# Enrichment of a Marine Methanotrophic Population and Its Kinetics of Methane and TCE Oxidation

Thesis by

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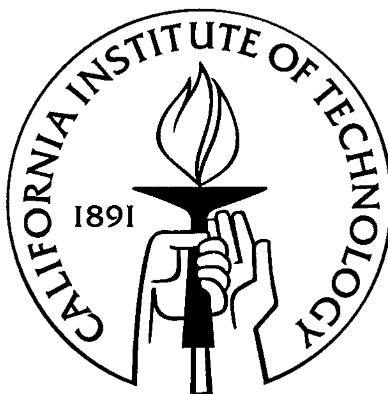
In partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

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## Abstract

Bacteria that grow on methane as their sole carbon and energy source (methanotrophs) are capable of degrading a variety of halogenated methanes and ethylenes, including trichloroethylene (TCE). These compounds are common pollutants in a variety of coastal and nearshore marine environments, and therefore, marine methanotrophs are attractive candidates for bioremediation of these contaminants. The enzyme which degrades TCE, the methane monooxygenase, occurs in two forms, the soluble, or cytoplasmic form (sMMO) and the particulate, or membrane-bound form (pMMO). These enzymes have different kinetics of methane and TCE degradation, and while all known methanotrophs produce the pMMO, only a few species of the Type II group produce the sMMO. The sMMO is only produced under conditions of copper limitation in these strains. No Type II methanotrophs have been isolated from the marine environment. Detection and identification of marine methanotrophs, as well as characterization of their rates of oxidation of methane and TCE, are important to our understanding of their response to attempts to stimulate them for in-situ bioremediation of TCE . Additionally, this information is necessary to the development of predictive models of bioremediation processes. The rate of methane oxidation by the native methanotrophic population of an estuarine sediment was measured, and the $K_s$ and $V_{max}$ values for methane of this population were determined from the Michaelis-Menten equation. The $K_s$ values (8-26 μM) suggest that the particulate methane monooxygenase is being used for methane oxidation. Several methanotrophs were isolated from this estuarine sediment after enriching it, and gene probes based on 16S rRNA sequences were used to identify the genus groups of these isolates. No Type II methanotrophs were isolated. The kinetics of methane and TCE oxidation by one of the methanotrophic isolates were measured, and it was found that the amount of copper added to the growth medium affects the kinetics of methane and TCE oxidation by this strain. When copper was added, the $K_s$ values were 10-13 μM, but when

no copper was added the results varied from a slightly elevated $K_s$ (16μM) to a high $K_s$ for which saturation with methane was not observed. When copper was added to cultures used for TCE degradation, $K_s$ values were 8-10 μM, but when no copper was added, TCE degradation was not observed. The kinetics of methane and TCE oxidation in enriched estuary sediment were measured, and based on the elevated $K_s$ values (48-53μM), the limited increase in the $V_{max}$ values (an indicator of a relatively small increase in the population), and the lack of TCE degradation in the enriched sediment, it is suggested that the methanotrophs may become limited for copper during enrichment. The data suggest that the sMMO is not being produced, even under copper limitation, consistent with the fact that no Type II methanotrophs were isolated. Therefore simple enrichment of marine methanotrophic populations may not result in enhanced TCE degradation rates. Nutrient addition, including bioavailable copper, will be necessary for the use of marine methanotrophs for in-situ TCE bioremediation. Gene probing was used to detect the presence of Type I methanotrophs in the sediment by probing slot blots of DNA extracted directly from the sediment. These blots were also used to estimate the number of methanotrophs present in the enriched sediment and this value was compared to estimates of methanotroph numbers based on $V_{max}$ values. These estimates were found to agree within the expected error of each estimation method, suggesting that gene probing will be a useful method for estimation of methantroph numbers in environmental samples.

# Table of Contents

**1. Introduction: Marine Methanotrophs and Bioremediation of TCE .... 1**

**1.1 Bioremediation for cleanup of TCE ........ 1**
**1.2 TCE in marine and coastal systems ........ 2**
**1.3 Bacterial degradation of TCE ........ 2**
**1.4 Methanotrophs for bioremediation of TCE ........ 3**
***1.4.1 Methane monooxygenase ........ 4***
**1.5 Oxidation of TCE by methanotrophs ........ 5**
**1.6 Characteristics of known methanotrophic strains ........ 7**
**1.7 Methanotroph phylogeny ........ 8**
***1.7.1 Methanotroph gene probes ........ 9***
**1.8 Occurrence of methanotrophs in aquatic environments ........ 10**
***1.8.1 Methanotrophs in sediments ........ 10***
***1.8.2 Methanotrophic isolates ........ 11***
**1.9 Significance of this work ........ 11**
**1.10 References ........ 16**

**2. Field Sampling and Methane Oxidation in Fresh Sediment ........ 21**

**2.1 Introduction ........ 21**
**2.2 Materials and methods ........ 21**
***2.2.1 Field Site ........ 21***
***2.2.2 Sample Collection ........ 22***
***2.2.3 Measurement of methane oxidation kinetics ........ 23***
**2.3 Results ........ 25**
***2.3.1 Mass transfer calculations ........ 26***
**2.4 Discussion ........ 28**
**2.5 Conclusions ........ 30**
**2.6 References ........ 37**

**3. Isolation and Characterization of Methanotrophs ........ 39**

**3.1 Introduction ........ 39**
**3.2 Materials and Methods ........ 39**
***3.2.1. Collection of sediment ........ 39***
***3.2.2. Enrichment conditions ........ 40***
***3.2.3. Isolation and purification of methanotrophic bacteria ........ 42***
***3.2.4. Characterization of isolates ........ 44***
**3.3. Results ........ 49**

*3.3.1. Types of isolates* ........ *49*
*3.3.2. Growth of isolates* ........ *49*
*3.3.3. Colony blotting results* ........ *50*
**3.4 Discussion** ........ **52**
**3.5 Conclusions** ........ **53**
**3.6 References** ........ **59**

**4. Substrate Oxidation Kinetics of Estuarine Isolate BB5.1** ........ **61**

**4.1 Introduction** ........ **61**
**4.2 Methane oxidation** ........ **62**
*4.2.1 Materials and methods* ........ *62*
*4.2.2 Results* ........ *64*
**4.3 TCE oxidation** ........ **66**
*4.3.1 Materials and methods* ........ *66*
*4.3.2 Results of TCE oxidation experiments* ........ *68*
**4.4 Discussion** ........ **70**
**4.5 Conclusions** ........ **72**
**4.6 References** ........ **84**

**5. Behavior of Sediment Enrichments** ........ **85**

**5.1 Introduction** ........ **85**
**5.2 Materials and methods** ........ **85**
*5.2.1 Enrichment conditions* ........ *85*
*5.2.2 Methane oxidation assays* ........ *86*
*5.2.3 TCE oxidation assays* ........ *87*
**5.3 Results** ........ **88**
*5.3.1 Methane oxidation results* ........ *88*
*5.3.2 TCE oxidation results* ........ *90*
**5.4 Discussion** ........ **90**
**5.5 Conclusions** ........ **93**
**5.6 References** ........ **99**

**6. Gene Probing of Estuarine Sediment** ........ **100**

**6.1 Introduction** ........ **100**
**6.2 Materials and methods** ........ **100**
*6.2.1 Enrichment conditions and sampling* ........ *100*
*6.2.2 Extraction and purification of DNA* ........ *100*
*6.2.3 Slot blots for hybridization of methanotroph probes* ........ *102*
*6.2.4 Hybridization experiments and image collection* ........ *103*
**6.3 Results** ........ **103**
**6.4 Discussion** ........ **106**

6.5 Conclusions ........................................ 107
6.6 References ........................................ 110

7. Conclusions ........................................ 111

7.1 Summary ........................................ 111
7.2 Kinetics experiments ........................................ 111
7.3 Results and copper effect ........................................ 113
7.4 Gene probing experiments ........................................ 114
7.5 Implications and significant new findings ........................................ 114
7.6 Further research ........................................ 114

8. Appendix A: Nonlinear regression results generated by TableCurve 2D (Jandel Scientific) ........................................ 117

9. Appendix B: The Michaelis-Menten Model ........................................ 124

10. Appendix C: Calculations of the Danckwerts criterion ........................................ 125

11. Appendix D: Nucleic acid extraction from sediment samples ........................................ 129

12. Appendix E: Additional Information Pertaining to Figure 5-3 ........................................ 131

# List of Tables

*Table 1-1. Characteristics of methanotrophic bacteria* ..... 8*
*Table 2-1: Sample collection for measurement of methane oxidation in fresh sediment ..... 23*
*Table 2-2: Summary of Michaelis-Menten parameters for methane oxidation in Newport Bay Estuary Sediment ..... 26*
*Table 2-3: Michaelis-Menten parameters for methane oxidation in enrvironmental samples ..... 28*
*Table 3-1: Characteristics of methanotrophic isolates ..... 49*
*Table 3-2: Results of probing colony blots with 16S rRNA probes ..... 51*
*Table 3-3: Results of probing digested DNA from control strains ..... 52*
*Table 4-1: Results of methane consumption experiments for the isolate BB5.1 ..... 65*
*Table 4-2: GC conditions for TCE quantification in pure cultures of isolate BB5.1 ... 67*
*Table 4-3:Results of TCE consumption experiments on the isolate BB5.1 ..... 70*
*Table 5-1: GC conditions for TCE quantification in enriched sediment ..... 88*
*Table 5-2 :Summary of methane oxidation kinetics for enriched sediment ..... 89*
*Table 6-1: DNA from M. marinus A45 and cell equivalents ..... 105*
*Table 7-1: Summary of kinetics experiments on sediment ..... 116*
*Table 7-2: Summary of kinetics experiments on isolate BB5.1 ..... 116*

# Table of Figures

*Figure 1-1: Methane oxidation by methanotrophs* ........ 13
*Figure 1-2: Aerobic TCE biotransformation* ........ 14
*Figure 1-3: Unrooted phylogenetic tree* ........ 15
*Figure 2-1: Southern California Coast and Newport Bay Estuary* ........ 32
*Figure 2-2: Methane oxidation in fresh sediment* ........ 33
*Figure 2-3: Methane oxidation in fresh sediment* ........ 34
*Figure 2-4: Methane oxidation in fresh sediment* ........ 35
*Figure 2-5: Methane oxidation in fresh sediment* ........ 36
*Figure 3-1: Colony blots with Type I probe* ........ 55
*Figure 3-2: Blots of gels with EUP probe* ........ 56
*Figure 3-3: Blots with Type I probe* ........ 57
*Figure 3-4: Blots with Type II probe* ........ 58
*Figure 4-1: Methane uptake velocity vs. cell mass* ........ 74
*Figure 4-2: Methane oxidation by isolate BB5.1* ........ 75
*Figure 4-3: Methane oxidation by isolate BB5.1* ........ 76
*Figure 4-4: Methane oxidation by isolate BB5.1* ........ 77
*Figure 4-5: Methane oxidation by isolate BB5.1* ........ 78
*Figure 4-6: Methane oxidation by isolate BB5.1* ........ 79
*Figure 4-7: TCE degradation vs. cell mass* ........ 80
*Figure 4-8: TCE oxidation by isolate BB5.1* ........ 81
*Figure 4-9: TCE oxidation by isolate BB5.1* ........ 82
*Figure 4-10: TCE concentration vs. time* ........ 83
*Figure 5-1: Methane oxidation by low-methane, low-nitrate enrichment* ........ 94
*Figure 5-2: Methane oxidation by low-methane, high nitrate enrichment* ........ 95
*Figure 5-3: TCE oxidation by low-methane, high nitrate enrichment* ........ 96
*Figure 5-4: TCE concentration vs. time, low methane-low nitrate enrichment* ........ 97
*Figure 5-5: TCE concentration vs. time, high methane enrichment* ........ 98
*Figure 6-1: Blots of control DNA and sample DNA hybridized to probe 1035-RuMP* ........ 108
*Figure 6-2: Blots of control DNA and sample DNA hybridized to probe 1034-Ser* .... 109

# 1. Introduction: Marine Methanotrophs and Bioremediation of TCE

## 1.1 Bioremediation for cleanup of TCE

An increasing number of environments are now known to be contaminated with hazardous wastes, due to methods of storage and disposal of these substances which, although considered to be safe at the time, have proved in the long run to be inadequate. One of the most common of these contaminants is trichloroethylene (TCE), a widely-used degreaser and organic solvent (Schaumberg, 1989). TCE has been classified by the United States Environmental Protection Agency (EPA) as a priority pollutant due to its toxicity, potential carcinogenicity, and ubiquitous pollution of natural water bodies. Under the Safe Drinking Water Act, a standard of 5 ppb or 38 nM has been established as a permissible level of TCE. Most of the current technology available for dealing with contaminated sites involves removing the contaminated material and either treating it in specialized facilities or placing it in a toxic waste dump for indefinite storage. Recent analyses have shown such strategies to be expensive and ineffective in the long term, as well as involving time-frames which are not considered acceptable. For example, systems involving pumping contaminated water out of the aquifer and removing the TCE by adsorption to activated carbon may require up to hundreds of years to reduce the TCE concentration in the aquifer to regulatory levels, due to slow desorption of the TCE from the aquifer materials (Travis and Doty, 1990). An alternative with potential to overcome these limitations is *in situ* remediation by bacteria. Although some *in situ* processes involve the addition of laboratory-grown bacteria to a contaminated site (Krumme, et al., 1994), in many cases such bacteria cannot compete well with indigenous populations and are not well maintained (Tiedje, et al., 1989). Bioremediation using indigenous populations of bacteria with biodegradative capabilities has been proposed as an alternative to the addition of engineered strains to a contaminated site (Morgan and Watkinson, 1989; Thomas and Ward, 1989). In order to develop a treatment system for use *in situ* with

indigenous populations of bacteria, it is necessary to better understand the population dynamics of these organisms, so that it becomes possible to stimulate these populations and optimize their degradative activities. Additionally it is desirable to be able to create predictive models of the behavior of these bacteria in such treatment systems, so that the usefulness of *in-situ* bioremediation can be assessed before treatment begins.

### 1.2 TCE in marine and coastal systems

The limited information which is available on TCE in marine and coastal systems is found primarily in documents published by government agencies, rather than the scientific literature. A study by Pearson and McConnell (1975) described TCE in estuarine and coastal waters, but concluded that the TCE was not accumulating and was not a hazard. A recent U.S. Department of Defense report (Defense Environmental Restoration Program, 1995) lists a number of coastal military sites which are at various stages of cleanup under federal legislative requirements. Many of these sites contain TCE, although the concentrations are not given. In those cases in which the treatment strategy being used or considered is given, none list bioremediation as a method for TCE removal. In general, extraction or treatment with granulated activated carbon are the proposed remediation methods. These are methods which may be subject to the pitfalls discussed for pump-and-treat systems as discussed above. The TCE contamination at the sites is listed as present in soils and groundwater; it seems likely that some of these sites would be candidates for bioremediation of TCE if this technology were available.

### 1.3 Bacterial degradation of TCE

Although no bacteria have been found which can grow on TCE as a sole source of carbon or energy, fortuitous degradation of TCE by a variety of bacteria has been described for both aerobic and anaerobic systems. Anaerobic degradation has been observed in methanogenic systems (Vogel and McCarty, 1985; Freedman and Gossett, 1989) and

other anaerobic systems (Kleopfer, et al., 1985; Barrio-Lage, et al., 1987). Anaerobic degradation of TCE is characterized by the formation of products in successive stages of dechlorination; some of the end products, such as vinyl chloride, a known carcinogen, are not desirable in the context of ground water cleanup. Additionally, anaerobic biotransformations are usually slower than aerobic ones, making the anaerobic degradation of TCE less desirable for cleanup operations, where time is often a significant factor in the cost of the cleanup (Vogel and McCarty, 1985). Aerobic degradation of TCE, typically faster than anaerobic degradation, has been observed in a variety of bacteria including toluene oxidizers, ammonia oxidizers, propane oxidizers, phenol oxidizers and methane oxidizers (Ensley, 1991; Gantzer and Wackett, 1991). These groups of bacteria are characterized by their production of monooxygenase and dioxygenase enzymes, which fortuitiously oxidize TCE to metabolizable products. The presence of heterotrophic bacteria can allow the complete mineralization (conversion to $CO_2$ and biomass) of TCE by further degrading the products of TCE oxidation (Newman and Wackett, 1991; Uchiyama, et al., 1992).

### 1.4 Methanotrophs for bioremediation of TCE

One of the most attractive classes of organisms for the biodegradation of TCE *in situ* is methanotrophs. It is known that methanotrophs (methane-oxidizing bacteria) can oxidize TCE by means of the methane monooxygenase (Wilson and Wilson, 1985; Fliermans, et al., 1988). Methanotrophs are part of the indigenous bacterial population of many environments that are contaminated with TCE, including marine environments and coastal aquifers. Currently, most bioremediation research on methanotrophs involves uncharacterized mixed cultures obtained from contaminated sites (Alvarez-Cohen and McCarty, 1991; Henry and Grbic-Galic, 1991; Alvarez-Cohen, et al., 1992; Broholm, et al., 1993). While the information gained from such studies is useful, it is site-specific, and as such lacks predictive capability. The factors that control biodegradation of halogenated solvents such as TCE by methanotrophs *in situ* are not well understood, especially

in marine and coastal environments. This study has addressed some of the issues surrounding use of methanotrophs for bioremediation of TCE in marine and coastal environments and is a first step towards the development of predictive models for such systems.

### *1.4.1 Methane monooxygenase*

Methanotrophs obtain energy and reducing power by oxidizing methane to $CO_2$ as shown in Figure 1-1. The first step is the oxidation of methane to methanol by the methane monooxygenase (MMO). Two different MMO enzymes are known, a membrane-bound or particulate form (pMMO), and a cytoplasmic, or soluble form (sMMO) (Prior and Dalton, 1985). In those strains capable of expressing both types of MMO, the ratio of initial copper concentration in the growth medium to the biomass produced determines the form of the MMO that is produced (Stanley, et al., 1983).

#### pMMO

All known methanotrophs express the pMMO, which appears to contain copper (Nguyen, et al., 1994) and can only degrade short-chain aliphatic compounds (Burrows, et al., 1984). The pMMO has been shown to oxidize TCE (DiSpirito, et al., 1992). The amount of copper added to the growth medium has been observed to affect the number of copper centers in the active site of the pMMO in *Methylococcus capsulatus* Bath (Nguyen,et al., 1994), and *Methylomicrobium albus* BG8 (Semrau and Zolandz, 1995). The pMMO has not been reproducibly purified, however, SDS-PAGE analysis shows two polypeptides of approximately 45 and 27 kDa are present in bacteria demonstrating pMMO activity (Dalton, 1992). Recent work on the genes encoding the pMMO (Semrau, et al., 1995) found significant homology between the N-terminal sequence of the 45 kDa polypeptide from three species of methanotrophs and between the methanotrophs and the N-terminal sequence of a similar polypeptide from *Nitrosomonas europaea.* That study also confirmed the presence of two copies of the genes encoding the polypeptides described above. Research is currently underway to purify and further describe the pMMO.

**sMMO**

The sMMO is distinct from the pMMO both in behavior and in polypeptide composition. The following is a summary of a description given in a recent review (Murrell, 1992): The active enzyme is composed of three component proteins, referred to as Proteins A, B, and C. Protein A, the hydroxylase, is made up of two copies each of three subunits α, β and γ. Protein A contains the binuclear iron center and is believed to be the site of oxygenation. Protein B is a regulator of electron transfer. Of these, only Protein C has independent catalytic activity. The active enzyme catalyzes the four-electron reduction of oxygen to water in the presence of a hydroxylatable substrate such as methane. In this process, Protein C acts as an NADH oxidoreductase. Protein A receives electrons from Protein C and is the site of substrate activation. Protein B regulates the flow of electrons to this site, causing the enzyme to function as an oxygenase rather than an oxidase. This enzyme has a broad substrate range that includes long-chain aliphatic compounds and aromatic compounds. Over 150 compounds have been shown to be oxidized by the sMMO, including chlorinated methanes, trichloroethylene, toluene, hexane and naphthalene. Since the enzyme acts by inserting an oxygen atom into the substrate, the products of these oxidations are alcohols, aldehydes and epoxides. Copper acts as a specific inhibitor of Protein C by interacting with the binuclear iron center (Pilkington and Dalton, 1990). The genes encoding the sMMO have been described by Stainthorpe, et al. (1990) and references therein. The genes encoding the subunits of Protein A and the genes encoding Proteins B and C were all found to be linked on the chromosomes of *M. capsulatus* Bath and *M. trichosporium* OB3b.

## 1.5 Oxidation of TCE by methanotrophs

Of the two types of methane monooxygenase, the sMMO is known to oxidize TCE at rates which are up to 100-fold higher than those of the pMMO. However, only a few

known species of methanotrophs have been shown to express the sMMO (Lidstrom, 1988). Both types of MMO have been shown to fortuitously degrade TCE by converting it to its epoxide as shown in Figure 1-2. The products of TCE oxidation by the MMO have been shown to include chloral (Newman and Wackett, 1991), however, no evidence has been obtained to indicate that chloral accumulates in the system, due to further breakdown by heterotrophic bacteria and abiotic reactions.

All of the methanotrophs isolated in this study apparently contain the pMMO only. It is believed that this enzyme may be important in the natural environment due to its prevalence in known strains under copper-sufficient conditions, since copper levels are not expected to be limiting in many environments. It has been shown by Berson and Lidstrom (1996) that *M. albus* BG8 has a very efficient copper uptake system, and it can be calculated from the publication by Stanley, et al. (1983) that only about $10^6$ atoms of Cu per cell are required to "switch on" production of the pMMO in strains that can produce both the sMMO and the pMMO. At the relatively low population densities that are typical of methanotrophs in the environment, these factors support the idea that the pMMO is important. In addition, the pMMO may be a useful enzyme for in-situ bioremediation due to its higher affinity for the substrates than the sMMO (Burrows, et al., 1984). This would allow the degradation of the pollutant to lower concentrations than could be achieved with the sMMO alone.

The fortuitous degradation of TCE, a non-growth substrate, by methane monooxygenase is referred to as cometabolism. Several aspects of bacterial metabolism are involved in the cometabolism of TCE. TCE and methane compete for the same active site on the MMO molecule, so they are competitive inhibitors (Fox, et al., 1990). The oxidation of both substrates requires reducing equivalents in the form of NADH, for which competition also occurs (Fox, et al., 1990; Henry and Grbic-Galic, 1991). The effects of competition were avoided in this study by measuring the oxidation of each substrate in the absence of the other. The products of TCE oxidation are toxic to the

cells, so as more TCE is oxidized, the toxic effects of these products become apparent (Henry and Grbic-Galic, 1991). Effects of toxicity were avoided in this study in most cases by limiting the time of the experiments to the initial period in which toxic effects of the products of TCE oxidation were not yet significant and by using low TCE concentrations. However, concentrations of TCE greater than 50 μM are toxic to most methanotrophs, and in one experiment toxicity at high TCE concentrations was observed. The concentration of copper also influences the kinetics of the degradation of TCE by bacteria producing the pMMO (Semrau, 1995). These studies show that while the maximum rate of degradation ($V_{max}$) is quite constant over the range of copper concentrations that were tested, the apparent half-saturation constant ($K_s$) for TCE increases dramatically as the amount of copper added to the medium for growth decreases. Data from electron paramagnetic resonance (EPR) studies suggest that the change in $K_s$ values at low copper is correlated with the loss of copper from the active site of the MMO. Work is in progress by others to delineate this mechanism (Nguyen, et al., 1994). Due to these observations, copper concentration was the principal parameter varied in experiments using pure cultures of methanotrophs in this study, in order to obtain the initial information which will be necessary to model the behavior of the bacteria with respect to copper.

### 1.6 Characteristics of known methanotrophic strains

Methanotrophs are a restricted group of Gram-negative rods, vibrio and cocci. The majority of strains that have been isolated are obligate methylotrophs, able to grow only on methane or methanol (Lidstrom, 1988). Three general groups of methanotrophs have been identified based on their patterns of internal membranes, carbon assimilation pathways, 16S rRNA sequences, percent G+C content, and predominant fatty acid chain length (Hanson, et al., 1988). Type I methanotrophs have stacks of membranes throughout the cytoplasm, while Type II strains have intracytoplasmic membranes in rings

around the periphery of the cell. Recent phylogenetic analyses have led to the proposal that the strain designated as Type X, *Methylococcus capsulatus* Bath (MC Bath), be categorized as a Type I, although it has characteristics of both types (Bowman et al., 1993). These analyses showed MC Bath to be most closely related to other members of the genus *Methylococcus*, all of which are designated as Type I. Based on results from similar analyses, the species known as *Methylobacter albus* BG8, along with two other strains formerly identified as *Methylobacter* strains, has now been designated as belonging to another genus altogether. The new genus is called *Methylomicrobium,* and the strain *M. albus* BG8 is now called *Methylomicrobium albus* BG8 (Bowman, et al. 1995). The characteristics of the methanotrophic genera are summarized in Table 1-1.

***Table 1-1. Characteristics of Methanotrophic Bacteria****

| Genus | Methylomonas | Methylobacter | Methylo-microbium | Methylococcus | Methylosinus | Methylocystis |
|---|---|---|---|---|---|---|
| Phylogenetic Position | gamma proteobacteria | gamma proteobacteria | gamma proteobacteria | gamma proteobacteria | alpha proteobacteria | alpha proteobacteria |
| Membrane Type | Type I | Type I | Type I | Type I | Type II | Type II |
| Metabolic Path | RuMP | RuMP | RuMP | RuMP/RuBP | Serine | Serine |
| Enzyme type | pMMO# | pMMO | pMMO | sMMO/pMMO | sMMO/pMMO | pMMO# |
| Major Fatty Acid | 16:1, 14:0 | 16:1 | 16:1 | 16:1,16:0 | 18:1 | 18:1 |
| $N_2$ fixation | No | No | No | Yes | Yes | Yes |

* after Murrell and Holmes, 1995
# one strain has been described with the sMMO in this genus

### 1.7 Methanotroph phylogeny

Ribosomal RNA (rRNA) can be used as an evolutionary chronometer for bacteria, due to the antiquity of the protein-synthesizing process. These molecules are functionally constant, universally distributed and well-conserved across large phylogenetic distances (Brock, 1991; Woese, 1987), and as such their sequences can serve as a basis for comparing evolutionary distances. One of the larger rRNA molecules, known as the 16S rRNA and containing about 1500 nucleotides, combines relative ease of handling with enough sequence variability to make it an especially useful tool for determining bacterial phylog-

eny. The use of 16S rRNA for this purpose was developed by Carl Woese (1987) and is used extensively today. Evolutionary distances are used to create phylogenetic trees which have revolutionized bacterial systematics.

The most far-reaching of these trees established the three-domain proposal, suggesting that all known life is divisible into three branches: the eucarya, the bacteria, and the archaea. Perhaps most startling was that the archaea, which is a group composed of many organisms thought to be quite primitive, is closer in evolutionary distance to the eucarya than to the bacteria.

The phylogeny of methanotrophs has been reevaluated in light of the new molecular techniques and the three-domain proposal. The current genera and species groupings of methanotrophs are based partly on 16S rRNA sequences and partly on previous classifications. The original groups of methanotrophs have held up remarkably well in the face of the new molecular data (Murrell and Holmes, 1994). As stated in Table 1-1, the known methanotrophs belong to the alpha and gamma subgroups of the proteobacteria, which falls into the bacterial domain. Figure 1-3 shows an unrooted phylogenetic tree for methanotrophs.

### *1.7.1 Methanotroph gene probes*

Sequences of methanotroph 16S rRNA have been used to develop gene probes for detection and identification of methanotrophs in environmental samples (Brusseau, et al., 1994 and Holmes, et al., 1995). These probes exploit the duplex nature of DNA and RNA molecules; an oligonucleotide labelled with $^{32}P$ (or other label) will hybridize to its complementary sequence in the sample of interest under conditions that are favorable for annealing. The radioactive label then makes it possible to detect the annealled molecules and hence the presence and/or location of sequences of interest in the samples. In the present study, probes based on 16S rRNA sequences were used to distinguish groups of methanotrophic genera. The 1035-RuMP probe detects genes specific to Type I

methanotrophs and the 1034-Ser probe detects genes specific to Type II methanotrophs (Brusseau, et al., 1994). These probes were developed based on 16S rRNA phylogenetic trees which showed that groups of methanotrophs which use the same metabolic pathways form clusters within the tree. These clusters can be used to identify sections of the rRNA molecule which are conserved within the cluster, and can then be used diagnostically for the members of that cluster. The metabolic clusters can be noted from Table 1-1, in which the carbon assimiliation pathway utilized by each genus is shown. The Type I group uses the ribulose monophosphate (RuMP) pathway, and the Type II group uses the serine (Ser) pathway. The development of the probes that were used for this study is described in Chapter 3.

## 1.8 Occurrence of methanotrophs in aquatic environments

Aerobic methane oxidation activity is typically concentrated at interfaces between oxic and anoxic regions in both freshwater and marine environments (Hanson, 1980; Ward, et al., 1989). Methanotrophs are present at these interfaces where their two main substrates, methane and oxygen, are at their maximum combination of concentrations. This is true whether the interface occurs in the water column, in the sediments, or in subsurface zones of waterlogged soils. Methanotrophs also occupy niches in association with the roots of plants (King,1994) and in unsaturated soils.

### *1.8.1 Methanotrophs in sediments*

Methanotrophic activity in marine and estuarine sediments was the focus of this study. In sediments, aerobic methane oxidation may occur in a zone only a few millimeters thick, which nonetheless may limit the methane flux out of the sediment to less than 10% of the amount produced (King, 1992 and references therein). Oxygen availability is thought to control sediment methane oxidation (Kuivila, et al., 1988; King, 1992), based on observations that methane oxidation is sensitive to changes in the depth of oxygen penetration

into the sediment. Similar observations in peat bogs and swamps have bolstered these ideas. Additional observations by King (1992) suggest that sediments may be relatively enriched for anoxia-tolerant methanotrophs which can survive well in fluctuating conditions of oxygen availability.

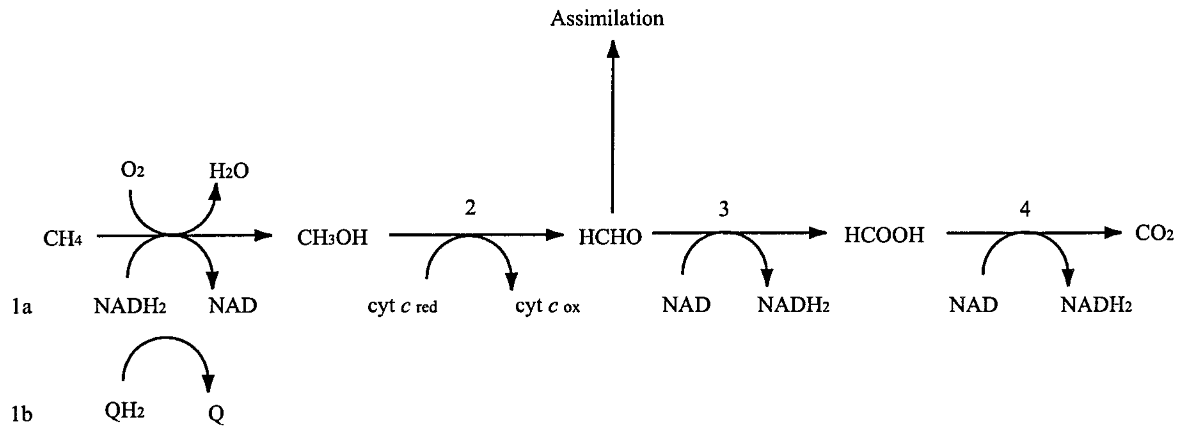
### *1.8.2 Methanotrophic isolates*

Methanotrophs have been isolated from a wide variety of habitats including terrestrial, freshwater, and marine. (Whittenbury, 1970; Lidstrom, 1988; Koh, et al., 1993) All the organisms isolated so far are similar and fall into the main categories discussed above. The methanotrophs isolated in this study do not appear to be exceptional in this regard. A recent review (King, 1992) has suggested that the similar enrichment and isolation techniques that have been used by investigators attempting to isolate methanotrophs has resulted in the isolation of similar species, in spite of the variety of environments sampled. The taxonomy and physiology of methanotrophs may be much more diverse than the current body of isolates indicates, as suggested by the King review, given that methanotrophs in the environment may be exposed to a variety of methane and oxygen regimes as well as variable temperature and pH. New enrichment and isolation techniques will give more information on the true diversity of methanotrophic bacteria, but were beyond the scope of this study. Gene probing techniques were used to obtain a first look at the population diversity of methanotrophs in an estuary sediment; these experiments will be described in subsequent chapters.

## 1.9 Significance of this work

This study has been the first direct comparison of the behavior of marine methanotrophs between the laboratory and the field. It has begun to address the major issues surrounding the optimization of the use of marine methanotrophs for in-situ bioremediation of TCE. These issues include the effect of copper concentration on the kinetics of methane and TCE oxidation by marine methanotrophs and the effect of enrichment on the popula-

tion of marine methanotrophs in an estuary sediment. This study is a first step in the development of predictive models for the use of marine methanotrophs to bioremediate marine and coastal environments which are contaminated with TCE. Such models will be based on the kinetic parameters for methane and TCE oxidation which have been determined here, and on the population dynamics under enrichment which have been examined for this estuary sediment. Additionally, this study has provided the field of methanotrophy with several new marine methanotrophic strains which may provide further insight into the ecology and genetics of marine methanotrophs and methanotrophs in general.




1a: sMMO (soluble methane monooxygenase)
1b: pMMO (particulate methane monooxygenase)
2: Methanol dehydrogenase
3: formaldehyde dehydrogenase
4: formate dehydrogenase
Q: quinol

***Figure 1-1: Methane oxidation by methanotrophs***

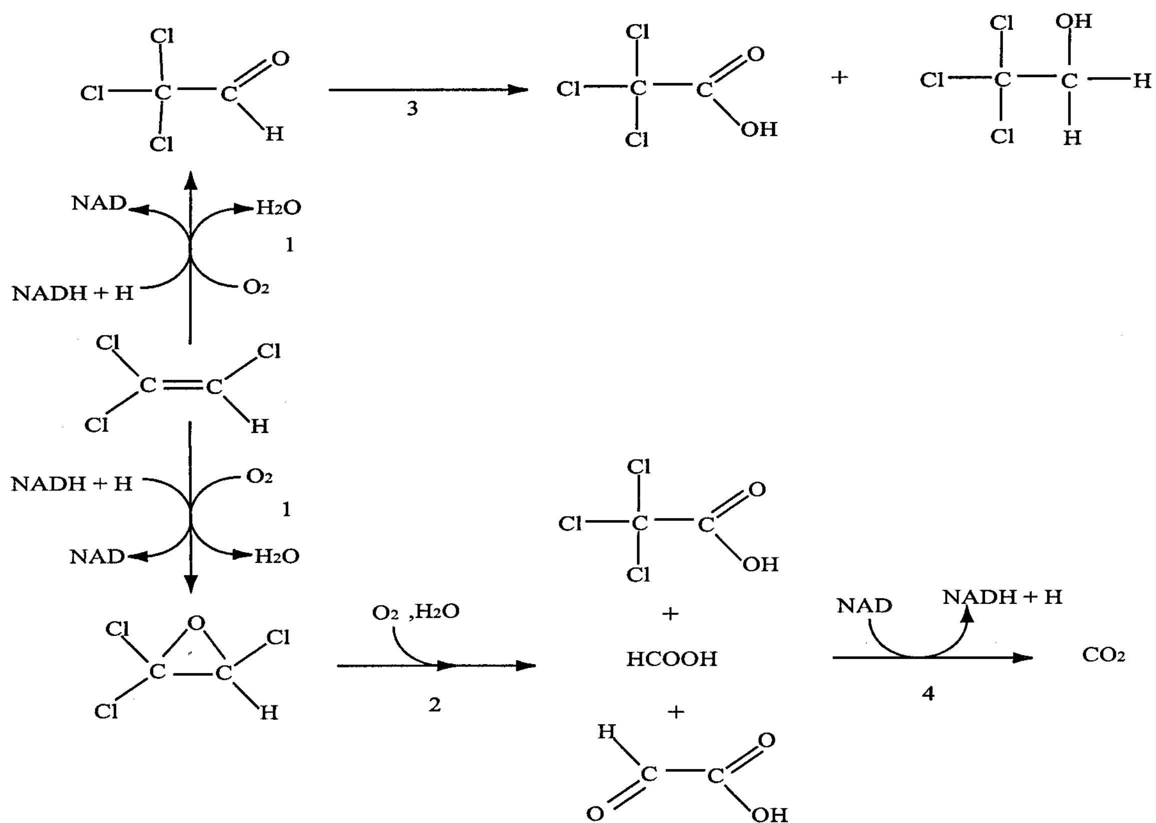



*Figure 1-2: Aerobic TCE biotransformation*

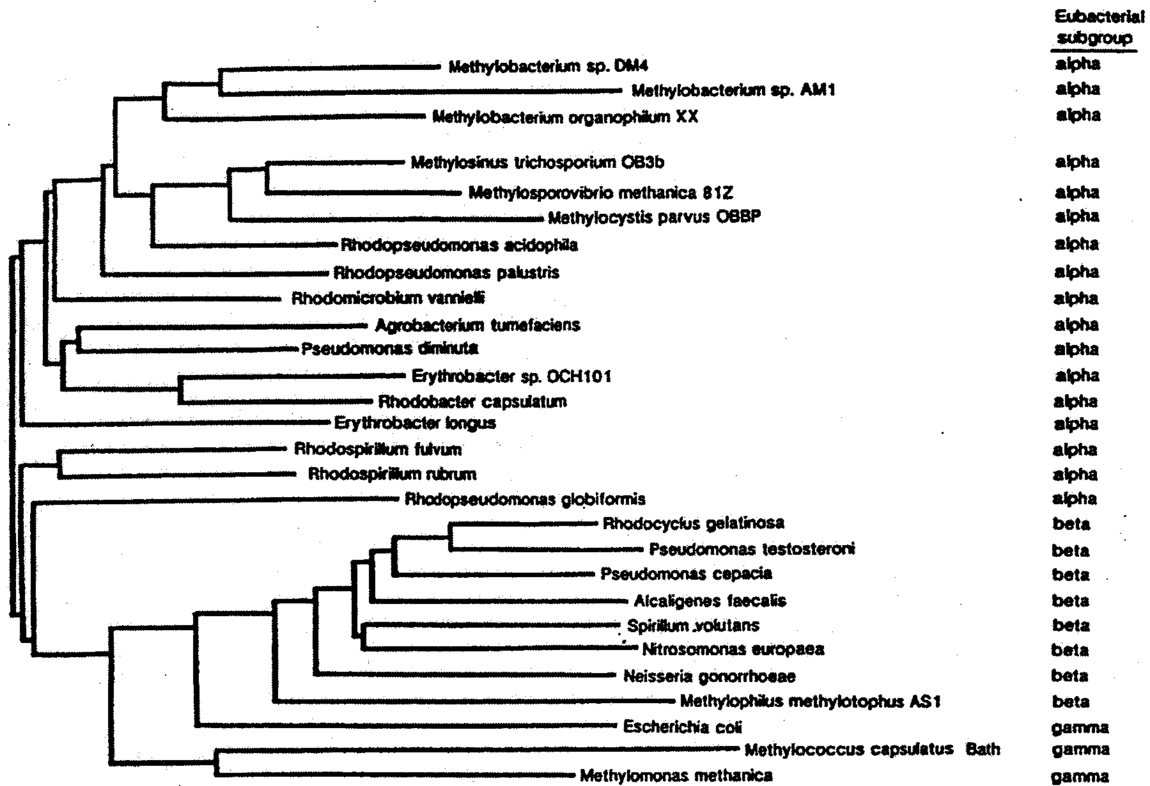



***Figure 1-3: Unrooted phylogenetic tree***

Reproduced with permission from : Hanson RS, Netrusov AI, Tsuji K (1988) The Obligate Methanotrophic Bacteria: *Methylococcus, Methylomonas,* and *Methylocystis*. In: Balows A, Truper H-G, Dworkin M, et al. The Prokaryotes. Springer-Verlag, New York, NY, pp. 2350-2354

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# 2. Field Sampling and Methane Oxidation in Fresh Sediment

## 2.1 Introduction

Although methanotrophs are an attractive class of bacteria for in-situ bioremediation of TCE, very little information is available about methanotrophs in coastal or marine environments, as discussed in Chapter 1. In this study, we began investigating the use of methanotrophs for bioremediation of TCE in coastal environments by selecting a coastal field site based on several criteria. First, methanotrophic activity was necessary, preferably at relatively high levels so that it could be easily measured. This activity was most likely to occur where high levels of organic material were present to support high levels of methane production (Lidstrom, 1988). Second, the site had to be accessible to Caltech so that samples could be collected and returned to the lab in a short period of time, and last, the area near the Kerckhoff Marine Laboratory was attractive since that laboratory facility would be available if necessary. Initially, the harbor outside of the marine lab was sampled by Dr. Wheeler North and by another graduate student, Kelly Goodwin, using scuba gear. These samples were successfully enriched with methane for the isolation of pure cultures of methanotrophic bacteria (described in Chapter 3), but repeated sampling of the harbor proved to be impractical for the purposes of this study, since trained scuba divers were necessary to collect the samples. A nearby estuary was selected for the rest of the sample collection, described in Section 2.2.1. Samples from the estuary were used to measure the kinetics of methane oxidation by the native methanotrophic population. These data were collected as a baseline for studies on the enrichment of the sediment for the purpose of enhanced TCE biodegradation.

## 2.2 Materials and methods

### *2.2.1 Field Site*

Samples for measurement of methane oxidation rates were collected from the Newport Bay estuary. Figure 2.1, reproduced from Manley, et al. (1992), shows the location of the

estuary on the Southern California coast. The estuary is tidal, and has input both from tidal ocean water and from inland freshwater. The estuary itself is legally protected as an ecological reserve, however the surrounding area is heavily developed with houses and other residential structures (e.g., culverts, parks, and streets). Due to this development, we assume that other inputs to the estuary will include nitrogen and hydrocarbon compounds from fertilizer and petroleum runoff. The site has not been identified as having any other pollution. The bacterial communities are therefore expected to have no prior exposure or adaptation to TCE. The bacterial communities were expected to include significant numbers of methanotrophs due to the high organic content of the sediments, typical of estuaries. The organic content usually results in anaerobic bacterial production of methane in the underlying sediments and high numbers of methanotrophs in the aerobic sediments into which the methane diffuses, as described in Chapter 1.

### *2.2.2 Sample Collection*

Samples were collected at low tide, on days in which the low tide occurred at mid-morning. This timing allowed the transport of the sediment to Caltech and performance of the methane consumption experiments on the same day. Bulk sediment was scooped into 1-L plastic bottles by hand, from sediments covered with shallow water up to about 10 cm depth. Only water-covered sediments were collected to ensure saturated conditions in the sediment. No attempt was made to maintain the original structure of the sediments since the methane oxidation rates were to be measured in bulk. The aerobic regions were assumed to consist of the light brown layer of sediment overlying a black layer which smelled of sulfide if disturbed. The brown layer was usually about 2-5 cm deep. Larger organisms such as snails were present on some sampling trips. Collection of such organisms was generally avoided, however, when these organisms did get into the samples that were brought to Caltech they were removed before the methane oxidation activity was measured. Table 2-1 shows the sample collection dates and characteristics of

the sediment samples.

***Table 2-1: Sample collection for measurement of methane oxidation in fresh sediment***

| Collection date | Sediment Temperature (°C) | Pore Water Total Carbon (ppm)[a] | Pore water total [Cu] (μmolal)[b] |
|---|---|---|---|
| 1 March 94 | 20 | 420 | 1.77 |
| 13 Apr 94 | 22 | 705 | 1.47 |
| 28 Nov 94 | 14.5 | 530 | 1.29 |
| 7 Apr 95 | 22 | 515 | 0.95 |
| 20 Jun 95 | 23.5 | 485 | 0.99 |

a) Measured by Shimadzu TOC-5000A; samples filtered with 0.45μm syringe filter and diluted 50x before analysis. Values are reported at full strength.
b) Measured by Perkin-Elmer ICP-MS; samples filtered with 0.45μm syringe filter and diluted 10x before analysis. Values are reported at full strength.

### *2.2.3 Measurement of methane oxidation kinetics*

Methane oxidation in the fresh sediment was measured by incubation with $^{14}CH_4$ and measurement of $^{14}C$-labelled products (carbonates and cell material) by scintillation counting. The method is a modification of the method of Rudd, et al. (1974), which consists of incubation with $^{14}CH_4$ and $CH_4$, and measurement of $^{14}C$-labelled products (carbonates and cell material) by scintillation counting. The rate of methane oxidation is measured as the rate of appearance of labelled products after incubation with $^{14}CH_4$ and $CH_4$ in a constant ratio. $^{14}C$-labelled products consist mostly of carbon dioxide and cell material, with small amounts of labelled methanol, formaldehyde and formate also occurring. To measure the overall rate of appearance of these products, the carbon dioxide must be dissolved so that all of the products are in solution. Therefore concentrated sodium hydroxide is added at various time points in the experiment to raise the pH, which stops the reactions by killing the bacteria and dissolves the carbon dioxide. Then

the residual $^{14}CH_4$ and $CH_4$ can be flushed out, and the remaining labelled compounds are all products of the bacterial action. These products can be quantified by scintillation counting and the rate of their appearance can be calculated. Since the stoichiometry of methanotrophy is such that each molecule of the labelled compounds must be produced as a result of the reaction of a molecule of $^{14}CH_4$, the rate of appearance of labelled products is equal to the rate of oxidation of $^{14}CH_4$; the overall rate of methane oxidation can be calculated from the ratio of $^{14}CH_4$ to $CH_4$ added.

By the time the sediment had been transported to the laboratory, the sediment had settled to the bottom of the bottles and the top 1/3 to 1/4 of the bottle contained water with very little sediment. This water was poured off, and the sediment was slurried with approximately 10% (by volume) of this overlying water to facilitate handling. Aliquots of 5.5 ml of this slurry were dispensed into sterile, acid-washed 35-ml serum vials (Pierce), which were closed with solid rubber stoppers (Bellco Glass Co.) and aluminum crimp seals. The serum vials were found to have an actual average volume of 37.5 ml when sealed. The serum vials were sterilized by autoclaving, and acid-washed according to the method of Semrau (1995). The acid-washing protocol consists of soaking the vials in 1N HCl overnight and removing the acid by repeated rinses in deionized-distilled water. Methane (reagent-grade, Matheson Gas Co.) was added through the stopper with gas-tight syringes (Series A-2, Dynatech Precision Sampling Co.) in varying amounts to give a range of dissolved methane concentrations. Partition coefficients were calculated from the model of Duan, et al. (1992). The vials were then spiked with $^{14}CH_4$ (synthetic, DuPont NEN, specific activity = 55mCi/mmol) in amounts calculated to give a constant ratio of $^{14}CH_4$ to $CH_4$ in each vial. The vials were incubated at room temperature with shaking (200 rpm). At various time points, vials were removed from the shaker and 50% (w/v) NaOH was added to a liquid concentration of 1N. As discussed above, the NaOH serves both to stop the methane consumption by raising the pH above the range tolerated by the bacteria, and also to dissolve the gas-phase $CO_2$ and $^{14}CO_2$ in the vial (Rudd, et al.,

1974). The vials were stored after the addition of NaOH at 4°C until all the time points were completed. If the experiment was completed during the night the vials were stored until the following day before subsampling. The vials were allowed to return to room temperature and then flushed with air to remove residual methane. Then 0.5 ml subsamples of the sediment slurry were dispensed into 7-ml scintillation vials containing 4.5 ml of scintillation fluid (Safety-Solve brand) and counted on a Packard liquid scintillation counter. Initially, the experimental time courses were run for up to 40 hours after the methane was added. This length of time appeared to be sufficient for the bacteria to begin growing, however, based on the exponential shape of the concentration versus time plots. Subsequent experiments were run for no longer than 24 hours in order to measure the initial (linear) methane consumption rate by the original in-situ population. The controls were carried out in the same manner as the experimental vials, except using autoclaved sediment.

### 2.3 Results

The $^{14}C$-labelled products were assumed to be produced in the same ratio to non-labelled products as the ratio of $^{14}CH_4$ to $CH_4$ added to the vials. The rate of $^{14}CH_4$ consumption was assumed to be equivalent to the rate of appearance of $^{14}C$-labelled products due to the stoichiometry of methane oxidation by methanotrophs (see Fig. 1-1). Plots of the velocity of methane uptake versus initial methane concentration are shown in Figures 2-1 to 2-4. Initial rates of uptake at each concentration were determined from linear regression of the scintillation counter data versus the elapsed time at which the NaOH was added. The data were modelled using the Michaelis-Menten model for enzyme kinetics. This model is described in Appendix B. The values of the uptake velocity at substrate saturation ($V_{max}$) and the half-saturation constant ($K_s$) were determined throughout this study by nonlinear regression using the software package TableCurve2D for Windows, version 2.0 (Jandel Scientific). This package uses the Levenburg-Marquardt algorithm

with matrix inversion by the Gauss-Jordan method for least-squares fitting. References for these methods are given in the user's manual for the package. The results of the nonlinear regression analyses are presented in Appendix A, including the 95% confidence limits for the $V_{max}$ and $K_s$ values. The $V_{max}$ and $K_s$ values are summarized in Table 2-2. All of the values are calculated on the basis of the volume of sediment in liters.

***Table 2-2: Summary of Michaelis-Menten parameters for methane oxidation in Newport Bay Estuary Sediment***

| Expt.# | Date | $K_s$ (μM) | $V_{max}$ (μM/hr) |
|---|---|---|---|
| 2 | 1 March 1994 | 8.9 | 1.0 |
| 4 | 13 April 1994 | 25.7 | 0.01 |
| 7 | 28 November 1994 | 8.2 | 2.3 |
| 8 | 7 April 1995 | 11.8 | 0.72 |

### *2.3.1 Mass transfer calculations*

As discussed in Danckwerts (1970), if a gas is dissolving into a liquid phase where it then undergoes an irreversible reaction, a criterion can be established for the situation to exist wherein the gas reacts slowly enough in the liquid that the dissolved concentration of the gas in the bulk of the liquid is nearly equal to the equilibrium concentration of the gas with respect to the headspace concentration. The existence of this situation is central to the assumptions of the substrate oxidation assays performed in this study, since the initial concentration of substrate used for the Michaelis-Menten model is A) calculated from Henry's Law, an equilibrium condition, and B) assumed to change less than 10% over the course of the assay (Robinson, 1985). The criterion established by Danckwerts relies on a measured mass transfer coefficient; for the purposes of this study the mass transfer coefficient $k_La'$ has been calculated from the film coefficient $k_L$, measured for oxygen transfer in a shake flask (van Suijdam, et al., 1978) multiplied by the specific surface area of the liquid in the serum vials. The specific surface area is calculated from the measured

surface area of the quiescent liquid divided by the total volume of liquid as shown:

$$a' = A/V \qquad (2 - 1)$$

Where : $a'$ = specific surface area, units of $m^{-1}$

$A'$ = measured surface area, units of $m^2$

$V$ = liquid volume, units of $m^3$

The film coefficient is shown by van Suijdam, et al. (1978) to depend on the orbiting speed of the flask; the value used for this study, 1.1 x $10^{-4}$ m/sec is calculated based on an orbiting speed of 220 rpm. This speed is close to the shaking speed used in this study, which was between 200 and 250 rpm. Since the vials were shaking during the experiments, the measured surface area is probably an underestimate of the actual surface area that was available for gas transfer. The film coefficient for oxygen transfer was taken to be a good approximation for that of methane since the Henry's Law coefficients for oxygen and methane differ by less than 20% (Stumm and Morgan, 1981) For the measurement of methane oxidation in the fresh sediment, a liquid volume of 5.5 ml was used. The surface area measured for this volume was 1.46 x$10^{-3}$ $m^2$, which gives a specific surface area of 265 $m^{-1}$. Using this value and the calculated film coefficient, for these experiments kLa' = 0.029 $s^{-1}$ or 104.8 $hr^{-1}$. Using this mass transfer coefficient, the observed rate of absorption of the gas into the liquid phase can be described as shown (Danckwerts, 1970):

$$\overline{R}a' = kLa'(A^* - A^o) \qquad (2 - 2)$$

Where : $\overline{R}a'$ = observed rate of absorption per unit volume of liquid

$kLa'$ = mass transfer coefficient, units of reciprocal time

$A^*$ = equilibrium dissolved concentration

$A^o$ = bulk dissolved concentration

Then if the bulk liquid is saturated with the dissolved gas,

$(A^* - A^o) << A^*$ (2-3)

and

$$\overline{R}a'/kLa' A^* << 1 \qquad (2\text{-}4)$$

If this criterion is true for the measured rates of methane uptake in these assays, then the measured rate can be taken to be equal to the rate of reaction in the liquid phase, in this case biological oxidation.

This criterion, which will be referred to as the Danckwerts criterion, was calculated for the measured rates of methane uptake in the fresh sediment. In all cases, the Danckwerts criterion was at least 3 orders of magnitude less than one. The calculated values are listed in Appendix C.

### 2.4 Discussion

The values that were found for $K_s$ for the fresh sediment are comparable to those reported in the literature for other environments (Table 2-3), while the $V_{max}$ values were much lower. The exceptions are experiment #4, in which a somewhat higher $K_s$ value and a very low $V_{max}$ value were found, and the study of King (1990) in which quite low $K_s$ values and very high $V_{max}$ values were found for a wetland sediment .

***Table 2-3: Michaelis-Menten parameters for methane oxidation in enrvironmental samples***

| Reference | Environment | $K_s$ (μM) | $V_{max}$(μM/hr) |
|---|---|---|---|
| Kuivila, et al., 1988 | freshwater sediment | 10 .0± 7.0 | 26.4±18 |
| Lidstrom and Somers, 1984 | freshwater sediment | 8.3-10.7 | 33-43 |
| Buccholz, et al., 1995 | freshwater sediment | 4.1-9.6 | 12.7-35.2 |
| Whalen, et al., 1990 | landfill soil | 4.1-8.3 | 15.9 |
| King, 1990 | wetland sediment | 2.2-3.7 | 663-1442 |

These results suggest that methane oxidation in the Newport Bay estuary is similar to methane oxidation in other sediments and landfill soil. The $K_s$ values suggest that the estuary organisms are using the pMMO or a similar enzyme for methane oxidation, since the $K_s$ values are lower than those reported for the sMMO (Oldenhuis, et al., 1991) . If methanotrophs capable of producing the sMMO are present, these data suggest that these organisms are not copper-limited, since, as discussed in Chapter 1, the copper level per cell determines whether the pMMO or the sMMO will be produced in organisms that are capable of producing both. Data from Berson and Lidstrom (1996) indicate that the determining factor in copper accumulation by the bacteria is the cupric ion concentration to which the bacteria are exposed rather than the total copper concentration. Further research will be necessary to determine the amount of copper available to the bacteria in this environment. The total copper in the porewater by itself should be capable of supporting a methanotroph population of $10^5$ to $10^6$ cells/ml if the fraction of bioavailable copper is high.

Measured values of $V_{max}$ can be used as an indicator of the total catalytic potential in a system. Previous research (Roslev and King, 1995; Lidstrom, 1996) has shown that the rate of methane oxidation per cell remains fairly constant in cells grown under a variety of conditions, including starved, nutrient-limited and nutrient excess. Therefore, the $V_{max}$ values can be used to estimate the number of methanotrophs in the sediment. Previous measurements have determined the approximate rate of methane oxidation per cell by laboratory methanotrophs to be $10^{-9}$ umol/hr/cell (Lidstrom, 1996). Using this value, the number of methanotrophs in the estuary sediment can be calculated to be on the order of $10^5$ to $10^6$ cells/ml. In contrast, the freshwater sediments shown in Table 2-3 can be estimated to contain on the order of $10^7$ cells/ml, and the wetland sediment can be estimated to contain up to $10^9$ cells/ml. A typical laboratory culture grown to late-expo-

nential phase will have $10^8$ to $10^9$ cells/ml. If the rate of methane oxidation per cell is similar in marine methanotrophs to that in strains from freshwater environments, the estuary sediment contains a somewhat lower population of methanotrophs than the environments discussed in the literature. Usually the population of methanotrophs is limited by the availability of methane and oxygen in a given environment (King, 1992), however the methane and oxygen fluxes in the estuary sediment have not been measured. These measurements were beyond the scope of this project, however, more information about methanotrophs in the estuary sediment was obtained from pure cultures of methanotrophic isolates from the sediment, and by the use of gene probing techniques. These experiments are discussed in subsequent chapters.

### 2.5 Conclusions

In order to use native populations of methanotrophs for *in-situ* bioremediation, it will be necessary to predict the rate of methane and TCE oxidation by the organisms. It is therefore necessary to know under what conditions the organisms exist before enrichment takes place. The measurement of the kinetic parameters for methane oxidation and the estimation of the number of methanotrophs present provide key data in this regard. These calculations and measurements suggest that the nethanotrophs in the estuary sediment have a methane monooxygenase with a similar substrate affinity (indicated by the $K_s$ values) to methanotrophs in other sediments, but that low numbers of organisms appear to be present relative to the other environments studied. This information may indicate that substantial enrichment of the population is necessary before significant rates of TCE degradation are achieved for *in-situ* bioremediation. The $K_s$ values also suggest that the estuarine population contains an MMO similar to the pMMO found in laboratory cultures. This enzyme, due to its greater affinity for TCE than the sMMO (Oldenhuis, 1991), has the potential to degrade TCE to lower concentrations. This is important due to the low regulatory level set for TCE (see Chapter 1). However, it has

been observed that methanotrophs which produce only the pMMO will not degrade TCE under low-copper conditions (Semrau, 1995; this study). If an enriched population of marine methanotrophs becomes limited for available copper, and if organisms producing the sMMO are either not present or are present in low numbers, TCE degradation may be incomplete. Since no sMMO-producing organisms have been isolated from or detected in the marine environment, it is possible that significant enrichment of marine or estuarine methanotrophic populations may actually interfere with TCE degradation by methanotrophs in these environments. These questions are addressed in subsequent chapters of this dissertation.

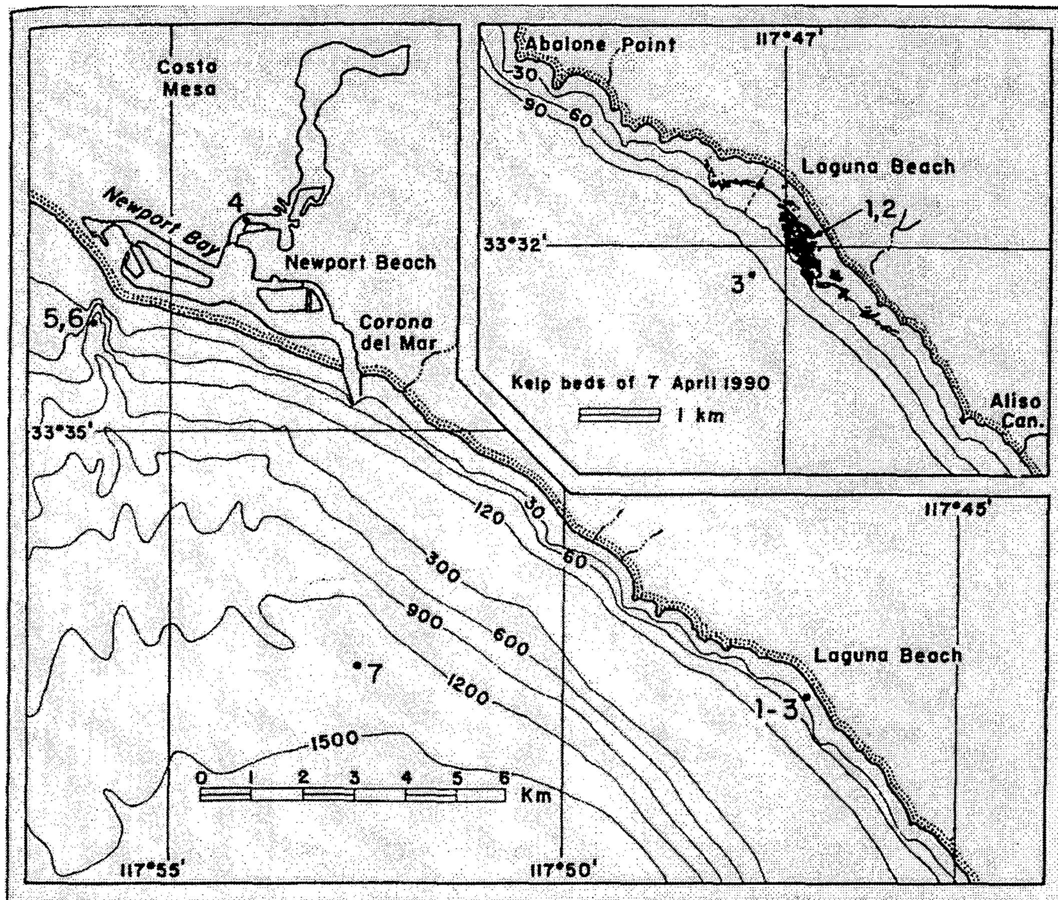
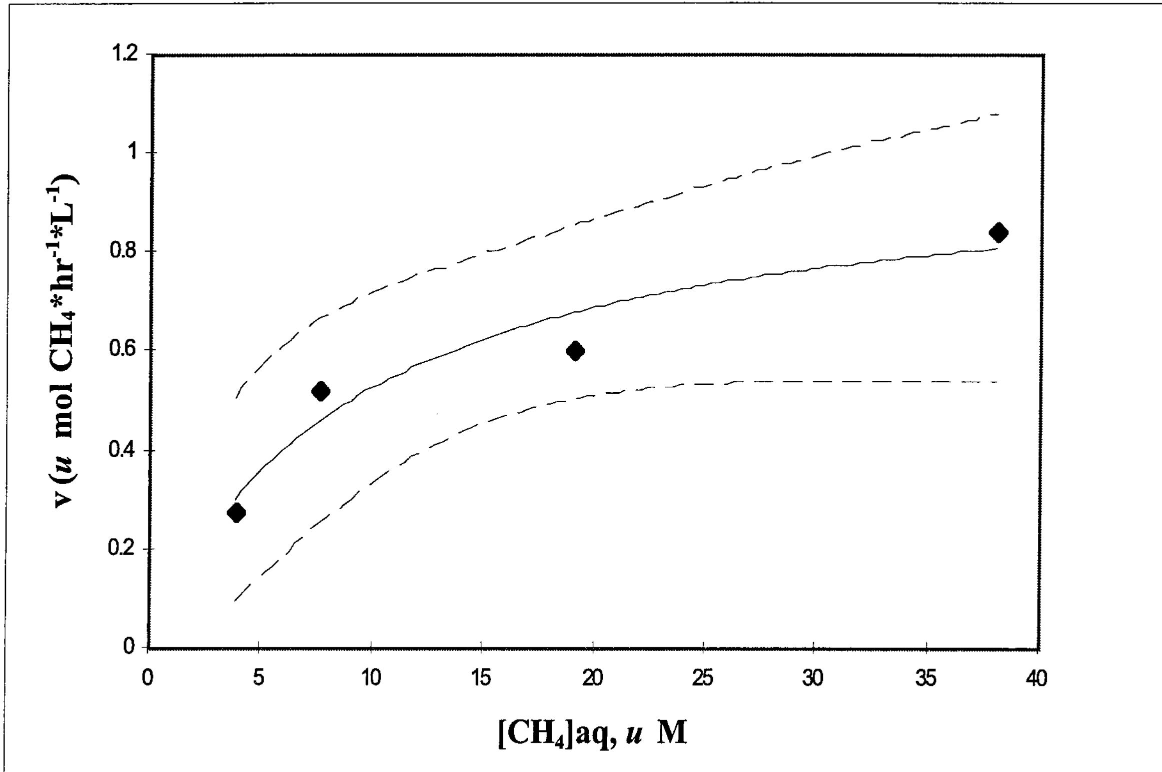



***Figure 2-1: Southern California Coast and Newport Bay Estuary***

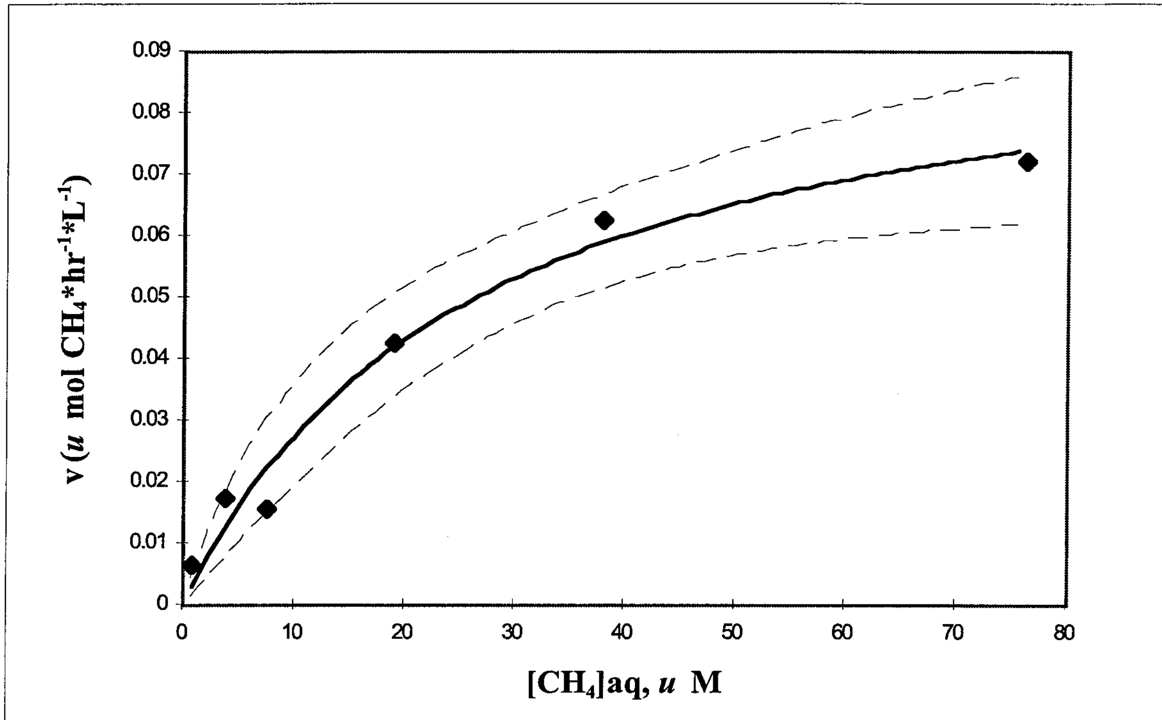
Reproduced with permission from: Manley SL, Goodwin K, North WJ (1992) Laboratory production of bromoform, methylene bromide and methyl iodide by macroalgae and distribution in nearshore southern California waters. Limnol Oceanogr 37:1652-1659




$K_s$ =8.9 μM
$V_{max}$ = 1.0 μM/hr

***Figure 2-2: Methane oxidation in fresh sediment***

Sample collection March 1, 1994
Points show measured velocities
Solid line shows fitted Michaelis-Menten curve
Dotted lines show 95% confidence limits generated by nonlinear regression




$K_s = 25.7\ \mu M$
$V_{max} = 0.01\ \mu M/hr$

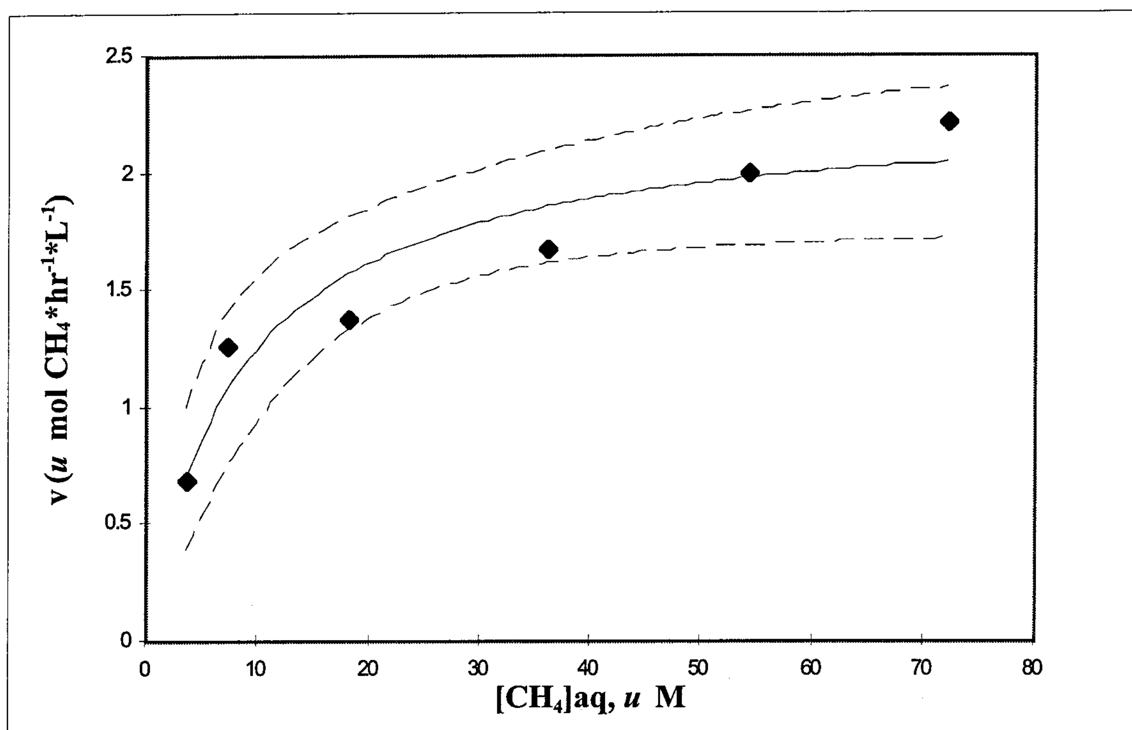
***Figure 2-3: Methane oxidation in fresh sediment***

Sample collection April 13, 1994

Points show measured velocities

Solid line shows fitted Michaelis-Menten curve

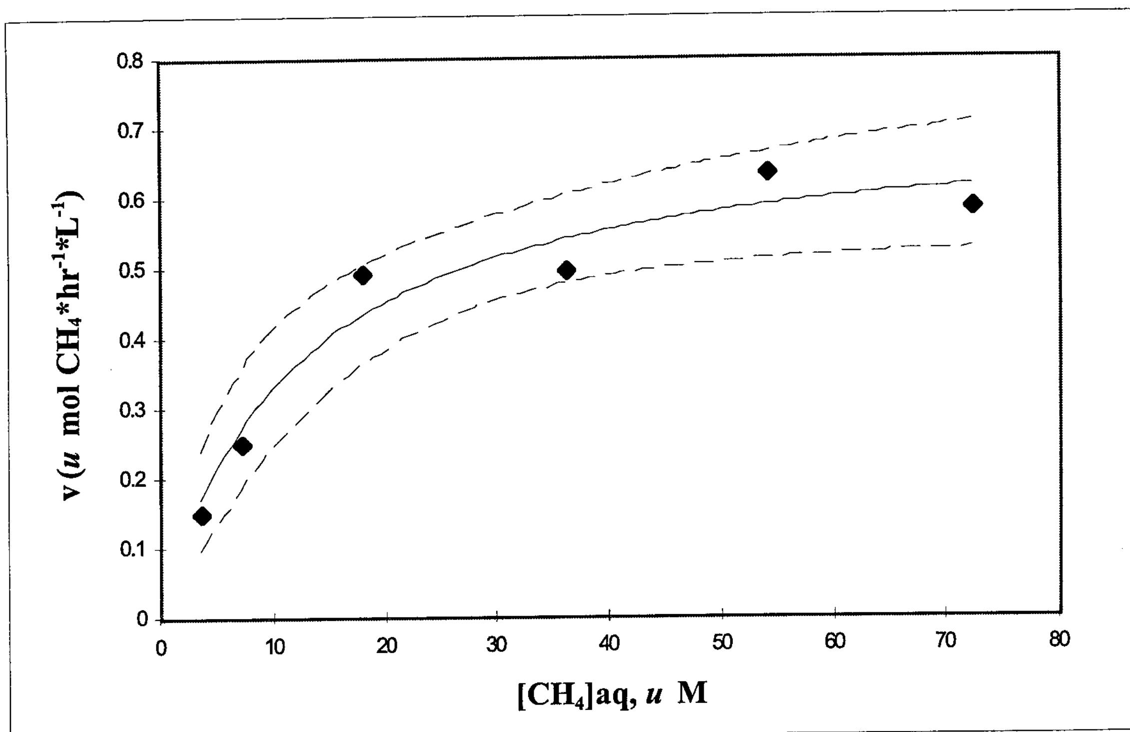
Dotted lines show 95% confidence limits generated by nonlinear regression




$K_s$ =8.2 μM
$V_{max}$ = 2.3 μM/hr

***Figure 2-4: Methane oxidation in fresh sediment***

Sample collection November 28, 1994
Points show measured velocities
Solid line shows fitted Michaelis-Menten curve
Dotted lines show 95% confidence limits generated by nonlinear regression




$K_s$ =11.8 μM
$V_{max}$ = 0.72 μM/hr

***Figure 2-5: Methane oxidation in fresh sediment***

Sample collection November 28, 1994
Points show measured velocities
Solid line shows fitted Michaelis-Menten curve
Dotted lines show 95% confidence limits generated by nonlinear regression

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# 3. Isolation and Characterization of Methanotrophs

## 3.1 Introduction

In addition to the studies of methanotrophic activity in fresh sediment, sediment samples were enriched in order to isolate methanotrophic bacteria. Since very few marine methanotrophs have been isolated, estuarine methanotrophs were of interest as new examples of salt-requiring strains, as well as for their potential use for bioremediation of TCE in coastal environments or other systems where salt-tolerant bacteria would be useful. The characteristics of these strains would also provide more information on the methanotrophic population in the estuary sediment, as discussed in Chapter 2.

## 3.2 Materials and Methods

### *3.2.1. Collection of sediment*

#### Sediment from Kerckhoff Marine Lab harbor

The first site used for collection of sediment for enrichment was the harbor at Kerckhoff Marine Laboratory, located at Corona Del Mar . Bulk sediment from this site was collected by hand by Dr. Wheeler North on 1 November 1993 and stored at the Marine Laboratory at 3-5°C overnight before transport (on ice) to Caltech. The sediment was stored in 1-L plastic bottles; after the sediment settled, about 50% of the volume of the bottles was filled with sediment; the remaining volume was taken up by water and a small amount of air headspace. No attempt was made to preserve the structure of the sediment layers. The water depth was 14-16 feet at the site of collection and the water temperature was 18°C. The top sediment layer varied from about 0.5 cm depth to 6 cm depth. This layer was presumed to be the aerobic layer due to its brown color and the black color of the layers beneath it. The presence of biological activity (tubeworms, snails, etc.) provided additional evidence for the oxygenation of the top sediment layer.

**Sediment from Newport Bay Estuary (Back Bay)**

Sediment from the estuary was collected by hand on 2 February 1994, as described for the kinetics experiments in Chapter 2. The tide was low, and the water covered the sediment to a depth of 2-3 cm. The temperature at the sediment surface was measured at 17°C. The sediment consisted of a thin brownish layer overlying deep black layers that smelled of sulfide. The brown layer was assumed to be the aerobic region; we avoided collecting the black layer as much as possible. This was the first sediment collection from the estuary; subsequent collections for kinetics experiments and gene probing were performed in an adjacent site with a deeper aerobic layer. The samples were transported to Caltech on ice and stored at 4°C overnight. The enrichments were set up the following day.

### *3.2.2. Enrichment conditions*

**Enrichment of sediment from Kerckhoff Marine Lab harbor**

The sediment was initially diluted 2-fold with Nitrate Mineral Salts (NMS) (Whittenbury, et al., 1970) to which 1.5% (by weight) NaCl was added. This medium was used for all enrichments and subsequent growth of isolated strains; it will be referred to as NMSS. Prior to methane addition, the sediment was diluted an additional 2-fold with NMSS, to which vitamins, phosphate buffer, and a trace elements solution (Whittenbury, et al., 1970) had been added. The amounts in the initial enrichments were sediment 0.25x, NMSS 0.75x, vitamins, buffer and trace elements 1x. This mixture was dispensed into serum vials which were sealed as described in Chapter 2. Methane was added (as described in Chapter 2) to 20% (by volume) of the headspace in the vials. The vials were incubated at room temperature with shaking at 200rpm. After 9 days of incubation these enrichments were diluted 100x with NMSS containing 2x vitamins, buffer and trace elements into fresh vials and re-incubated with the same amount of methane. The higher

levels of vitamins, buffer and trace elements were used to ensure that none of these elements would be reduced to limiting amounts during further enrichment. After 8 more days of incubation, the enrichments were spread-plated on solid medium containing 1.5wt% Difco Bacto-agar and NMSS, with 10μM Cu (added as a sterile solution of $CuSO_4$). Vitamins, phosphate buffer, and trace elements were added at 1x concentrations. In addition, Nystatin (Sigma Chemical Co.) was added to the plates to a final concentration of 100mg/L to inhibit the growth of fungi (Whittenbury, et al., 1981). After 11 days of incubation at 30°C, the plates were covered with a dense lawn of yellowish bacteria; no individual colonies could be distinguished. The same enrichments were plated again at this time, after a total of 19 days incubation at the final dilution in the serum vials. The liquid samples were diluted 100x before spread plating on the same media described above. The colonies observed on these plates are described in Section 3.2.3.1. It was not possible to ascertain whether the methanotrophs grew to a culture density in the enrichments which would result in visible turbidity since the presence of the sediment made the liquid turbid from the beginning.

**Enrichment of sediment from Newport Bay Estuary (Back Bay)**

Enrichments from the estuary samples (designated BB since the estuary is locally known as the Back Bay) were set up according to a different protocol, in order to assess the effect of dilution on the types of bacteria found in the plated samples. The enrichment was initially prepared by diluting the sediment 10x with NMSS. Vitamins were added, and the mixture was dispensed into serum vials, which were sealed as described in Chapter 2. Methane was added as described in Chapter 2 to 25% (by volume) of the headspace. The vials were incubated at room temperature with shaking at 200rpm. After 12 days serial dilutions of one of the enrichments were plated on the same NMS-based medium used for the KML enrichments. The concentrations of sediment plated, including

the initial dilution, were 0.1%, 0.01%, 0.001% and 0.0001%. All of the plates were incubated at 30°C. After 17 days many of the plates had vigorous growth; the higher concentration samples resulted in dense lawns of bacteria, and the lower two concentrations had fewer, but similar, colonies. No apparent correlation of the type of bacteria growing with the dilution was observed. Similar types of bacteria were observed compared to the KML enrichments except fewer types of bacteria that appeared to be agar-degraders were seen and the pink colonies described below were obtained.

In a later set of experiments, an attempt was made to select for Type II methanotrophs in the enrichments by omitting nitrate from the additions made to the sediment during enrichment. These enrichments were diluted with nitrate-free NMSS, prepared with an equivalent concentration of potassium chloride instead of potassium nitrate. These enrichments were plated on a different medium, Ammonium Mineral Salts (Whittenbury, 1981) which contains no added nitrate. Although this medium contains ammonium, it has been found to encourage the growth of Type II methanotrophs. This medium was also prepared with 1.5% NaCl (w/v) and a total concentration of 10μM Cu, added as a sterile solution of $CuSO_4$. The elimination of nitrate from the media preparations was done in an attempt to encourage the growth of nitrogen-fixing methanotrophs, since all of the methanotrophs described with this capability so far are Type II methanotrophs.

### *3.2.3. **Isolation and purification of methanotrophic bacteria***

#### Samples from Kerckhoff Marine Laboratory harbor

The plates were incubated for 26 days at 30°C under a 50% methane, 50% air atmosphere in Difco anaerobic jars equipped with valves and pressure gauges for addition and removal of gases. At the end of this period several different types of colonies were seen. These consisted of large tan or brown colonies, several types of thin, spreading colonies,

small, opaque tan or brown colonies, and smaller yellow colonies that appeared to be degrading the agar and forming pits under the colonies. Large tan or brown colonies were immediately tested for methane-dependence and growth by streaking the same colony on 2 different plates, one of which was placed in an air atmosphere to which 40% methane was added ($CH_4$(+)) and the other in an atmosphere to which no methane was added ($CH_4$(-)). Smaller tan or brown colonies were transferred and incubated in the $CH_4$(+) atmosphere prior to checking for methane dependence. The other types of colonies were not checked further since fast-growing, opaque colonies had been found to be marine methanotrophs in previous work (Lidstrom, 1988). Colonies that demonstrated methane-dependent growth were further purified. Many of the strains were contaminated with one or two other bacteria which could be seen on the $CH_4$ (-) plates as thin, white or clear colonies; usually these bacteria could be eliminated by careful selection of single colonies of the opaque strain for transfer. Cultures were determined to be pure by criteria adapted from Lidstrom, 1988 for purification of marine methanotrophs. The criteria that were used are: 1) No secondary growth rings (evidence of a co-culture) were observed around the colonies after at least 4 weeks incubation on the media described above; 2) No growth was observed on nutrient agar plates; 3) Microscopic analysis revealed only one morphological type from both plate and liquid cultures. Strains purified from these enrichments were designated KML to indicate the sampling location near Kerckhoff Marine Lab.

### Samples from Newport Bay Estuary (Back Bay)

Several of the bacteria produced large tan or brown colonies, similar to the KML enrichments and the freshwater bacteria in the lab. Others produced bright pink colonies. All of these isolates were designated with BB to indicate the origin of the sediment in the estuary. These isolates were purified as described above, with the exception of the pink

strain. The pink strain could not be purified; it would not form transferrable colonies on plates in the absence of a second culture of bacteria. The second culture of bacteria formed thin white colonies or simply grew in the area surrounding the pink colonies. The pink strain is believed to be the methanotrophic strain since it did not grow under $CH_4$(-) conditions and the other strain did. These bacteria are currently being maintained as a co-culture. Such co-cultures have been described in the literature (Harrison, 1978, Lidstrom, et al., 1983) and generally involve bacteria that grow on methanol excreted by the methanotrophs or bacteria that grow on cell lysis products of the methanotrophs (Lidstrom, et al., 1983).

Several isolates, designated NF, were obtained by spread-plating from the nitrate-free enrichments, and were checked for methane-dependent growth and growth in pure culture as described above. Since the primary objective of isolating these additional strains was to try to obtain Type II methanotrophs, these isolates were next characterized by gene probing.

### *3.2.4. Characterization of isolates*

#### Growth curves

All of the methanotrophs were tested for growth at two different temperatures. The strains were incubated on plates at 30°C and 37°C; those that appeared to grow faster at 37°C were maintained at that temperature. Strains that grew better at 30°C and strains that appeared to grow at about the same rate at both temperatures were maintained at 30°C. Of the strains that could be isolated in pure culture, four strains which appeared to be representative of the methanotrophic isolates were chosen for further characterization. Two strains were used from each collection site, one strain from each site which appeared to grow best at 30 °C and one that appeared to grow best at 37 °C. Replicate cultures of each strain were grown at the previously-determined temperature with and without added NaCl in the NMS media. All of the cultures had 10μM Cu added to the medium as a

sterile solution of $CuSO_4$ immediately before inoculation. The cultures were inoculated from plates to minimize salt carry-over into the new cultures. Growth rates were determined by measuring the change in optical density over time of a liquid culture incubated at either 30°C or 37°C. Optical density measurements were taken using a Klett colorimeter. These data were fitted to an exponential growth equation to determine the specific growth rate ($\mu$). The doubling time ($t_d$) represents the time for the culture to double in density and is calculated from the specific growth rate by the equation $t_d = \mu / \ln(2)$. When examined with a light microscope under 1000x magnification, all of the strains appeared to be motile rod-shaped bacteria, typical of many methanotrophic strains (Hanson, et al., 1992). The strains were further characterized by gene probing, as described below.

### Gene probing

Gene probing of colony blots was used as a screening tool for the newly isolated methanotrophic bacteria. As described in Chapter 1, oligonucleotides corresponding to genes encoding diagnostic regions of the 16S rRNA were synthesized by the Caltech DNA synthesis facility. These were used to identify the group of genera to which each isolate belongs. The probe sequences and the published references from which they were obtained are listed below:

1034-Ser [a]

5'-CCA TAC CGG ACA TGT CAA AAG C-3'

1035-RuMP [a]

5'-GAT TCT CTG GAT GTC AAG GG-3'

Eubacterial Universal Probe (EUP) [b]

5'-GCT GCC TCC CGT AGG AGT-3'

a) Brusseau, *et al.*., 1994
b) Distel and Cavanaugh, 1994

The method used is a modification of the protocol for colony blotting described in Sambrook, et al. (1989). For these experiments, nitrocellulose membranes (S&S NC, Schleicher and Schuell) were placed on plates containing NMSS, bacto-agar (Difco) and 10μM Cu (added as a sterile solution of $CuSO_4$) and 100mg/L Nystatin (Sigma Chemical Co.). With the membranes in place, the plates were inoculated with the methanotrophic isolates and incubated at the appropriate growth temperature for each strain (described above) in an atmosphere of 40% methane and 60% air (by volume). Inoculation of the plates with the membranes in place ensures that the bacterial colonies grow on the membrane so that their DNA can be obtained and fixed without further transfers. When large colonies were visible on the membranes (after 5-7 days incubation), they were prepared for probing by sequential treatment with 10% (w/v) sodium dodecyl sulfate (SDS), denaturing solution (150mM NaCl, 0.5M NaOH), and 0.5M Tris Base (pH 8.0). All chemicals are reagent grade from Sigma Chemical Co. This procedure serves to lyse the bacterial cells, denature the DNA, and neutralize the pH. The DNA was fixed to the membranes by drying in an 80°C vacuum oven for 2 hours. The membranes were then placed in washing and hybridization solutions as follows:

Prewash solution (0.05M Tris Base, pH 8.0; 1M NaCl; 10mM EDTA; 0.01% SDS); incubated at 60°C for 1 hour.

Hybridization solution(5x Denhardt's reagent, 6x SSC, 0.01%SDS, 10μg/ml Salmon Testes DNA); incubated at 60°C for at least 4 hours. Denhardt's reagent is prepared as a 50x stock consisting of 10g/L Ficoll detergent, 10 g/L polyvinyl pyrrolidone, and 10g/L bovine serum albumin. SSC is prepared as a 20x stock consisting of 3.0 M NaCl and 0.3 M sodium citrate (Sambrook, et al., 1989). Salmon Testes DNA serves as a blocking agent which binds to non-specific DNA binding sites on the membranes.
The oligonucleotide probes were labeled with $^{32}P$ γ-ATP (DuPont NEN) by phosphorylation at the 5' end using polynucleotide kinase (Boeringer Mannheim Biochemicals). Solutions containing about 6pg of each synthetic oligonucleotide were mixed with 5 μl of 10x phosphorylation buffer (Boeringer Mannheim Biochemicals) and enough water to make the final volume of each reaction mixture 50 μl. 50μCi of the $^{32}P$ γ-ATP and 10 units of polynucleotide kinase were added to each reaction, and the mixtures were incubated at 37°C for 45-60 min. The reactions were stopped by incubation for 5-10 min. at 70 °C, and finally, the probes were denatured by incubation at 100°C for 5 to10 min. just prior to addition to the hybridization buffer. 25 μl of each oligonucleotide probe was added to the hybridization solutions containing the membranes and the mixtures were incubated with the membranes overnight. The temperature for hybridization was 50°C for all probes used. The membranes were rinsed in 0.5x SSC + 0.1%SDS three times for 20 minutes each at the same temperature, then exposed to x-ray film for up to 3 days. The autoradiograms were developed using a Kodak X-Omat developer.
The strains designated NF were also characterized by gene probing, however, a different protocol was used due to poor hybridization of the Type II probe. These strains were grown on nylon membranes (Amersham Hybond N) which were placed on plates with the same medium formulation as described above. The membranes were processed by sequential treatment with 10% (w/v) sodium dodecyl sulfate (SDS), denaturing solution (150mM NaCl, 0.5M NaOH), and 0.5M Tris Base (pH 8.0), with the addition of a final treatment with 6x SSC. The membranes were air dried, and then the DNA was fixed by

exposing the membranes to UV light as described in Sambrook, et al. (1989). The filters were then placed in the following hybridization solution and pre-hybridized at the hybridization temperature for 1 hour.

Hybridization solution for nylon membranes: 10mM sodium phosphate buffer (pH=6.8), 6x SSC, 0.5% SDS, 5mg Salmon Testes DNA, 5x Denhardt's reagent (described for nitrocellulose membranes above).

The probes were labelled as described for the nitrocellulose filters, except that appx. 25x the amount of oligonucleotide was used, in an attempt to improve the hybridization signal. 25 µl of each oligonucleotide probe was added to the hybridization solutions containing the membranes and the mixtures were incubated with the membranes overnight. The temperature for hybridization was 42°C for all probes used. The membranes were rinsed in 0.5x SSC + 0.1%SDS three times for 20 minutes each at the same temperature, then exposed to x-ray film for 6 days. The autoradiograms were developed using a Kodak X-Omat developer.

The specificity of the probes was ascertained by hybridizing the probes to DNA from a series of positive and negative control bacteria under the same experimental conditions. Chromosomal DNA from each organism was digested with the restriction enzyme *Hin*d III (Boeringer Mannheim Biochemicals) and run in a 0.7% agarose gel. The gels were processed by denaturing and neutralization as described for colony blots, then dried in a vacuum gel drier for 1-2 hours. The gels were then placed in a hybridization solution (6x SSC, 10mM EDTA, 0.5% SDS, 5mg/ml dry milk powder) to which 25 µl of the radiolabelled probe was added. The probes were labelled as described for the colony blots on nitrocellulose membranes. The gels were hybridized, rinsed, and exposed to x-ray film as described for colony blots.

All of the liquid media had 10µM Cu added as described above. The results clearly show the dependence of the isolates on added NaCl, since none of the strains would grow in medium to which no salt was added. All of the strains grew with a doubling time less All

## 3.3. Results

### *3.3.1. Types of isolates*

With the exception of the pink methanotroph growing in co-culture, all of the isolates produced tan or brownish colonies on plates. Large single colonies of 3-5mm in diameter were produced by all isolates in 7-10 days on NMSS-agar with 10μM Cu (added as described for isolation of strains) when incubated in a 20% methane, 80% air (by volume) atmosphere at one of the temperatures described below.

### *3.3.2. Growth of isolates*

Table 3-1 shows the growth rates and growth with and without 1.5% (w/v) NaCl added to the NMS medium for the isolates.

***Table 3-1: Characteristics of methanotrophic isolates***

| Strain | Temp(°C) | NaCl added | μ ($hr^{-1}$) | $t_d$ (hrs) |
|---|---|---|---|---|
| KML E-2 | 30 | 1.5 wt% | 0.212 | 3.06 |
| | 30 | 1.5 wt% | 0.227 | 3.27 |
| | 30 | none | NG | NG |
| | 30 | none | NG | NG |
| BB5.1 | 30 | 1.5 wt% | 0.182 | 3.82 |
| | 30 | 1.5 wt% | 0.220 | 3.14 |
| | 30 | none | NG | NG |
| | 30 | none | NG | NG |
| BBA6 | 37 | 1.5 wt% | 0.192 | 3.61 |
| | 37 | 1.5 wt% | 0.186 | 3.73 |
| | 37 | none | NG | NG |
| | 37 | none | NG | NG |
| KML E-1 | 37 | 1.5 wt% | 0.195 | 3.55 |
| | 37 | 1.5 wt% | 0.192 | 3.61 |
| | 37 | none | NG | NG |
| | 37 | none | NG | NG |

NG = no growth observed

of the liquid media had 10 μM Cu added as described above. The results clearly show the dependence of the isolates on added NaCl, since none of the strains would grow in medium to which no salt was added. All of the strains grew with a doubling time less than 4 hours. This is a relatively rapid growth rate compared to laboratory strains of freshwater methanotrophs, which typically have doubling times of about 8 hours.

### *3.3.3.* ***Colony blotting results***

Figures 3-1a and 3-1b show the results of the colony blotting experiments. Table 3-2 summarizes these results. All of the isolates tested hybridized to the probe 1034-RuMP. This probe corresponds to Type I methanotrophs as described in Chapter 1. All of the isolates, as well as all of the laboratory strains, also hybridized to the eubacterial probe (EUP), which was used as a positive control during the probing experiments. The control experiments confirmed the specificity of the probes for Type I and Type II bacteria as shown in Figures 3-2 to 3-4. In the control experiments, DNA from several methylotrophic strains was used, as well as a range of other eubacterial species, including *N. europaea*, which produces an enzyme similar to the pMMO (Semrau, 1995). The 1035-RuMP and 1034-Ser probes used in this study hybridized only to DNA from Type I and Type II methanotrophs, respectively, and were therefore considered to be specific for these bacteria. DNA from the Type X strain, *Methylococcus capsulatus* Bath, did not hybridize to either probe, as reported previously (Brusseau, et al., 1994). In these experiments, the DNA tested was digested with a restriction enzyme and run on a gel, which allows an assessment of the number of 16S rRNA gene copies. In the methanotrophs the results suggest the presence of 2-3 copies. This information will be useful for interpretation of future probing studies.

***Table 3-2: Results of probing colony blots with 16S rRNA probes***

| Strain | Probe | | |
|---|---|---|---|
| | 1034-Ser | 1035-RuMP | EUP |
| *Methylobacter marinus* A45 | (-) | (+) | (+) |
| *Methylosinus trichosporium* OB3b | (+) | (-) | (+) |
| *Methylococcus capsulatus* Bath | (-) | (-) | (+) |
| KML E-1 | (-) | (+) | (+) |
| BBA6 | (-) | (+) | (+) |
| KML E-2 | (-) | (+) | (+) |
| BB5.1 | (-) | (+) | (+) |
| NF-1 | (-) | (+) | (+) |
| NF-2 | (-) | (+) | (+) |
| NF-4 | (-) | (+) | (+) |

Table 3-3 shows the strains used in the control experiments. The lane numbers correspond to those marked on Figures 3-2 to 3-4.

*Table 3-3: Results of probing digested DNA from control strains*

| Lane # | Strain | Probe | | |
|---|---|---|---|---|
| | | 1034-Ser | 1035-RuMP | EUP |
| 1 | Methylobacillus flagellatum KT | (-) | (-) | (+) |
| 2 | Methylobacter albus BG8 | (-) | (+) | (+) |
| 3 | Vibrio fischeri | (-) | (-) | (+) |
| 4 | Methylococcus capsulatus Bath | (-) | (-) | (+) |
| 5 | Methylobacterium extorquens AM1 | (-) | (-) | (+) |
| 6 | Methylomonas sp MN | (-) | (-) | (+) |
| 7 | Pseudomonas aeruginosa | (-) | (-) | (+) |
| 8 | Methylomonas methanica S1 | (-) | (-) | (+) |
| 9 | Methylophilus sp W3A1 | (-) | (-) | (+) |
| 10 | Methylosinus trichosporium OB3b | (+) | (-) | (+) |
| 11 | Nitrosomonas europaea | (-) | (-) | (+) |
| 12 | Paracoccus denitrificans | (-) | (-) | (+) |
| 13 | Bacillus subtilis | (-) | (-) | (+) |

### 3.4 Discussion

Enrichment of sediment from both the Newport Bay Estuary and the Kerckhoff Marine Laboratory harbor resulted in the isolation of several strains of fast-growing methanotrophs. Those that were tested all required salt for growth, making them poten-

tially useful for bioremediation in saline environments. In addition, these bacteria may be useful for genetic studies on methanotrophs and marine methanotrophs in particular due to their rapid growth rate. High rates of growth are desirable for genetic studies, as discussed by Lidstrom (1988). The four isolates from the Newport Bay Estuary sediment and the Kerckhoff Marine Laboratory harbor that were characterized have been found to be Type I methanotrophs. These results are consistent with previous reports of the isolation of marine methanotrophic bacteria in that no Type II bacteria were found (Lidstrom, 1988; Sieburth, et al., 1989; Murrell and Holmes, 1994). Subsequent attempts to isolate Type II bacteria from the estuary sediment by carrying out nitrate-free enrichments and plating on a nitrate-free medium also failed; all isolates that were obtained by this procedure were also found to be Type I methanotrophs by gene probing.

Since in general the sMMO is found only in Type II methanotrophs, and the sMMO is capable of high rates of TCE oxidation, it is important to determine whether Type II methantotrophs are present in marine sediments and whether the production of sMMO can be achieved in marine environments. However, the results of this study support other studies that failed to obtain evidence of Type II methanotrophs. The inability to isolate these strains is not definitive evidence of their absence. It is well known that conventional culture techniques cannot be assumed to result in the isolation of the bacteria which are important to the system *in situ* (Giovannoni, et al., 1990; Ward, et al., 1990; King, 1992). Gene probing techniques can overcome some of the limitations of culturing techniques and will be discussed in subsequent chapters.

### 3.5 Conclusions

Several pure cultures of methanotrophs were obtained from enrichments of sediment from the harbor at Kerckhoff Marine Laboratory and from the Newport Bay Estuary. Most of these isolates are similar in colony morphology to laboratory freshwater strains. At least one methanotrophic organism was obtained which is pink-pigmented but which

will not grow in pure culture, and is being maintained as a consortium. All of the purified isolates tested required added NaCl for growth, grew rapidly at mesophilic temperatures, and grow well on liquid and solid media containing NMS with added salt and copper. These bacteria are potentially useful for bioremediation of TCE in saline environments. They may also be particularly useful for genetic studies on methanotrophs due to their rapid growth rate, which facilitates genetic research. Gene probing with oligonucleotide probes based on 16S rRNA sequences suggests that all of the isolates tested are Type I methanotrophs. It is therefore expected that they will produce only the pMMO for methane oxidation. No Type II methanotrophs were obtained from these enrichments and isolations, consistent with previous studies. Information about Type II methanotrophs in the estuary sediment may be obtained by using gene probing techniques, which are capable of overcoming some of the limitations of conventional culturing techniques for obtaining information about bacterial diversity.

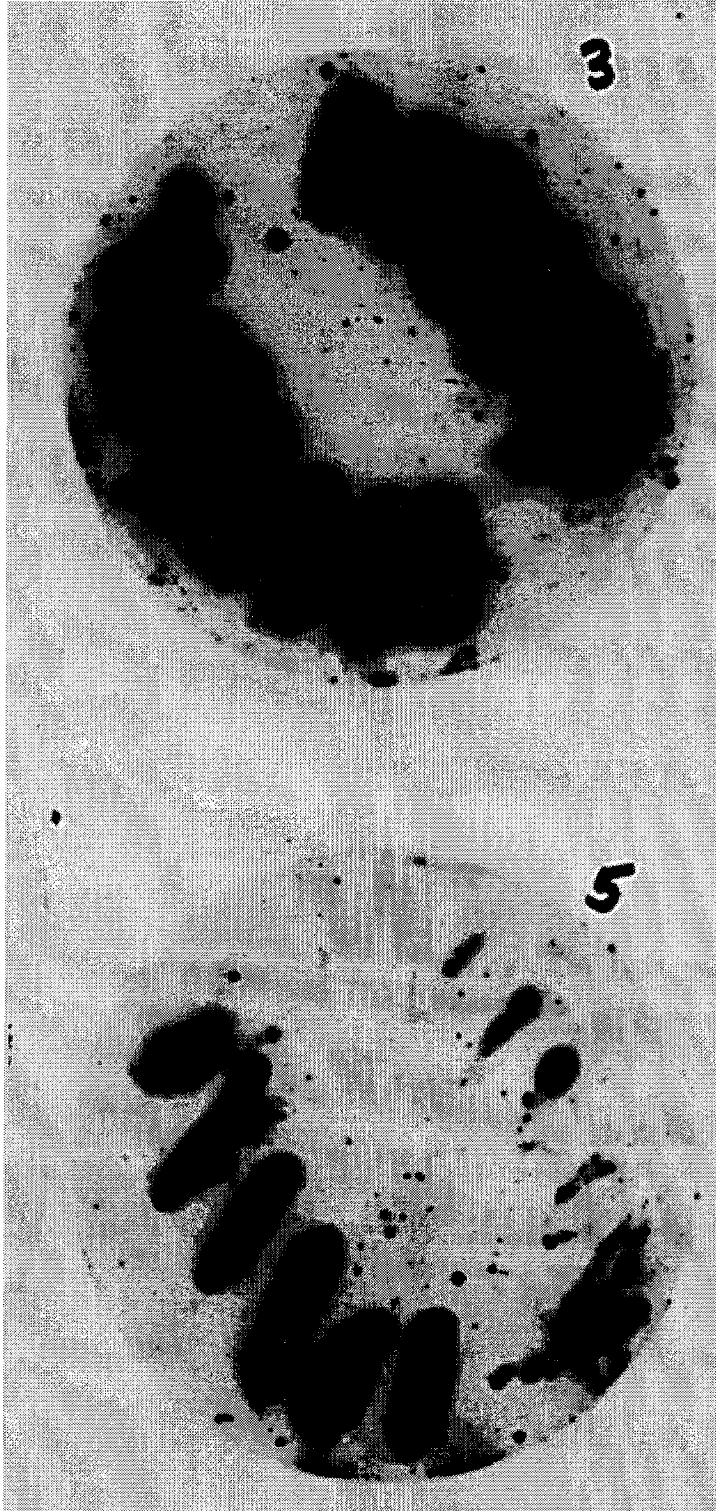


***Figure 3-1: Colony blots with Type I probe***

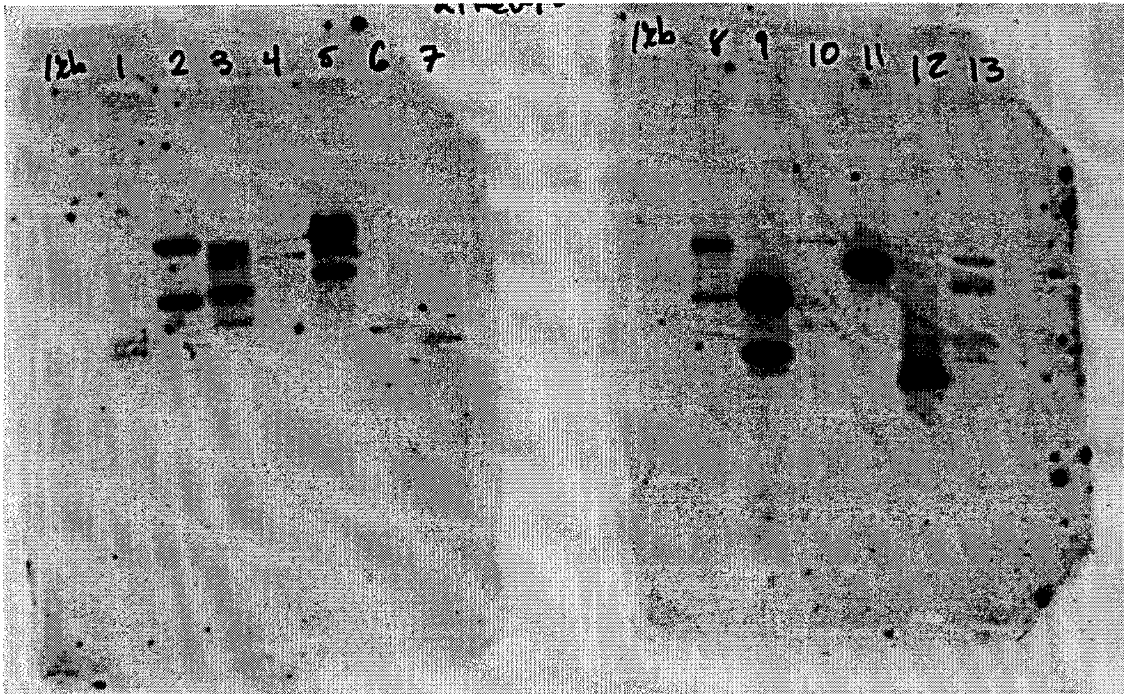


***Figure 3-2: Blots of gels with EUP probe***

All lanes show hybridization; all lanes contain eubacterial DNA digested with *Hin*d III

Strains are listed in Table 3-3

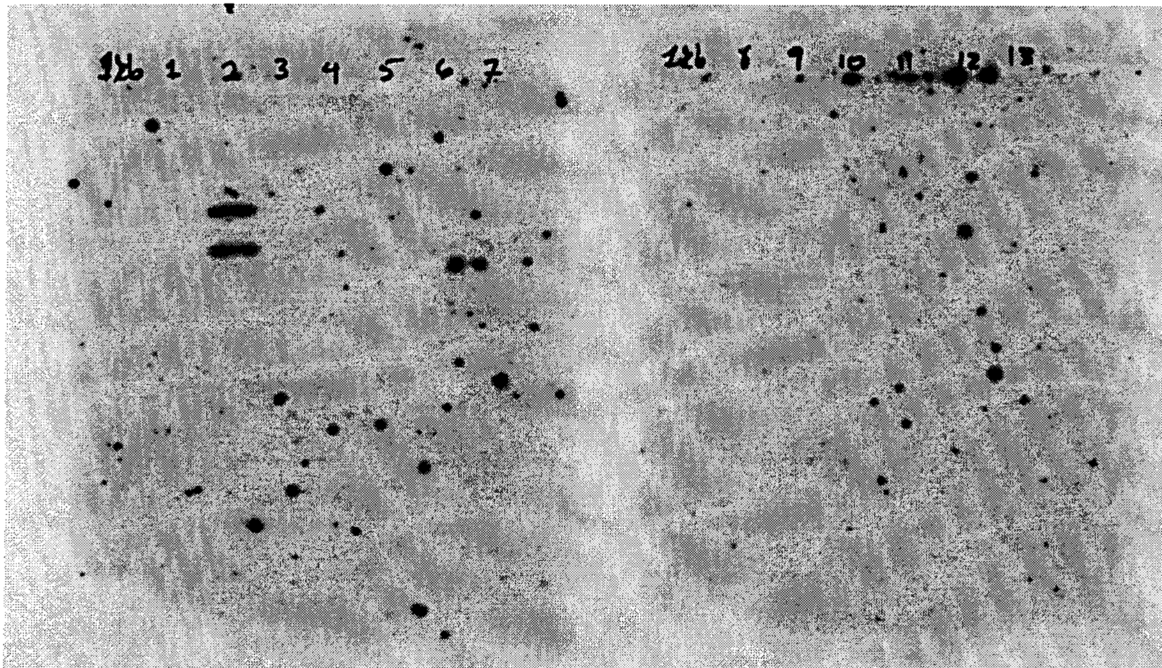


***Figure 3-3: Blots with Type I probe***

Blots of Fig 3-2 probed with 1035-RuMP

Lane 2 shows hybridization with DNA from *M. albus* BG8

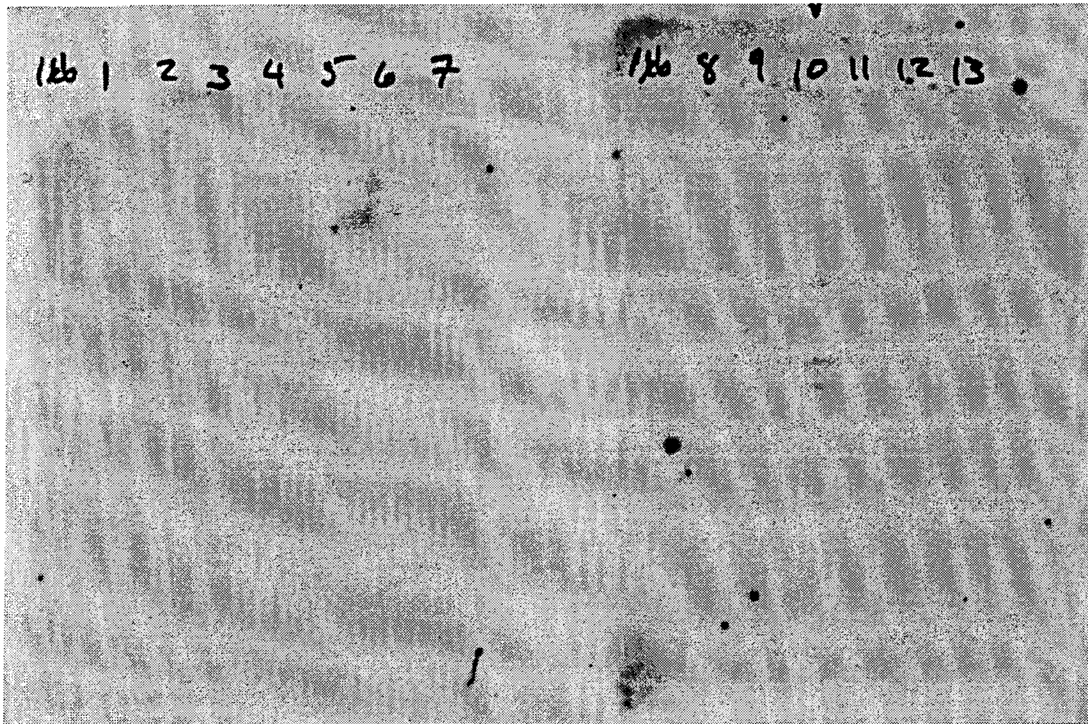


***Figure 3-4: Blots with Type II probe***

Blots of Fig 3-2 probed with 1034-Ser

Lane 10 shows hybridization with DNA from *M. trichosporium* OB3b

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# 4. Substrate Oxidation Kinetics of Estuarine Isolate BB5.1

## 4.1 Introduction

As discussed in Chapter 1, the enzyme in methanotrophs that catalyzes the initial step in the oxidation of methane to carbon dioxide is the methane monooxygenase. This enzyme is known to occur in two different forms, the particulate form, or pMMO and the soluble form, or sMMO. The pMMO is found in all known methanotrophs, while the sMMO has been found in only a few strains. Both forms of the MMO have been shown to fortuitously oxidize TCE by converting it to its epoxide, which is then rapidly broken down by other processes. The kinetics of methane and TCE oxidation were measured for the estuarine methanotrophic isolate BB5.1 to provide the initial information necessary for future development of predictive models of the behavior of marine or estuarine methanotrophs for *in-situ* bioremediation of TCE. As discussed in Chapter 3, the estuarine isolate BB5.1 requires NaCl for growth, has a fairly rapid doubling time of three to four hours, and has been determined to be a Type I methanotroph based on gene probing of colony blots. Since it is a Type I methanotroph, it is expected to produce only the pMMO, as discussed in Chapter 1. The kinetics of the oxidation of each substrate were examined separately to avoid competition effects, for comparison with the behavior of the methanotrophs in the sediment, and to establish boundaries for the expected behavior of an estuarine methanotroph during *in-situ* bioremediation of TCE. The behavior of the bacteria while in the presence of both substrates would involve the effects of competition by the substrates for the enzyme; examination of these effects was beyond the scope of this work and was not undertaken in this study.

### 4.2 Methane oxidation

#### *4.2.1 Materials and methods*

Unless otherwise noted, materials are the same as described in Chapter 2 for measurement of methane oxidation in the estuary sediment. All glassware was acid-washed by the method of Semrau (1995) and then autoclaved. Methane additions were determined for a range of dissolved methane concentrations based on partition coefficients calculated from the model of Duan, et al. (1992) for Nitrate Mineral Salts (Whittenbury, et al., 1981) with 1.5% (w/v) NaCl added. This medium will be referred to as NMSS. Unless otherwise noted, the experiments were performed at room temperature.

Liquid cultures of the isolate BB5.1 were grown in 100ml of NMSS. Inocula were obtained from agar plates containing NMSS and 10µM copper. The cultures were grown in stoppered 1-liter Erlenmyer flasks under an atmosphere of 20% (v/v) methane, at 30°C. In order to examine the effect of two levels of copper on the behavior of the bacteria, two culture conditions were used; a high copper condition in which 25 µM copper was added, and a low copper condition in which no copper was added. When copper was added, it was added to both the solid and liquid media as a filter-sterilized 0.1M solution of $CuSO_4(5H_2O)$. The background copper present in an uninoculated sample of the low copper media was measured by Ion Coupled Plasma Mass Spectrometry (ICP-MS) on a Perkin-Elmer Sciex Elan 5000. This background total copper was 3.2 µM. The initial copper concentration in the high-copper medium can be calculated to be approximately 28 µM. Cultures were grown at 30°C for 16-18 hours with shaking at 200 rpm.

The cultures were first evacuated to remove residual methane, and then were diluted in NMSS to an $OD_{600}$ of no more than 0.05. This cell density was chosen based on the results of the mass-transfer experiment described below. Optical density measurements were taken on a Hewlett-Packard model 8453A diode array spectrophotometer.

The diluted culture was dispensed in 0.5-ml aliquots into 20-ml serum vials and the vials were sealed with blue rubber stoppers (Bellco Glass Co.) and aluminum crimp seals (Hewlett-Packard). Controls were killed cultures consisting of identical vials to which 0.04 ml of a 50% (w/v) solution of NaOH had been added prior to the addition of culture. The amount of NaOH was calculated to bring the final NaOH concentration to approximately 1 M. Methane consumption was measured by adding $^{14}CH_4$ and measuring the rate of appearance of $^{14}C$-labelled products, as described in Chapter 2. Methane spiked with $^{14}CH_4$ was added to each vial as described in Chapter 2. The vials were shaken longitudinally at 250rpm. At time points vials were removed from the shaker and 50% (w/v) NaOH was added, also to a final concentration of 1M. The addition of NaOH served to kill the bacteria by raising the pH and also to dissolve the carbonate products of methane oxidation, as described in Chapter 2. A typical time course for these experiments consisted of samples taken at 0 hr (killed controls), 1.5 hr, 2.5 hr and 3.5 hr. To remove residual methane, the vials were flushed with air by inserting needles through the stopper as an inlet and outlet. The vials were flushed for at least 1 minute at 7 ml/sec. The vials were then opened and the liquid was subsampled for scintillation counting. Scintillation counting was performed as described in Chapter 2.

Velocities of methane oxidation were calculated by linear regression of the scintillation counter time course data. The velocities were normalized to the total protein concentration of the culture and plotted against the calculated initial dissolved methane concentrations. The data were fitted to the Michaelis-Menten model by non-linear regression using the software package TableCurve 3D v.2 (Jandel Scientific). The Michaelis-Menten model is described in Appendix B. The protein concentrations were determined using the DC Protein Assay (Bio-Rad). This assay is based on the reaction of protein with an alkaline copper tartrate solution and a Folin reagent, resulting in color development proportional to the amount of protein in the sample. Color development is quantified at the wavelength of maximum absorption (750 nm) as specified by the manu-

facturer. Absorbances were measured on a Hewlett-Packard model 8453A Diode Array spectrophotometer and the protein concentration was calculated using a standard curve of serial dilutions of bovine serum albumin which were subjected to the same assay. Final total copper concentrations in the media were measured by ICP-MS on a Perkin-Elmer Sciex Elan 5000. The medium was sterile-filtered to remove cell material before copper measurements were made.

### *4.2.2 Results*

#### **Mass transfer experiment**

The determination of the kinetic parameters of methane oxidation by whole cells depends upon measurement of the bacterial rate of methane oxidation. Since methane is a sparingly soluble gas, the rate at which methane dissolves in the liquid phase may become significant compared to the rate of bacterial consumption of the dissolved methane. To ensure that the measured rates of methane oxidation in this experimental system were limited by bacterial consumption only, the relationship between the number of bacterial cells and the measured rate of methane oxidation was determined. This relationship was determined by measuring the rate of methane oxidation by serial dilutions of a culture of the estuarine methanotrophic isolate BB5.1. The experimental protocol was identical to that for the kinetics experiments except that the same amount of methane was added to all of the vials, while the amount of bacteria was varied. The amount of methane added was calculated to result in an equilibrium aqueous phase concentration of 10μM. The results of this experiment (Figure 4-1) showed a linear relationship between the rate of methane oxidation per unit volume of liquid and the amount of cells, up to a cell density of about $OD_{600} = 0.1$. At higher cell densities the rate of methane oxidation did not show a linear relationship to cell density. Results of this type are typically obtained when the rate of oxidation of a gaseous substrate begins to become limited by the rate of mass transfer of

the gaseous substrate between the gas and liquid phases (Danckwerts, 1970; Robinson, 1982). These results suggested that the cell densities used for the kinetics experiments should be below $OD_{600} = 0.1$, and the cell densities used in this study were between $OD_{600} = 0.02$ and $OD_{600} = 0.05$.

### Results of kinetics experiments

Earlier preliminary results from the Lidstrom laboratory had suggested that the kinetics of methane oxidation by methanotrophs might be affected by the copper levels used in the media for growing the cells (Semrau, 1995).. Therefore, two copper conditions were tested, high copper (25µM) and low copper (none added, 3µM). The results from this study are summarized in Table 4-1. Figures 4-2 to 4-6 show the Michaelis-Menten plots and the fitted curves from these experiments. The values of the uptake velocity at sub-strate saturation ($V_{max}$) and the half-saturation constant ($K_s$) were determined by nonlin-ear regression using the software package TableCurve2D for Windows, version 2.0 (Jandel Scientific), as described in Chapter 2. The results of the nonlinear regression analyses are presented in Appendix A, including the 95% confidence limits for the $K_s$ and $V_{max}$ values.

***Table 4-1: Results of methane consumption experiments for the isolate BB5.1***

| Copper level | $K_s$ [a] | $V_{max}$ [b] | Added [Cu] [c] | Final [Cu] [d] |
|---|---|---|---|---|
| High | 13.1 | 6.6 | 25µM | 4.2 |
| High | 9.7 | 5.4 | 25µM | 4.7 |
| Low | >50 | no data | none | no data |
| Low | 81.4 | 6.1 | none | 1.2 |
| Low | 16.4 | 1.7 | none | 1.0 |

a) µM dissolved $CH_4$
b) nmole $CH_4 * hr^{-1} * ug$ total $protein^{-1}$
c) residual Cu level in medium with no Cu added is 3µM
d) µM, measured in medium by ICP-MS

As may be seen from Table 4-1, the kinetics of methane oxidation by this strain are not always similar under high-copper and low-copper growth conditions. In some cases, only a slight elevation of the $K_s$ was seen when the bacteria were grown under low-copper conditions. This result is similar to results obtained for this strain are similar to those obtained by Udell (1996) for the strain *M. albus* BG8, a strain containing only the pMMO. In other cases, a strong elevation of the $K_s$ was observed when the bacteria were grown under low-copper conditions. This result was similar to those obtained by Semrau (1995) for a number of methanotrophs producing the pMMO. Since our evidence suggests that the isolate BB5.1 (a Type I methanotroph) cannot produce the sMMO, all of the measured rates can be attributed to the pMMO. The inconsistencies in all of these results can probably be attributed to variable levels of copper contamination in the medium and slight differences in growth protocols, such that the pMMO becomes limited for copper in some cases and not in others. Although the level of total copper in the medium at the end of the experiments was similar, this value may not provide much information about the amount of bioavailable copper present during the growth of the cell culture or during the experiment, due to complex speciation of the copper compounds in the medium (Berson and Lidstrom, 1996). In addition, the copper pool used for synthesizing the active pMMO is not yet clear. Therefore, it is possible that small changes in the concentration of a specific copper species will result in large changes in the kinetics of the pMMO. Further research will be necessary to explore this issue.

## 4.3 TCE oxidation

### *4.3.1 Materials and methods*

Cultures of the isolate BB5.1 for TCE oxidation experiments were grown under the same conditions described for methane oxidation. The same two copper levels were used, that

is, a high copper level in which 25μM Cu was added as $CuSO_4$ and a low copper level in which no copper was added to the cultures. The cultures were dispensed in 1 ml aliquots into 20 ml headspace sampler vials (Hewlett-Packard), after which varying initial concentrations of TCE were added as aliquots of a TCE-saturated water solution. The saturated water solution was made according to the method of Semrau (1995). The vials were sealed with Teflon-lined septa and aluminum crimp seals immediately after addition of TCE. Controls for these experiments consisted of identical vials to which 80 μl of a 50% (w/v) NaOH solution was added before the bacterial culture was dispensed. At time points, the consumption of TCE was stopped by the addition of 80 μl of 50% (w/v) NaOH. In these experiments, the addition of NaOH served only to kill the bacteria, but was used in order to make the experimental conditions between the methane and TCE oxidation experiments as similar as possible. The rate of TCE oxidation was determined by the disappearance of TCE over time in the gas phase of the vials with a Hewlett-Packard 7694 Headspace Autosampler in conjunction with a Hewlett Packard 5890 Series II Plus gas chromatograph equipped with an Electron Capture Detector. The conditions under which the GC analyses were run are listed in Table 4-2.

***Table 4-2: GC conditions for TCE quantification in pure cultures of isolate BB5.1***

| Parameter | Value |
|---|---|
| Column | HP-624 |
| Column Length | 30 m |
| Column Inner Diameter | 0.32 mm |
| Film Thickness | 1.8 μm |
| Inlet Temperature | 200 °C |
| Detector Temperature | 300 °C |
| Column Temperature | 95°C |
| Run Time | 3.5 min |
| TCE Retention Time | 2.1 min |

Velocities of TCE oxidation were calculated by linear regression of the TCE-disappearance time course data. TCE concentrations were determined from the peak areas of the chromatograms by comparing them to a standard curve. The standard curve was determined for each experiment by plotting the peak areas obtained for the killed controls versus the amount of TCE added to each vial. Liquid phase concentrations were calculated according to Henry's law, using a dimensionless partition coefficient of 0.36 (Gossett, 1987). The velocities were normalized to the total protein concentration of the culture and plotted against the calculated initial dissolved TCE concentrations. The data were fitted to the Michaelis-Menten model by non-linear regression using the software package TableCurve 3D v.2 (Jandel Scientific). The protein concentrations were determined using the DC Protein Assay (Bio-Rad), and the final total copper concentrations in the media were measured by ICP-MS, both as described for the methane oxidation experiments.

### *4.3.2 Results of TCE oxidation experiments*

**Mass transfer experiment**

As described for the methane oxidation experiments, the relationship between the TCE oxidation rate and the cell mass was determined by measuring the rate of TCE oxidation by serial dilutions of a culture of the isolate BB5.1. The experimental protocol was identical to that for the kinetics experiments except that the same amount of TCE was added to all of the vials, while the number of bacteria was varied. The amount of TCE added was calculated to result in an equilibrium aqueous phase concentration of 7μM. Although there was some variability in the measured rates of TCE oxidation, the results of this experiment (Figure 4-5) showed a generally linear relationship between the rate of TCE oxidation per unit volume of liquid and the number of cells, up to the highest cell density used for the kinetics experiments, about $OD_{600}$ = 1.15. The plot of the

TCE oxidation rates vs the cell densities did not show the behavior that was seen in the similar experiment which was performed for methane oxidation, in which the oxidation rates clearly ceased to be linearly related to the culture density. This result indicates that the measured rate of TCE oxidation was not limited by the rate of mass transfer of gaseous substrate between the gas and liquid phases in the vials (Danckwerts, 1970; Robinson, 1982). The cultures of the isolate BB5.1 were used without dilution ($OD_{600}$ = 0.8 to $OD_{600}$= 1.15) to measure the kinetics of TCE oxidation.

**Results of kinetics experiments**

The kinetics of TCE oxidation by the estuarine isolate BB5.1 were measured for cultures grown under the two copper levels discussed above. The results of these experiments are shown in Figures 4-6 to 4-9 and are summarized in Table 4-3. Cultures grown under the high copper condition resulted oxidized TCE at detectable rates. The measured rates of TCE disappearance were fitted to the Michaelis-Menten model by nonlinear regression as described for the methane kinetics experiments. The velocity versus initial substrate concentration plots and the fitted curves are shown in Figures 4-6 and 4-7. The experiments performed on cultures grown under the low copper condition (no copper added) resulted in no measurable TCE disappearance. Rates of TCE disappearance were measured over a 9-fold range in the mass-transfer experiment, which was set up in the same way as the kinetics experiments. Since a TCE oxidation rate as low as 0.1 mM/hr was detected in the mass transfer experiment, the rate of TCE oxidation in the low-copper cultures, if any, must be less than this. Typical rates of TCE oxidation (at the same TCE concentration used for the mass-transfer experiment) in the high-copper cultures were 0.8 to 1.0 mM/hr. Plots showing the TCE concentration over time are shown in Figures 4-8 and 4-9. These results are consistent with data reported by Semrau (1995), in which no TCE oxidation was observed for freshwater Type I methanotrophs grown under low copper conditions without added reducing equivalents. These results also confirm the

lack of the production of the sMMO under the low-copper growth condition, since this enzyme oxidizes TCE at high rates (DiSpirito, et al., 1992). As described in Semrau, (1995), toxicity effects were not apparent in any experiments in which TCE oxidation was observed, since linear plots of disappearance over time were obtained. It should be noted that a much lower maximum concentration TCE was used in this study than was used by Semrau (1995). In this study, the dissolved TCE concentrations were 3μM to18 μM, whereas in the study by Semrau the TCE concentrations used were 1μM to 125μM. The lower concentrations were used because saturation kinetics were achieved at less than18μM dissolved TCE. Final copper levels in the media were similar to those obtained in the methane oxidation experiments.

***Table 4-3:Results of TCE consumption experiments on the isolate BB5.1***

| Copper level | $K_s$ [a] | $V_{max}$ [b] | Added [Cu][c] | Final [Cu][d] |
|---|---|---|---|---|
| High | 8.1 | 6.1 | 25μM | 4.9 |
| High | 10.4 | 9.0 | 25μM | 4.4 |
| Low | ND[e] | ND[e] | none | 1.2 |
| Low | ND[e] | ND[e] | none | 1.0 |

a) μM dissolved TCE
b) nmole TCE*$hr^{-1}$ *mg total $protein^{-1}$
c) μM, measured in medium by ICP-MS
d) residual Cu level in medium with no Cu added is 3μM
e) ND = no disappearance observed

### 4.4 Discussion

The results of the measurement of the kinetics of methane and TCE oxidation by the estuarine isolate BB5.1 indicate that the copper concentration used for growth of the bacteria has a strong effect on the apparent affinity for methane in some cases, and at low copper levels the cells do not oxidize TCE at detectable rates. Estimates of the Michaelis-

Menten parameters $K_s$ and $V_{max}$ can be compared for the oxidation of methane and TCE by this strain for those experiments in which saturation kinetics were observed.

The $V_{max}$ values for methane oxidation by this isolate are approximately 2 to 3 orders of magnitude higher than those for TCE oxidation. A previous report of TCE oxidation by methanotrophs producing the pMMO, including the marine methanotroph *Methylobacter marinus* A45, (DiSpirito, et al., 1992) found $V_{max}$ values for TCE oxidation 2-6x higher than reported for this strain. Semrau (1995) did not observe saturation kinetics in studies of TCE oxidation by methanotrophs producing the pMMO under high copper conditions; the $V_{max}$ for that study was estimated to be approximately 250x higher than the value found for the estuarine isolate BB5.1 in this study. Under low copper conditions (2μM added copper), Semrau observed saturation kinetics for TCE oxidation if reducing equivalents in the form of formate were added to the assay. The $K_s$ for TCE obtained under these conditions was 25μM. The effect of the addition of formate was not examined in this study, so that result cannot be directly compared to the results of this study. The apparent affinity for substrate of this isolate, $K_s$, is similar for both substrates, ranging from 8.1 to 10.4 μM for TCE and 9.7 to 16.4 for methane. According to the Michaelis-Menten model of enzyme kinetics, a lower $V_{max}$ for TCE and a similar $K_s$ for these two substrates may indicate that the enzyme is less efficient in "turning over" TCE than methane after the substrate has bound.

The results obtained in this study have confirmed the copper effect seen by Semrau (1995) on TCE oxidation in the absence of added formate. Further examination of this effect is beyond the scope of this work, however it can be noted that the measured copper levels in the media at the end of all of the low-copper kinetics experiments are consistently lower than the copper levels in the media from the high-copper experiments. The measured copper levels are similar within each group of experiments, that is, the measured levels from the low-copper experiments are all similar to each other, and the same for the high-copper experiments. The lower copper levels measured in the media

from the low-copper experiments are consistent with the effects observed, that is, the available copper was clearly low enough to affect the substrate oxidation kinetics compared to the high-copper experiments. The results of Berson and Lidstrom (1996) indicate that in the Type I methanotroph *M. albus* BG8 most of the copper making up the difference between the starting and ending levels in the medium is sorbed to the bacterial cell surfaces. This sorbed copper does not appear to be available for use by the bacteria.

The consequences of these results for the use of the estuarine isolate BB5.1 for bioremediation of TCE are twofold: first, copper limitation may become problematic during *in-situ* TCE degradation. If the bacterial population is enriched to increase rates of TCE degradation, the supply of available copper may be depleted to the point that TCE oxidation will stop. It is worth noting that the total copper levels measured in the filtered pore water from the field experiments described in Chapter 2 were similar to those found in the laboratory medium at the end of the kinetics experiments, that is 1-2 μM. It is not known what fraction of that copper is bioavailable or whether additional copper bound to organic material in the sediments would be available to the bacteria. It is possible that the addition of bioavailable copper complexes as part of the biostimulation would overcome this problem. Second, the determination of the Michaelis-Menten parameters for the two substrates is important for the future development of predictive models of TCE biodegradation. These parameters are required for predicting the effects of substrate competition on enzyme activity, as well as in estimating the extent of TCE removal that can be achieved *in-situ* and the rate at which it would occur.

### 4.5 Conclusions

Saturation kinetics were observed for the oxidation of methane and for the oxidation of TCE by the estuarine isolate BB5.1. A strong effect on methane oxidation was observed in some cases when low-copper conditions were used for growth of the bacterial culture, in which a very high $K_s$ value was observed and saturation was not always achieved. The

results suggest that subtle changes in copper concentration may have a strong effect on methane oxidation at low copper levels, which may explain the inconsistent results obtained by Semrau (1995) and Udell (1996) for similar methane oxidation experiments. No TCE oxidation was observed for cultures grown in media under low copper conditions, confirming previous observations of the importance of copper concentration for TCE oxidation. This copper effect may limit in-situ bioremediation of TCE, since enriched populations of methanotrophs can deplete the supply of available copper. The addition of bioavailable copper complexes or an alternative source of reducing power during biostimulation may overcome this problem. Where saturation kinetics were observed the Michaelis-Menten kinetic parameters were determined for each substrate and compared. The apparent affinity constants ($K_s$) for the two substrates are similar whereas the maximum velocity of oxidation ($V_{max}$) was lower for TCE than for methane. Therefore the effective amount of enzyme activity present for TCE oxidation is lower than that for methane, although the affinity of the enzyme for both substrates appears to be similar. These data will provide important input to the development of predictive models for TCE bioremediation.

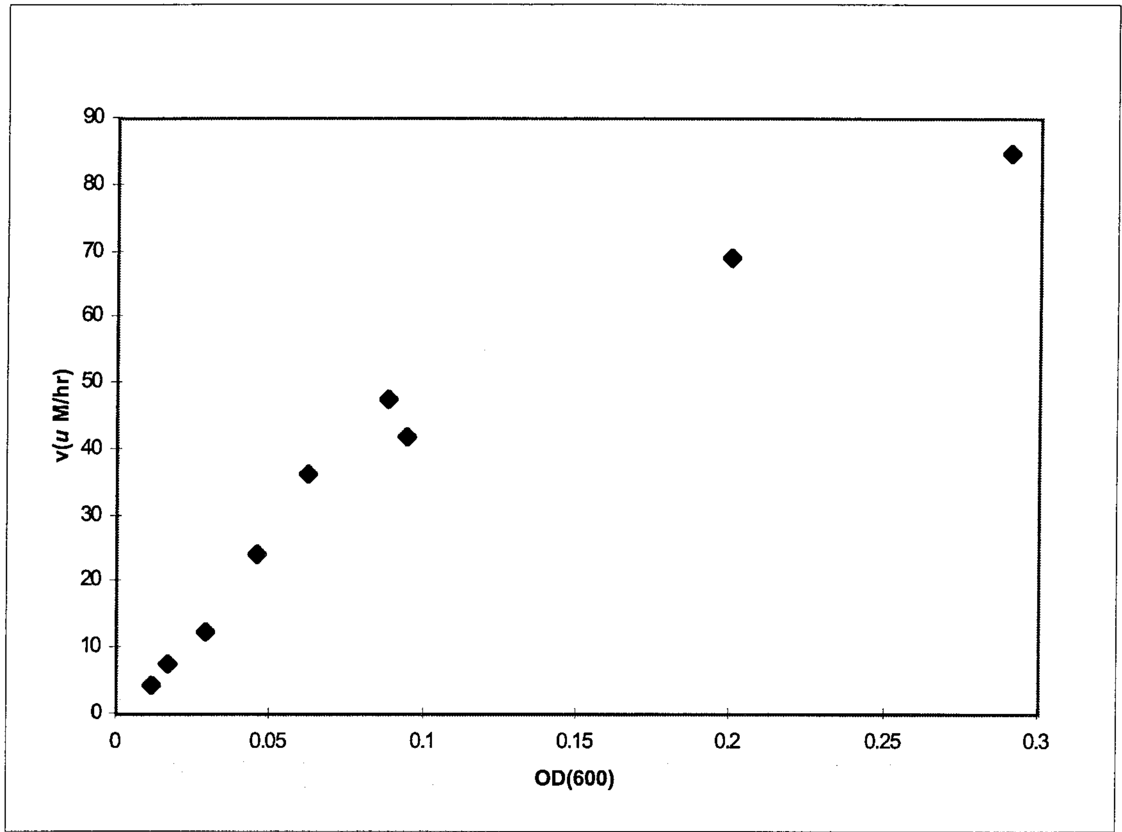
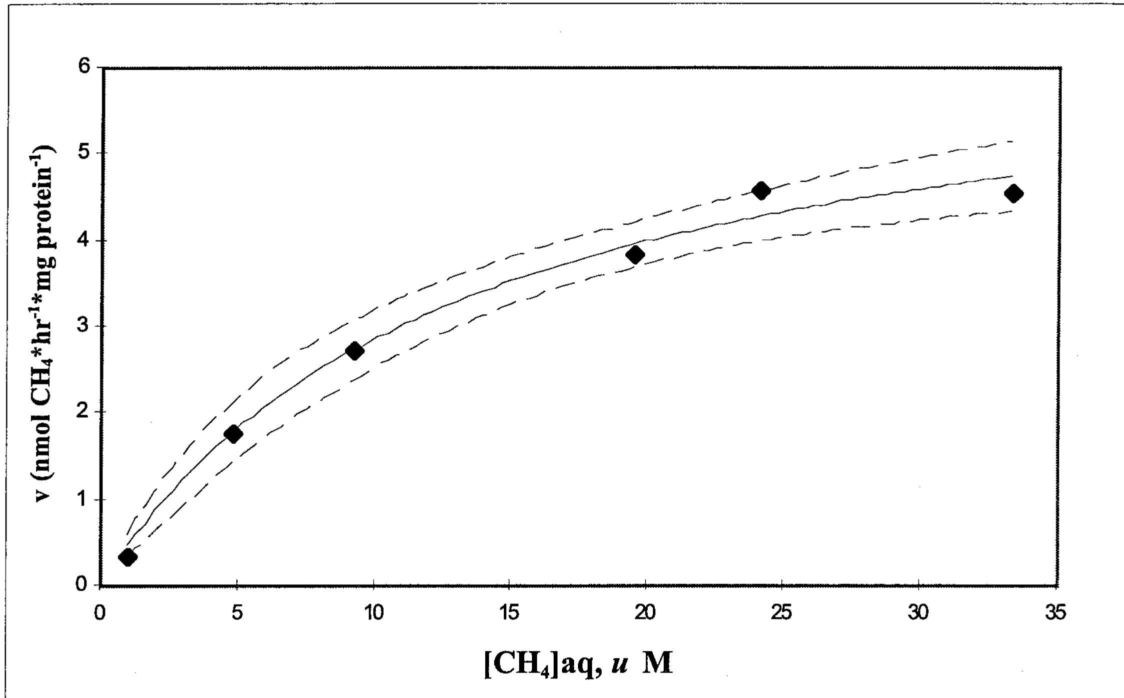



***Figure 4-1: Methane uptake velocity vs. cell mass***




$K_s$ =13.1 μM

$V_{max}$ = 6.6 nmol*hr$^{-1}$*μg total protein$^{-1}$

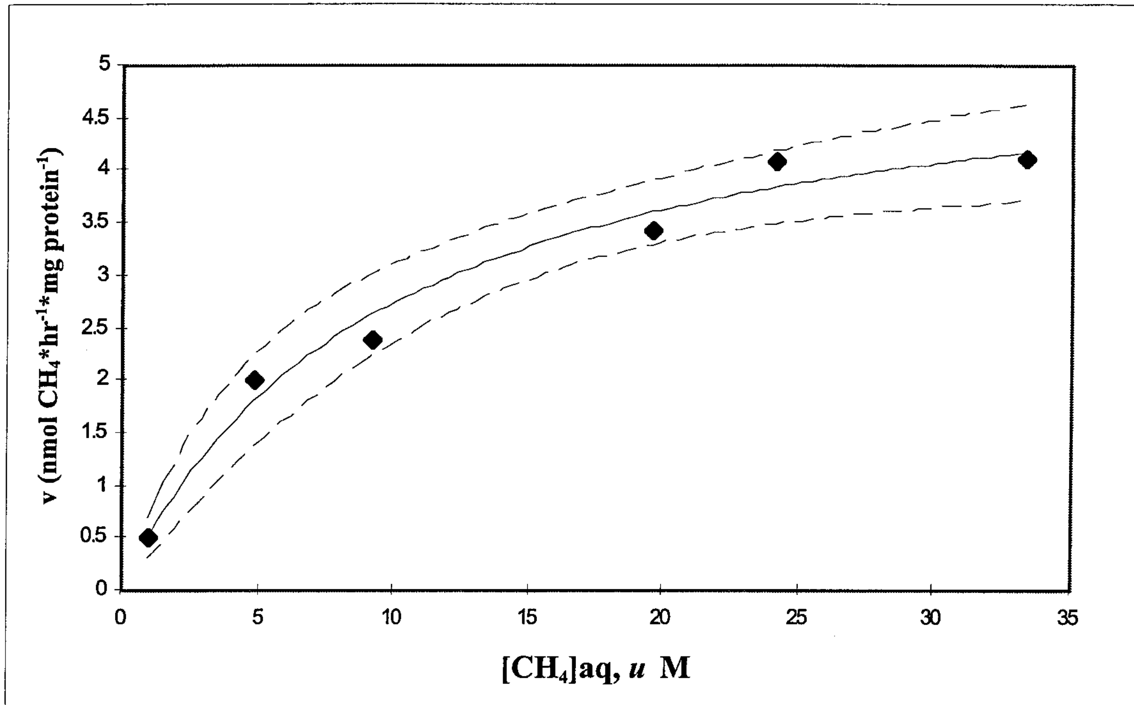
***Figure 4-2: Methane oxidation by isolate BB5.1***

High copper experiment #1

Points show measured velocities

Solid line shows fitted Michaelis-Menten curve

Dotted lines show 95% confidence limits generated by nonlinear regression




$K_s$ =9.7 μM

$V_{max}$ = 5.4 nmol*$hr^{-1}$*μg total $protein^{-1}$

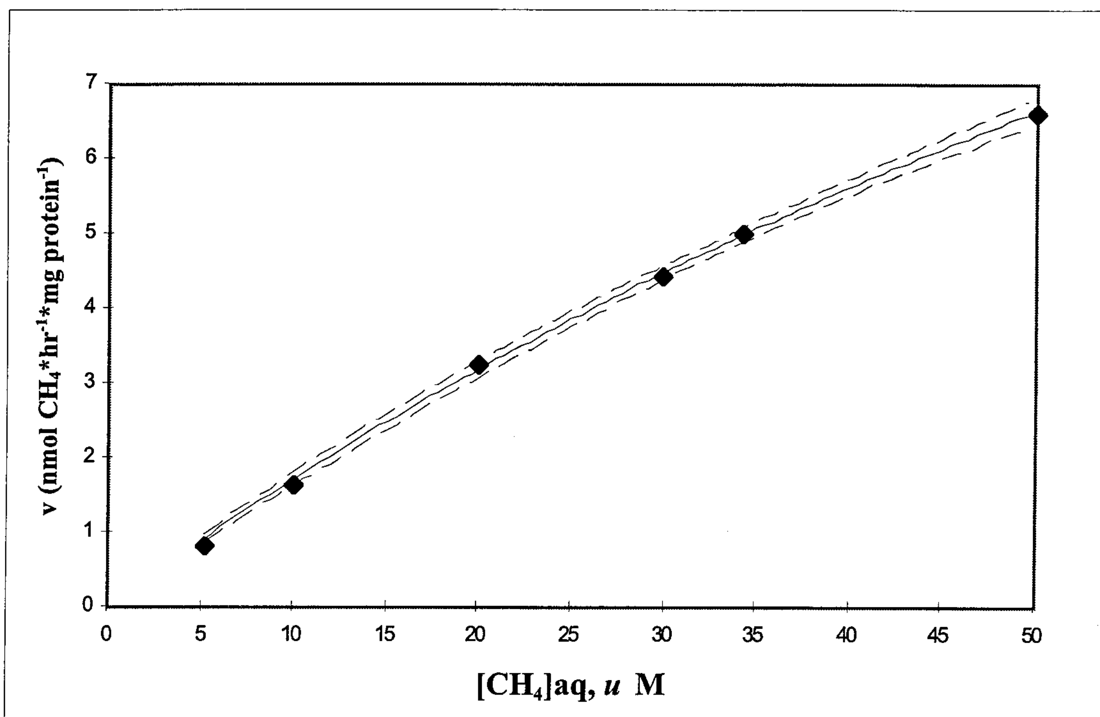
***Figure 4-3: Methane oxidation by isolate BB5.1***

High copper experiment #2

Points show measured velocities

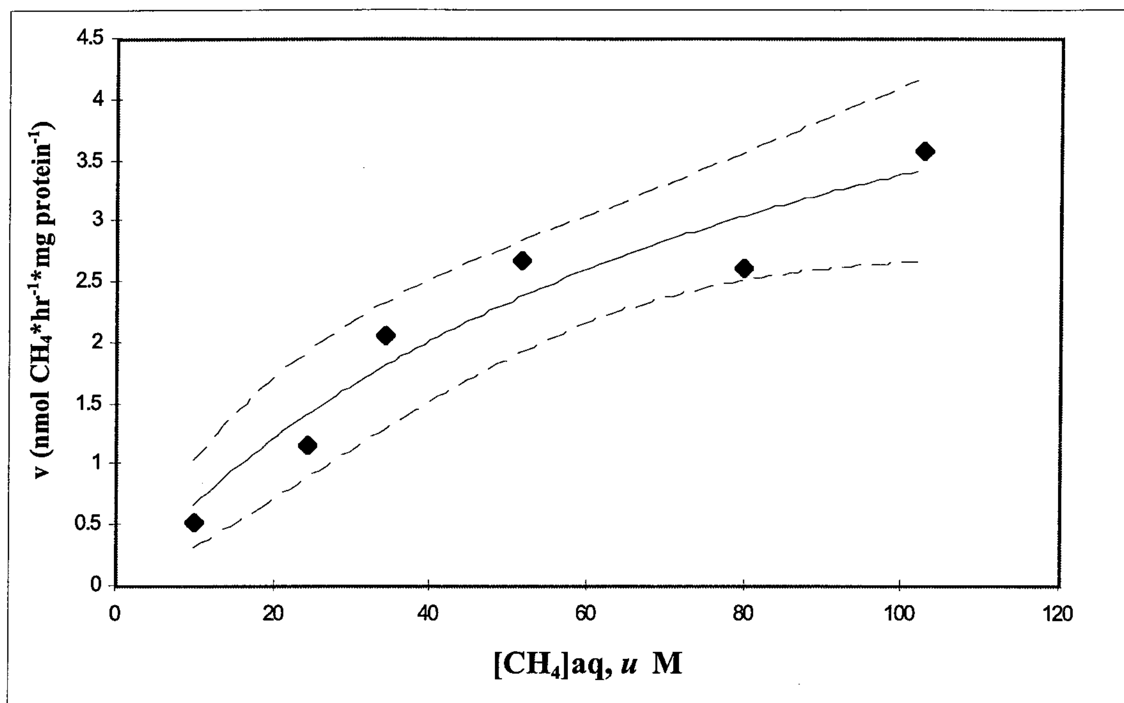
Solid line shows fitted Michaelis-Menten curve

Dotted lines show 95% confidence limits generated by nonlinear regression




$K_s$ > 50 µM
$V_{max}$ = unknown

***Figure 4-4: Methane oxidation by isolate BB5.1***
Low copper experiment #1
Points show measured velocities
Solid line shows fitted Michaelis-Menten curve
Dotted lines show 95% confidence limits generated by nonlinear regression




$K_s = 81.4\ \mu M$

$V_{max} = 6.1$ nmol*hr$^{-1}$*ug total protein$^{-1}$

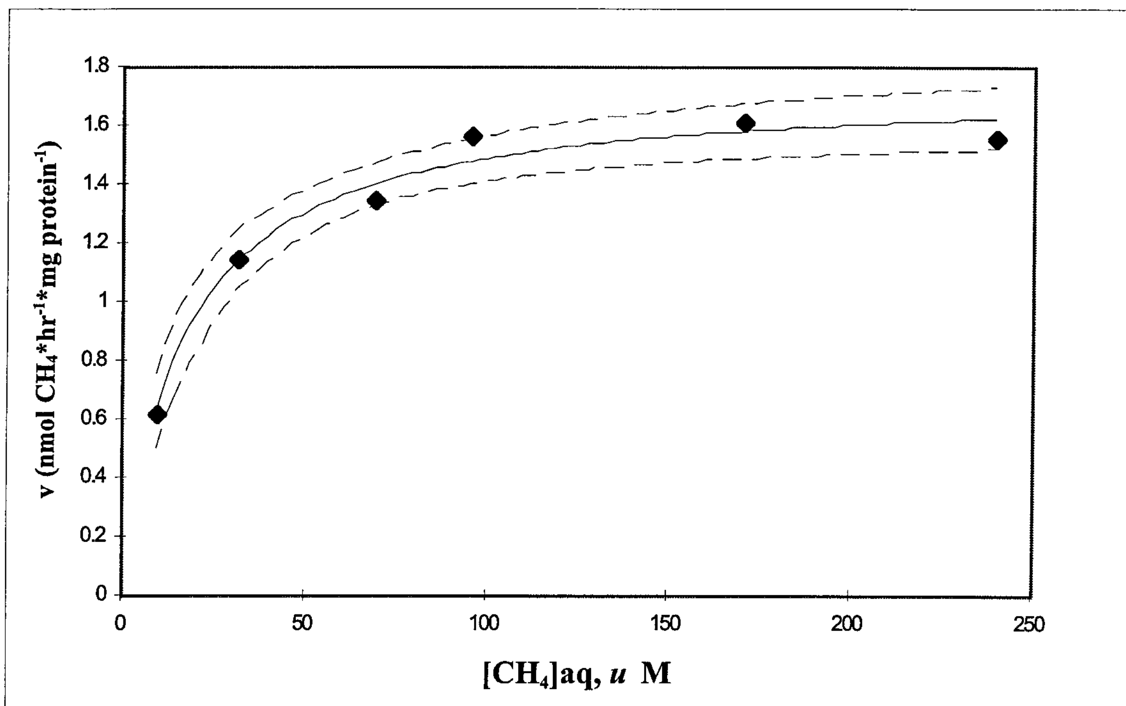
***Figure 4-5: Methane oxidation by isolate BB5.1***

Low copper experiment #2

Points show measured velocities

Solid line shows fitted Michaelis-Menten curve

Dotted lines show 95% confidence limits generated by nonlinear regression




$K_s$ =16.4 μM

$V_{max}$ = 1.7 nmol*hr$^{-1}$*μg total protein$^{-1}$

***Figure 4-6: Methane oxidation by isolate BB5.1***

Low copper experiment #3

Points show measured velocities

Solid line shows fitted Michaelis-Menten curve

Dotted lines show 95% confidence limits generated by nonlinear regression

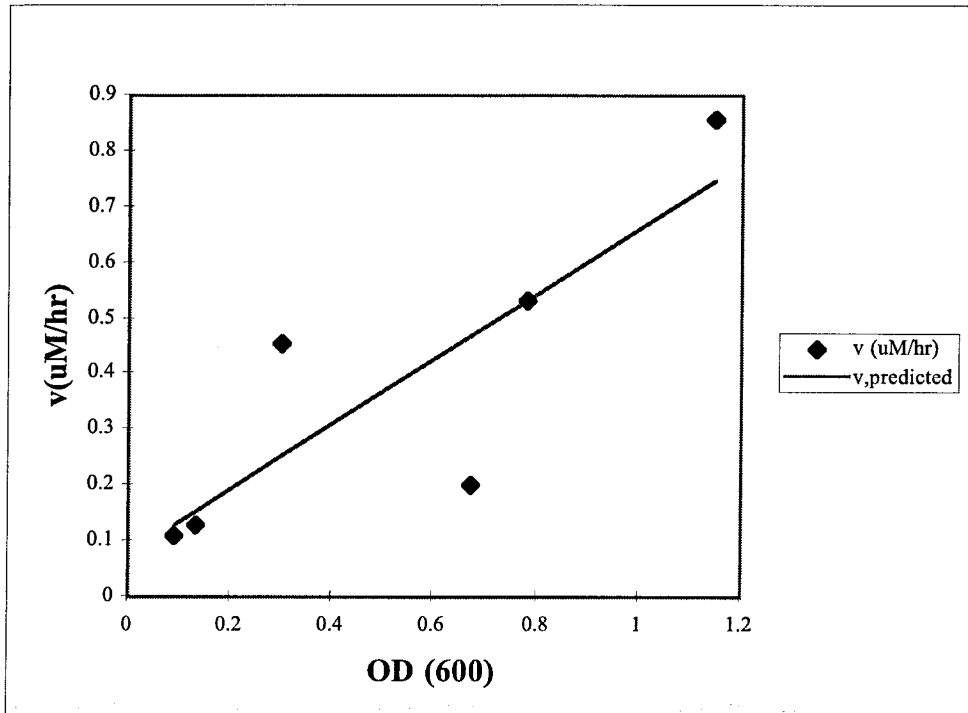
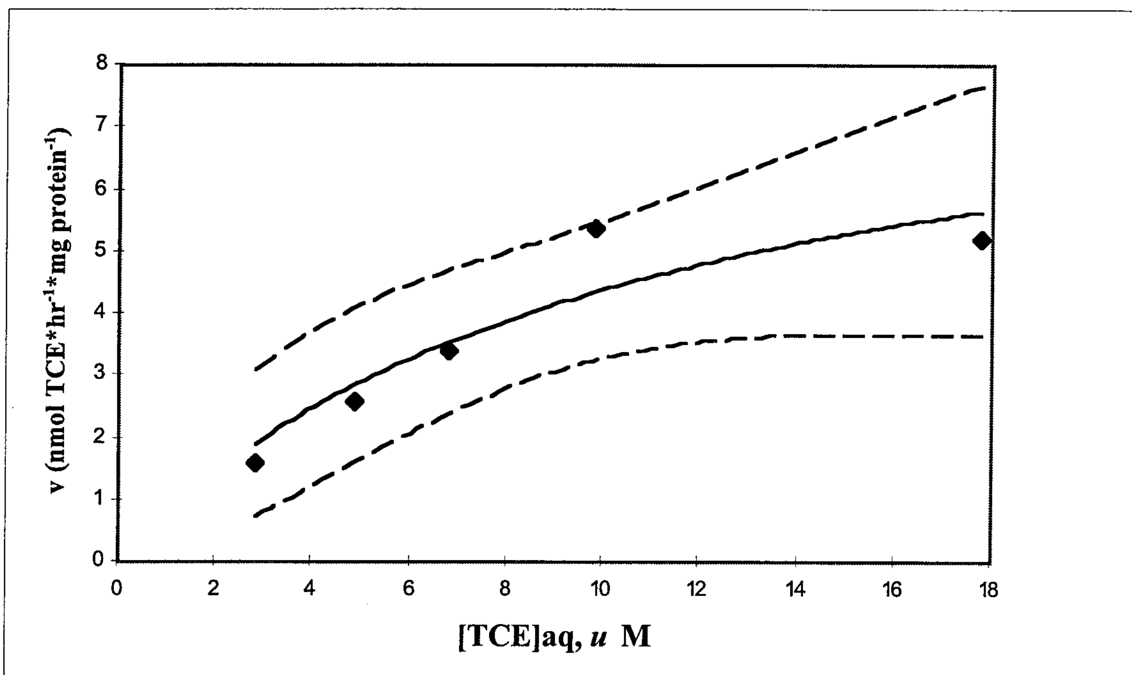



*Figure 4-7: TCE degradation vs. cell mass*




$K_s = 8.1\ \mu M$

$V_{max} = 6.1$ nmol*hr$^{-1}$*mg total protein$^{-1}$

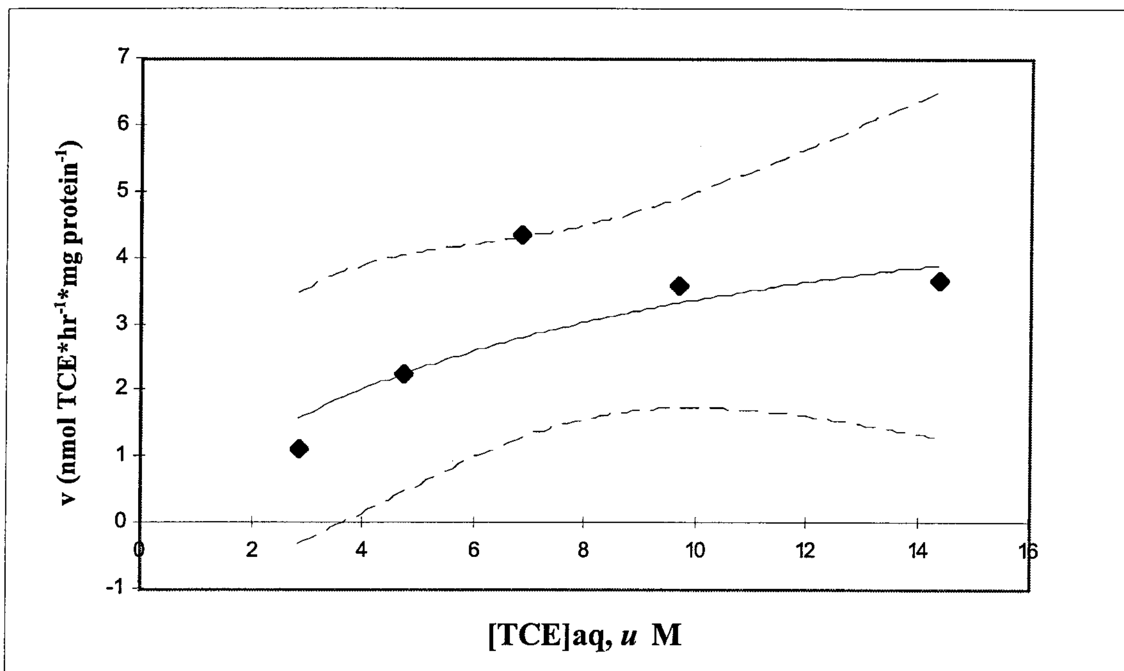
***Figure 4-8: TCE oxidation by isolate BB5.1***

High copper experiment #1

Points show measured velocities

Solid line shows fitted Michaelis-Menten curve

Dotted lines show 95% confidence limits generated by nonlinear regression




$K_s$ = 10.4 μM

$V_{max}$ = 9.0 nmol*hr$^{-1}$*mg total protein$^{-1}$

***Figure 4-9: TCE oxidation by isolate BB5.1***

High copper experiment #2

Points show measured velocities

Solid line shows fitted Michaelis-Menten curve

Dotted lines show 95% confidence limits generated by nonlinear regression

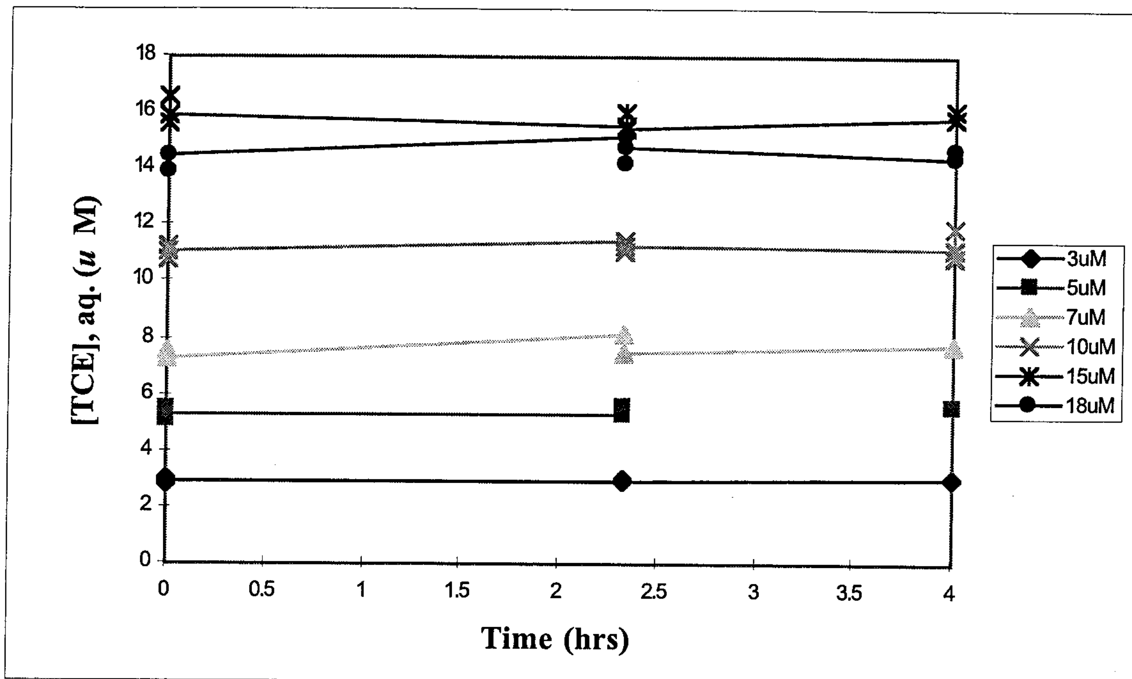



***Figure 4-10: TCE concentration vs. time***
Low copper experiment #1

### 4.6 References

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# 5. Behavior of Sediment Enrichments

## 5.1 Introduction

In-situ bioremediation of TCE by methanotrophs is an attractive method for cleanup of TCE in contaminated environments. The rate of TCE degradation by these bacteria will depend on the number of bacteria present, therefore it has been suggested that the population of methanotrophs should be increased by the addition of methane and oxygen to the site in order to increase the rate of TCE oxidation (Wilson and Wilson, 1985; Semprini, et al., 1990). The addition of these substrates would have an unknown effect on the population distribution of methanotrophs in the system, and an as-yet unpredictable effect on the kinetics of methane and TCE oxidation by the methanotrophic population. In this study, estuary sediment was enriched in batch systems in the laboratory in order to study the effect of enrichment of estuary sediment on the kinetics of methane and TCE degradation by the estuarine methanotrophic population. The effect of enrichment on the relative numbers of different groups of methanotrophs was also examined; these experiments are described in subsequent chapters.

## 5.2 Materials and methods

### *5.2.1 Enrichment conditions*

Sediment was collected from the Newport Bay Estuary as described in Chapter 2 and brought back to Caltech on the same day. The sediment was diluted approximately 10% by volume with Nitrate Mineral Salts + 1.5% (w/v) NaCl (NMSS, described in Chapter 2) as described below for each enrichment condition. 600 ml of this mixture was placed in a 2-liter Erlenmyer flask. The flasks were sealed and methane was added as described below for each enrichment condition. Three enrichment conditions were established; a high and a low methane condition (60% methane and 20% methane by volume of headspace, with the remainder supplied by air), and a low-nitrate condition to which nitrate-free NMSS was added in order to select for nitrogen fixation in the enrichment.

The low-nitrate flask was supplied with 20% methane by volume. The low-nitrate condition was chosen based on the characteristics of Type II methanotrophs. The genera in this group are capable of using atmospheric nitrogen under low-oxygen conditions (Murrell, 1994; see also Chapter 1); since no marine Type II methanotrophs have been found, this condition was used in an attempt to selectively enrich Type II organisms if any were present (Whittenbury, 1981). The different methane concentrations were used to determine the effect, if any, of the amount of methane on the enriched methanotrophic population. The flasks were placed on a rotary shaker at 250 rpm and incubated at 28°C for a total of 8 weeks. The flasks were removed and supplied with fresh air and methane every 8-10 days. At the end of the 8 week period, the enrichments were removed from the shaker and the methane and TCE oxidation kinetics of the enriched sediment were measured.

### *5.2.2 Methane oxidation assays*

Enriched sediment was dispensed in 2 ml aliquots into 20 ml serum vials (Pierce Co.) and sealed with butyl rubber stoppers (Bellco Glass Co.) and aluminum crimp seals (Hewlett Packard) as described for the other methane oxidation assays in Chapters 2 and 4. The rate of methane oxidation in these assays was measured by following the disappearance of methane in the headspace of the vials, so $^{14}CH_4$ was not used. Unlabelled methane (maximum purity, Matheson Gas Co.) was added to the vials with gastight syringes (Dynatech Precision Sampling, Series A2). The amounts of methane added were calculated to create a range of dissolved methane concentrations using Henry's law. Partition coefficients were calculated from the model of Duan, et al. (1992). Control vials contained killed cells, and were set up the same way as the experimental vials, except 200µl of 50% NaOH was added to each vial before the bacterial culture was dispensed. Triplicate vials of live and triplicate vials of dead bacteria were established at each methane concentration. The vials were shaken longitudinally at 200 rpm and room temperature.

At time points, 100 μl samples of the headspace gas were removed from each vial and analyzed for methane content on a Hach-Carle Gas Chromatograph equipped with a flame-ionization detector (FID) and a Haysep-Q 80/100 column (EG&G Engineering). The column temperature was 140°C. Methane concentrations were determined by comparison to methane standards. No more than four time points were taken, in order to minimize the change in pressure in the vial headspaces. Somewhat different time courses were used for each enrichment condition. Velocities of methane consumption were determined from the slope of the concentration versus time plots for each initial methane concentration, in triplicate. The data were fitted to the Michaelis-Menten model by nonlinear regression using the software package TableCurve 3D v.2 (Jandel Scientific) as described in Chapter 4. The Michaelis-Menten model is described in Appendix B. This assay differs from the methane consumption assays described previously in that the decrease in methane concentration in the vial headspaces was monitored in the same vials over the course of the experiment. Therefore consumption rates were calculated for each initial methane concentration in triplicate, and the Michaelis-Menten model was fitted to the entire data set. The $^{14}CH_4$ assays described previously involved separate sets of vials for each time point as well as each initial concentration, so consumption rates were calculated for each initial methane concentration by linear regression of all concentration versus time data, and the Michaelis-Menten model was fitted to the series of ( $[CH_4]_{inital}$, consumption rate) data pairs. The nonlinear regression results for this set of experiments are shown in Appendix A, including the 95% confidence limits for the Michaelis-Menten parameters.

#### *5.2.3 TCE oxidation assays*

TCE oxidation assays were carried out as described in Chapter 4, except that the vials were incubated for longer periods of time (12-20 hours). Therefore, non-biological TCE loss was monitored by establishing "dead" vials, set up the in the same way as the controls noted in Section 5.2.2, which were sampled at the same time as the "live" experi-

mental vials. Biological TCE degradation was determined from the difference in concentration between the "live" and "dead" vials at each time point. The rate of TCE oxidation was determined by measuring the disappearance of TCE over time as described in Chapter 4. The conditions under which the GC analyses were run are listed below:

***Table 5-1: GC conditions for TCE quantification in enriched sediment***

| Parameter | Value |
|---|---|
| Column | HP-624 |
| Column Length | 30 m |
| Column Inner Diameter | 0.32 mm |
| Film Thickness | 1.8 μm |
| Inlet Temperature | 200 °C |
| Detector Temperature | 300 °C |
| Column Inital Temperature | 80 °C |
| Column Final Temperature | 105 °C |
| Temperature Ramp | 4 °C/min |
| Run Time | 9 min |
| TCE Retention Time | 3.4 min |

A protocol different from that used in Chapter 4 was used for these analyses. This protocol involved a temperature ramp, which resulted in better peak separation on the chromatograms. Velocities of TCE oxidation were calculated as described in Chapter 4.

## 5.3 Results

### *5.3.1 Methane oxidation results*

Methane oxidation rates were measured for the low-nitrate condition and the low-methane condition. It was not possible to obtain usable data for the high-methane condition since a substantial fraction of the methane had been consumed by the time the first samples were taken, and the enrichments provided enough sample for only one set of experiments. However, this information suggests that the rates were higher than in the other two experiments. Figures 5-1 and 5-2 show the results of the methane oxidation experiments on the low-nitrate and low-methane enrichments. The values obtained for the Michaelis-Menten parameters are summarized in Table 5-2.

***Table 5-2 :Summary of methane oxidation kinetics for enriched sediment***

| Enrichment | $K_s$ [a] | $V_{max}$ [b] |
|---|---|---|
| Low methane, high nitrate | 48.3 | 5.9 |
| Low methane, low nitrate | 52.8 | 8.6 |

a) µM dissolved $CH_4$
b) µM $CH_4$/hr

The results are very similar for the two enrichment conditions, suggesting that the lack of added nitrate did not have a substantial effect on the kinetics of methane oxidation by the enriched population. The 95% confidence limits for the $V_{max}$ values overlap, suggesting that the two values are not statistically different. However, the values are listed with one decimal point for clarity. The$V_{max}$ values are about 3-10x higher than the $V_{max}$ values for methane oxidation in fresh sediment, suggesting an approximately 10-fold enrichment of the methanotrophic population. Perhaps the most striking result shown is the increase in the $K_s$ values relative to the fresh sediment. Although a Ks value of 26 µM was measured in one experiment on fresh sediment, $K_s$ values of 8-12 µM were typical. The values measured in the enrichments may represent a very different methanotrophic population than that detected in the fresh sediment, since the $K_s$ value is an indicator of the affinity of the MMO for methane.

**Mass transfer calculations**

As discussed in Danckwerts (1970), if a gas is dissolving into a liquid phase where it then undergoes an irreversible reaction, such as consumption by bacteria, a criterion can be established for the situation to exist wherein the gas reacts slowly enough in the liquid that the dissolved concentration of the gas in the bulk of the liquid is nearly equal to the equilibrium concentration of the gas with respect to the headspace concentration. In this case, the rate of reaction of the dissolved gas is much slower than the rate of dissolution of the gas into the liquid (the mass transfer rate), and the measured rate of reaction of the

gas, measured by its disappearance from the headspace of a closed vial, can be attributed to the liquid phase reaction only. In this case the system is not mass-transfer limited. The derivation of the criterion for this situation was described in Chapter 2. This criterion, which will be referred to as the Danckwerts criterion, was calculated for this experimental system and these measured rates of methane uptake in the enriched sediment. The mass transfer coefficient used was calculated from the film coefficient used by van Suijdam, et al. (1978) and the calculated specific surface area of the liquid phase in the vials. In all cases, the Danckwerts criterion was at least 2.5 orders of magnitude less than one, suggesting that this system was not mass-transfer limited. The calculated values are listed in Appendix C.

### *5.3.2 TCE oxidation results*

TCE oxidation was detected only in the low-methane enrichment (Figure 5-3). In this case, the rate of oxidation appears to increase up to 50μM dissolved TCE, but it decreases at higher concentrations, suggesting inhibition or toxicity may have occurred. Because of this apparent inhibition, the kinetics of TCE oxidation cannot be determined for this experiment, but the $K_s$ appears to be high since the rate of oxidation increased linearly up to 50μM. TCE oxidation was not detected at all in the low-nitrate and high-methane enrichments (Figures 5-4 and 5-5). The limit of detection in these assays is less than 0.017 μM/hr, so if TCE oxidation occurred, it was at a rate less than this. These results also indicate that no production of the sMMO occurred, as discussed in Chapter 4.

## 5.4 Discussion

The enrichment of the estuary sediment, as indicated by the elevated methane oxidation rates for all the enrichment conditions, shows that addition of methane and incubation results in an increase in methanotrophic bacterial populations in the estuary sediment. This implies that enrichment of estuary sediment in-situ for bioremediation of TCE would also result in increased numbers of methanotrophs and increased methane oxida-

tion rates, although at in-situ temperatures the increase would probably be slower than at laboratory temperatures. The limited level of enrichment (approximately 10-fold) is surprising, since the amount of methane and air present should have allowed enrichment of many orders of magnitude. These results suggest that a nutrient other than methane or oxygen may have become limiting in these enrichments. The results of this chapter also indicate that TCE oxidation is not necessarily enhanced by enrichment of the methanotrophic population. Since no TCE oxidation was detected in some enrichments it appears that bioremediation of TCE in these sediments by methanotrophs may require the addition of nutrients other than methane and oxygen. The methanotrophic isolate from these sediments, a Type I methanotroph referred to as isolate BB5.1, also exhibits variable behavior in the presence of TCE; the isolate showed no oxidation of TCE when grown under conditions of low copper concentration. It is possible that the estuary sediment contains a relatively low level of bioavailable copper, such that an enriched methanotrophic population becomes limited for copper in the same way that the isolate appeared to be. This idea is consistent with the limited amount of enrichment obtained. Also, it was observed by Semrau (1995) that TCE oxidation was stimulated in a Type I methanotroph grown under low copper conditions when reducing equivalents in the form of formate ions were added to the reaction mixture. Therefore it may be that in the event that adding copper to an in-situ bioremediation site is not practical, the addition of formate may stimulate the TCE oxidation.

TCE oxidation was observed in one enrichment, the one in which low-methane conditions were used. Since the $K_s$ for TCE oxidation appeared to be high, it is possible that the TCE was oxidized by the sMMO. However, this does not appear to be the case, based on the following analysis of TCE and methane oxidation rates. One of the differences between the sMMO and pMMO regarding TCE oxidation is the much higher rate of oxidation of TCE by the sMMO compared to the pMMO. Therefore, a distinctive property of sMMO activity is a ratio of $V_{max}$ values for methane and TCE of approxi-

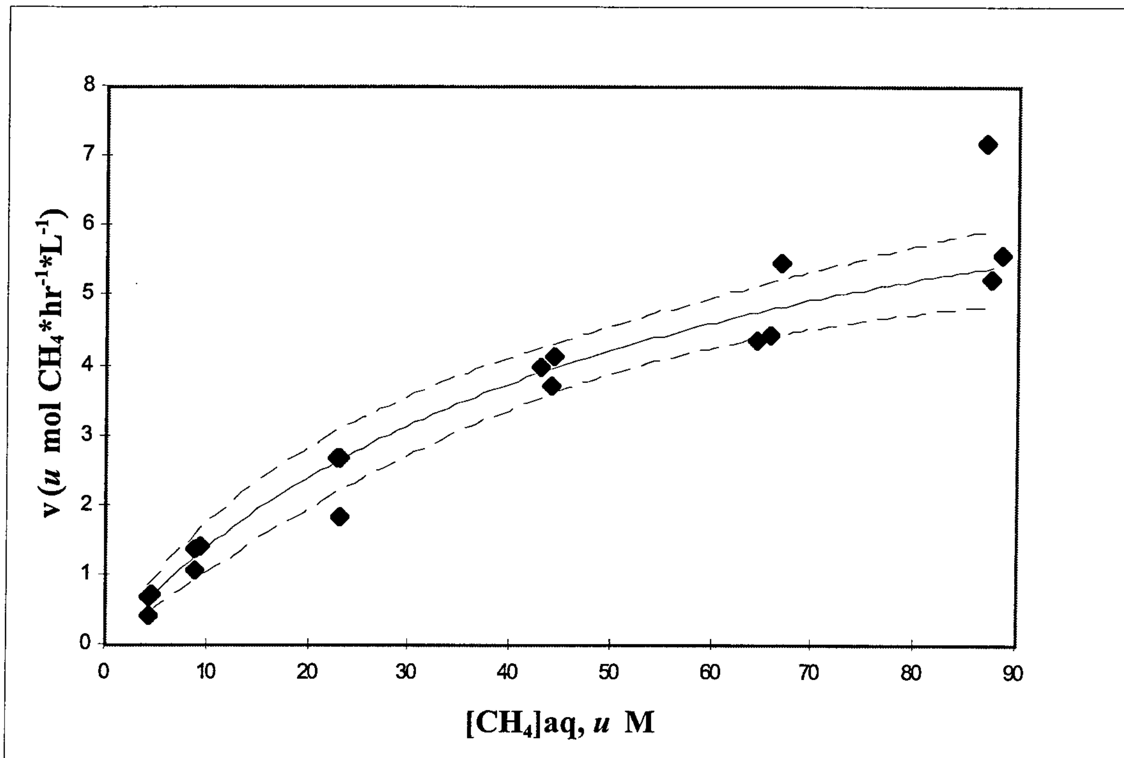
mately 1.25 (Oldenhuis, 1992). The same ratio for pMMO from the isolate BB5.1 can be calculated from the data in Chapter 4 to be about 791. Although it is not possible to calculate a $V_{max}$ for TCE directly for the low-methane enrichment, an estimated $V_{max}$ can be calculated for the case in which TCE oxidation occurs by the sMMO. In the Oldenhuis study, the $K_s$ for oxidation of TCE by the sMMO (in whole cells) was measured as 145 μM. Assuming the sMMO was produced in the sediment enrichments, with a similar $K_s$ for TCE oxidation, the measured rates of TCE oxidation can be used to calculate a Vmax value of about 0.4 μM/hr. The ratio of the Vmax for methane oxidation in the enriched sediment to this Vmax is about 14.8, a ratio indicative of the pMMO, not the sMMO. This result suggests that the enzyme responsible for the TCE oxidation in the low-nitrate enrichment is not the sMMO. Therefore, it is not yet clear why this enrichment oxidized TCE and the others did not. If copper and/or reducing power are limiting factors for TCE oxidation in these enrichments, then it is possible that the low-methane enrichment somehow resulted in more efficient utilization of these nutrients than the other enrichment conditions. Further experiments will be necessary to assess this phenomenon.

The kinetics of methane oxidation by the enriched sediment were different from the fresh sediment, but similar to the isolate BB5.1. Methane oxidation assays in the latter two sets of experiments showed similar half-saturation constants ($K_s$), varying not more than 2-3x under conditions of copper sufficiency. The $K_s$ values for the enriched sediment, however, are similar to the results for the isolate under low-copper conditions, that is, 4-5x higher than all of the other values measured. Since the $K_s$ value can be seen as a measure of the affinity of the enzyme for the substrate, in an inverse relationship, it appears that the enriched methanotrophic population may produce a methane-oxidizing enzyme that has a lower affinity for methane than the original population or the isolate under copper sufficiency. The $K_s$ values measured for the enriched sediment fall between the reported $K_s$ values for methane for the pMMO and the sMMO (see chapter 1); the

enriched sediment may contain methanotrophic bacteria producing a new enzyme, or the pMMO produced may be in a copper-insufficient condition, as discussed in Chapter 1. No sMMO-producing bacteria have so far been isolated from the marine environment, and the results presented here support previous findings in this regard.

### 5.5 Conclusions

Enrichment of the estuary sediment by the addition of methane and nutrients and incubation at laboratory temperatures with shaking resulted in an increase in methanotrophic activity in the sediment. Fitting the velocity data to the Michaelis-Menten model resulted in calculated Vmax values which were about 10x higher than those found in the fresh sediment suggesting that further growth of methanotrophs was limited by a nutrient other than methane or oxygen. The enrichment conditions used did not appear to have a significant effect on the degree of methanotrophic activity that was detected, although the oxidation rate for the high-methane enrichment appeared to be higher than for the two low-methane enrichments. In spite of the increase in methane-oxidizing activity, TCE oxidation was only detected in one enrichment and not at all in the others. The use of enriched populations of methanotrophic bacteria in estuary sediment for TCE bioremediation may require the addition of nutrients other than methane and oxygen to stimulate TCE oxidation. Previous research indicates that the addition of reducing equivalents in the form of formate ions stimulates TCE oxidation by methanotrophs, so formate may be an attractive candidate for such nutrient addition. The enrichment also resulted in increased values of the half-saturation constant for methane, $K_s$, which suggests that the enriched methanotrophic population had a reduced affinity for methane.



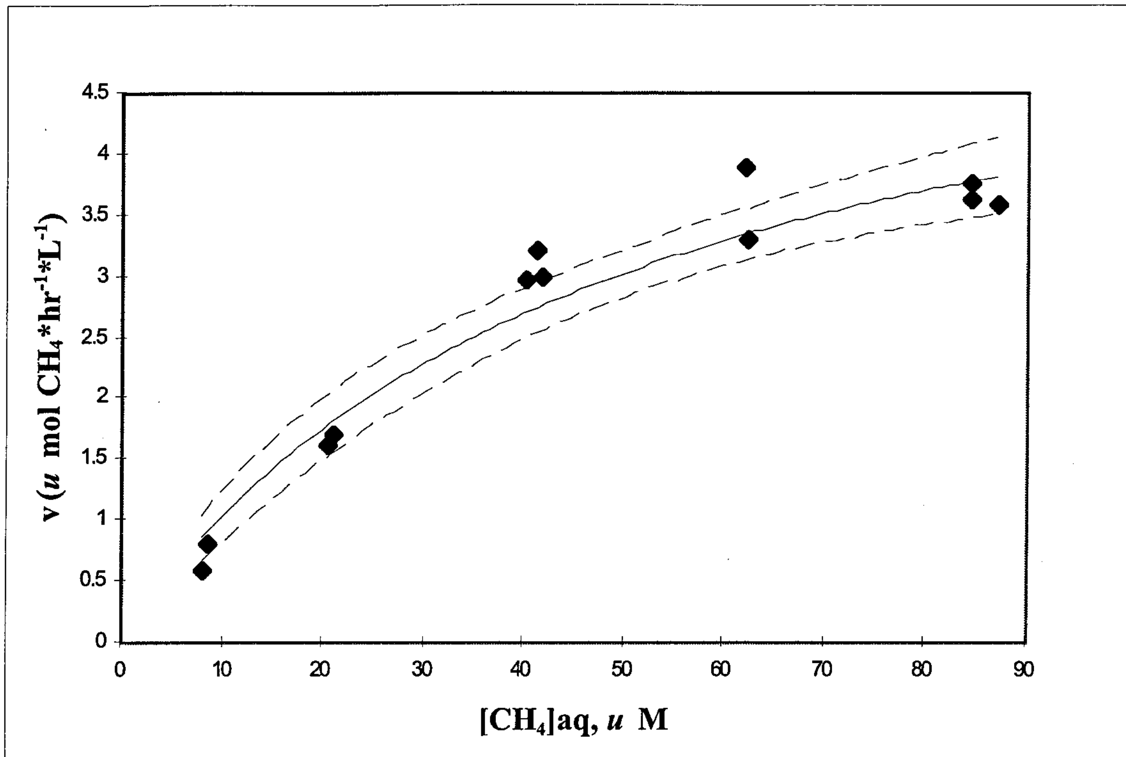

$K_s$ = 52.8 μM
$V_{max}$ =8.6μM/hr

***Figure 5-1: Methane oxidation by low-methane, low-nitrate enrichment***

Points show measured velocities

Solid line shows fitted Michaelis-Menten curve

Dotted lines show 95% confidence limits generated by nonlinear regression




$K_s$ = 48.3 μM
$V_{max}$ =5.9μM/hr

***Figure 5-2: Methane oxidation by low-methane, high nitrate enrichment***
Points show measured velocities
Solid line shows fitted Michaelis-Menten curve
Dotted lines show 95% confidence limits generated by nonlinear regression

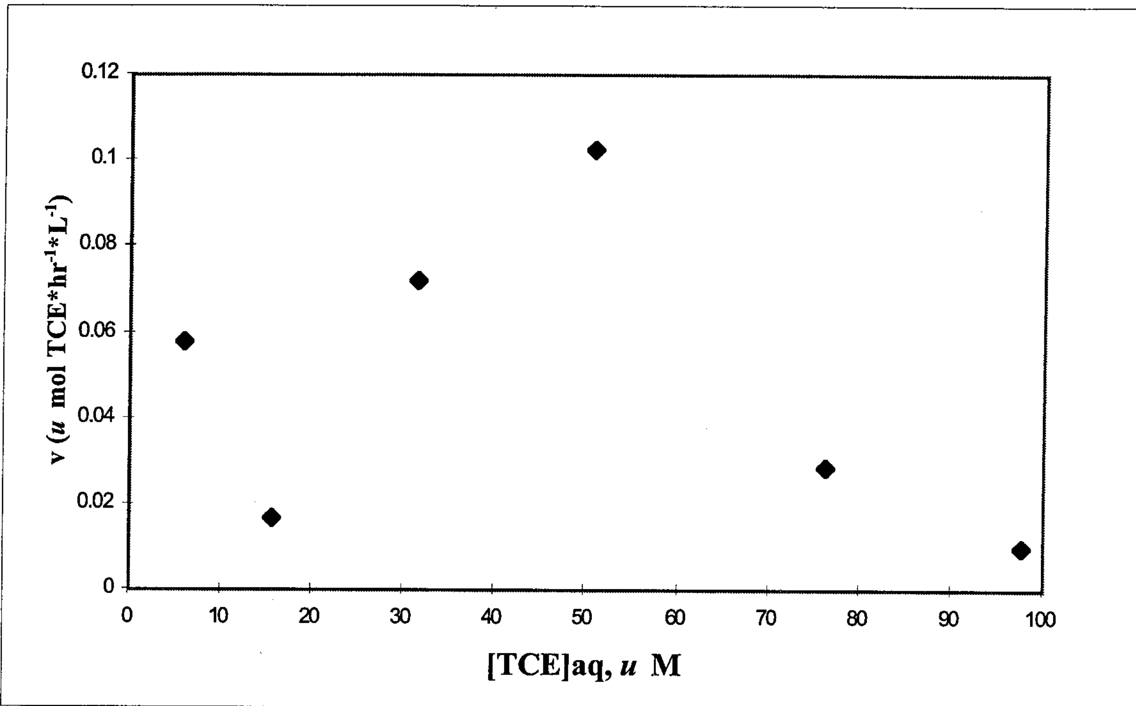



***Figure 5-3: TCE oxidation by low-methane, high nitrate enrichment***

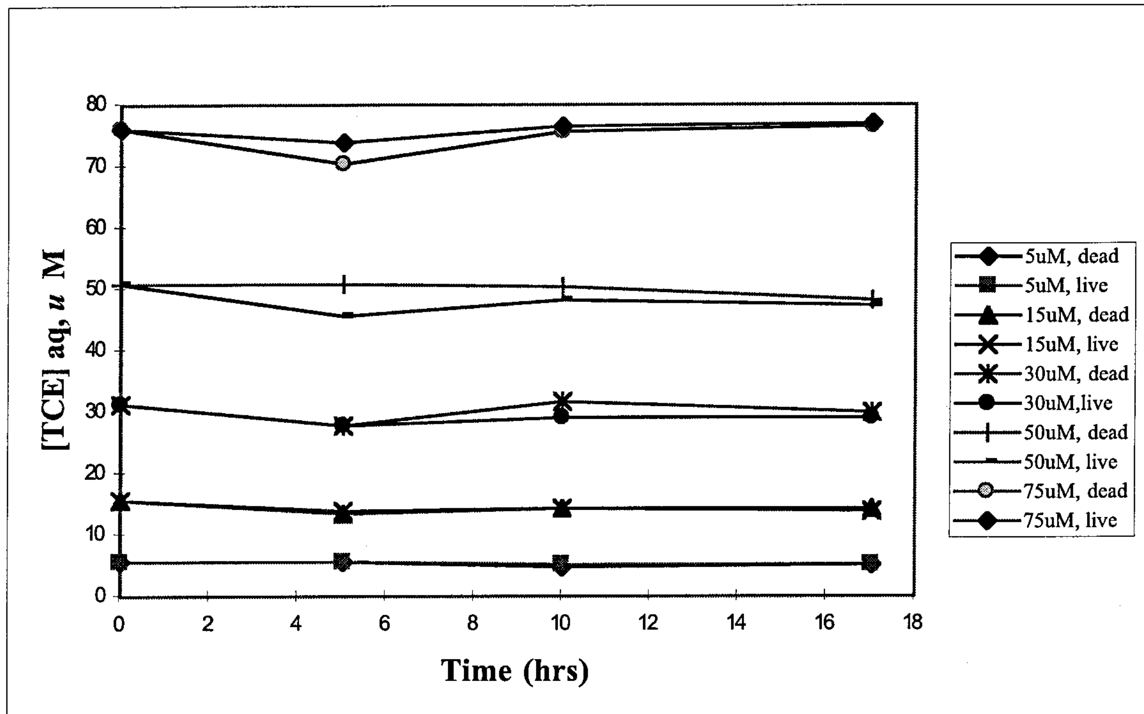



*Figure 5-4: TCE concentration vs. time, low methane-low nitrate enrichment*

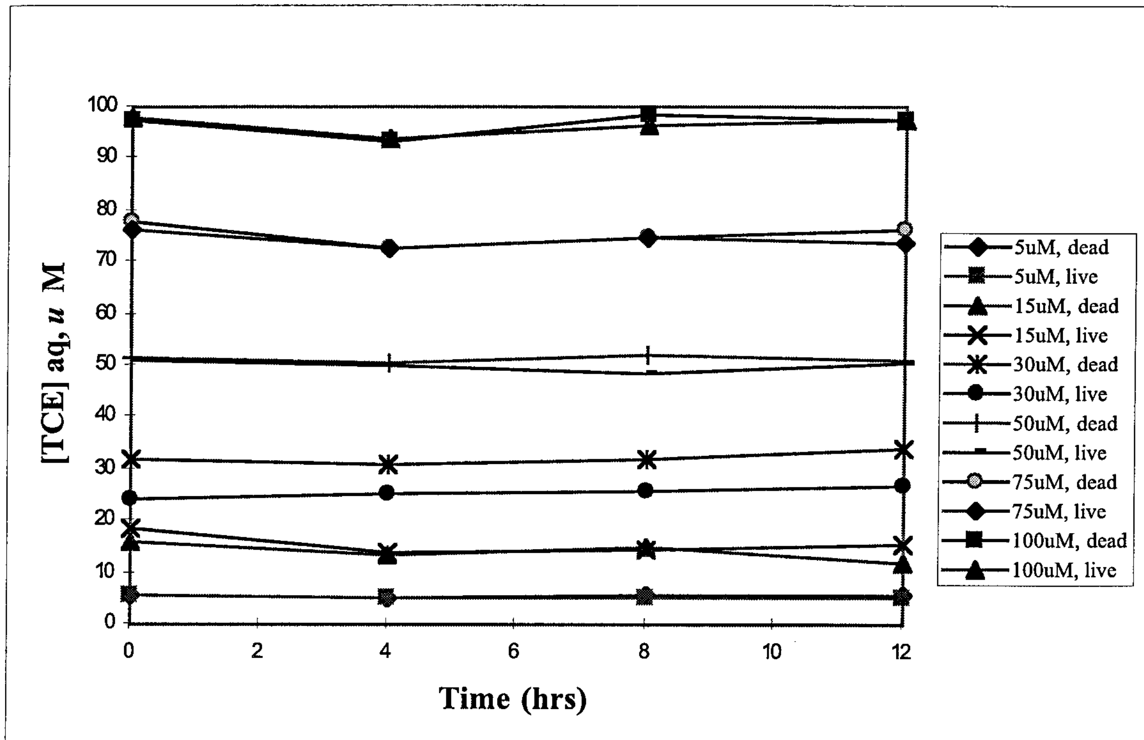



***Figure 5-5: TCE concentration vs. time, high methane enrichment***

## 5.6 References

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# 6. Gene Probing of Estuarine Sediment

## 6.1 Introduction

As previously discussed, conventional culturing techniques have proved to be limited in their ability to provide information about the structure of bacterial communities *in-situ* (King, 1992; Ward, et al., 1992; Lidstrom 1996). Molecular techniques, including gene probing, have the potential to overcome some of the limitations posed by culturing techniques and to provide insights into bacterial community dynamics that were previously unavailable. In this study we have used gene probing to provide information on the methanotrophic population in an estuary sediment as it underwent enrichment with methane. These results will provide valuable information for modelling the community dynamics of methanotrophs in systems where *in-situ* bioremediation is being considered for TCE removal.

## 6.2 Materials and methods

### *6.2.1 Enrichment conditions and sampling*

The sediment was collected by hand as described in Chapter 2 from the Newport Bay Estuary on June 6, 1995. The enrichments used were those set up for the kinetics experiments described in Chapter 5. Each enrichment was sampled at 8-10 day intervals and the samples were stored at -70°C until the DNA extraction was performed.

### *6.2.2 Extraction and purification of DNA*

The total community DNA was extracted from the sediment samples by an electrophoretic method based on the method described by Rochelle and Olson (1991) for extraction of DNA from sediment. This method consists of three parts: immobilization of the sediment, lysis of the bacterial cells, and extraction of the DNA. All chemicals used were reagent-grade from Sigma Chemical Co. The sediment was immobilized in agarose by

mixing 0.5 ml of sediment with 0.5 ml of a solution of 1.5% (w/v) agarose (Difco) in TE buffer. (TE buffer consists of 10mM Tris, pH= 8.0 and 1 mM EDTA, as given by Sambrook, et al., 1989). The mixtures of sediment and agarose were drawn into 1-ml disposable syringes and allowed to solidify at 4°C. The immobilized sediment was removed from the syringes by trimming the nozzle end off the syringe with a razor blade and expelling the cylinder of sediment and agarose with the syringe plunger. For lysis of the bacterial cells, the immobilized sediment was rinsed with TE buffer, then submerged in lysozyme solution (15mg/ml, also in TE buffer) and incubated at 37°C for 6-9 hours. The immobilized sediment was then removed from the lysozyme and rinsed in TE buffer, and submerged in a lysis solution consisting of: 2% sodium dodecyl sulfate (SDS), 1M NaCl, 10mM Tris (pH=8.0), 50mM EDTA, and 0.5% Triton X-100 (Sigma Chemical Co.). The immobilized sediment was incubated in the lysis solution for 12-18 hours at 37°C. The immobilized sediment was then removed from the lysis solution, rinsed with TE buffer, and stored at 4°C in TE buffer for 10-30 minutes while the electrophoretic procedure was set up. The DNA was extracted from the immobilized sediment by placing slices of the sediment in the wells of an agarose gel (1% agarose in TAE buffer, with 0.2 mg ethidium bromide stain added), sealing the wells of the gel with more agarose solution and running the gel at about 80 volts in a standard electrophoresis unit (Gibco-BRL). TAE buffer consists of 40mM Tris-acetate and 2mM EDTA, as given by Sambrook, et al., (1989). As the electrophoresis proceeded, a thick band of brown compounds could be seen running in the gel, sometimes followed by thinner bands of other brown compounds. Based on reports in the literature on extracting DNA from soil and sediment samples (Rochelle and Olson, 1991; Tebbe and Vahjen, 1993), these compounds are believed to be humic acids and other organic material. The literature also reports that such organic material tends to co-purify with DNA (Rochelle and Olson, 1991; Tebbe and Vahjen, 1993) in standard DNA purification protocols. In this protocol, the DNA molecules were relatively large compared to the colored compounds and ran very late in the

gel. They were visible at the top of the gel when the gel was exposed to UV light, usually completely separated from any of the brown material. This separation overcomes many of the problems associated with DNA co-purifying with organic material and is the principal advantage of this extraction method. The gels ran for 2-2.5 hours. The bands of large DNA molecules were excised from the gels and the DNA was purified using the GeneClean kit (Bio101) according to the manufacturer's instructions for purifying DNA from agarose gels.

#### *6.2.3 Slot blots for hybridization of methanotroph probes*

The DNA was blotted onto nylon membranes (Schleicher and Schuell) using a slot-blot apparatus (Schleicher and Schuell). To enhance the efficiency of hybridization, all of the samples were adjusted to 100µl at 0.4 N NaOH and 10mM EDTA, as recommended by Duong, et al. (1994) and incubated at 100°C for 10 min. before blotting. The blots were made using 6x SSC as a transfer buffer, as recommended by the manufacturer of the apparatus. Since previous work by Grant (1992) also determined that the degree of hybridization to blotted DNA was significantly less in the column of slots closest to the vacuum outlet on the slot-blotting aparatus, only the final two columns of slots were used. The samples were blotted onto the nylon membrane in slots on the lower half of the apparatus, in chronological order by sample date during the enrichment. DNA from *M. trichosporium* OB3b and *M. marinus* A45 was blotted onto the membrane in slots on the upper half of the apparatus to serve as hybridization controls for the gene probing experiments. DNA from these laboratory strains was blotted as a dilution series to provide a measure of the amount of DNA in each sediment sample which hybridized to the probes in the gene probing experiments. The same volume of sample was blotted in each slot (100µl) for both the sediment samples and the DNA from laboratory strains, to control for any effects of sample volume on hybridization. The blots were dried at room temperature and the DNA was fixed on the membrane by exposing the blots to UV light

for 3 minutes as recommended by the membrane manufacturer.

### *6.2.4 Hybridization experiments and image collection*

For the hybridization experiments with the methanotroph-specific probes, DNA was extracted from the enrichments under the low-nitrate condition and the high-methane condition (see chapter 5 for descriptions of these conditions). The blots were hybridized to the gene probes 1035-RuMP and 1034-Ser to determine the presence of Type I and Type II methanotroph DNA in the DNA from the sediment samples. The hybridizations were carried out as described for the probing experiments using nylon membranes in Chapter 3. The hybridization and rinsing temperatures used were 45°C and 42°C for the RuMP and Ser probes, respectively. After hybridization and rinsing, the blots were sealed in plastic wrap and exposed to the imaging emulsion in a Molecular Dynamics Phosphorimager Storage Cassette. The blots were exposed for 6 days, then the images were collected on a Molecular Dynamics Phosphorimager (Model 425E) and processed with the manufacturer's imaging software, ImageQuant v.3.1.

## 6.3 Results

The results of these experiments are shown in Figures 6-1 and 6-2. It can be seen that the Type I probe hybridized to all of the DNA samples extracted from the sediment, as well as the control DNA from *M.marinus* A45. The Type II probe, however, did not hybridize to the control DNA from *M. trichosporium* OB3b, although there was some background hybridization to the sample DNA and the other control DNA. This probe has been unreliable in our hands, and further work will be needed to optimize its use for natural samples. For the Type I probe, differences in hybridization between samples from different collection dates during the enrichments are slight. The first sample, as shown in the figures, was collected on June 16, 1995, 10 days after the enrichments were set up. The results suggest that the numbers of Type I methanotrophs in the enrichments remained fairly constant during the rest of the enrichment period. The number of methanotrophic cells

per ml of sediment can be estimated for the enriched sediment, based on calculations of the rate of methane oxidation per cell by the isolate BB5.1. The DNA blots can also be used to obtain an estimate of methanotroph number. The amount of DNA present in a typical bacterial cell is known. By correcting this value for methanotrophs the number of cell-equivalents of control DNA on the blot can be calculated, and the amount of hybridization to the control DNA can be used for comparison to the sample DNA to estimate the number of cell-equivalents in the sample DNA on the blot. Since the sample DNA consists of the DNA extracted from 1ml of sediment, the cell-equivalents per ml as determined from $V_{max}$ values and from the gene probing can be compared.

Neidhardt, et al. (1990) give estimates of the proportions of various cell components for the Gram-negative species *E. coli*. The methanotrophs of interest are about the same size and mass as *E.coli*, although they grow much more slowly and therefore have less DNA per cell. To calculate the $V_{max}$ for methane consumption on a per cell basis from data in moles/time*mass protein, an estimate of the amount of protein per cell is necessary. From Neidhardt, et al., the relevant estimates are: total dry mass of a bacterial cell = $2.84 \times 10^{-13}$g. Percentage of dry mass which is protein = 55%. Therefore, mass of protein/cell = $1.56 \times 10^{-13}$g/cell or $1.56 \times 10^{-10}$ mg/cell. Since it appears (based on the lack of TCE degradation by the enriched sediment) that the enrichments may contain copper-limited methanotrophs, the $V_{max}$/cell is calculated from the low-copper pure culture in which saturation kinetics was observed, 6118 nmol* $hr^{-1}$*mg $protein^{-1}$. This value gives a $V_{max}$/cell of $9.54 \times 10^{-7}$ nmol*$hr^{-1}$*$cell^{-1}$, or $0.95 \times 10^{-9}$ µmol*$hr^{-1}$*$cell^{-1}$. This value is very similar to the average value of $1 \times 10^{-9}$ µmol*$hr^{-1}$*$cell^{-1}$ used by Lidstrom (1996). The range of these values reported in the literature is 0.1 - $1.6 \times 10^{-9}$ µmol*$hr^{-1}$*$cell^{-1}$. From Chapter 5, the Vmax for the enriched sediment is 8.6 µM/hr, so the enriched sediment is then estimated to have $5.4 \times 10^{6}$ to $8.6 \times 10^{7}$ bacteria/ml at the end of the enrichment period when the methane oxidation kinetics were measured.

This cell number estimated from the methane oxidation rate per cell can now be

compared to the cell number estimated from gene probing. Table 6-1 shows the amounts of control DNA from *M. marinus* A45 added to the slot blots, and the cell equivalents of this DNA based on an estimate of $3.0 \times 10^{-15}$ g DNA/cell. This estimate is based on the value of $9.0 \times 10^{-15}$ g DNA/cell for *E.coli* (Neidhardt, et al., 1990) and adjusted for slower-growing bacteria, as discussed above. The sediment bacteria are assumed to have an average of 3 copies of the 16S rRNA genes per cell, the same as the pure cultures used for the controls. Unfortunately, the dilution series of control DNA from M. marinus A45 did not produce hybridization signals which were linearly related to the amount of DNA blotted and so a standard curve could not be generated. However, the lower dilutions appear to be more linear, and a visual inspection of Figure 6-1 suggests that the final sample of DNA from the sediment enrichments contains between 0.06 and 0.18 μg of methanotroph DNA. Since this is the amount extracted from 1 ml of sediment, this value represents about $3 \times 10^{7}$ cells/ml of sediment.

***Table 6-1: DNA from M. marinus A45 and cell equivalents***

| Slot # | Mass DNA (μg) | Cell equivalents ($x10^{-8}$) |
|---|---|---|
| 1 | 1.2 | 4.0 |
| 2 | 0.9 | 3.0 |
| 3 | 0.6 | 2.0 |
| 4 | 0.3 | 1.0 |
| 5 | 0.18 | 0.6 |
| 6 | 0.06 | 0.2 |
| 7 | 0.03 | 0.1 |

This estimate falls within the range of estimates based on the measurement of $V_{max}$. At this time, these calculations represent only rough estimates, because there is still significant uncertainty concerning the efficiency of DNA recovery from the sediment samples and the proportion of methanotrophs *in situ* that hybridize to the probe. Further research should address these uncertainties, to provide a more precise estimate of bacterial numbers.

### 6.4 Discussion

It is apparent from these results that Type I methanotrophs were present in all of the sediment samples from the enrichments. This result correlates well with the fact that Type I methanotrophs were isolated from enrichments of this sediment in all attempts, as described in Chapter 3. These results provide little information about the presence of Type II methanotrophs in the estuary sediment. The Type II probe has been unreliable in our hands, often hybridizing at a much lower level to control DNA than the Type I probe. In these experiments the hybridization was too low to detect. However, previous work has suggested that the marine environment may not contain Type II methanotrophs. Probing of 16S rDNA libraries by Holmes (1995) detected no Type II methanotrophs from marine samples, and additional probing with a functional gene probe for the genes encoding the soluble methane monooxygenase (sMMO) did not detect these genes in samples from the marine environment (Holmes, 1995). This enzyme is produced mainly by a few known species of Type II methanotrophs and the presence of the genes encoding it would be strong evidence of the presence of Type II methanotrophs. Future experiments on these samples will include optimizing hybridization with the Type II probe, and hybridizing the DNA blots with the probe for the sMMO genes. If these results are negative as expected, it would provide further supportive evidence for the lack of Type II strains in these environments.

The hybridization experiments with the eubacterial probe demonstrate the success of the DNA extraction method for the estuary sediment. The calculations of cell number based on the DNA probe method and the Vmax/cell method agreed within the level of uncertainty for these methods, which is about an order of magnitude for both. Traditional plating and culture methods fail to detect a much larger proportion of natural populations (King, 1992; Lidstrom 1996) and so the methods presented here are a major advance over

currently available techniques. Further work will be necessary to optimize these methods and improve their precision. However, these preliminary results are encouraging and suggest these methods will become a useful technique for quantifying natural populations of methanotrophs.

### 6.5 Conclusions

Gene probing of DNA extracted from enrichments of estuary sediment demonstrated the presence of Type I methanotrophs in the sediment during the enrichments. No information was obtained about the presence of Type II methanotrophs. However, previous research suggests that Type II methanotrophs may not be present in the marine environment. Further research will be necessary to improve the reliability of the Type II probe and to hybridize the sediment DNA samples with a probe encoding genes for the sMMO before conclusions can be drawn about the existence of Type II methanotrophs in the estuary sediment. The methanotroph Type I probe can be used to detect methanotroph DNA extracted from sediment samples and may be useful for quantitation of bacterial populations in enriched estuary sediment.

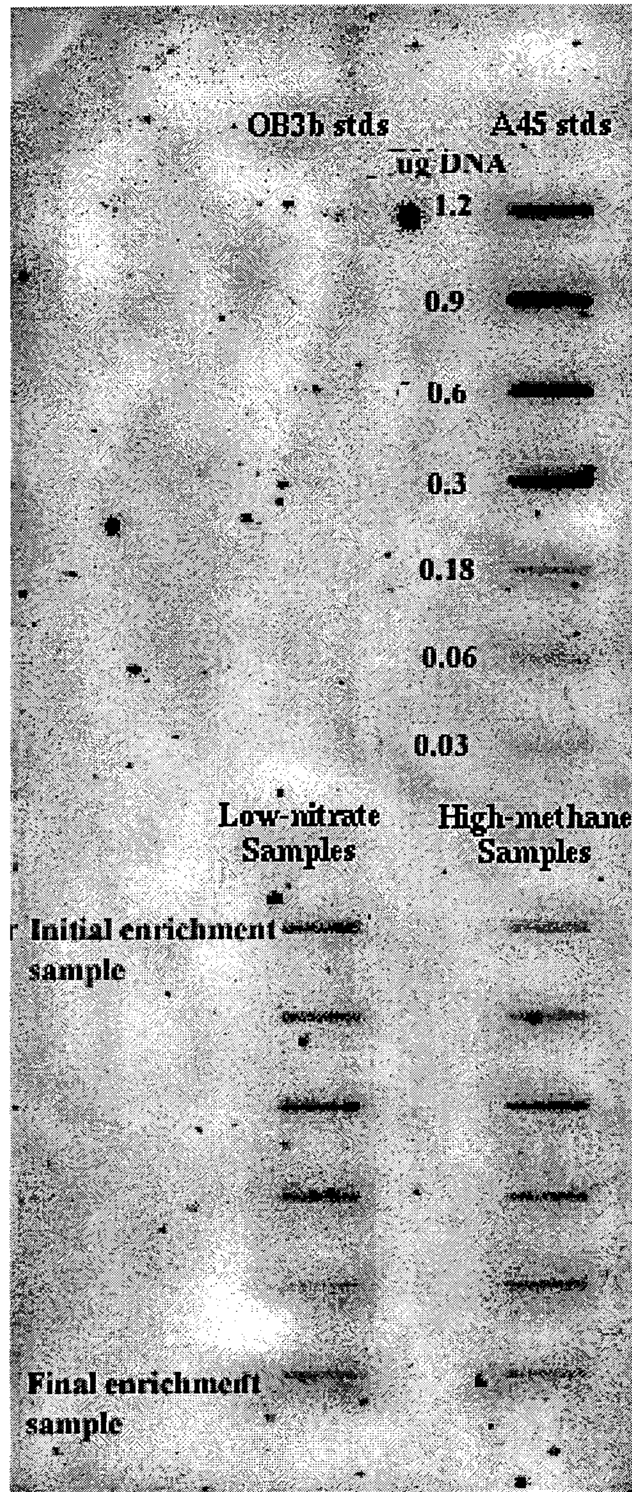


***Figure 6-1: Blots of control DNA and sample DNA hybridized to probe 1035-RuMP***

OB3bstds = dilution series of DNA from M. trichosporium OB3b
A45stds = dilution series of DNA from M. marinus A45
Low-nitrate samples = chronolgical series of DNA from low-nitrate enriched sediment
High-methanesamples = chronolgical series of DNA from high-methane enriched sediment

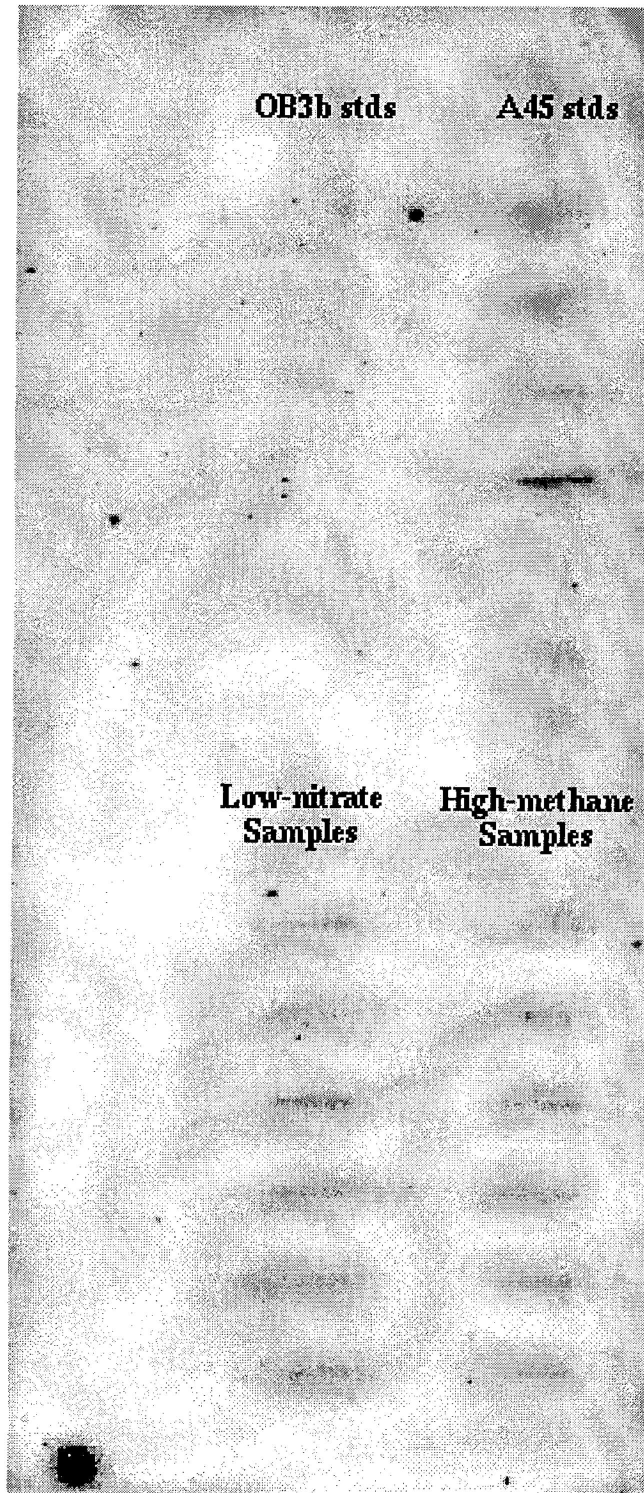


***Figure 6-2: Blots of control DNA and sample DNA hybridized to probe 1034-Ser***

OB3bstds = dilution series of DNA from M. trichosporium OB3b
A45stds = dilution series of DNA from M. marinus A45
Low-nitrate samples = chronolgical series of DNA from low-nitrate enriched sediment
High-methane samples = chronolgical series of DNA from high-methane enriched sediment

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# 7. Conclusions

## 7.1 Summary

Methanotrophs are an attractive class of bacteria for *in-situ* bioremediation of TCE. However, all previous studies of TCE utilization by methanotrophs have focused on strains that will not grow in the presence of elevated NaCl. Since it is known that TCE contamination exists in marine and coastal environments, it is of interest to obtain information on biodegradation of TCE by marine methanotrophs. This study presents research on the use of marine methanotrophs for cleanup in these systems. In order to obtain mechanistic information on this process, this study has compared the kinetics of methane oxidation by estuarine methanotrophs in fresh sediment samples, in enriched sediment, and in pure cultures of estuarine methanotrophic isolates. In addition, the kinetics of TCE oxidation were examined both in enriched sediment and in pure cultures. To complement the kinetic studies, gene probing methods were used to analyze the *in-situ* methanotrophs to obtain population data. In addition, an electrophoretic method for extraction of DNA from sediment samples was used to collect bacterial DNA for gene probing, and gene probing was used to identify the presence of methanotrophs in enriched sediment. Gene probing was also used to estimate methanotroph population numbers based on comparison of probe hybridization to dilution series of control DNA. This study has been the first direct comparison of methanotrophic activity between the laboratory and the field and has provided information on the use of methanotrophs for *in-situ* bioremediation of TCE in estuarine or marine environments.

## 7.2 Kinetics experiments

The kinetics of methane oxidation were measured in fresh and estuary sediment. These measurements suggest that the methanotrophs in the fresh estuary sediment have a methane monooxygenase with a similar substrate affinity (indicated by the $K_s$ values) to methanotrophs in other sediments, but that low numbers of organisms appear to be

present relative to the other environments studied. This information may indicate that substantial enrichment of the population will be necessary to achieve significant rates of TCE degradation for *in-situ* bioremediation. The $K_s$ values also suggest that the estuarine population contains an MMO similar to the pMMO found in laboratory cultures. These values also indicate that this pMMO is copper-sufficient.

Methane oxidation was also measured in enriched estuary sediment. The uptake velocity at saturation ($V_{max}$) was found to have increased approximately 10-fold in the enriched sediment, indicating an approximately 10-fold increase in the number of methanotrophs in the sediment. The concentrations at half-saturation ($K_s$) in the enrichments increased also, indicating a reduced affinity for methane by the enriched population.

Several pure cultures of methanotrophs were isolated from enriched estuary sediment and from sediment in a nearby harbor. All of these isolates were found to depend on added salt in the growth medium and to grow with relatively fast doubling times of approximately 4 hours. All of the isolates appear to be Type I methanotrophs based on gene probing of colony blots. This is consistent with previous reports in the literature in which only Type I and no Type II methanotrophs were isolated from the marine environment.

One of these isolates, BB5.1, was used to obtain kinetic parameters for comparison to the data from fresh and enriched sediment (see Tables 7-1 and 7-2 for a summary of these data). The concentration of copper used for growth was found to affect the kinetics of methane and TCE oxidation by this isolate. When high copper was supplied to the growth medium, $K_s$ values for methane averaged about 10μM. However, when the isolate was grown with no added copper, results were variable, with a high $K_s$ found in two cases and a $K_s$ of 16 μM found in a third case. Previously-obtained data from the Lidstrom laboratory also contain conflicting results on the effect of copper on methane oxidation by pure cultures of freshwater methanotrophs, in some cases showing a strong

effect and in others only a small effect (Semrau, 1995; Udell, 1996). It now seems likely that these variable results are due to variations in available copper concentration in the growth medium used for the kinetics experiments, probably due to low levels of copper contamination in the other components of the medium. Other research (Berson and Lidstrom, 1996) has shown that small changes in the available copper level cause dramatic effects on the behavior of methanotrophs. Therefore copper limitation of the pMMO may occur in some cases but not in others. In cases where copper limitation of the pMMO appears to have occurred, it is characterized by an elevated $K_s$ for methane oxidation and a lack of TCE degradation.

### 7.3 Results and copper effect

These results can be applied to the data obtained for both fresh sediment and enriched sediment (Table 7-1). The methanotrophs in fresh sediment are probably not copper-limited, since the $K_s$ values obtained generally are in the range of the copper-sufficient pMMO values. However, it appears that enriched populations of methanotrophs in estuary sediment may have become limited for copper, since two of the enrichments exhibited no TCE degradation. Consistent with this result is the finding that these enrichments had $K_s$ values that were around 50μM. As noted above, cultures containing copper-limited pMMO are characterized by high $K_s$ values for methane and no detectable TCE degradation.

In the enrichment in which TCE degradation was observed, the $K_s$ for TCE appeared to be elevated relative to that found in pure cultures, although no $V_{max}$ could be determined due to toxicity of the TCE above concentrations of 50μM. This degradation did not appear to be caused by the sMMO. Since no sMMO-producing organisms have been isolated from or detected in the marine environment, significant enrichment of marine or estuarine methanotrophic populations may actually interfere with TCE degradation by methanotrophs in these environments, due to copper limitation of the pMMO

and its subsequent effect on TCE degradation.

### 7.4 Gene probing experiments

The gene probing data generally support the conclusions from the kinetics experiments regarding the methanotrophs present, since the data indicate substantial numbers of Type I methanotrophs were present in the enrichments. Type I methanotrophs usually contain on the pMMO, and the kinetics data showed that no sMMO expression occurred in the enrichments. Further research will be necessary to optimize the use of gene probing to estimate methanotroph populations more precisely, but the results presented here suggest this will be a valuable tool for future experiments.

### 7.5 Implications and significant new findings

The results from this study show that simply increasing the population of methanotrophs in a marine or estuarine environment does not necessarily result in enhanced rates of TCE degradation. This is in direct opposition to the usual suggestions for the use of these bacteria for *in-situ* bioremediation, in which the rate of TCE breakdown is assumed to be directly proportional to the number of methanotrophs present. This study indicates that the use of methanotrophs for in situ bioremediation of TCE in estuarine sediments will require the addition of nutrients in addition to methane and oxygen, most likely bioavailable copper and/or a source of reducing equivalents. This project now suggests a new set of experiments that should be carried out for optimizing enhanced bioremediation of TCE in marine sediments.

### 7.6 Further research

In summary, this research has been the first direct comparison of methanotrophic activity between the laboratory and the field, and has provided important background information for the use of marine methanotrophs for bioremediation of TCE. It has

provided further evidence of the effect of copper on the oxidation of both methane and TCE. It has pointed out the limitations involved in simply enriching the bacterial population in an environment for *in-situ* bioremediation; that is, nutrient limitation may result in depressed, rather than enhanced, rates of biodegradation. It has provided the initial work on the use of gene probing with marine methanotrophic populations to discover the types of methanotrophs present and to estimate their numbers in estuarine sediment.

Future research on these areas should involve determination of the available copper threshhold which produces the kinetic effects observed on methane oxidation and TCE degradation, determination of an available copper complex which could be supplied to the bacteria *in-situ*, and further optimization of the use of gene probing to determine whether Type II methanotrophs or the genes encoding production of the sMMO are present in the marine or estuarine environments. The assumptions surrounding the use of gene probing for estimation of bacterial numbers should also be examined and this aspect of the gene probing techniques should be further optimized.

*Table 7-1: Summary of kinetics experiments on sediment*

| Methanotrophs | Substrate | Copper condition | $K_s$ (µM) | $V_{max}$ (µM/hr) | Implied copper status |
|---|---|---|---|---|---|
| Fresh sediment | methane | ND[a] | 8-26 | 0.1-2.3 | sufficient |
| **Enriched sediment:** | | | | | |
| Low methane | methane | ND[a] | 48.3 | 5.9 | limiting |
| | TCE | ND[a] | >50 | unknown | sufficient? |
| Low nitrate | methane | ND[a] | 52.8 | 8.6 | limiting |
| | TCE | ND[a] | NA[b] | NA[b] | limiting |
| High methane | methane | ND[a] | unknown | unknown | unknown |
| | TCE | ND[a] | NA[b] | NA[b] | limiting |

*Table 7-2: Summary of kinetics experiments on isolate BB5.1*

| Methanotrophs | Substrate | Copper condition | $K_s$ (µM) | $V_{max}$ (nmol*hr$^{-1}$*mg protein$^{-1}$) | Implied copper status |
|---|---|---|---|---|---|
| Isolate BB5.1 | methane | high | 13.1 | 6600 | sufficient |
| | methane | high | 9.7 | 5400 | sufficient |
| | methane | low | >50 | unknown | limiting? |
| | methane | low | 81.4 | 6100 | limiting |
| | methane | low | 16.4 | 1700 | sufficient? |
| | TCE | high | 8.1 | 6.1 | sufficient |
| | TCE | high | 10.4 | 9.0 | sufficient |
| | TCE | low | NA[b] | NA[b] | limiting |
| | TCE | low | NA[b] | NA[b] | limiting |

a) No Data
b) Not Applicable (no degradation observed)

# 8. Appendix A: Nonlinear regression results generated by TableCurve 2D (Jandel Scientific)

Algorithms listed in Chapter 2

Field expt #2 — [Form2Hyp_] y=ax/(b+x)

| | | Parameters | Values | Std Error | T Value | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| Eqn | | a | 0.995173 | 0.140714 | 7.072332 | 0.411724 | 1.578622 |
| Eqn # | 8108 | b | 8.921243 | 3.58022 | 2.491814 | -5.92363 | 23.76612 |
| r 2 | 0.933408 | | | | | | |
| DF Adj r2 | 0.800223 | | | | | | |
| Fit Std Err | 0.073977 | | | | | | |
| F-stat | 28.03347 | | | | | | |

| XY # | X Observed | Y Observed | Y Predicted | Y Residual | Y Residual% | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| 1 | 3.809374 | 0.273574 | 0.297785 | -0.02421 | -8.84978 | 0.092289 | 0.503281 |
| 2 | 7.618748 | 0.521026 | 0.458402 | 0.062623 | 12.01925 | 0.251023 | 0.665781 |
| 3 | 19.04687 | 0.605203 | 0.677734 | -0.07253 | -11.9844 | 0.502532 | 0.852936 |
| 4 | 38.09374 | 0.840638 | 0.806336 | 0.034302 | 4.08048 | 0.537559 | 1.075112 |

Field expt #4 — [Form2Hyp_] y=ax/(b+x)

| | | Parameters | Values | Std Error | T Value | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| Eqn | | a | 0.098936 | 0.011886 | 8.323669 | 0.065894 | 0.131977 |
| Eqn # | 8108 | b | 25.71349 | 7.502273 | 3.427426 | 4.858345 | 46.56863 |
| r 2 | 0.974343 | | | | | | |
| DF Adj r2 | 0.957239 | | | | | | |
| Fit Std Err | 0.004869 | | | | | | |
| F-stat | 151.904 | | | | | | |

| XY # | X Observed | Y Observed | Y Predicted | Y Residual | Y Residual% | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| 1 | 0.762076 | 0.006306 | 0.002848 | 0.003458 | 54.83825 | 0.001438 | 0.004258 |
| 2 | 3.809374 | 0.017201 | 0.012766 | 0.004436 | 25.78624 | 0.007441 | 0.01809 |
| 3 | 7.618748 | 0.015712 | 0.022614 | -0.0069 | -43.9224 | 0.014901 | 0.030327 |
| 4 | 19.04687 | 0.042727 | 0.0421 | 0.000627 | 1.467857 | 0.033609 | 0.050591 |
| 5 | 38.09374 | 0.062482 | 0.059066 | 0.003416 | 5.467367 | 0.051509 | 0.066622 |
| 6 | 76.18748 | 0.072097 | 0.07397 | -0.00187 | -2.59867 | 0.06183 | 0.086111 |

Field expt #7

| | | Parameters | [Form2Hyp_] y=ax/(b+x) Values | Std Error | T Value | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| Eqn | | a | 2.269541 | 0.182111 | 12.46237 | 1.763299 | 2.775782 |
| Eqn # | 8108 | b | 8.168582 | 2.564806 | 3.184873 | 1.038823 | 15.29834 |
| r 2 | 0.913694 | | | | | | |
| DF Adj r2 | 0.856157 | | | | | | |
| Fit Std Err | 0.180494 | | | | | | |
| F-stat | 42.3468 | | | | | | |

| XY # | X Observed | Y Observed | Y Predicted | Y Residual | Y Residual% | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| 1 | 3.616431 | 0.690239 | 0.696447 | -0.00621 | -0.89941 | 0.389605 | 1.003289 |
| 2 | 7.232862 | 1.256456 | 1.065827 | 0.190629 | 15.17197 | 0.738264 | 1.39339 |
| 3 | 18.08216 | 1.381699 | 1.563316 | -0.18162 | -13.1444 | 1.32105 | 1.805581 |
| 4 | 36.16431 | 1.67856 | 1.851365 | -0.1728 | -10.2948 | 1.61213 | 2.090601 |
| 5 | 54.24647 | 1.997366 | 1.972514 | 0.024852 | 1.244225 | 1.686991 | 2.258037 |
| 6 | 72.3392 | 2.213825 | 2.039266 | 0.17456 | 7.884983 | 1.71585 | 2.362682 |

Field Expt #8

| | | Parameters | [Form2Hyp_] y=ax/(b+x) Values | Std Error | T Value | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| Eqn | | a | 0.716078 | 0.059718 | 11.99099 | 0.550071 | 0.882085 |
| Eqn # | 8108 | b | 11.7795 | 3.381308 | 3.483711 | 2.379994 | 21.17901 |
| r 2 | 0.950008 | | | | | | |
| DF Adj r2 | 0.916679 | | | | | | |
| Fit Std Err | 0.047973 | | | | | | |
| F-stat | 76.01209 | | | | | | |

| XY # | X Observed | Y Observed | Y Predicted | Y Residual | Y Residual% | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| 1 | 3.616431 | 0.151538 | 0.168203 | -0.01667 | -10.9977 | 0.096478 | 0.239928 |
| 2 | 7.232862 | 0.249328 | 0.272417 | -0.02309 | -9.26041 | 0.186248 | 0.358587 |
| 3 | 18.08216 | 0.49076 | 0.433607 | 0.057153 | 11.64576 | 0.362768 | 0.504446 |
| 4 | 36.16431 | 0.49481 | 0.540142 | -0.04533 | -9.16148 | 0.477107 | 0.603177 |
| 5 | 54.24647 | 0.634843 | 0.588325 | 0.046518 | 7.327499 | 0.511697 | 0.664952 |
| 6 | 72.3392 | 0.585645 | 0.615803 | -0.03016 | -5.1494 | 0.525606 | 0.706 |

Isolate High Cu #1 [Form2Hyp_] y=ax/(b+x)

| | | Parameters | Values | Std Error | T Value | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| Eqn | | a | 6.586829 | 0.516182 | 12.76067 | 5.151924 | 8.021734 |
| Eqn # | 8108 | b | 13.09328 | 2.536134 | 5.162694 | 6.043229 | 20.14334 |
| r 2 | 0.989455 | | | | | | |
| DF Adj r2 | 0.982425 | | | | | | |
| Fit Std Err | 0.193769 | | | | | | |
| F-stat | 375.3242 | | | | | | |

| XY # | X Observed | Y Observed | Y Predicted | Y Residual | Y Residual% | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| 1 | 0.965031 | 0.349585 | 0.452152 | -0.10257 | -29.3396 | 0.315994 | 0.588309 |
| 2 | 4.825155 | 1.762428 | 1.77373 | -0.0113 | -0.64127 | 1.424064 | 2.123396 |
| 3 | 9.190772 | 2.712943 | 2.716653 | -0.00371 | -0.13673 | 2.371305 | 3.062 |
| 4 | 19.53039 | 3.835776 | 3.943251 | -0.10747 | -2.8019 | 3.681375 | 4.205127 |
| 5 | 24.12578 | 4.581313 | 4.26965 | 0.311663 | 6.802921 | 3.977837 | 4.561463 |
| 6 | 33.31655 | 4.55292 | 4.728533 | -0.17561 | -3.85715 | 4.320514 | 5.136552 |

Isolate High Cu expt. #2 [Form2Hyp_] y=ax/(b+x)

| | | Parameters | Values | Std Error | T Value | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| Eqn | | a | 5.362165 | 0.460941 | 11.63308 | 4.080821 | 6.64351 |
| Eqn # | 8108 | b | 9.659852 | 2.347544 | 4.114875 | 3.134047 | 16.18566 |
| r 2 | 0.978628 | | | | | | |
| DF Adj r2 | 0.96438 | | | | | | |
| Fit Std Err | 0.229139 | | | | | | |
| F-stat | 183.1629 | | | | | | |

| XY # | X Observed | Y Observed | Y Predicted | Y Residual | Y Residual% | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| 1 | 0.965031 | 0.506345 | 0.487032 | 0.019313 | 3.814169 | 0.29198 | 0.682084 |
| 2 | 4.825155 | 2.012686 | 1.786211 | 0.226475 | 11.25236 | 1.351877 | 2.220545 |
| 3 | 9.190772 | 2.373675 | 2.614367 | -0.24069 | -10.14 | 2.221881 | 3.006852 |
| 4 | 19.53039 | 3.415063 | 3.587678 | -0.17261 | -5.05451 | 3.279741 | 3.895615 |
| 5 | 24.12578 | 4.091598 | 3.829037 | 0.262562 | 6.41709 | 3.481069 | 4.177004 |
| 6 | 33.31655 | 4.115829 | 4.156906 | -0.04108 | -0.99803 | 3.693422 | 4.620389 |

Isolate Low Cu expt#1 [Form2Hyp_] y=ax/(b+x)

| | | Parameters | Values | Std Error | T Value | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| Eqn | | a | 23.88366 | 2.207874 | 10.81749 | 17.74611 | 30.0212 |
| Eqn # | 8108 | b | 130.4101 | 15.42969 | 8.451891 | 87.51797 | 173.3022 |
| r 2 | 0.999194 | | | | | | |
| DF Adj r2 | 0.998657 | | | | | | |
| Fit Std Err | 0.068471 | | | | | | |
| F-stat | 4960.352 | | | | | | |

| XY # | X Observed | Y Observed | Y Predicted | Y Residual | Y Residual% | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| 1 | 5.138012 | 0.829507 | 0.905321 | -0.07581 | -9.13968 | 0.84826 | 0.962382 |
| 2 | 9.99058 | 1.63823 | 1.699505 | -0.06127 | -3.7403 | 1.609952 | 1.789057 |
| 3 | 19.98116 | 3.254519 | 3.173211 | 0.081308 | 2.498305 | 3.063703 | 3.282719 |
| 4 | 29.97174 | 4.433717 | 4.463316 | -0.0296 | -0.66759 | 4.365624 | 4.561008 |
| 5 | 34.25342 | 5.004971 | 4.968295 | 0.036676 | 0.732791 | 4.872427 | 5.064162 |
| 6 | 49.9529 | 6.5943 | 6.61476 | -0.02046 | -0.31028 | 6.440244 | 6.789277 |

Isolate low Cu #2 [Form2Hyp_] y=ax/(b+x)

| | | Parameters | Values | Std Error | T Value | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| Eqn | | a | 6.118063 | 1.787752 | 3.422209 | 1.148392 | 11.08773 |
| Eqn # | 8108 | b | 81.40563 | 42.92045 | 1.896663 | -37.9065 | 200.7177 |
| r 2 | 0.927662 | | | | | | |
| DF Adj r2 | 0.879437 | | | | | | |
| Fit Std Err | 0.33384 | | | | | | |
| F-stat | 51.296 | | | | | | |

| XY # | X Observed | Y Observed | Y Predicted | Y Residual | Y Residual% | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| 1 | 9.99058 | 0.519814 | 0.668769 | -0.14896 | -28.6555 | 0.310688 | 1.026851 |
| 2 | 24.26284 | 1.160696 | 1.404786 | -0.24409 | -21.0297 | 0.88293 | 1.926641 |
| 3 | 34.25342 | 2.053249 | 1.811917 | 0.241332 | 11.75367 | 1.291603 | 2.33223 |
| 4 | 51.38012 | 2.673571 | 2.367324 | 0.306248 | 11.45462 | 1.909646 | 2.825001 |
| 5 | 79.92464 | 2.602779 | 3.03095 | -0.42817 | -16.4505 | 2.506548 | 3.555352 |
| 6 | 102.7602 | 3.583056 | 3.413736 | 0.16932 | 4.725584 | 2.66094 | 4.166532 |

| Isolate low Cu #3 | | | [Form2Hyp_] y=ax/(b+x) | | | | |
|---|---|---|---|---|---|---|---|
| | | Parameters | Values | Std Error | T Value | 95% Conf Limit | 95% Conf Limit |
| Eqn | | a | 1.729293 | 0.051938 | 33.29547 | 1.584914 | 1.873672 |
| Eqn # | 8108 | b | 16.4113 | 2.409032 | 6.812404 | 9.714569 | 23.10803 |
| r 2 | 0.979602 | | | | | | |
| DF Adj r2 | 0.966003 | | | | | | |
| Fit Std Err | 0.060687 | | | | | | |
| F-stat | 192.0932 | | | | | | |

| XY # | X Observed | Y Observed | Y Predicted | Y Residual | Y Residual% | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| 1 | 9.325437 | 0.615844 | 0.626591 | -0.01075 | -1.74516 | 0.500843 | 0.75234 |
| 2 | 31.97293 | 1.147086 | 1.14274 | 0.004346 | 0.378881 | 1.041628 | 1.243851 |
| 3 | 69.27467 | 1.347546 | 1.398085 | -0.05054 | -3.75044 | 1.324818 | 1.471351 |
| 4 | 95.91878 | 1.562743 | 1.476645 | 0.086097 | 5.509356 | 1.40073 | 1.552561 |
| 5 | 170.5223 | 1.606121 | 1.577475 | 0.028646 | 1.783581 | 1.483913 | 1.671037 |
| 6 | 239.797 | 1.556802 | 1.618524 | -0.06172 | -3.96466 | 1.513654 | 1.723394 |

| Isolate BB5.1 TCE | | | [Form2Hyp_] y=ax/(b+x) | | | | |
|---|---|---|---|---|---|---|---|
| High Copper | | Parameters | Values | Std Error | T Value | 95% Conf Limit | 95% Conf Limit |
| Eqn | | a | 8.964145 | 2.853673 | 3.141265 | -0.05015 | 17.97844 |
| Eqn # | 8108 | b | 10.41443 | 6.431961 | 1.619168 | -9.9031 | 30.73195 |
| r 2 | 0.868373 | | | | | | |
| DF Adj r2 | 0.736746 | | | | | | |
| Fit Std Err | 0.692356 | | | | | | |
| F-stat | 19.79164 | | | | | | |

| XY # | X Observed | Y Observed | Y Predicted | Y Residual | Y Residual% | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| 1 | 2.82093 | 1.584676 | 1.910581 | -0.32591 | -20.566 | 0.73462 | 3.086543 |
| 2 | 4.885715 | 2.577375 | 2.862474 | -0.2851 | -11.0616 | 1.617772 | 4.107177 |
| 3 | 6.811984 | 3.389502 | 3.544767 | -0.15527 | -4.58076 | 2.391339 | 4.698195 |
| 4 | 9.838901 | 5.373958 | 4.354708 | 1.01925 | 18.96646 | 3.249999 | 5.459418 |
| 5 | 17.79895 | 5.222079 | 5.655203 | -0.43312 | -8.29407 | 3.634478 | 7.675928 |

| Isolate BB5.1 TCE | | | [Form2Hyp_] y=ax/(b+x) | | | | |
|---|---|---|---|---|---|---|---|
| High Copper | | Parameters | Values | Std Error | T Value | 95% Conf Limit | 95% Conf Limit |
| Eqn | | a | 6.057251 | 3.875378 | 1.563009 | -6.18444 | 18.29894 |
| Eqn # | 8108 | b | 8.080778 | 10.58471 | 0.763439 | -25.3546 | 41.51617 |
| r 2 | 0.585426 | | | | | | |
| DF Adj r2 | 0.170852 | | | | | | |
| Fit Std Err | 0.954583 | | | | | | |
| F-stat | 4.23634 | | | | | | |

| XY # | X Observed | Y Observed | Y Predicted | Y Residual | Y Residual% | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| 1 | 2.837007 | 1.138783 | 1.573988 | -0.43521 | -38.2167 | -0.30439 | 3.452362 |
| 2 | 4.744391 | 2.260884 | 2.240748 | 0.020136 | 0.890623 | 0.458788 | 4.022707 |
| 3 | 6.846164 | 4.335766 | 2.778127 | 1.55764 | 35.92536 | 1.266021 | 4.290232 |
| 4 | 9.696133 | 3.58389 | 3.303831 | 0.280059 | 7.814389 | 1.725741 | 4.881921 |
| 5 | 14.34893 | 3.677006 | 3.874998 | -0.19799 | -5.38459 | 1.285705 | 6.464291 |

| Low CH4 enrichment kinetics data | | | [Form2Hyp_] y=ax/(b+x) | | | | |
|---|---|---|---|---|---|---|---|
| | | Parameters | Values | Std Error | T Value | 95% Conf Limit | 95% Conf Limit |
| Eqn | | a | 5.926805 | 0.679953 | 8.716489 | 4.427131 | 7.426478 |
| Eqn # | 8108 | b | 48.34173 | 11.70077 | 4.131501 | 22.53506 | 74.14841 |
| r 2 | 0.946182 | | | | | | |
| DF Adj r2 | 0.935419 | | | | | | |
| Fit Std Err | 0.280941 | | | | | | |
| F-stat | 193.3937 | | | | | | |

| XY # | $[CH_4]aq$, $u$ M | v, measured | v, predicted | Y Residual | Y Residual% | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| 1 | 8.070192 | 0.585651 | 0.847878 | -0.26223 | -44.7753 | 0.660265 | 1.035492 |
| 2 | 8.433152 | 0.807083 | 0.880348 | -0.07326 | -9.07773 | 0.688018 | 1.072678 |
| 3 | 20.42934 | 1.613012 | 1.760634 | -0.14762 | -9.152 | 1.5066 | 2.014669 |
| 4 | 20.48571 | 1.614177 | 1.764046 | -0.14987 | -9.2846 | 1.510008 | 2.018084 |
| 5 | 21.00093 | 1.713856 | 1.794976 | -0.08112 | -4.7332 | 1.54098 | 2.048972 |
| 6 | 40.28107 | 2.975941 | 2.693867 | 0.282074 | 9.478489 | 2.484864 | 2.90287 |
| 7 | 41.3373 | 3.201608 | 2.731944 | 0.469664 | 14.66964 | 2.525404 | 2.938484 |
| 8 | 41.91325 | 2.995972 | 2.752332 | 0.243641 | 8.132271 | 2.547053 | 2.95761 |
| 9 | 62.02768 | 3.885661 | 3.330868 | 0.554793 | 14.27796 | 3.11934 | 3.542396 |
| 10 | 62.21384 | 3.294656 | 3.335239 | -0.04058 | -1.2318 | 3.123194 | 3.547284 |
| 11 | 84.30157 | 3.627529 | 3.766786 | -0.13926 | -3.83889 | 3.463847 | 4.069725 |
| 12 | 84.50562 | 3.751774 | 3.770104 | -0.01833 | -0.48857 | 3.466187 | 4.07402 |
| 13 | 86.99475 | 3.576188 | 3.80977 | -0.23358 | -6.5316 | 3.493878 | 4.125662 |

NO3- free enrichment kinetics data [Form2Hyp_] y=ax/(b+x)

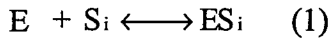
| | | Parameters | Values | Std Error | T Value | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| Eqn | | a | 8.644901 | 1.321255 | 6.542944 | 5.838211 | 11.45159 |
| Eqn # | 8108 | b | 52.76959 | 16.54448 | 3.189559 | 17.62481 | 87.91437 |
| r 2 | 0.929622 | | | | | | |
| DF Adj r2 | 0.920238 | | | | | | |
| Fit Std Err | 0.552683 | | | | | | |
| F-stat | 211.344 | | | | | | |

| XY # | $[CH_4]$aq, *u* M | v, measured | v, predicted | Y Residual | Y Residual% | 95% Conf Limit | 95% Conf Limit |
|---|---|---|---|---|---|---|---|
| 1 | 4.13031 | 0.421579 | 0.627525 | -0.20595 | -48.8512 | 0.431595 | 0.823456 |
| 2 | 4.244644 | 0.670123 | 0.643603 | 0.02652 | 3.957468 | 0.443425 | 0.843781 |
| 3 | 4.598954 | 0.740739 | 0.693019 | 0.04772 | 6.442243 | 0.480025 | 0.906013 |
| 4 | 8.682632 | 1.072963 | 1.221445 | -0.14848 | -13.8385 | 0.893914 | 1.548976 |
| 5 | 8.856452 | 1.366803 | 1.242383 | 0.12442 | 9.102994 | 0.911155 | 1.573611 |
| 6 | 9.260215 | 1.403832 | 1.290567 | 0.113265 | 8.068254 | 0.951073 | 1.630061 |
| 7 | 22.75344 | 2.680338 | 2.60452 | 0.075818 | 2.828691 | 2.164346 | 3.044693 |
| 8 | 22.96988 | 2.693415 | 2.621782 | 0.071633 | 2.659576 | 2.181785 | 3.061779 |
| 9 | 23.097 | 1.847722 | 2.631874 | -0.78415 | -42.4389 | 2.191996 | 3.071751 |
| 10 | 42.81364 | 3.996995 | 3.872224 | 0.124771 | 3.121615 | 3.509393 | 4.235055 |
| 11 | 43.86408 | 3.719289 | 3.924105 | -0.20482 | -5.50684 | 3.564956 | 4.283254 |
| 12 | 44.23358 | 4.122123 | 3.942087 | 0.180036 | 4.367566 | 3.584153 | 4.300021 |
| 13 | 64.30089 | 4.358991 | 4.748207 | -0.38922 | -8.92903 | 4.372662 | 5.123751 |
| 14 | 65.61014 | 4.455067 | 4.791303 | -0.33624 | -7.54728 | 4.409006 | 5.1736 |
| 15 | 66.79395 | 5.45826 | 4.829458 | 0.628802 | 11.5202 | 4.440604 | 5.218312 |
| 16 | 86.7536 | 7.209817 | 5.37528 | 1.834537 | 25.44498 | 4.83661 | 5.913951 |
| 17 | 87.46655 | 5.258577 | 5.391903 | -0.13333 | -2.53539 | 4.847176 | 5.93663 |
| 18 | 88.4361 | 5.5949 | 5.414239 | 0.180661 | 3.229024 | 4.861249 | 5.967229 |

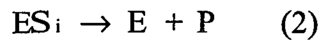
# 9. Appendix B: The Michaelis-Menten Model

**Michaelis-Menten model of enzyme kinetics**

The consumption of a given substrate in batch culture can be modeled by the following equations:



$$E + S_i \longleftrightarrow ES_i \qquad (1)$$



$$ES_i \rightarrow E + P \qquad (2)$$

where: E = enzymic catalyst; in this case whole methanotroph cells containing MMO
$S_i$ = substrate i ; methane or TCE
$ES_i$ = substrate-catalyst complex

Strictly, the $ES_i$ complex is the enzyme molecule with bound substrate; however since we do not yet have the purified MMO available to us, E must be taken to refer to the whole cell, including the MMO to which the substrate is bound. By assuming the $ES_i$ complex to be at steady-state, these equations can be solved to obtain the Michaelis-Menten equation for disappearance of the substrate $S_i$:

$$\frac{-dS_i}{dt} = V_i = \frac{V_{max_i} \times S_i}{K_{S_i} + S_i} \qquad (3)$$

where: $K_{Si}$ = ith substrate concentration at half the maximum uptake rate
$V_{maxi}$ = maximum uptake rate of ith substrate

This equation can be used to describe the ability of the methanotrophs to oxidize either methane or TCE.

# 10. Appendix C: Calculations of the Danckwerts criterion

Danckwerts criterion for fresh sediment methane oxidation experiment

| | | | |
|---|---|---|---|
| Liquid volume: | 0.0055 L | Surface area= | 0.001456 m2 |
| Film coeff(kL): | 1.10E-04 m/s | Specific SA (a')= | 264.7273 m-1 |
| | | kLa'= | 104.832 hr-1 |

| #2 | 1-Mar-94 | |
|---|---|---|
| CH4 conc, | v (umolesCH4 | Ra'/(A*kLa') |
| 3.809374 | 0.2735742 | 0.0006851 |
| 7.618748 | 0.52102577 | 0.0006524 |
| 19.04687 | 0.60520346 | 0.0003031 |
| 38.09374 | 0.84063772 | 0.0002105 |

Danckwerts criterion for fresh sediment methane oxidation experiment

| | | | |
|---|---|---|---|
| Liquid volume: | 0.0055 L | Surface area= | 0.001456 m2 |
| Film coeff(kL): | 1.10E-04 m/s | Specific SA (a')= | 264.7273 m-1 |
| | | kLa'= | 104.832 hr-1 |

| #4 | 13-Apr-94 | |
|---|---|---|
| CH4 conc, | v (umolesCH4 | Ra'/(A*kLa') |
| 0.762076 | 0.00630573 | 7.893E-05 |
| 3.809374 | 0.01720139 | 4.307E-05 |
| 7.618748 | 0.01571244 | 1.967E-05 |
| 19.04687 | 0.04272725 | 2.14E-05 |
| 38.09374 | 0.06248199 | 1.565E-05 |
| 76.18748 | 0.07209687 | 9.027E-06 |

Danckwerts criterion for fresh sediment methane oxidation experiment

| Liquid volume: | 0.0055 | L | Surface area= | 0.001456 | m2 |
|---|---|---|---|---|---|
| Film coeff(kL): | 1.10E-04 | m/s | Specific SA (a')= | 264.7273 | m-1 |
| | | | kLa'= | 104.832 | hr-1 |

#7 28-Nov-94

| CH4 conc, v | (umolesCH4 | Ra'/(A*kLa') |
|---|---|---|
| 3.616431 | 0.69023895 | 0.0018206 |
| 7.232862 | 1.25645606 | 0.0016571 |
| 18.08216 | 1.38169933 | 0.0007289 |
| 36.16431 | 1.67856042 | 0.0004428 |
| 54.24647 | 1.99736577 | 0.0003512 |
| 72.3392 | 2.21382543 | 0.0002919 |

Danckwerts criterion for fresh sediment methane oxidation experiment

| Liquid volume: | 0.0055 | L | Surface area= | 0.001456 | m2 |
|---|---|---|---|---|---|
| Film coeff(kL): | 1.10E-04 | m/s | Specific SA (a')= | 264.7273 | m-1 |
| | | | kLa'= | 104.832 | hr-1 |

#8 7-Apr-95

| CH4 conc, v | (umolesCH4 | Ra'/(A*kLa') |
|---|---|---|
| 3.616431 | 0.15153762 | 0.0003997 |
| 7.232862 | 0.24932835 | 0.0003288 |
| 18.08216 | 0.4907602 | 0.0002589 |
| 36.16431 | 0.49481018 | 0.0001305 |
| 54.24647 | 0.6348428 | 0.0001116 |
| 72.3392 | 0.58564541 | 7.723E-05 |

Danckwerts criterion for Low-methane enrichment methane oxidation experiment

| | | | | |
|---|---|---|---|---|
| Liquid volume: | 0.002 L | | Surface area= | 0.000774 m2 |
| Film coeff(kL): | 1.10E-04 m/s | | Specific SA (a')= | 387 m-1 |
| | | | kLa'= | 153.252 hr-1 |

| A* Co(uM) | Ra' v(uM/hr) | Ra'/(A*kLa') |
|---|---|---|
| 8.070192 | 0.58565125 | 0.0006922 |
| 8.433152 | 0.80708298 | 0.0009129 |
| 20.48571 | 1.61417671 | 0.0007516 |
| 21.00093 | 1.71385553 | 0.0007785 |
| 20.42934 | 1.6130116 | 0.0007532 |
| 40.28107 | 2.9759412 | 0.0007047 |
| 41.91325 | 2.99597213 | 0.0006819 |
| 41.3373 | 3.20160815 | 0.0007388 |
| 62.02768 | 3.88566105 | 0.0005976 |
| 62.21384 | 3.29465553 | 0.0005052 |
| 84.50562 | 3.75177352 | 0.0004235 |
| 86.99475 | 3.57618775 | 0.0003921 |
| 84.30157 | 3.62752921 | 0.0004105 |

Danckwerts criterion for Low-nitrate enrichment methane oxidation experiment

| | | | |
|---|---|---|---|
| Liquid volume: | 0.002 L | Surface area= | 0.000774 m2 |
| Film coeff(kL): | 1.10E-04 m/s | Specific SA (a')= | 387 m-1 |
| | | kLa'= | 153.252 hr-1 |

| Co (uM) | v (uM/hr) | Ra'/(A*kLa') |
|---|---|---|
| 4.13031 | 0.42157879 | 0.0009736 |
| 4.244644 | 0.67012285 | 0.001506 |
| 4.598954 | 0.74073938 | 0.0015364 |
| 8.682632 | 1.07296251 | 0.0011788 |
| 9.260215 | 1.40383204 | 0.0014461 |
| 8.856452 | 1.36680308 | 0.0014722 |
| 22.96988 | 2.69341503 | 0.0011185 |
| 22.75344 | 2.68033832 | 0.0011237 |
| 23.097 | 1.84772176 | 0.0007631 |
| 42.81364 | 3.99699462 | 0.0008905 |
| 43.86408 | 3.71928946 | 0.0008088 |
| 44.23358 | 4.12212345 | 0.0008889 |
| 64.30089 | 4.35899116 | 0.0006467 |
| 66.79395 | 5.45826017 | 0.0007795 |
| 65.61014 | 4.4550666 | 0.0006477 |
| 86.7536 | 7.20981719 | 0.0007928 |
| 88.4361 | 5.59489952 | 0.0006035 |
| 87.46655 | 5.25857734 | 0.0005735 |

# 11. Appendix D: Nucleic acid extraction from sediment samples

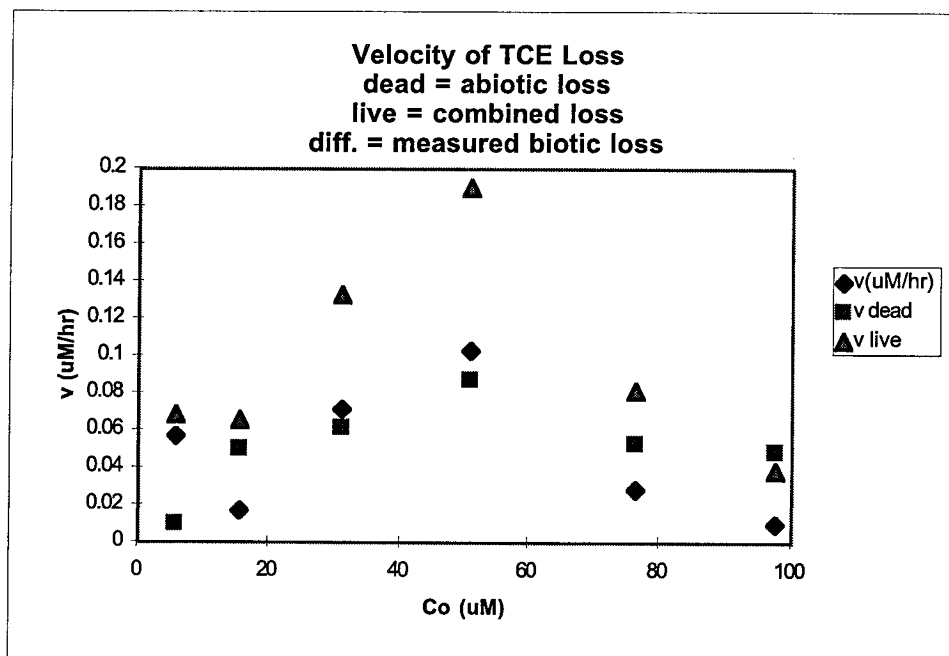
Nucleic acid extraction methods consist of two basic types: removal of the bacteria from the sample matrix, lysis, and purification of nucleic acids; and lysis of the bacteria in-situ followed by extraction and purification of the nucleic acids. This study has used the latter method, due to indications in the literature that it resulted in better DNA recovery (Tebbe and Vahjen, 1993. Appl. Environ. Microbiol. 59(8):2657-2665). Crude preparations of nucleic acids from natural samples are usually contaminated with humic acids and other humic substances which coextract from the soil matrix with the nucleic acids. Most methods in the literature for removing the humic substances, such as the use of hydroxyapatite columns or cesium chloride-ethidium bromide density centrifugation are time-consuming and limit the number of samples which can be processed. They may also result in the loss of significant amounts of DNA and reduced rates of recovery. Since this study required processing a large number of samples, and efficient recovery was desirable (methanotrophs are a relatively small fraction of the total bacterial population), a less well-known method for DNA extraction was used. Rochelle and Olson (BioTechniques 11(6):724:728; 1991) described the use of electrophoresis in agarose gels to separate nucleic acids from a sediment matrix after lysis of the cells *in-situ*. This method was further developed for this study. It has the further advantage that the lysis is carried out by lysozyme and detergent treatments, rather than freeze-thaw cycles or bead-mill homogenization. Both of these methods, when tried, did not result in the recovery of any detectable DNA, probably due to shearing.

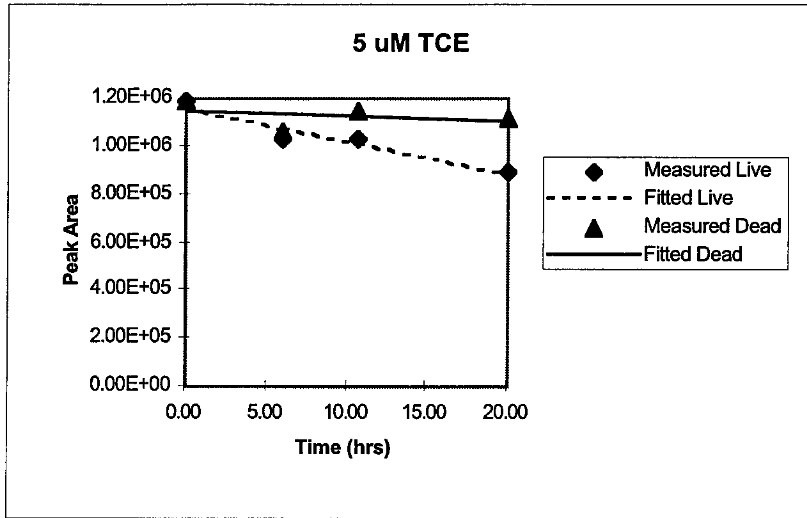
The method described by Rochelle and Olsen was modified in relatively minor respects. In their protocol, the environmental samples were immobilized in agarose by mixing the sediment and agarose in a disposable cuvette. In this study, disposable plastic 1-ml syringes were used, both for their greater ease of handling, and because they are cheaper. In this study, the commercial detergent preparation in the second lysis step was

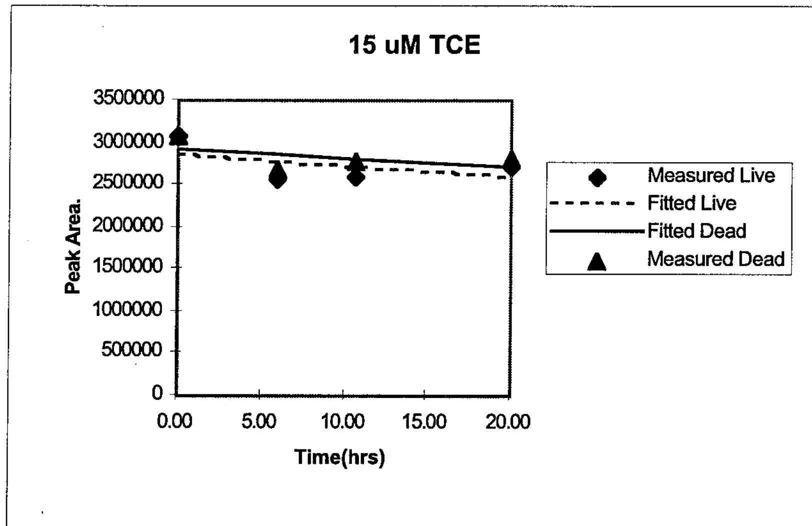
Triton X-100, whereas Rochelle and Olson used Brij 58. The Triton detergent was found to have a similar composition and was on hand in the laboratory when this study was begun, so it was used instead. Finally, several protocols were tried to scale-up the method so that a full gram of sediment could be processed at once. It was found that: a) the largest fraction of sediment that could be immobilized successfully with agarose was 50% by volume; b) DNA is extracted most successfully during electrophoresis if slices of immobilized sediment are placed in the wells of the gel with their flat sides perpendicular to the current; c) it is easiest to load the gel with the slices if the wells are slightly wider than normal (accomplished by taping the teeth of the comb used for casting the gel) and if the gel is not covered with buffer while loading; d) a full gram of sediment can be extracted in one large (200 ml ) gel if three slices of sediment are loaded vertically into each well. Processing a full gram of sediment produced enough DNA for detection of the methanotrophs with an oligonucleotide probe even from fresh (not enriched) sediment. Also, repeated freezing and thawing of the sediment samples seems to shear the humic substances so that more of them are present in the bands of DNA. No advantage was obtained by using low melting-point agarose either for immobilization or for the gels; it is simply too difficult to handle. It was not possible to digest the DNA before extracting it from the sediment; humic substances are known to interfere with endonucleases. A pair of fine-point forceps or tweezers is an indispensible tool for handling the immobilized sediment and the slices of agarose with DNA in them. After electrophoresis, any method for purifying DNA from agarose can be used; if the humics have not completely separated from the DNA in the gel, another gel extraction can be run before purification. In general, this method can be used to obtain DNA pure enough for Southern blots in 2 days, and does not involve solvents or chromatography or overnight centrifugation. It has the potential to be useful on a routine basis for monitoring bacterial populations in sediments.

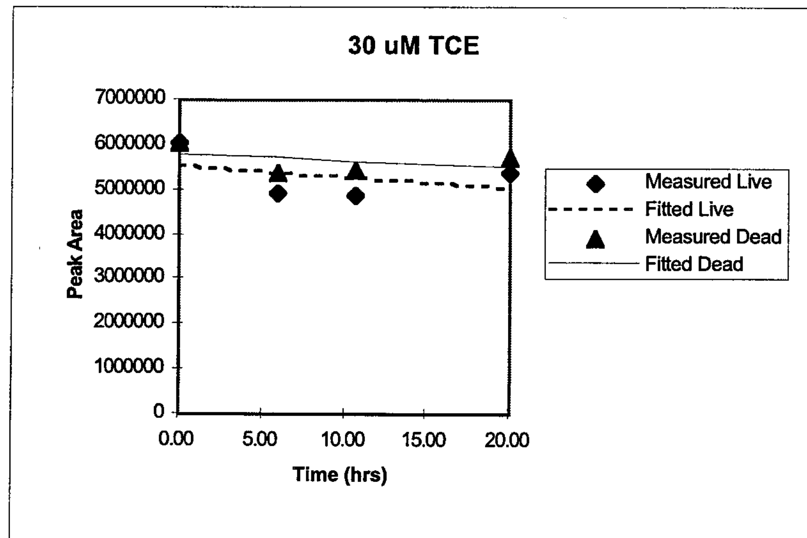
# 12. Appendix E: Additional Information Pertaining to Figure 5-3

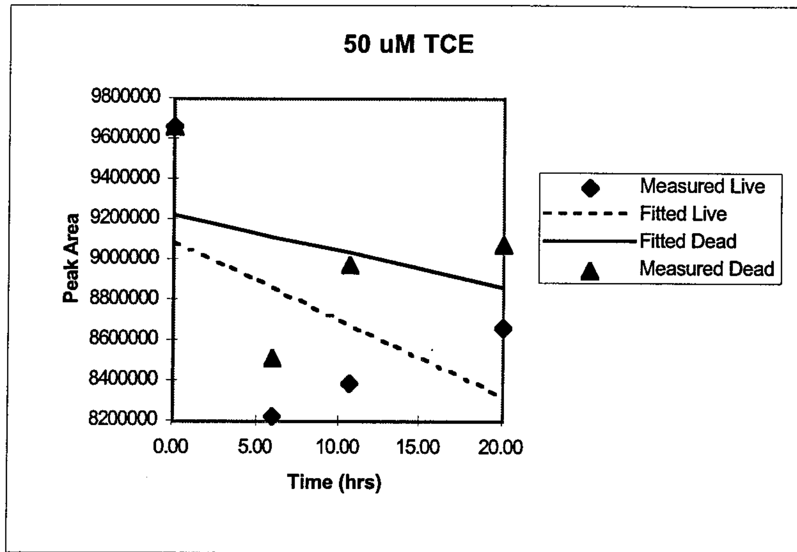
Figure 5-3 presents the velocities of bacterial oxidation of TCE in the sediment enrichment in which TCE oxidation was observed. This appendix contains supplementary information about that experiment. The chart titled Velocities of TCE Loss shows the velocities of TCE loss measured in vials containing killed enriched sediment (abiotic losses), live enriched sediment (combined losses) and the differences between these velocities, which are taken to be the rates of loss due to bacterial activity. These last values are the values which are shown in Figure 5-3. The additional charts show the time series at each aqueous TCE concentration, in which the peak areas are the raw data obtained from the GC analyses. These graphs show the great variability which is present when working with TCE and illustrate the difficulty inherent in detecting bacterial activity on this substrate. These data do not alter the conclusion which was drawn concerning Figure 5-3, which was that bacterial degradation occurred up to 50 μM dissolved TCE; at higher concentrations toxicity effects become apparent.

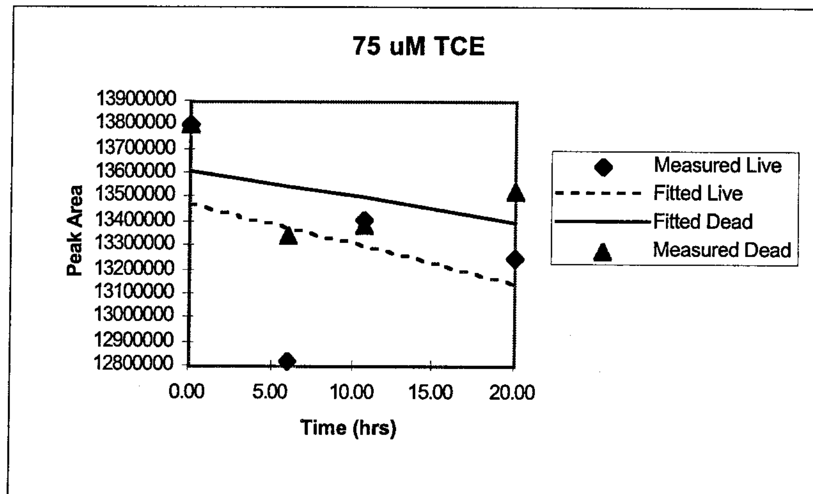


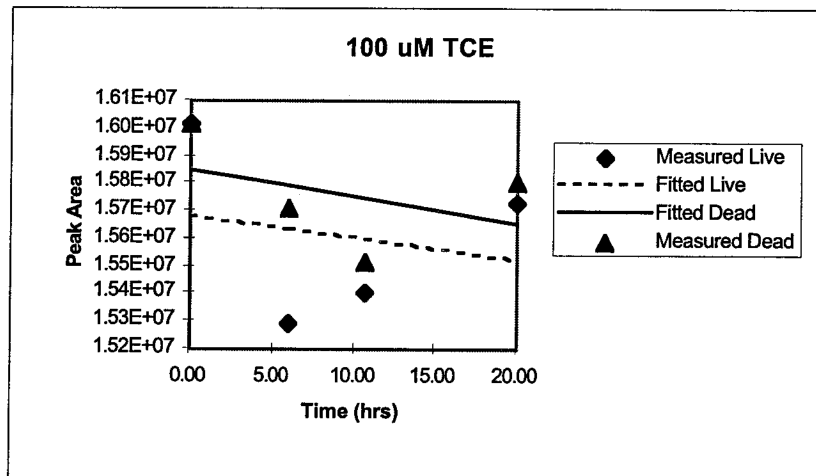

5 uM TCE
1.20E+06
1.00E+06
8.00E+05
6.00E+05
4.00E+05
2.00E+05
0.00E+00
Peak Area
0.00
5.00
10.00
15.00
20.00
Time (hrs)
Measured Live
Fitted Live
Measured Dead
Fitted Dead


15 uM TCE
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
Peak Area.
0.00
5.00
10.00
15.00
20.00
Time(hrs)
Measured Live
Fitted Live
Fitted Dead
Measured Dead


30 uM TCE
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
Peak Area
0.00
5.00
10.00
15.00
20.00
Time (hrs)
Measured Live
Fitted Live
Measured Dead
Fitted Dead


50 uM TCE
Peak Area
9800000
9600000
9400000
9200000
9000000
8800000
8600000
8400000
8200000
0.00
5.00
10.00
15.00
20.00
Time (hrs)
Measured Live
Fitted Live
Fitted Dead
Measured Dead


75 uM TCE
Peak Area
13900000
13800000
13700000
13600000
13500000
13400000
13300000
13200000
13100000
13000000
12900000
12800000
0.00
5.00
10.00
15.00
20.00
Time (hrs)
Measured Live
Fitted Live
Fitted Dead
Measured Dead


100 uM TCE
Peak Area
1.61E+07
1.60E+07
1.59E+07
1.58E+07
1.57E+07
1.56E+07
1.55E+07
1.54E+07
1.53E+07
1.52E+07
0.00
5.00
10.00
15.00
20.00
Time (hrs)
Measured Live
Fitted Live
Fitted Dead
Measured Dead