

Fluorescent Antibody Application in Bioremediation Procedures at the Savannah River Site (U)

by

R. L. Brigmon

Westinghouse Savannah River Company

Savannah River Site

Aiken, South Carolina 29808

B. S. Richardson

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R.L. Brigmon, B.S. Richardson, M.M. Franck

**Environmental Science Section
Savannah River Technology Center
Westinghouse Savannah River Company
Bldg. 704-8T
Aiken, SC 29808
Phone (803) 557-7715
Fax (803) 557-7223
EMAIL R03.brigmon@srs.gov**

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(Name and Title)
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Abstract

Direct Fluorescent Antibodies (DFA) and Most Probable Number (MPN) techniques are currently being employed at the Savannah River Site to monitor methanotrophic bacteria for the bioremediation of trichloroethylene (TCE) in field studies. Direct Fluorescent Antibodies were developed against various methanotrophic bacteria isolated from SRS (Brigmon et al, 1995) as well as methanotrophic bacteria acquired from the American Type Culture Collection (ATCC). DFA's are anticipated to be more efficient for monitoring methanotroph activity than MPN's because of shorter processing time, lower cost, and the direct nature of the assay. The DFA method is a direct technique, in that samples are processed immediately and can be enumerated within an hour. The MPN method is indirect, since samples must be cultured for 6-8 weeks before measuring methane consumption and carbon dioxide production. Indirect methods are not highly selective and have limited application. The greatest advantage of a faster assay, is that bioremediation procedures utilizing methanotrophic bacteria could be amended. These amendments would be based on environmental monitoring with results in real time (1 hour). The elimination of the MPN technique and the use of DFA's will save significantly on both materials and labor. The data obtained from the DFA's and MPN's were statistically compared to each other and to total bacterial counts (AODC). The statistical analysis used was Analysis of Variants (ANOVA). Using this analysis, groundwater samples were found to be not significantly different; whereas soil were significantly different. These methods were employed on soil samples from the Southern Sector and ground water samples from the TCE-contaminated Sanitary Landfill at SRS. Acridine Orange Direct Counts were compared to show relative differences between total bacterial and methanotroph population.

Introduction

For effective bioremediation strategies it is necessary to understand the effects that pollutants and remediation techniques have on subsurface microbial communities. Therefore, detailed characterization of these microbial communities is essential. This research is on monitoring processes used in specific types of bioremediation procedures at the Savannah River Site (SRS). In the areas observed, trichloroethylene (TCE) is an environmental contaminant of both subsurface soil and groundwater. Methods for remediation have been developed using naturally occurring bacteria in these areas. A percentage of these bacteria have been found to be methanotrophic and important for bioremediation of the solvent. Quantification of these methanotrophic bacteria is necessary to estimate the rate of contaminant degradation. These methanotrophs produce an enzyme, methane monooxygenase (MMO), which will dechlorinate TCE (Brigmon et al, 1995). Once this has occurred, other bacteria in that environment can biodegrade the remaining hydrocarbon chain. Therefore, one can see the essential nature of having the methanotrophs present as well as closely monitoring their populations.

Current methods used to quantify bacterial populations in environmental samples include both direct and indirect techniques. A direct method detects bacteria found in an environmental sample soon after the sample is collected, where very few changes have occurred that would alter the bacterial population, thus the assay does not require cellular growth. The direct methods examined were Direct Fluorescent Antibodies (DFA) for methanotrophs and Acridine Orange Direct Counts (AODC) for total bacterial counts. An indirect method measures the number of bacteria that will grow in an artificial (media) environment. The indirect method used was the Most Probable Number technique for methanotrophs. These artificial methods are often selective in the type of organism that can grow. Because not all bacteria can survive in a select artificial environment, there can be variations in the results using these indirect method types.

In addition to various monitoring techniques, two types of environmental samples from SRS were examined in this project; soil and groundwater samples. The soil samples were obtained from the Southern Sector, where there is the potential for TCE contamination due to a subsurface plume moving toward the area. The sampling of this area was to establish whether naturally occurring methanotrophs exist and to what degree. The second type of samples were groundwater from the SRS Sanitary Landfill, where known TCE contamination exists. In both of these sites, the amount of TCE that can be biodegraded is dependent on the subsurface microbial population present.

Materials and Methods

Fluorescent Antibodies (DFAs):

Antibodies in this project were developed against bacteria isolated from the Sanitary Landfill of the Savannah River Site and obtained from the American Type Culture Collection (ATCC). Polyclonal Antibodies (PABs) were developed in New Zealand White rabbits at the Medical College of Georgia (MCG), Augusta GA. (Fliermans and Schmidt, 1975). Immunoglobins were isolated by ammonium sulfate precipitation from rabbit antisera having agglutination titers >1280 and conjugated with fluorescein-isothiocyanate (FITC). The DFA's were separated from unconjugated FITC on Sephadex G-25 columns and stored at -70 °C.

Fluorescent Antibody Technique (DFA)

Soil

One to two grams of soil were added to 5 ml. of Homogenizing buffer, mixed well, 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 phosphate buffered saline buffer (FA). Two ml. of the diluted supernatant was filtered through a 0.2 micron filter (Nucleopore, Pleasantview, CA) in a vacuum manifold. Ten microliters of a 1:20 dilution of the antibody were used to flood the filter, the filter apparatus was then covered with aluminum foil and allowed to incubate for 30 min. at room temperature. Following incubation, 20 ml. of FA buffer were filtered through the filter to remove any unattached antibody (background). 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 (Rodriguez, 1960). Methanotrophic bacteria labeled with the specific DFA are then counted using an epifluorescent microscope (Axioscope, Carl Zeiss Inc., NY, NY).

Groundwater

An initial volume of sample (usually double that used in AODC's) was filtered through a 0.2 micron filter (Nucleopore, Pleasantview, CA) in a vacuum manifold. With vacuum off and stopcocks closed, 10 microliters of 1:20 dilution of antibody was placed in the center of the filter. The vacuum apparatus is covered with aluminum foil and allowed to incubate for 30 min. at room temperature. After incubation, the DFA was filtered and the filter washed with 20 ml. of FA buffer to remove unconjugated antibody (background). The filter is then mounted on a microscope slide using a drop of elvanol with another drop of elvanol on top of the filter and a cover slip applied (Rodriguez 1960). A control using 1 ml. of filter sterilized water was prepared first to assure that antibody had not coagulated and that the FA buffer was

not contaminated. Methanotrophic bacteria are then quantified using an epifluorescent microscope (Axioscope, Carl Zeiss Inc., NY, NY).

Most Probable Number

Soil

Methanotrophic bacteria from soil samples were quantified using the MPN technique. The first dilution contained 3 g of soil to 27 ml. minimal salts media (MSM). A series of 7 dilutions were made with triplicate tubes for each dilution. A set of serial dilutions requires 28 tubes plus four controls for a total of 32 tubes. MSM was supplemented with 10 % methane 90 % air in the headspace of Balch tubes sealed with black butyl rubber stoppers. Tubes were incubated for 6 to 8 weeks at ambient temperature. The concentration of methane and carbon dioxide in the headspace of control tubes were determined where half the average methane volume represented the lower limit of methane removal and carbon dioxide production needed to count as a positive MPN tube.

Groundwater

Methanotrophs from water samples were enumerated using the MPN technique. The first dilution contained 0.5 ml of sample to 4.5 ml of MSM. A series of 7 dilutions were made with triplicate tubes for each dilution. A set of serial dilutions requires 28 tubes plus four controls for a total of 32 tubes. MSM was supplemented with 10 % methane 90 % air in the headspace of Balch tubes sealed with black butyl rubber stoppers. Tubes were incubated for 6 weeks at ambient temperature. The concentration of methane and carbon dioxide in the headspace of control tubes were determined and half the average methane volume represented the lower limit of methane removal and carbon dioxide production needed to count as a positive MPN tube.

Acridine Orange Direct Counts

Soil

Total bacterial counts were accomplished by the Acridine Orange Direct Count Method (AODC). One to 2 grams of soil were added to 5 ml. Homogenizing Buffer, mixed well, sonicated for 30 sec. and vortexed for 30 sec. The mixture was then centrifuged at 3000 rpm for 5 min. The supernatant was diluted 1:10. One ml. of this dilution was mixed with 0.5 ml. Acridine Orange, incubated for 2 minutes in a vacuum manifold, and then filtered through a

0.2 micron filter (Nucleopore, Pleasantview, CA). The filter was then mounted on a microscope slide and counted using an epifluorescent microscope (Axioscope, Carl Zeiss Inc., NY, NY).

Groundwater

One ml of sample was mixed with 0.5 ml. of acridine orange and allowed to incubate for 2 min. The sample is then filtered through a 0.2 micron filter (Nucleopore, Pleasantview, CA). The filter was then mounted of a microscope slide and viewed by an epifluorescent microscope (Axioscope, Carl Zeiss Inc., NY, NY)

Results and Discussion

The MPN and DFA methanotroph data from the Sanitary Landfill groundwater samples were found to be not significantly different. A log plot of the data for each of three select wells sampled during an 8 day testing period was done and are Figures 1a, 1b, and 1c. In Figure 1a the initial counts from the DFA's show no methanotrophs whereas the MPN's have substantial counts. Halfway through the sampling time though, the populations seem to coincide indicating a possible population shift. It appears that the methanotrophs indicated by the MPN's are of a type for which we maybe have no antibody, thus no counts were possible. However, as the injection of methane progressed, the methanotrophs we had DFA's for increased in population. The initial assumption is that the methanotrophs that corresponded with the DFA's were the ones selected in the environment. Additional testing on this theory will be to run DFA's on the populations found in the MPN tubes to see if any variation occurs. On a long range scale, if the methanotrophs in the MPN tubes prove to be different, these bacteria would then be cultured and used to produce new antibodies that would make further use of DFA's more complete. In Figure 1b the variation in the results does not seem to have affected the statistical correlation of the two methods as they are still not significantly different. In Figure 1c the differences are proportional between the two methods. This difference is not unusual, and were even expected, when comparing an indirect method to a direct one.

A comparison of the ratio of methanotrophs to total bacterial population was made. Figures 2a, 2b, and 2c show the percent of the total microbial population that was methanotrophic bacteria. Over the sampling period it appears that the percent methanotroph population increased with methane injection relative to the overall population. In addition to water samples, gas samples were taken from vadose zone piezometers to measure the contaminant gas content as well as to track injection gasses. These piezometer readings show the percent, of the gas samples taken, that were TCE and its derivatives. Figures 3a, b, and c show the changes in gas content of the vadose zone as related to the sample dates. In comparing Figures 1 and 3 for each well, it can be seen that as the methanotroph populations increase the DCE content in the vadose zone decreases. Since the methane injection is increasing the overall methanotroph population it correlates with a decrease in contaminant volume. In conclusion, the use of DFA's in the Sanitary Landfill gives quite accurate results and could replace the MPN's.

In the Southern Sector however, the comparison of DFA's to MPN's showed the two methods to be significantly different. This variation compared to water samples is attributed to

the rigorous sample prep required to run a DFA on a soil sample. For further tests, plans are to vary the procedure used in sample preparation. Our most likely problem is the method used to separate the bacteria from the sediment in solution. A variety of surfactants and treatments such as varying the amount of sonication and vortex time. Until comparable results can be achieved on soil samples DFA's cannot replace the use of MPN's. This shall be pursued, as the use of a direct method for quantification is desired over the indirect MPN method.

In summary, DFA results for groundwater samples from the Sanitary Landfill were not significantly different from MPN with the results in 1 day versus 8 weeks at a fraction of the cost. Southern Sector samples were found to be significantly different and still require the use of the MPN technique. Future modification of the soil preparation procedure should allow the replacement of MPN's in soil samples as well as water samples. This would allow for savings in cost and labor as well as receipt of results in a timely fashion.

Figure 1a
**LOG 10 FA VS MPN (WELL
2S)**

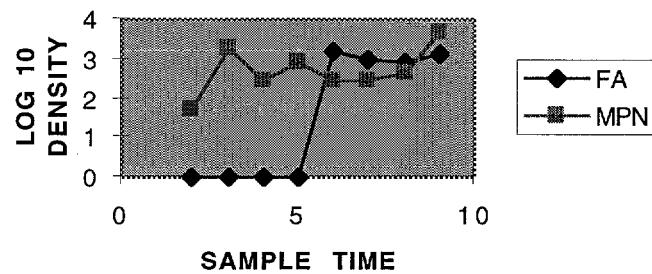


Figure 1b
LOG 10 FA VS MPN (WELL 9S)

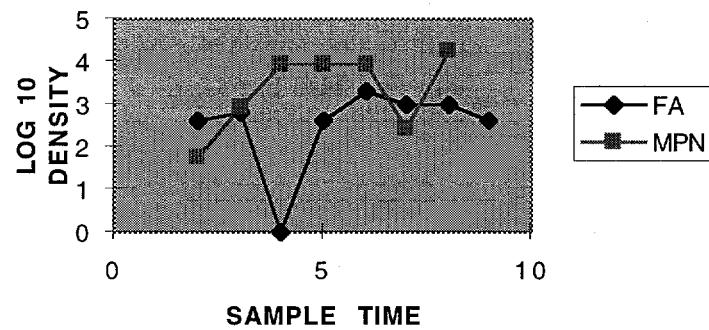


Figure 1c
**LOG 10 DFA VS MPN (WELL
14S)**

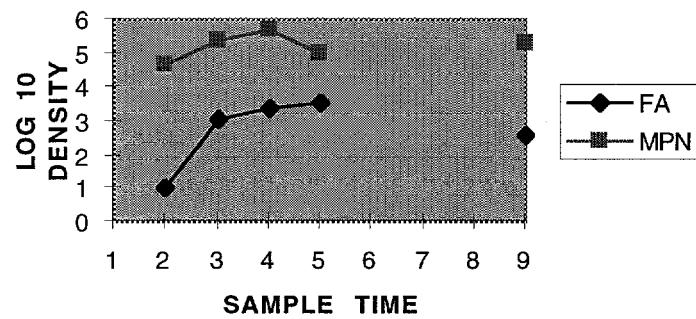


Figure 2a
Ratio of Methanotrophs to Total Counts

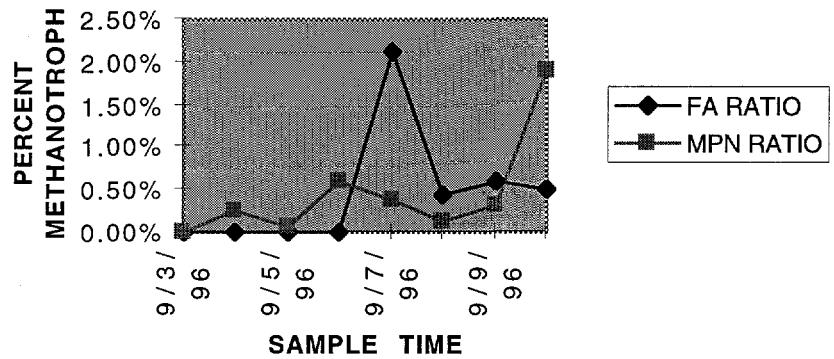


Figure 2b
**ratio of Methanotrophs to
Total Counts (Well 9S)**

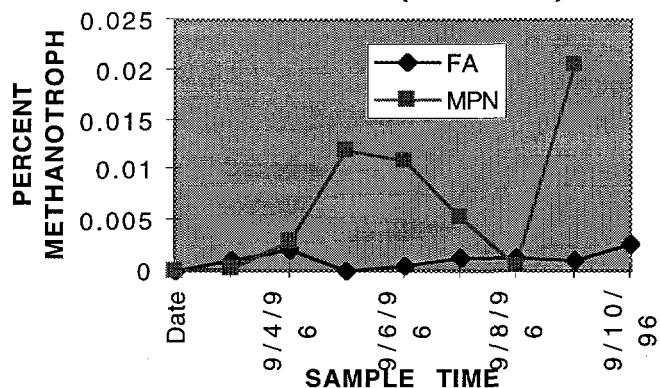
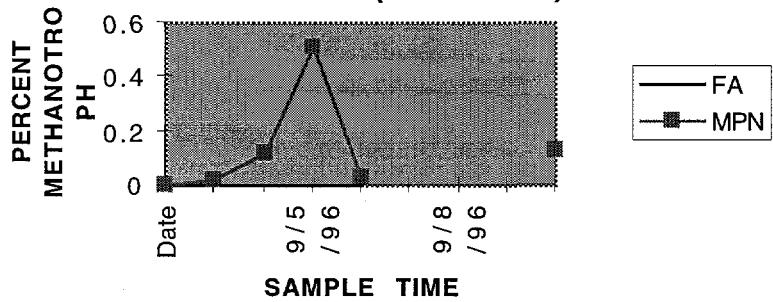


Figure 2c
**Ratio of Methanotrophs to Total
counts (Well 14S)**



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Sanitary Landfill Supplemental Test Saturated Zone Piezometer

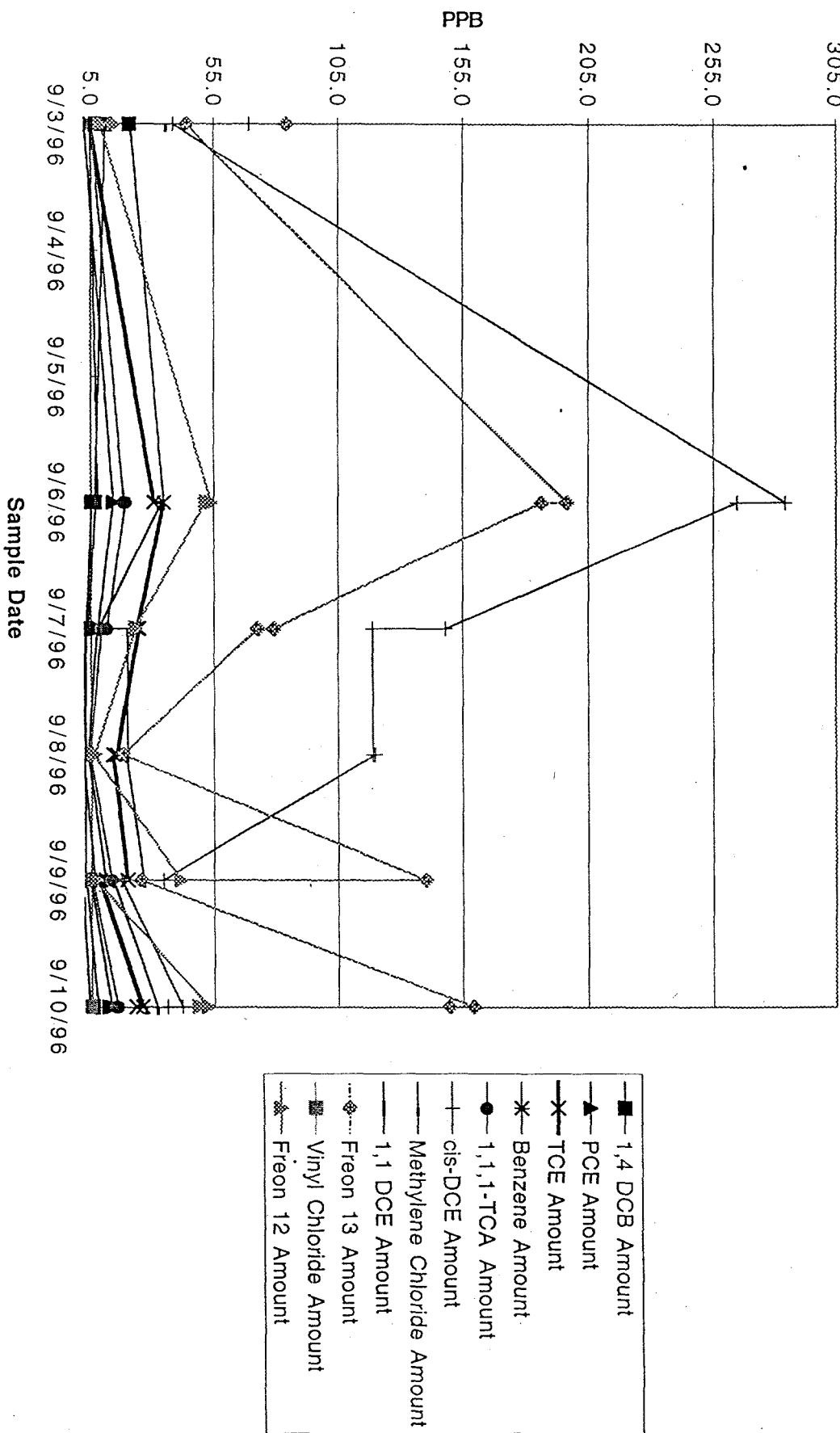
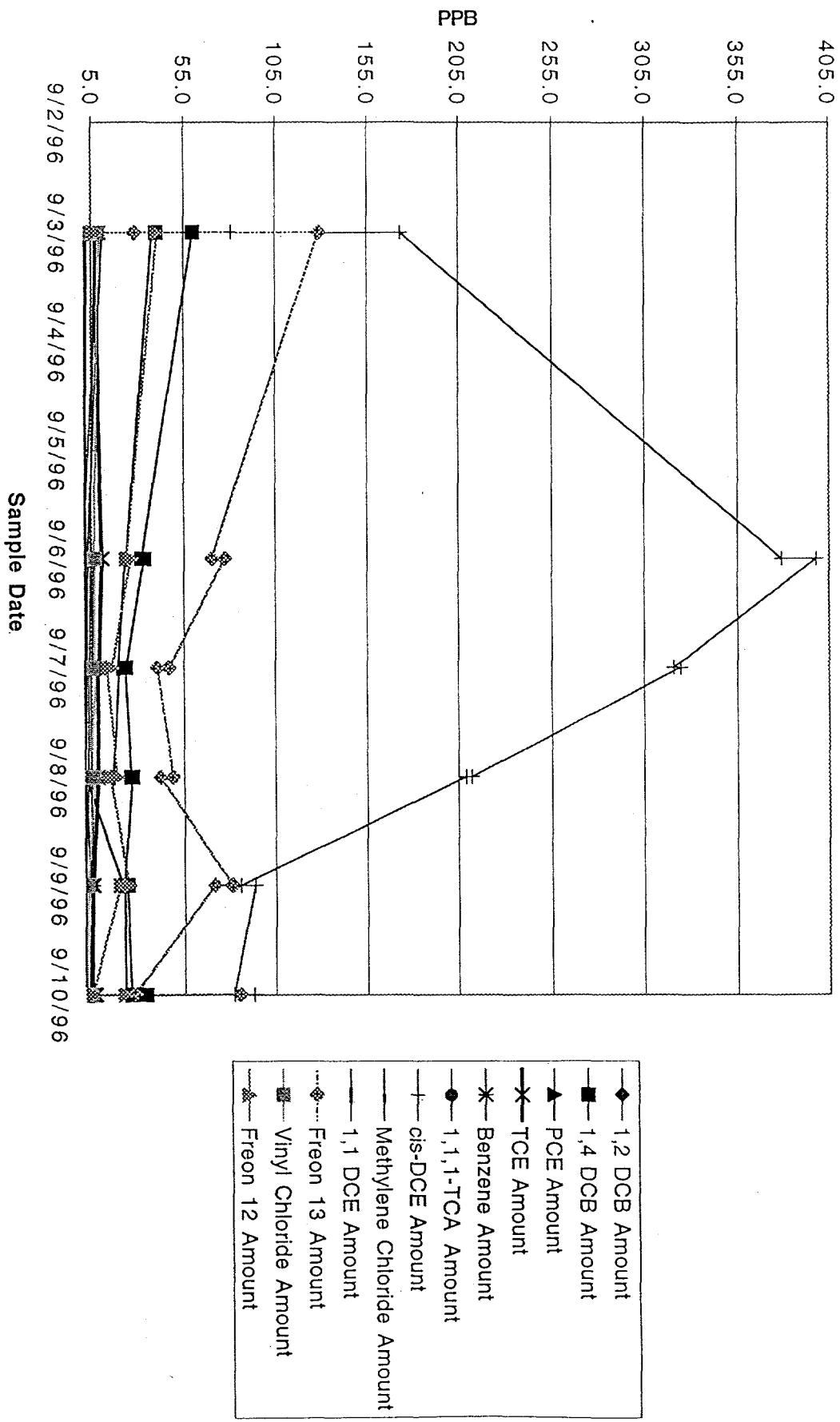


Fig 3a

Fig 36

Sanitary Landfill Supplemental Test
 Saturated Zone Piezometer
 9 Shallow



Sanitary Landfill Supplemental Test
Saturated Zone Piezometer
14 Shallow

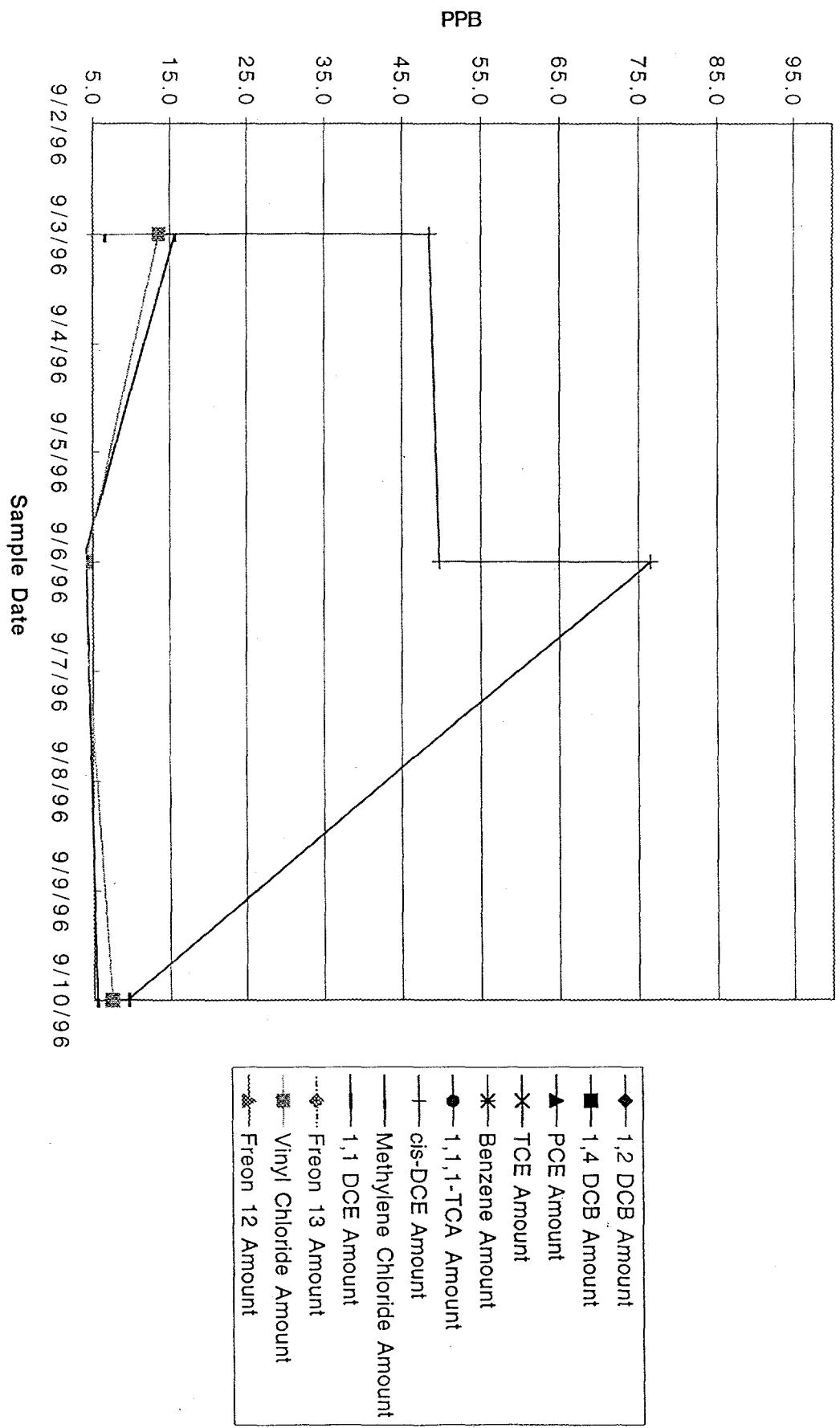


Fig 3c