Monitoring: Evaluation of the Abundance, Diversity, and Activity of Methanotroph Populations in Groundwater
July 1998 - June 2000

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IV. Project Summary

Title: Monitoring: Evaluation of the Abundance, Diversity, and Activity of Methanotroph Populations in Groundwater

Project I.D.: UW-WRI #99-SAM-6

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Contract: July 1, 1998 through June 30, 2000

Background/Need: Groundwater monitoring

Objectives:

The objectives of this work are to develop methods that can be applied to the assessment of bioremediation by methanotrophs. These methods are for the detection of methanotrophs, evaluation of methanotroph activity, estimation of the size of the methanotroph population, assessment of population diversity in methanotrophic bacteria.

Methods:

Methods based on the polymerase chain reaction (PCR) were developed. Primer design and PCR conditions were optimized. Direct PCR (DPCR), in which environmental samples were used directly as a template without DNA extraction, was employed.

Results and Discussion:

Primers and PCR conditions were optimized to achieve specific amplification of the *pmoA*, which encodes a protein of the particulate methane monooxygenase. The presence of methanotrophs was detected by PCR amplification of *pmoA* using DNA extracted from groundwater as the template. The activity of methanotrophs in groundwater was indicated by detection of *pmoA* RNA by reverse transcriptase PCR. Methanotrophs were quantified in groundwater samples by DPCR in conjunction with Most Probably Number analysis or Competitive PCR. Methods to distinguish among methanotroph populations were evaluated.

Conclusions and Recommendations:

PCR-based methods for the detection of methanotrophs and methanotroph activity in groundwater have been developed. Two quantitative methods have been developed and applied to groundwater samples. Both of these used DPCR which is a rapid and simplified approach to sample preparation for PCR that allows detection and quantification of particular bacterial groups without isolation of DNA.

DPCR should be applicable to the detection of other bacteria in groundwater, including pathogens. This application of DPCR should be explored. DPCR should also be applicable for PCR-based methods for examination of methanotroph populations. This will require further investigation and empiric optimization.

Related Publications:

Cheng, Y. S., J. L. Halsey, K. A. Fode, C. C. Remsen and M. L. P. Collins. 1999. Detection of methanotrophs in groundwater by PCR. Appl. Environ. Microbiol. *65:* 648-651.

Drought, J. F., E. A. Buc, T. J. Grundl, K. A. Fode, M. L. P. Collins. 1999. Fate of tetrachloroethene and benzene at a dry cleaning facility. Proceedings of the 5th In Situ and On-site bioremediation symposium. Battelle Press, pp. 253-258.

Key words: bioremediation, TCE, MMO, methanotrophs, PCR

V. Introduction

Bioremediation is an emerging technology in the suite of approaches that can be applied to remediation of contaminated groundwater. Bioremediation can be used to effect the *in situ* degradation of persistent chemicals; this approach can be minimally intrusive and cost-effective. Among the most promising of organisms for use to remediate pollution by a variety of organic contaminants are the methanotrophic bacteria. Among the compounds that are degraded by methanotrophs is trichloroethylene (TCE) which is an Environmental Protection Agency-priority pollutant that contaminates the water supply of communities across the country including many in Wisconsin.

Prerequisites for the application of bioremediation are 1) an understanding of the biological process/es involved and 2) a means to monitor these processes. The importance of monitoring microbial activity in this process was emphasized in a National Research Council Report that stated: "The most elemental criterion for success of an *in situ* bioremediation effort is that microorganisms are mainly responsible for the clean-up. Without evidence for microbial involvement, there is no way to verify that the bioremediation project was actually a bioremediation" [National Research Council, 1993].

This research project developed methods using polymerase chain reaction (PCR) technology that can be applied to the assessment of bioremediation by methanotrophs. This assessment requires methods to detect methanotrophs, to evaluate methanotroph activity, to estimate the size of the methanotroph population, and to distinguish among populations of methanotrophic bacteria. Progress was made toward all of these goals.

VI. Procedures and Methods

Methods of bacterial culture, primer design, PCR, gel analysis, and site sampling are detailed in Cheng et al. [1999], Drought et al. [1999], and Fode-Vaughan et al. [2001].

VII. Results and Discussion

A. Detection of Methanotrophs

In methanotrophic bacteria, the first enzyme in the pathway of methane oxidation is methane monooxygenase (MMO). This enzyme can also co-metabolize a variety of substrates, including groundwater contaminants such as TCE. MMO exists in two distinct forms which are encoded by different sets of genes a soluble methane monooxygenase (sMMO) found in the cytoplasm and a particulate methane monooxygenase (pMMO) localized in the membrane fraction of the cell. The pMMO has been found in all known methanotrophic bacteria, with one possible exception [Dedysh 2000], while the sMMO is restricted to a subset of methanotrophs [Murrell, 2000]. Because pMMO is widespread in methanotrophs, we used a portion of a gene

encoding a protein of pMMO, *pmoA*, as a marker for the detection of these bacteria in environmental samples.

Primers (pmof1 and pmor) were designed for the PCR amplification of a fragment of *pmoA*. Primer design and PCR conditions were empirically optimized. Specificity of the primers for *pmoA* was tested by using DNA prepared from methanotrophs and other bacteria. The latter served as negative controls. These primers amplified a product of 330 bp from methanotrophic lab strains *Methylomicrobium album* BG8, *Methylococcus capsulatus* (Bath), and *Methylosinus trichosporium* OB3b as well as environmental methanotrophs (Fig. 1). No product was obtained with the negative controls *Escherichia coli* and *Nitrosomonas europaea*. Additionally, as shown in Fig. 1, these *pmoA* primers could be used in multiplex PCR for the simultaneous detection of sMMO and 16S rDNA. The latter, amplified with universal Bacterial 16S primers, encodes a ribosomal RNA which is common to all Bacteria and served as a control for the adequacy of the template for PCR.

The *pmoA* primers have proven quite useful and have been employed by other research teams [Wise et al., 1999; Carini et al., 2000]. While these primers were designed when sequence information was available from only 4 methanotroph species, they would hybridize to and should prime PCR amplification of *pmoA* from many other organisms. Sequences that match these primers are present in 86 (64 are perfect matches and 22 have 1 - 3 mismatches) authentic *pmoA* sequences deposited in Genbank by November 2000, including cultured as well as uncultured methanotrophs. This includes some organisms that are quite divergent on the basis of their 16S or *pmoA* sequences [Bodrossy, 1999; Henckel, 2000; Holmes, 1999]. This suggests that these primers should be useful in identifying uncultured methanotrophs in the environment.

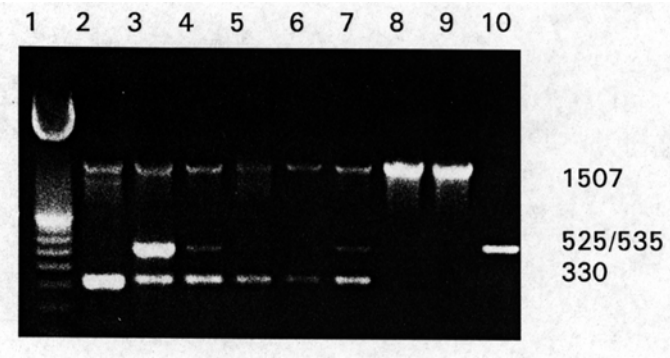

Fig. 1. Multiplex PCR. For lanes 2 - 9, the 16S, *mmoX*, and pmof1/r primer pairs were used. For lane 10 the amo/pmo primer pair was used. DNA was obtained from 2, *M. album* BG8; 3, *M. capsulatus* (Bath); 4, *M. trichosporium* OB3b; 5, groundwater enrichment culture; 6, isolate from sediment in Green Bay, Lake Michigan; 7, isolate from Yellowstone Lake, Yellowstone National Park; 8, E. coli; 9, 10, *N. europaea*. Lane 1, 100 bp ladder (Amersham Pharmacia). PCR products are identified by their size: 16S, 1507 bp; *mmoX*, 535 bp; *amo/pmo*, 525 bp; *pmo*A, 330 bp. From Cheng et al. 1999.

DNA was extracted from groundwater and used as a template for PCR with the *pmoA*-specific primers. These results (Fig. 2) indicate that this PCR method may be used to detect methanotrophs in groundwater. These results are described in detail in Cheng et al. [1999].

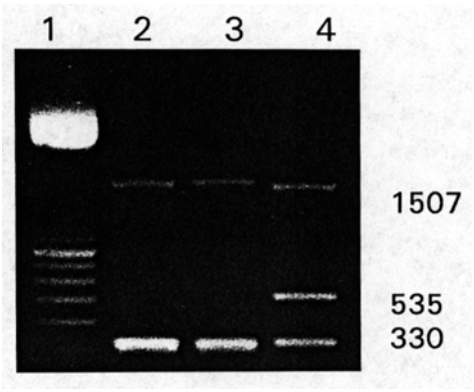

Fig. 2. Multiplex PCR analysis of groundwater. Primer pairs were 16S, mmoX, and pmof1/r. DNA was prepared from groundwater at these sites 2, CAP; 3, GLRF; 4, LAPHAM. Lane 1, 100 bp ladder (Amersham Pharmacia). The 16S PCR product (1507 bp) and *pmo*A PCR product (330 bp) were observed in lanes 2 - 4; the *mmox* PCR product (535 bp) was detected in lane 4. No product was obtained when distilled water was substituted for groundwater (not shown). From Cheng et al. [1999].

B. Assessment of methanotroph activity

The detection of *pmoA* by PCR with DNA extracted from groundwater is indicative of the presence of methanotrophs. To demonstrate activity of methanotrophs, it is necessary to detect mRNA specific to methanotrophs. A *pmoA* mRNA is detected by first making a cDNA reverse transcript from the RNA. The coupled procedure is referred to as RT-PCR.

RNA was extracted from methanotroph lab strains and groundwater and used in RT-PCR. A product of the correct size was obtained with cDNA that had been reverse-transcribed from RNA prepared from lab strains, groundwater and a groundwater enrichment culture (Fig.3). The identity of these PCR products was confirmed by nested PCR (not shown). No product was obtained when the RNA preparations were used directly without a reverse transcriptase step (not shown). This is described in detail in Cheng et al. [1999].

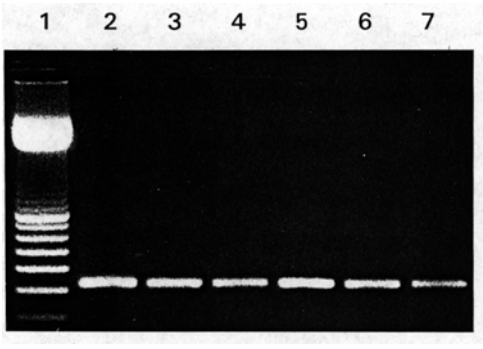

Fig. 3. RT-PCR using pmof1/pmor primer pair. RNA was obtained from 2, *M. album* BG8; 3, *M. capsulatus* (Bath); 4, *M. trichosporium* OB3b; 5, groundwater enrichment; 6, GLRF groundwater; 7, LAPHAM groundwater. Lane 1, 100 bp ladder (Amersham Pharmacia). A 330 bp product was detected in lanes 2 - 8. From Cheng et al. [1999].

C. Quantitative PCR

In addition to its use in detecting microbes, PCR may be used to quantify template DNA thus providing an estimate of a particular bacterial population. However, a major limitation in quantitative analysis based on PCR is the efficiency of DNA extraction. An alternative approach eliminating the need for DNA extraction was developed (see a.). This was used in two different quantitative PCR approaches (see b. and c.).

1. Direct PCR

Purity and recovery are problems encountered in the extraction of DNA from environmental samples. Several DNA extraction methods have been developed for preparation of DNA from natural samples. The approaches used in most molecular ecological studies involve the direct extraction of DNA from these samples [Henckel, 2000; Holmes, 1999; Khan, 1998; Knaebel, 1995; More, 1994] or the recovery of cells from samples followed by DNA extraction [Cheng, 1999; Duarte, 1998]. Contamination of DNA by substances of environmental origin that inhibit the PCR, assumed to be humic acids and phenolic compounds, has necessitated the use of extensive procedures to obtain DNA of adequate purity [Khan, 1998; Duarte, 1998; Knaebel, 1995; More, 1994]. Several investigators found that even after elaborate purification steps, it was still necessary to dilute the DNA preparations to minimize the inhibitory effect of remaining contaminants on PCR [More, 1994; Picard, 1992].

An alternative approach that may avoid some of the problems associated with DNA extraction is the use of environmental samples directly in the PCR without prior isolation of DNA or pretreatment of the sample. We have developed and applied this alternative approach to environmental samples. The strategy involves serial dilution of samples and use of these diluted samples directly in PCR without isolation of DNA.

The Direct PCR (DPCR) method was first applied to lab strains to optimize a protocol to obtain the lowest detection limit by the PCR with the *pmoA* primers. Using the optimized protocol, suspensions of cells (10^7cells) were decimally diluted to extinction. The detection limit for each strain was determined as the lowest number of cells that when used as a template, yielded a PCR product that could be detected on an agarose gel. For *M.album* BG8 (Fig. 4) and *M. capsulatus* (Bath), 10 cells could consistently be detected. While for *M. trichosporium* OB3b, *M. parvus* OBBP, and EPA, the results were more variable. For the *M. trichosporium* OB3b and *M. parvus* OBBP, 10 - 100 cells were required for detection and for EPA, 100 - 1000 cells were required. These results imply that there is some variability in the detection limit among methanotrophic strains. These results are reported in Fode et al. [1999a] and Fode-Vaughan et al. [2001].

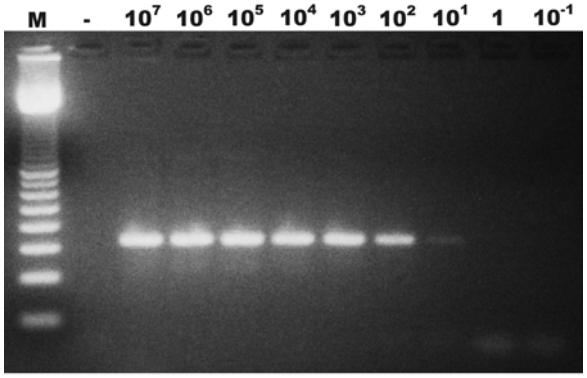

Fig 4. PCR with *M. album* BG8 cells in water. Primers used were pmof1 and pmor. Serial dilution of *M. album* BG8 in sterile distilled water. M, Amersham Pharmacia Biotech 100 bp ladder; -, no DNA control; 10^7 - 10^1 indicate the number of cells in the PCR reaction. From Fode-Vaughan et al. [2001].

2. DPCR-MPN

Most probable number (MPN) PCR has been used by other investigators to quantify organisms after the extraction of DNA [Degrange & Bardin, 1995; Picard, et al., 1992; Recorbet et al., 1993; Rosado, et al. 1996]. This approach involves serial dilution of DNA prior to PCR; quantitative estimates are based on statistical analysis of replicate series in which the template is diluted to extinction. In the present study, most probable number in conjunction with DPCR (MPN-DPCR) was applied to natural samples.

To apply statistical analysis to the determination of bacterial numbers by dilution to extinction, MPN-DPCR was used to quantify the number of methanotrophic cells using the results of the five replicate dilution series of each methanotroph strain. The number of cells in the original sample was determined by MPN-DPCR and compared to the direct count (Table 1). For *M. album* BG8 and *M. capsulatus* (Bath), the direct count was 8 times greater than the MPN (Table 1). This is consistent with the observation that the detection limit for *M. album* BG8 is approximately 10 cells (Fig. 4). In contrast, the MPN determinations for *M. trichosporium* OB3b and *M. parvus* OBBP and EPA are a greater underestimate of bacterial number (Table 1).

The detection limit determined with the *pmoA* primers using the five strains of methanotrophs demonstrates that the limit is cell-specific. This could reflect differences in the susceptibility of the cells to lyse under the hot start conditions of the PCR, factors affecting the binding of the primers with the particular templates, and/or PCR efficiency including differences in sequence, genome size, and copy number of the target gene [Farrelly et al, 1995; Polz & Cavanaugh, 1998; Suzuki & Giovannoni, 1996].

Table 1. Comparison of the number of methanotrophs in a 1 ml sample determined by Direct Count and DPCR.

Strain	Direct Count	MPN-DPCR[a]	95% confidence limits
M. album BG8	2×10^8	2.40×10^7	7.25×10^6 - 7.90×10^7
M.capsulatus (Bath)	2×10^8	2.40×10^7	7.25×10^6 - 7.90×10^7
M. trichosporium OB3b	2×10^8	7.90×10^6	2.40×10^6 - 2.61×10^7
M. parvus OBBP	2×10^8	4.93×10^6	1.50×10^6 - 1.63×10^7
EPA	2×10^8	4.93×10^5	1.50×10^5 - 1.63×10^6

[a] Because the copy number of *pmoA* in these methanotrophs has been shown to be or is assumed to be 2, the population estimated by MPN-PCR was divided by 2.

Before application of this method to environmental samples, initial experiments were performed with seeded samples to determine if the sensitivity of the method would be affected by constituents in the sample. *M. album* BG8 cells were serially diluted in filter-sterilized GLRF ground water (Fig. 5) and compared to the same cells diluted in

deionized water (Fig. 4). Reagents for PCR were added to the dilution series, PCR performed, and samples analyzed. No PCR product was obtained from 10^7 cells in undiluted filter-sterilized ground water (Fig. 5). This is assumed to be due to inhibition of the PCR by constituents present in the groundwater which was overcome with dilution. The end-point (10 *M. album* BG8 cells) was not affected by the presence of materials in filter-sterilized ground water (Fig. 4).

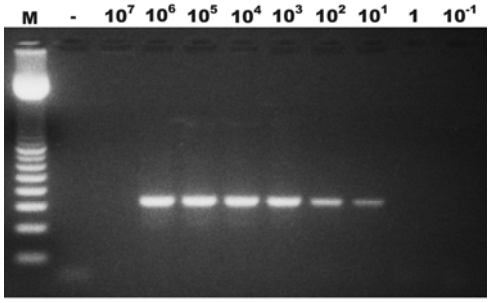

Fig 5. PCR with *M. album* BG8 cells in groundwater. Primers used were pmof and pmor. Serial dilution of *M. album* BG8 in filter-sterilized GLRF water. M, Amersham Pharmacia Biotech 100 bp ladder; -, no DNA control; 10^7 - 10^1 indicate the number of cells in the PCR reaction.

The DPCR protocol was then applied to a variety of environmental samples. Examples of the analysis of a groundwater is shown in Fig. 6. Both undiluted and 10^{-1} diluted GLRF groundwater did not yield a PCR product. The difference in the extent of dilution required to obtain a PCR product from filtered (Fig. 5) and unfiltered (Fig. 6) GLRF water implied that some inhibitors were associated with the particulate material removed by filtration. A PCR product was obtained at the 10^{-2} and 10^{-3} dilutions of the GLRF water.

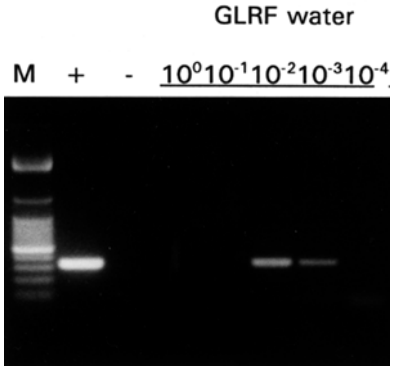

Fig. 6. PCR with serial dilutions of groundwater. Primers used were pmof1 and pmor. M, Promega 100 bp ladder; +, positive control, *M. album* BG8 DNA; - negative control, no DNA; dilutions of GLRF water indicated.

For the purpose of quantifying methanotrophs in environmental samples, MPN-DPCR was determined using five replicate dilution series. Because methanotrophs may vary in the number of cells required to obtain a PCR product, quantification of methanotrophs in environmental samples cannot be made on an absolute basis but rather in comparison to a lab strain. For this purpose, the greatest dilution that was adequate as a template for the amplification of a *pmoA* product was equated to 10 *M. album* BG8 cells (the observed detection limit on agarose gels - Fig. 4, 5). Using this approach, methanotrophs were quantified as *M. album* BG8 equivalents in natural water samples (Table 2).

Table 2. Quantification of methanotrophs in environmental water samples by MPN-DPCR

Sample	greatest dilution yielding PCR product	*M. album* BG8 equivalents/ml	95% confidence limits
groundwater			
GLRF	10^{-2} (3), 10^{-3} (2)	4.93×10^4	1.50×10^4 - 1.63×10^5
MW-1	10^{-1} (2), 10^{-2} (3)	7.90×10^3	2.40×10^3 - 2.62×10^4
lake water			
YS7	10^{-1} (5)	2.40×10^3	7.25×10^2 - 7.90×10^3
YS21	10^{-2} (5)	2.40×10^4	7.25×10^3 - 7.90×10^4
YS27	10^{-2} (5)	2.40×10^4	7.25×10^3 - 7.90×10^4

While the use of DPCR is limited to those samples that have a sufficiently high concentration of methanotrophs or other target group to withstand the dilution necessary to eliminate the effect of inhibitors, a diverse array of environments tested meet this criterion, suggesting that this method should be widely applicable. Moreover, the detection of methanotrophs at TCE-contaminated sites being assessed for natural attenuation (MW-1, Table 2), suggests that this method will be suitable for monitoring bioremediation. This is reported in Drought et al. [1999].

These results indicate that DPCR is a simple and rapid method that may be used quantitatively to assess changes in the magnitude of the methanotrophs population over time or in response to changes in the environment. These results are reported in Fode et al. [1999a] and Fode-Vaughan et al. [2001].

3. Competitive PCR

Competitive PCR is another technique that can be used to quantify target DNA. This technique involves construction of a competitive template that has the same priming sites as the native template. The product amplified from the competitive template should be distinguishable from the product amplified from the native template on an agarose gel. The native template and the competitor are assumed to compete equally for primers and the signal strength is determined by the ratio of the templates in a PCR reaction. By making a dilution series in which the concentration of the competitor is known, the concentration of the unknown may be determined by comparing the strength of the signal with that of the competitor.

A competitive *pmoA* template was constructed by amplifying a copy with an internal deletion. Use of this template (pCOMPET) resulted in a PCR product of 194 bp. Competitive PCR was evaluated with DNA and whole cells (DPCR) of *Methylomicrobium album* BG8. Equivalent PCR products were obtained with 10^3 cells competing with 10^2 copies of pCOMPET. This is consistent with the detection limit of 10 cells for *M. album* BG8 and that of 1 copy for pCOMPET (not shown).

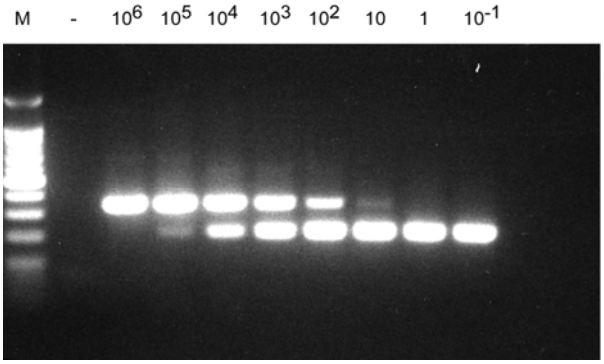

Fig 7. Competitive PCR.
M. album BG8 cells were diluted and included in PCR reactions with 10^2 copies of pCOMPET. Equivalent products were formed in the reaction containing 10^3 cells.

Competitive PCR was applied to environmental samples using DPCR and the methanotroph population estimated (Table 3). These results were determined in 5 replicates. The agreement in the values determined by MPN-DPCR (Table 2) and Competitive PCR (Table 3) indicates the validity of both methods. These results are reported in Fode et al. [1999b].

Table 3. Determination of size of the methanotroph population in groundwater samples using competitive PCR

groundwater	*M. album* BG8 equivalents/ml
GLRF	6×10^4
MW1	6×10^3

D. Evaluation of methanotroph populations

Analysis of methanotroph population diversity is important in bioremediation. Anthropogenic activities affect microbial diversity in environments**.** Chemically-impacted environments would be expected to have altered microbial populations. Furthermore, manipulation of conditions (e.g. methane or oxygen) in enhanced *in situ* bioremediation or bioreactors would be expected to affect methanotroph populations. Methods to evaluate methanotroph population diversity would be useful in addressing several questions. Are certain methanotroph populations more likely to be present in contaminated environments? Is the presence of certain methanotroph populations correlated with improved rates of degradation of problem chemicals? What environmental conditions are correlated with the presence of these degraders?

Methanotroph population diversity was initially evaluated by denaturing gel electrophoresis (DGGE). This method may be used to distinguish between PCR products amplified from templates with differences in sequence [Muyzer, 1993]. Such differences imply differences in the population. The results of these initial DGGE experiments with *pmoA* PCR products were not encouraging. Therefore an alternative approach was explored.

Single-stranded conformational polymorphism (SSCP), which provides a DNA fingerprint of PCR products amplified from a mixture of templates, was originally developed for medical application. More recently, it has been applied to environmental samples. The SSCP method is based on the principle that single-stranded DNA has a folded structure that is determined by intramolecular interactions based on its primary sequence. When this folded structure is subjected to electrophoresis on a non-denaturing gel, molecules that differ by even

a single base can be resolved. For the evaluation of a microbial community, the total genomic DNA is used as a template for PCR amplification with specific primers, the PCR products are denatured into single strands and loaded onto a gel for electrophoretic separation.

Use of SSCP for evaluation of methanotroph populations will be developed with the support of funds from the Wisconsin Department of Natural Resources.

VIII. CONCLUSIONS AND RECOMMENDATIONS

PCR-based methods for the detection of methanotrophs and methanotroph activity in groundwater have been developed. Two quantitative methods have been developed and applied to groundwater samples. Both of these used DPCR which is a rapid and simplified approach to sample preparation for PCR that allows detection and quantification of particular bacterial groups without isolation of DNA.

DPCR should be applicable to the detection of other bacteria in groundwater, including pathogens. This application of DPCR should be explored. DPCR should also be applicable for PCR-based methods for examination of methanotroph populations. This will require further investigation and empiric optimization.

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Fode-Vaughan, K. A., C. F. Wimpee, J. Maki, and M. L. P. Collins. 2001. Detection of bacteria in environmental samples by Direct PCR without DNA extraction (submitted for presentation at the General Meeting of the American Society for Microbiology, 21-25 May 2001)

These additional reports of research on methanotrophs are indirectly related to this project:

Brantner, C. A. 1999. The relationship of copper-containing particulate methane monooxygenase and intracytoplasmic membrane formation in *Methylomicrobium album* BG8. Intracytoplasmic membrane development in bacteria. Ph.D. thesis, University of Wisconsin-Milwaukee.

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