends on physical and chemical properties of the soil adsorption processes (Reinhart, 1996; Strachan *et al*. 2000; Yuen *et al*. 2001 Zain *et al*. 2002). When sewage sludge is applied to soil, the heavy metals concentrate in the topsoil layer where these can either leach through the soil or remain in the soil matrix. The time dependent factors are soil type where a high permeability soil allows this percolation to occur rapidly and soil pH where an increase in the soil acidity renders heavy metals more soluble in the soil (Zain *et al.* 2002). Zain *et al*. (2002) found that an increased volume of applied sludge decreased the soil pH especially at 284L/m^2 to 341L/m^2.

Future considerations

Future work is aimed at optimising soil conditions for indigenous methanotrophic bacteria in order to possibly minimise CH_4 emissions into the atmosphere. This includes the use of a higher permeability soil than soils currently used in South African landfill cover as well as the possible elimination of the clay layer. This may allow for greater gas and air permeability whereby CH_4 availability to methanotrophs in the soil is enhanced for subsequent CH_4 oxidation. Leachate recycling procedures may be applied to new landfills in South Africa together with an efficient basal liner, so as to reduce the lifespan (and so maintenance costs) of these landfills. The Johannesburg region is a semi-arid region and evaporation may hinder efficient bacterial action on the emitted CH_4. Whalen *et al.* (1990) showed that waterlogged soils can inhibit CH_4 oxidation processes,

suggesting that over saturation of soils inhibits methanotrophs. Therefore, soil moisture content consistent with current landfill moisture levels needs to be evaluated in relation to CH_4 oxidation. Culture techniques for methanotrophs are also being reconsidered so as to efficiently isolate a wider variety of strains. Laboratory grown strains cannot cope with environmental stresses, such as those occurring in a landfill, suggesting that indigenous landfill cover soil methanotrophs are better suited to current South African soil conditions. Soil conditions therefore need to be optimised according to the growth requirements of the indigenous methanotroph population.

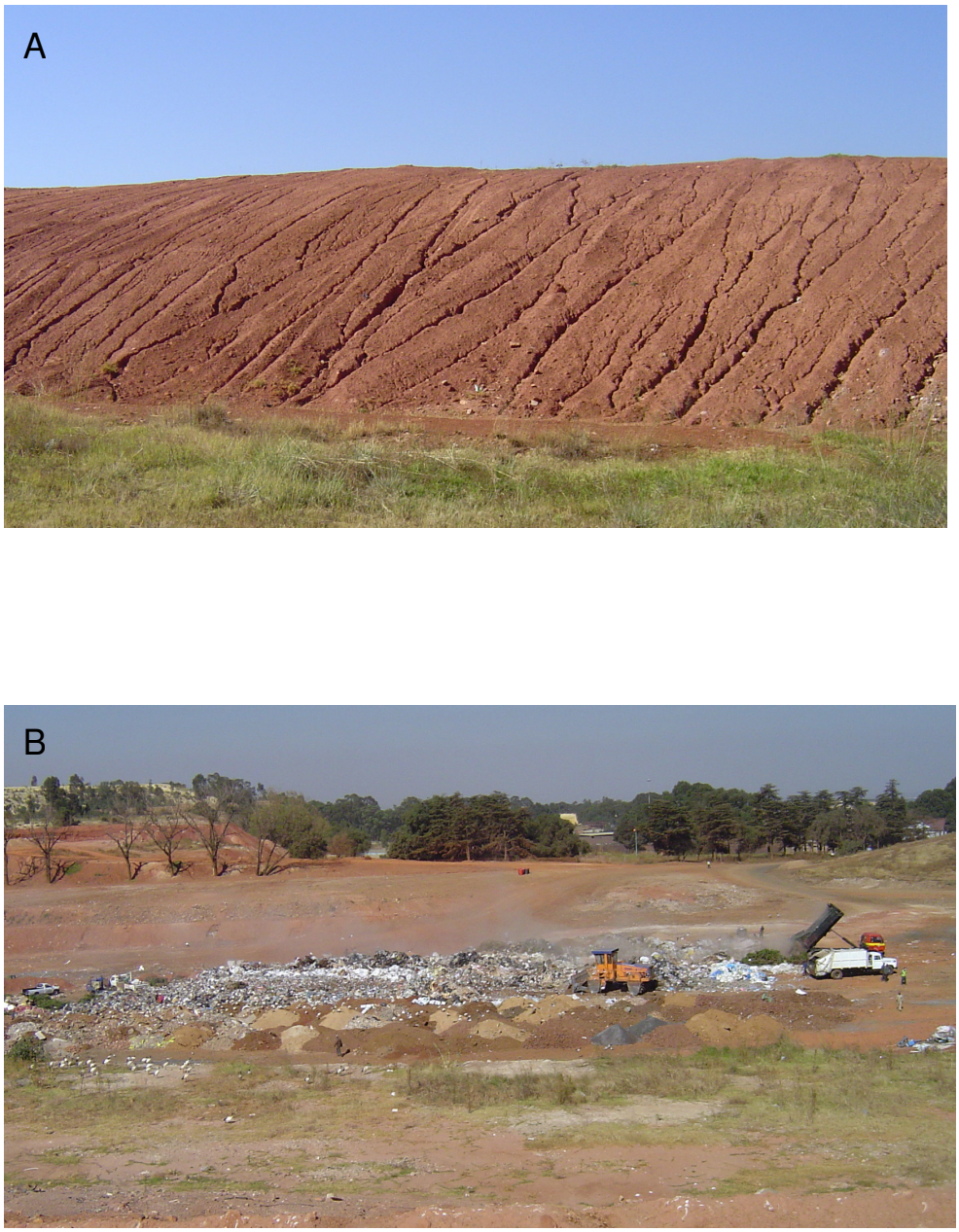

Figure 1.1. A typical soil cover layer (A) and (B) the waste compaction process.

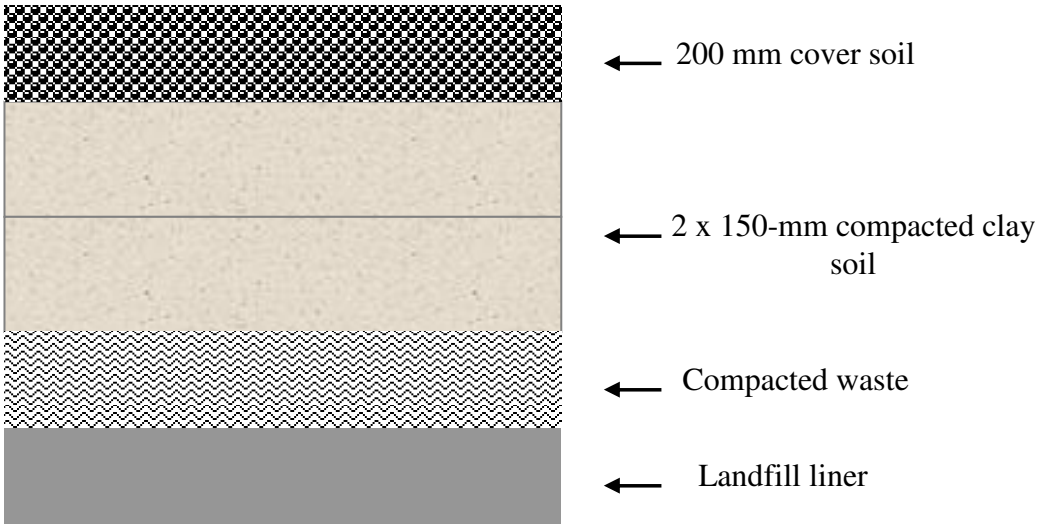

Figure 1.2. Design of a landfill as set out by the Minimum Requirements for Waste Disposal by Landfill, South Africa, 1998. The landfill liner below the waste body separates wastes from the land surface below.

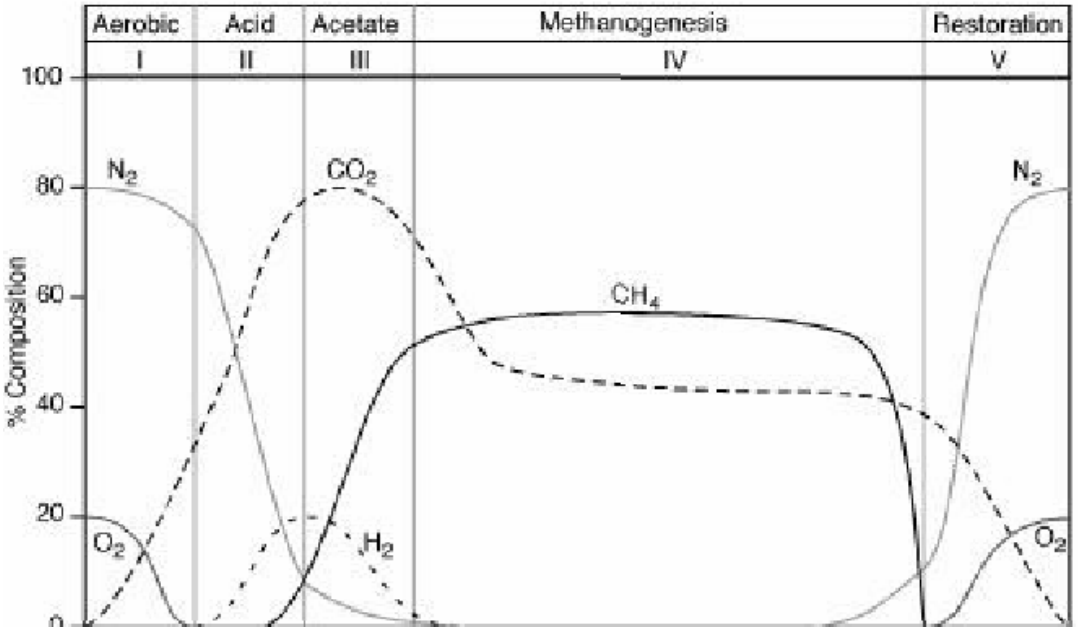

Figure 1.3. The 5 phases of gas generation and biodegradation in a landfill. The diagram shows the levels of the various gases at different degradation stages in the anaerobic component of a landfill (Farquhar and Rovers, 1973).

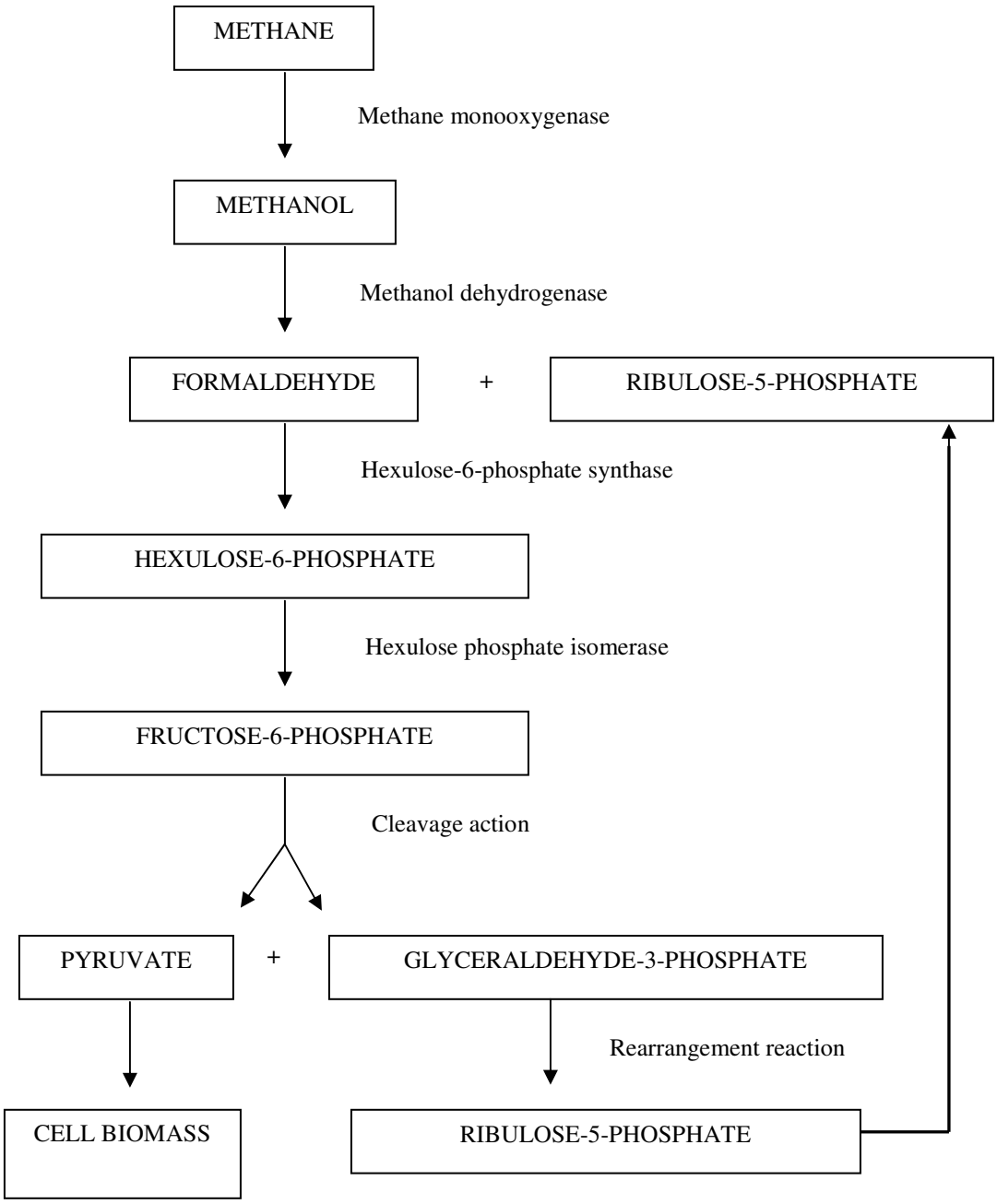

Figure 1.4. The ribulose monophosphate (RuMP) pathway of formaldehyde assimilation in methanotrophs.

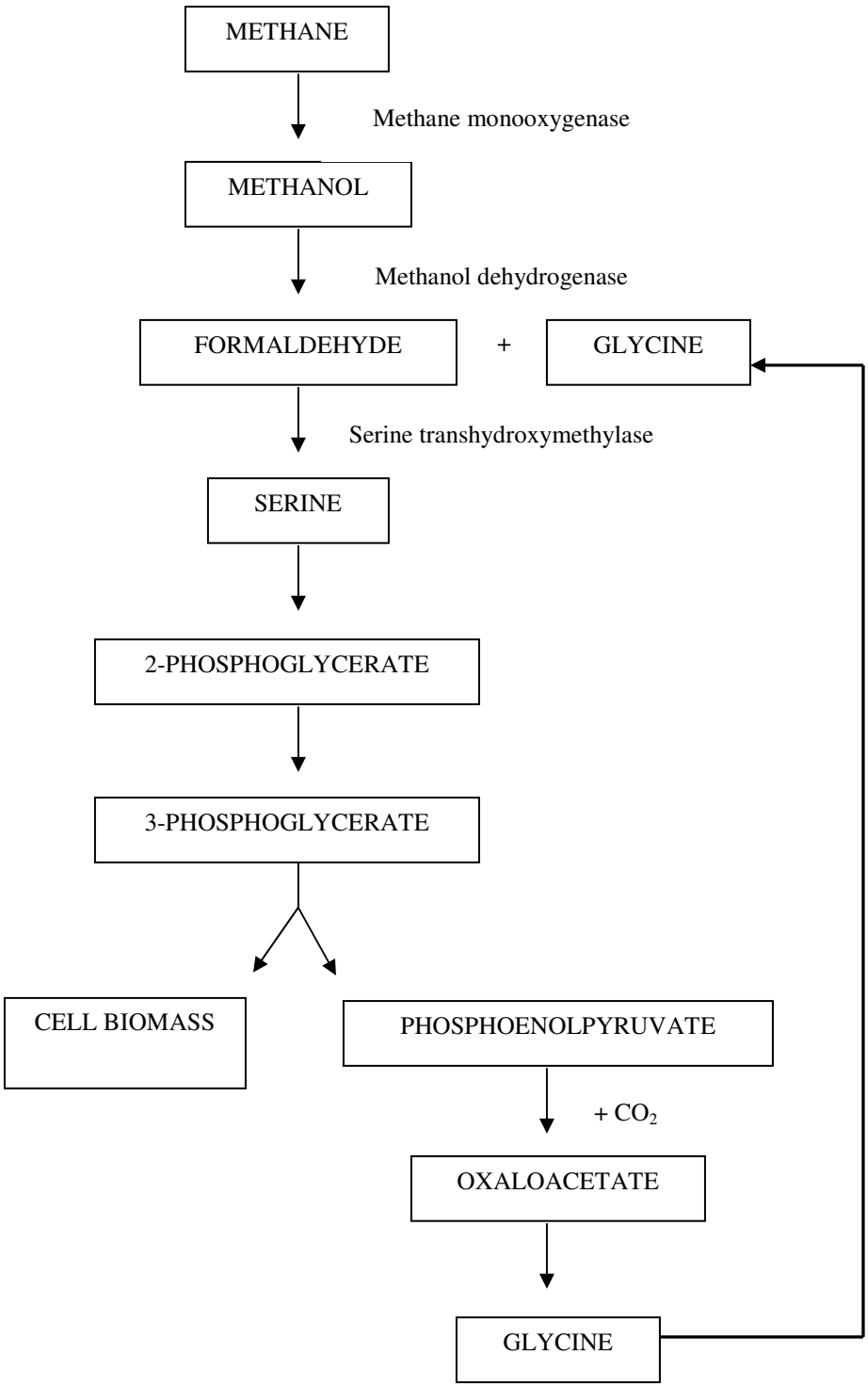

Figure 1.5. The serine pathway for formaldehyde assimilation during in methanotrophs.