Direct Immunofluorescence and Enzyme-Linked Immunosorbent Assays for Evaluating Chlorinated Hydrocarbon Degrading Bacteria

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LIST OF KEY WORDS

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Abstract

Immunological procedures were developed to enumerate chlorinated hydrocarbon degrading bacteria. Polyclonal antibodies (Pabs) were produced by immunizing New Zealand white rabbits against 18 contaminant-degrading bacteria. These included methanotrophic and chlorobenzene (CB) degrading species. An enzyme-linked immunosorbent assay (ELISA) was used to test for specificity and sensitivity of the Pabs. Direct fluorescent antibodies (DFAs) were developed with these Pabs against select methanotrophic bacteria isolated from a trichloroethylene (TCE) contaminated landfill at the Savannah River Site (SRS) and cultures from the American Type Culture Collection (ATCC). Analysis of cross reactivity testing data showed some of the Pabs to be group specific while others were species specific. The threshold of sensitivity for the ELISA is 10^5 bacteria cells/ml. The DFA can detect as few as one bacterium per ml after concentration. Results from the DFA and ELISA techniques for enumeration of methanotrophic bacteria in groundwater were higher but not significantly different ($P>0.05$) compared to indirect microbiological techniques such as MPN. These methods provide useful information on *in situ* community structure and function for bioremediation applications within 1- 4 hours of sampling.

Keywords: Direct immunofluorescence; Enzyme-linked immunosorbent assays; Methanotrophs communities

1. Introduction

Historically, methods used to identify methanotrophic bacteria in environmental samples have been inadequate because isolation and identification procedures are time-consuming and expensive. Current microbiological techniques such as the most probable number (MPN) procedure are indirect and often fail to separate specific bacteria from other environmental microorganisms [1]. Methanotrophic bacteria have been found to be particularly difficult to enumerate due to particle attachment compared to the majority of soil bacteria [2]. Wilson and Wilson [3] demonstrated that trichloroethylene (TCE), the most frequently observed volatile organic contaminant at Resource Conservation and Recovery Act (RCRA) sites [4] and groundwater [5], is susceptible to cometabolism by soil communities enriched with natural gas. Methanotrophic bacteria have been isolated and characterized from TCE-contaminated soils at the U.S. Department of Energy Savannah River Site (SRS) [6]. Fliermans and others [7] demonstrated that cultures isolated from SRS enriched with methane and propane could cometabolically degrade a wide variety of chlorinated aliphatic hydrocarbons including TCE; 1,2-cis-dichloroethylene (c-DCE); 1,2-trans-dichloroethylene (t-DCE); and vinyl chloride (VC).

It is now well recognized that TCE and other chlorinated aliphatic compounds can be degraded by selected methanogens [8] methanotrophs [9], *Pseudomonas* and *Burkholderia* spp. [10]. Rates of TCE degradation by methanotrophs can vary significantly depending on the species and culture conditions [11]. At SRS the population of methanotrophic bacteria was correlated with the TCE biodegradative potential of a specific site [12]. Characterization of selected microorganisms in the natural setting is important for the evaluation of bioremediation potential and its effectiveness. This realization has necessitated techniques that are direct, selective, sensitive and easily

applicable to soils, sediments, and groundwater that can identify and quantify microbial types *in situ* in real time [13].

The number of organisms present in groundwater, soil and sediment samples can be determined directly using immunoassay procedures including direct fluorescent antibodies (DFA) and enzyme-linked immunosorbent assay (ELISA). Additionally, any potential cross reactions can be eliminated by employing a modified ELISA technique and carefully selecting antibodies as previously described [14]. Immunoassay techniques are capable of detecting, identifying, and quantifying bacteria in very complex environments. Immunological methods have proven to be advantageous over traditional microbiological methods for detection of certain bacteria in environmental samples which show problems of slow growth rate and difficulties in isolation and purification [15]. Bacteria can retain their antigenic abilities throughout different growth phases in mixed ecosystems [16].

We determined that immunodetection techniques can provide a rapid, reliable, sensitive and inexpensive methodology for monitoring groundwater bacteria capable of degrading contaminants. This report describes immunological procedures for selective enumeration of TCE and chlorobenzene (CB) degraders in environmental samples.

2.0 Materials and Methods

2.1 Bacteria and culture conditions.

The bacteria used in this project were obtained from the American Type Culture Collection (ATCC) as well as methanotrophic bacteria isolated from environmental samples at the SRS (Table 1). Methanotrophic bacteria were grown in minimal salts media with 10 % methane as described [17]. All other bacteria were grown on Peptone-Trypticase-Yeast-extract-Glucose (PTYG) [18]. Bacteria for immunoassays and immunizations were inactivated in log phase by overnight incubation at 4° C with 10 % formalin in phosphate buffered saline (PBS). Bacteria cells were then washed 3X by centrifugation and diluted to standard densities with PBS for immunization.

2.2 Polyclonal Antibodies.

Polyclonal antibodies (Pabs) were developed in male New Zealand White rabbits at the Medical College of Georgia (MCG), Augusta, GA according to established protocol [19]. Hunter's Titermax (Sigma Chemical Co., St. Louis MO) was employed as an adjuvant. Serum was collected and frozen at -70°°C in 100 µl aliquots.

2.3 DFA.

Immunoglobulins were isolated by ammonium sulfate precipitation from rabbit antisera having agglutination titers>1280 and conjugated with fluorescein isothiocyanate (FITC). All DFAs were separated from unconjugated FITC on Sephadex G-25 columns, filtered with 0.2 µm filter, and frozen in 100 µl aliquots at

-70°C. For analysis with DFAs, 10 µl aliquots of bacteria suspension were pipetted onto slides and dried for 10 min at 65° C. A 2% hydrolyzed gelatin solution in 10 µl PBS was then layered over the dried bacteria and allowed to dry for 10 min at 65° C. The DFAs were then pipetted over the bacteria in 10 µl aliquots diluted 1:32 in PBS. The slides were incubated 30 min at room temperature in a moist chamber, washed with distilled water, incubated overnight in filtered PBS, rinsed in 5% sodium pyrophosphate (NPP) and air dried. The slides with fixed samples (both pure cultures and groundwater samples) were mounted using a drop of SlowFade (Molecular Probes Inc., Eugene, OR) and examined with a LSM 310 Laser Scanning Microscope (Carl Zeiss, Inc., Thornwood, NY).

Both methanotrophic and chlorobenzene-degrading bacteria were enumerated groundwater using the DFA technique. The DFAs were pooled and tested separately for the methantrophic bacteria and CB degraders. One ml of groundwater was added to 5 ml. of NPP, vortexed, sonicated for 30 seconds and vortexed for 30 sec. The mixture was then centrifuged at 3000 rpm for 5 minutes. The supernatant was diluted 1:10 in PBS buffer. Two ml of the diluted supernatant was filtered through a 0.2 micron filter (Millipore, Bedford, MA) in a vacuum manifold. Ten µl of a 1:20 dilution of the DFA were used to flood the filter, covered with aluminum foil and allowed to incubate for 30 min at room temperature. Following incubation, 20 ml. of PBS were filtered through the manifold to remove unattached DFA (background). Positive control included immunogens and negative controls included sterile water. The filter was then mounted on a microscope slide using drop of elvanol with another drop of elvanol placed an top of the filter and a cover slip applied. Bacteria labeled with the specific DFAs were then counted with an epifluorescent microscope.

2.4 ELISA.

For ELISA 100 µl of a 10^7 organisms/ml suspension or media from MPN tubes, were added to immunoassay plate wells previously treated with polylysine. ELISA plates were treated sequentially for 1 h each with PBS containing 1% bovine serum albumen (PBSA), 100 µl of Pab, 100 µl of a 1:1000 dilution in PBS of affinity-purified horse radish peroxidase goat anti-rabbit immunoglobulins [20]. After incubation with each reagent, ELISA plates were washed 6X with PBS containing 0.05% Tween-20 on an ELISA plate washer reader. Negative controls included PBSA and normal rabbit serum. Bound conjugate was observed by addition of 100 µl of substrate (1 mg/ml 2, 2'-Azino-bis(3-Ethylbenz-thiazoline-6-Sulfonic Acid) (ABTS), in citrate buffer with 3% hydrogen peroxide). The plates were read on a ELISA plate reader at 405 nm after 30 min incubation at room temperature (Biotek Instruments, Inc., Winooski, VT).

2.5 Environmental Sampling.

The Savannah River Site (SRS) is an 320 square mile facility located in a rural area along the Savannah River, principally in the Aiken and Barnwell counties of South Carolina. The SRS is owned by the U.S. Department of Energy and operated by Westinghouse Savannah River Company. In the early 1970's, solid waste was consolidated into a single Sanitary Landfill near the center of the site. Aerobic and anaerobic ground waters at the Sanitary Landfill were contaminated with TCE, VC, and chlorobezene (CB) as a result of some of these activities. As part of an ongoing bioremediation project work at the Sanitary Landfill, groundwater samples collected from monitoring wells were evaluated with immunological and microbiological techniques for the presence of TCE, and CB degraders.

2.6 Determination of microbial biomass.

Total heterotrophic bacteria were enumerated using the heterotrophic plate count technique that provides an estimate of the total number of viable aerobic and facultatively anaerobic bacteria present in the soils. Low (1%) and high (100%) concentrations of PTYG media were used to culture bacteria adapted to oligotrophic and eutrophic [21]. Groundwater samples (1 ml) were added directly into 15 ml sterile conical centrifuge tubes containing 9 ml of NPP. Subsequent serial dilutions were made in PBS. Each dilution (0.1 ml) was inoculated onto a corresponding plate of 1 % and full strength formulation of PTYG, respectively [21]. The inoculum was evenly spread over duplicate agar plates and incubated at room temperature for 1 week prior to counting.

Methanotrophic bacteria from environmental samples were quantified in groundwater samples using the MPN enumeration method. Minimal salts media (MSM) were supplemented with 10 % methane 90 % air headspace in Balch tubes sealed with black butyl rubber stoppers [17]. Triplicate tubes were run for each dilution. The first dilution contained 1 ml of groundwater into 20 ml MSM with 3 subsequent 1 to 10 dilutions. Tubes were incubated for 6 weeks along with a set of 4 control tubes. After gas chromatographic determination of MPN tube methane and carbon dioxide concentrations, samples were taken in 100 µl duplicates and tested with the ELISA.

Total bacterial concentrations were accomplished by the Acridine Orange Direct Count (AODC). One ml of groundwater was added to 5 ml NPP, mixed well, sonicated for 30 sec. and vortexed for 30 sec. The mixture was then centrifuged at 3000 rpms for 5 min. The supernatant was diluted 1:10. One ml of this dilution was mixed with 0.5 ml acridine orange for 2 minutes on a 0.2 µm pore size filter (Millipore, Bedford, MA) in a vacuum manifold, and then filtered. The filter was then mounted on the slide on top of 1 drop of immersion oil. One drop of immersion oil added on top of the filter and a cover slip applied.

Dichlorobenzene degraders were enumerated by a plate count procedure. This method provided an estimate of the number of viable aerobic and facultatively anaerobic bacteria capable of growth on chlorobenzene (CB) as a carbon and energy source. From each sample 1 ml of groundwater was diluted in 9 ml NPP. Subsequent serial dilutions were made in PBS. Appropriate dilutions were spread on minimal salts medium solidified with 1.8 % agar (w/v) and supplemented with yeast extract (10 mg/l) [22]. The CB was supplied in the vapor phase to cultures in desiccators at room temperature. Control plates containing PBS were incubated in the presence of CB. Plates were incubated for 6-8 weeks or more prior to counting.

3.0 Results and Discussion

The specificity of the ELISA utilizing the 18 Pabs developed for the ATCC and SRS bacteria is shown in Table 2. Two of the Pabs reacted in both readings only with their antigen, SC29 and SC30. The Pab preparations for the SRS isolate SC11 bound all other SRS isolates except SC30 (Table 2). Other SRS isolate preparations showed similar activity (SC12, SC13, SC14, SC15, SC18). The Pabs SC14 and SC19, seemed to bind the*Pseudomonas* and *Rhodochochrous* spp tested. The Pabs against SC24 (*Pseudomonas* sp.) showed reactivity against other antigens in Table 2. The detection limit of the ELISA is 10^5 bacteria cells/ml. A panel of the antibodies was employed for the ELISA in testing environmental samples. Table 3 compares the MPN and ELISA method for methanotrophs with respect to positives and negatives. There was a 76% agreement between the two tests. The ELISA took 4 hours to complete while the MPN method takes 6 weeks.

Based on crossreactivity information from the ELISA all the DFAs for methanotrophic bacteria were pooled for environmental testing with the exception of SC14 and SC19. Similarly, the DFAs for SC23 and SC 26 was pooled for microbial

chlorobenzene degrader analysis. Table 4 summarizes the results from enumerating the total heterotrophic bacteria (1% and regular PYTG plates), total counts (AODC), chlorobenzene degraders from plate counts, and DFA counts for both methanotrophic and chlorobenzene-degrading bacteria. Slides for both AODC and DFA were made simultaneously from groundwater samples within 2 hours sampling. The results of cell counts calculation taken from slides were compared to plate counts with analysis of variance (ANOVA). The DFA counts were significantly higher by an order of magnitude for the CB degraders as compared to viable plate count. The counts by DFA for methanotrophic bacteria were higher but not significantly different ($P>0.05$) compared to the MPN technique as (Table 4).

Identification of *bacteria* with pooled DFA generated against methanotrophic bacteria is readily evident in pure culture for *Methylobacterium extorquens* (ATCC 43645) (Fig. 1A) and detection of methanotrophic bacteria in a groundwater sample from the Sanitary Landfill (Fig. 1B). Immunofluorescent binding of DFAs generated against CB degrading bacteria are shown for *Rhodococcus rhodochrous* (ATCC 14347) in pure culture (Figure 1C) and chlorobenzene degrading bacteria in groundwater (Fig. 1D).

The immunological procedures described in this paper permit the selective enumeration of methanotrophic and chlorobenzene degrading bacteria. These methods can be used for characterization of TCE and CB degrading bacteria and for monitoring the response of subsurface environments to contamination. Present methods are tedious or incompatible for field applications. The method for enumeration of CB degrading bacteria by plate counts requires the use of CB in the laboratory as a carbon source. Use of CB requires additional specific safety precautions and also generates more hazardous waste. This is a problem in laboratories throughout the world. Both the CB and methanotroph microbial enumeration techniques can take up to of 6 weeks for completion as well as being labor intensive. The ELISA and DFA techniques can be accomplished in 1 day.

There were differences in enumeration results when comparing indirect microbiological and direct immunological procedures tested here. These differences could be explained in part by the fact that DFA and ELISA techniques react the same with

live/active/inactive and dead bacterial cells while the microbial techniques measure culturable microorganisms. Not all viable bacteria will grow on any specific type of media. As demonstrated in this study less than 1% of the total population could be cultured using high and low nutrient sources (Table 4). Thus direct techniques are always greater than indirect for environmental microbiological monitoring where possible [12]. However, the Pabs may not recognize certain bacteria in the environmental samples. But we also saw in the ELISA for methanotrophs that there were as many false negatives as positives compared to the MPN method (Table 3). The relative numbers of total bacteria to CB and TCE degraders are of interest regarding the structure of the microbial community in bioremediation applications (Table 4). This information indicates the potential for intrinsic bioremediation of TCE at this landfill by the number of methanotrophic bacteria .

The results shown here demonstrate the effective use of immunoassays for the purpose of studying select microorganisms in groundwater samples. Preliminary work with these DFAs and ELISA indicate they can be used to monitor methanotrophic bacteria in active TCE bioremediation work [23]. While the ELISA is more useful for rapid screening of multiple samples, a limitation is the sensitivity that may be improved by amplification techniques.

There is evidence for biodegradation of TCE and CB at the SRS Sanitary Landfill as the result of a bioremediation test [24]. This evidence includes increases in populations of degrading bacteria (i.e. methanotrophs and CB degraders) and production of chloride in the saturated zone as a result of methane and air biostimulation. A reduction in mass of contaminants was demonstrated after stimulation of indigenous organisms. The immunological techniques described here, as well other antibody preparations under development, will be used to further evaluate the microbial populations associated with contaminant biodegradation at the Sanitary Landfill. These methods should provide valuable analytical tools for monitoring bioremediation applications.

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Table 1

Bacteria employed for antibody preparation and testing

ATCC Cultures	Species
43882	*Methylobacterium rhodiesianum*
35070	*Methylosinus trichosporium*
35068	*Methylomonas agile*
29832	*Unidentified bacterium*
31483	*Pseudomonas flourescens*
12633	*Pseudomonas flourescens*
14347	*Rhodococcus rhodochrous*
35066	*Methylocystis parvis*
35069	*Methylosinus sporium*
43645	*Methylobacterium extorquens*
SRS Cultures	
S4A/1Bd	methanotroph
S3C/2AB	methanotroph
S3A/1Q	methanotroph
S2C/1b	methanotroph
S3A/1C	methanotroph
S4A/1BC	methanotroph
S3C/1b	methanotroph
S4B/1Aa	methanotroph

Table 2

ELISA crossreactivity

BACTERIA	ID	ANTIBODY PROBE																	
		SC 11	SC 12	SC 13	SC 14	SC 15	SC 16	SC 17	SC 18	SC 19	SC 20	SC 22	SC 23	SC 24	SC 26	SC 27	SC 28	SC 29	SC 30
S4A/1Bd	SC11	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
S3C/2AB	SC12	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
S3A/1Q	SC13	+	+	+	+	-	-	+	+	+	-	-	-	+	-	-	-	-	-
S2C/1b	SC14	+	+	+	+	+	+	+	+	+	-	-	-	+	-	-	-	-	-
S3A/1C	SC15	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	+	-	-
43882	SC16	-	+	+	+	-	+	+	+	-	-	-	-	+	-	-	-	-	-
35070	SC17	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-
S4A/1BC	SC18	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	-
S3C/1b	SC19	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-	+	-	-
35068	SC20	-	-	-	+	-	-	-	-	-	+	+	-	+	-	+	+	-	-
29882	SC22	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-
31483	SC23	+	-	-	+	-	-	-	-	+	-	+	-	+	-	-	-	-	-
12663	SC24	-	-	-	+	-	-	-	-	+	-	+	-	+	-	-	-	-	-
14347	SC26	-	-	-	+	-	-	-	-	+	-	+	+	+	+	-	-	-	-
35066	SC27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35069	SC28	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
43645	SC29	-	-	-	+	-	-	-	-	+	+	+	-	-	-	+	+	+	-
S4B/1Aa	SC30	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	+	-	+

Table 3

Detection of methanotrophic bacteria in environmental samples by culture/biochemical and ELISA methods

		MICROBIOLOGICAL	
		Positive[a]	Negative
ELISA	Positive[b]	73	13
	Negative	12	4

[a]Samples were positive if growth on methane as sole carbon and energy source
[b]Samples were positive if absorbance $\geq$ 0.20 over controls

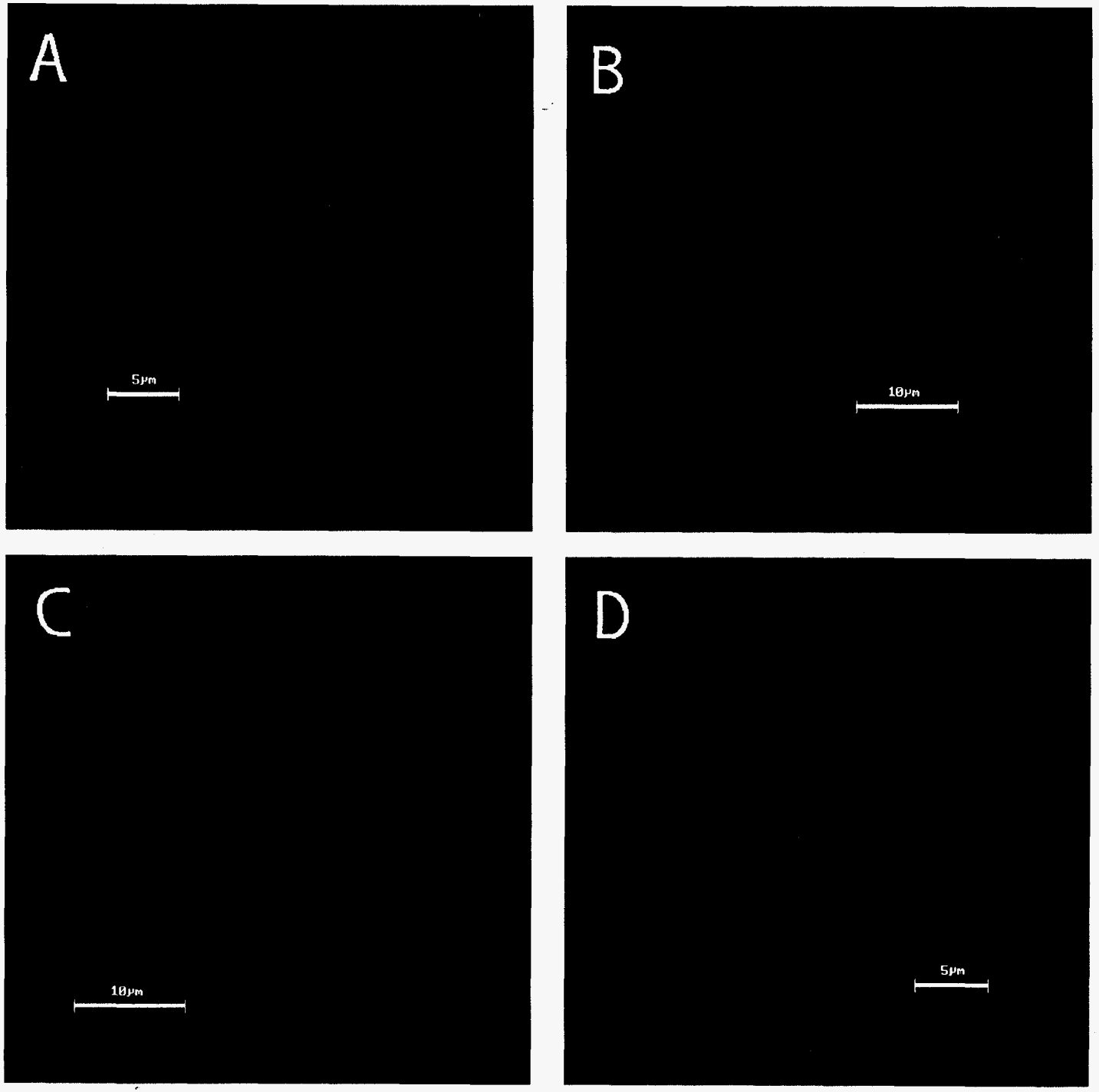
Table 4

Results from enumerating the total heterotrophic bacteria (1% and 100% PYTG plates), total counts (AODC), chlorobenzene degraders from plate and DFAs, and methanotrophic bacteria from MPN and DFA counts.

MICROBIAL COUNT	N	Concentration (cells ml^{-1})	% of TOTAL COUNT
AODC (Cells/ml)	215	$1.67x10^6$	100
Heterotrophs (1% PYTG) (CFU/ml)	105	$3.18x10^4$	0.1
Heterotrophs (PYTG) (CFU/ml)	105	$1.42x10^4$	0.1
Chlorobenzene Degraders (CFU/ml)	95	$2.43x10^3$	0.01
Chlorobenzene Degraders (DFA) (Cells/ml)	63	$2.39\ x10^4$	0.1
Methanotrophs (MPN) (Cells/ml)	79	$1.01x\ 10^3$	0.01
Methanotrophs (DFA) (Cells/ml)	79	$2.90x\ 10^3$	0.01

FIGURE CAPTIONS:

Fig. 1. Identification of *bacteria* with DFA generated against bacteria including A) *Methylobacterium extorquens* (ATCC 43645) B) and methanotrophic bacteria in groundwater. Immunofluorescent binding of DFAs generated against C) *Rhodococcus rhodochrous* (ATCC 14347) in pure culture and D) chlorobenzene degrading bacteria in groundwater.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