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Immunological Techniques as Tools to Characterize the Subsurface Microbial Community at a Trichloroethylene Contaminated Site (U)

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DISCLAIMER

Immunological Techniques as Tools to Characterize the Subsurface Microbial Community at a Trichloroethylene Contaminated Site.

C. B. FLIERMANS*, J. M. DOUGHERTY, M. M. FRANCK, P. C. McKINZEY and T. C. HAZEN. Savannah River Technology Center, Aiken, SC.

ABSTRACT

Effective *in situ* bioremediation strategies require an understanding of the effects pollutants and remediation techniques have on subsurface microbial communities. Therefore, detailed characterization of a site's microbial communities is important. Subsurface sediment borings and water samples were collected from a trichloroethylene (TCE) contaminated site, before and after horizontal well *in situ* air stripping and bioventing, as well as during methane injection for stimulation of methane-utilizing microorganisms. Subsamples were processed for heterotrophic plate counts, acridine orange direct counts (AODC), community diversity, direct fluorescent antibodies (DFA) enumeration for several nitrogen-transforming bacteria, and Biolog® evaluation of enzyme activity in collected water samples. Plate counts were higher in near-surface depths than in the vadose zone sediment samples. During the *in situ* air stripping and bioventing, counts increased at or near the saturated zone, remained elevated throughout the aquifer, but did not change significantly after the air stripping. Sporadic increases in plate counts at different depths as well as increased diversity appeared to be linked to differing lithologies. AODCs were orders of magnitude higher than plate counts and remained relatively constant with depth except for slight increases near the surface depths and the capillary fringe. Nitrogen-transforming bacteria, as measured by serospecific DFA, were greatly affected both by the *in situ* air stripping and the methane injection. Biolog® activity appeared to increase with subsurface stimulation both by air and methane. The complexity of subsurface systems makes the use of selective monitoring tools imperative.

BACKGROUND

The Integrated Demonstration Project at Savannah River Site (SRS) provides a unique blend of collaborative partners from industry, academia and government, while building on the funding of related research projects. The Gas Research Institute (GRI) in collaboration with SRTC (Savannah River Technology Center) has been funding research and development of a methanotrophic treatment process for trichloroethylene contaminated groundwater for the past 4 years. University and industry investigators funded by GRI have been integrated with the experience and expertise of several national laboratories (DOE and EPA) to provide the greatest experience and resource of bioremediation expertise ever assembled for any bioremediation demonstration.

During this project, indigenous microorganisms were stimulated through the use of dual horizontal wells to degrade TCE, tetrachloroethylene (PCE) and their daughter products *in situ* by the addition of gaseous nutrients to the contaminated zone. Horizontal wells form the basis for the SRS Integrated Demonstration Project and have provided a significant advantage over vertical wells and conventional bioremediation techniques. The increased surface area has allowed better delivery of nutrients and more efficient recovery of gas and water, as well as minimizing formation clogging and plugging phenomena. Biodegradation is a highly attractive remediation technology because contaminants are destroyed, not simply moved to another location or immobilized. Bioremediation has been shown to be among the least costly technologies where applicable.

Since air/methane mixtures have been shown to stimulate selected members of the indigenous microbial community that have the capability to degrade TCE, the principal nutrients supplied via the horizontal wells was methane (1-4%) and nitrogen in air. While the lower horizontal well provided a very efficient delivery of gas throughout the contaminated region, a vacuum was applied to the upper well (vadose zone). Such a combination encouraged air/methane movement through the upper saturated zone and lower vadose zone while inhibiting the spreading of the organic plume. An extensive monitoring program using existing monitoring wells and soil borings has served to determine the biological response in the soil, sediment, and groundwater systems following the injection of air/methane. Data from Phase I (air injections) and Phase II (1% and 4% methane injection) of the Integrated Demonstration Project have illustrated the effectiveness of *in situ* bioventing and methane injection for the bioremediation of TCE and PCE.

This manuscript centers on the biological effects of the remediation actions on selected microbiological components in the zones of influence of the horizontal wells. The data are derived from the use of serospecific fluorescent antibodies to detect and enumerate selected microbial populations. Additionally, the analyses included the use of Biolog® technology on groundwater samples to determine the degradative capability of the microbial populations with respect to 95 different carbon and energy sources.

MATERIALS AND METHODS

Soil, sediment and groundwater samples were collected aseptically as previously described (Phelps *et al.* 1989; Fliermans and Balkwell, 1989; Fliermans and Hazen, 1990). Viable bacterial densities were determined by plating samples on laboratory medium as described by Balkwell and Ghiorse (1985). Total bacterial densities in each soil, sediment and groundwater sample were measured by direct epifluorescence microscopy using AODC techniques of Balkwell and Ghiorse (1985).

Selected bacterial strains were enumerated using a modified direct immunofluorescent technique with serospecific polyvalent fluorescent antibodies (Fliermans *et al.*, 1992). These antibodies had been prepared as described by

Schmidt, 1968 and Fliermans *et al.*, 1974. These bacterial isolates included the following:

- *Azotobacter chroococcum* ,a free-living nitrogen fixer that lives under aerobic conditions;
- *Nitrosolobus multiformis* and *Nitrosomonas europea*; chemolithotrophs that obtain their energy from oxidizing ammonia to nitrite while fixing CO_2;
- *Nitrobacter agilis* and *Nitrobacter winogradski*, chemolithotrophs that secure their energy by oxidizing nitrite to nitrate while fixing CO_2;
- *SRL-MIIF*, a Type II methanotroph isolated from SRS with trichloroethylene (TCE) degradative capabilities;
- *Methanobacterium formicicum* , a strict anaerobe that produces CH_4;
- *Thiobacillus ferrooxidans* a chemolithotroph that gets its energy by oxidizing ferrous iron to ferric iron while fixing CO_2;
- *Legionella pneumophila* Serogroup 1, a human pathogen and etiological agent of Legionnaires' Disease as well as an aquatic and terrestrial bacterium;
- *Bradyrhizobium japonicum* ,a symbiotic nitrogen-fixer that is present in association with soybean nodulation and thus its niche is primarily close to the soil surface.

Unconcentrated groundwater samples were loaded (150µl/well) into each well of GN Biolog® plates and incubated at the *in situ* groundwater temperature, 23°C. The plates were read after 2 weeks incubation and the optical density of the reduced color in each of the wells was recorded. The data were expressed as optical density for each of the 95 organic compounds tested and grouped into reactive groups as previously described (Gorden, *et al.*, in press). The enzyme activity associated with each of the degradative activities was expressed in terms of the location of the sampled well along with the perturbation occurring at the time of sampling.

RESULTS

The positioning of the monitoring wells sampled during this investigation for the Integrated Demonstration Project is shown in Figure 1. Generally the lower numbered wells, MHT 1-7, are more affected by the changes being incorporated into the horizontal wells, while the higher numbered wells, MHT 8-11, are less affected. Water samples were collected on a bimonthly basis from each of these wells, while sediment samples were collected during the initial drilling of the wells.

In this manuscript the data from the groundwater sampling will center on the use of DFA and Biolog® techniques for assessing selected bacterial populations in the groundwater. The techniques provide data necessary to determine the population densities of the selected nitrogen cycling bacteria in

the subsurface and the extent that other selected microbial populations are affected by the perturbations made during bioventing and methane injection investigations.

The data in Figure 2 show the total bacterial population density in well MHT-2C as measured by AODC with respect to the DFA concentrations of *Azotobacter chroococcum* . The data for MHT-2C indicate that the densities of *A. chroococcum* are greatest just after the beginning of the air injection and the densities fall off rather dramatically after the start of the methane injections. The AODCs for MHT-2C are relatively constant as one might suspect. There was an appreciable dip in total bacterial counts after the 4% methane injection began but the systems recovered to preinjection levels. Wells MHT-4C and 9C showed the same kind of response in that the free-living nitrogen fixing *Azotobacter* had the highest consistent levels before the bioventing stopped and was greatly reduced after the methane injections began. MHT-9C was less affected by the perturbations in the horizontal wells because of its juxtaposition, but the *Azotobacter* populations were highly variable being at preinjection levels at one sampling time and below detectable limits the next. Air injection for the bioventing consisted of 70% nitrogen so that the utilization of this air as a nitrogen source and subsequent growth by the free-living nitrogen fixers is consistent with the data.

The data in Figure 3 show the total bacterial population density in MHT-2C as measured by AODC with respect to DFA concentrations of *Nitrosomonas europea*, and *Nitrosolobus multiformis*. The first step of nitrification after the fixation of nitrogen is the oxidation of the ammonia to nitrite. Thus if the population densities of the free-living nitrogen fixers were to increase, it is reasonable to assume that the bacterial populations of the chemoautotrophic nitrifiers might increase as well. The data for MHT-2C indicates that the densities of *N. europea* were greatest just after the beginning of the air injection and the densities fall off dramatically after the start of the methane injections. The AODCs for MHT-2C are relatively constant as one might expect. The MHT-4C well samples remain fairly constant during the bioventing but drop off again sharply with the injection campaign of methane. MHT-9C demonstrates the same phenomenon in that once methane injections began the concentrations of 86% of the samples are below detectable limits of 1 *Nitrosomonas europea* per milliliter of groundwater. *Nitrosolobus multiformis* was never detected in very large concentrations in any of the samples and appears to play a rather minor role, numerically speaking, from the standpoint of ammonia oxidation.

The data in Figure 4 show the total bacterial population density in MHT-2C as measured by AODC with respect to DFA concentrations of *Nitrobacter agilis* and *Nitrobacter winogradski* . These *Nitrobacter* populations appear to follow the same pattern as the ammonia-oxidizing bacteria in that once the methane injections began the densities of the nitrogen transforming bacteria declined dramatically. MHT-2C,4C and 9C demonstrate the same phenomenon in that once methane injections began, the concentrations of 70% of the samples are below detectable limits of 1 *Nitrobacter agilis* and *Nitrobacter winogradski* per milliliter of groundwater.

The data in Figure 5 show the total bacterial population density in MHT-2C as measured by AODC with respect to DFA concentrations of *SRL-MIIF*. The data indicate that the organism is not readily observed until after the methane injections had begun. Of the 12 samples collected before methane injection only 25% showed detectable levels of the bacterium while 36% showed its presence after the methane injections had begun.

The data in Figure 6 show the total bacterial population density in MHT-2C as measured by AODC with respect to DFA concentrations of *Methanobacterium formicicum*. The data from the MHT-2C seems to indicate that the organism was present more often before the methane injections than after its injections. This is somewhat difficult to understand since the organism is a strict anaerobe and seemingly the bioventing would cause an increase in aerobic rather than anaerobic niches. On the other hand the presence of methane during the injection cycles may have caused a feed back inhibition for *Methanobacterium formicicum*. As has been shown previously in soil microbiology, niches of anaerobiosis exist even in aerated soils and sediments such as those present at SRS.

The data in Figure 7 show the total bacterial population density in MHT-2C as measured by AODC with respect to DFA concentrations of *Thiobacillus ferrooxidans*. The data indicate that this particular strain of *Thiobacillus ferrooxidans* is not affected either by the air or methane injections in that the population densities of the organism did not change with respect to either one of these perturbations in the horizontal wells.

Finally, the data in Figure 8 show the total bacterial population density in MHT-2C as measured by AODC with respect to DFA concentrations of *Legionella pneumophila* Serogroup 1. The data indicate that the perturbations made in the Integrated Demonstration Project did not affect the densities of *Legionella pneumophila* Serogroup 1.

Bradyrhizobium japonicum, was not found in any of the groundwater samples, although it was observed in the shallow soil samples from the site (data not shown).

Biolog® plates were used to determine the activity of the microbial consortia present in the groundwater samples collected from the wells. Groundwater samples from each sampling period were inoculated into triplicate GN Biolog® test plates. Plate were read using a Biolog® plate reader and the optical density of the tetrazolium dye recorded. The Biolog® automated plate reader has been factory programmed to sense a threshold optical density value for each plate based on the color of the reference well (A-1) in the Biolog® plate. Therefore, optical density readings which determined each inoculum pattern, were significantly above the threshold level. The density of the tetrazolium reactions, a measure of the ability of the microorganisms to metabolize the individual compounds listed in Table 1, were plotted with respect to the perturbations made in the horizontal wells.

The reactions were grouped based on their chemical relationships and plotted. The data in Figure 9a and 9b show the reaction of the microbial

community in MHT-2C with respect to ester and amine utilization, respectively over the duration of the Integrated Demonstration Project. While very little change in the ester utilizing population was observed over the course of the investigations, substantial variability was observed with respect to the amine utilization. Both the amine and amide utilization data appear to demonstrate that each perturbation (bioventing, 1% CH_4 and 4% CH_4) increased the ability of the population to utilize the amine group of compounds. Once this surge of utilization was completed, the population of microorganisms or their enzyme systems responsible for the utilization would fall to a lower level. Previous studies (Fliermans and Balkwell, 1989; Fliermans and Hazen, 1990) on the subsurface microbial populations at SRS have demonstrated the stimulation of microbial populations to nitrogen additions. This same phenomenon was observed for glycerol; 2,3 butanediol; bromosuccinic acid; polymer group; phosphorylated, and aromatic compounds, but not for carboxylic acids or alcohols.

DISCUSSION

There are two basic approaches to the study of microbial systems in nature. They are the synecological and the autecological approaches. Both have their merits and short comings. The synecological approach is one in which the entire microbial community is investigated in its relationship to a given habitat. In this approach there is little concern about the type of microorganisms involved in the functioning of the habitat, but rather the extent of microbial processing that takes place. This approach provides the investigator with process-oriented data and information for an overall picture of the functioning of the habitat. Such data are highlighted by information on the cycling of nutrients through an ecosystem and the transformation of chemical components of the system by microorganisms intrinsic to the ecosystem without regard to the microorganisms involved. Such investigations often rely on the mineralization or transformation of particular compounds which have been isotopically labeled thus providing a marker for transformation assessments. The markers can thus be followed during various stages of incubation, degradation, and mineralization enabling one to measure breakdown products, intermediates and rates.

On the other hand, the autecological approach focuses on a particular microorganism and asks questions with regard to that organism and how it functions in the studied habitat. The data and information gained from this perspective are exceedingly valuable particularly when one is dealing with an organism that is potentially pathogenic or is greatly involved in a functional aspect of the ecosystem. Such an approach allows one to pursue questions about the organism, its particular functioning in a given ecological niche, and the potential control of the organism that could not otherwise be made. The disadvantage of the autecological approach is that it requires specialized techniques for the identification and assessment of the bacterium. Few, if any, bacteria can be reliably characterized by their morphological structure even at the genus level. Many bacteria are pleomorphic depending on their nutritional

status and thus morphological considerations are not acceptable means of assessment.

The apparent lack of techniques for autecological investigations is overcome through the use of specific serological and genetic probes which can identify the bacterium to a particular species and subgroup without culturing the organism. Under appropriate conditions the DFA technique can evaluate a bacterium as to its viability, state of physiological health, and transformation of selected isotopically labeled compounds using cytochrome activity (Fliermans *et al.* 1981), fluorescent probe (Rodriguez *et al.* 1992), and microautoradiographic (Fliermans and Schmidt, 1975) techniques, respectively.

Our discussion centers on the autecological approach to the ecology of these selected bacteria. Specific polyvalent and/or monoclonal fluorescent antibody probes have been developed to view, enumerate and access the role of these bacteria in complex ecological environments that are neither sterile, monoculture, nor easily sampled. Immunofluorescent techniques were initially used in diagnostic medical microbiology and only in the last few decades have they been employed in the areas of microbial ecology for agriculture, terrestrial and aquatic microbiology investigations. More recently DFA has been used to define the ecology of microorganisms associated with the degradation of toxic and hazardous wastes.

As with any technique there are pros and cons with which one must deal. The DFA technique is capable of visualizing a bacterium in very complex habitat media of soils, metal corrosion piping, sediments, vascular plant material, sewage sludge, and virtually any aquatic habitat. If one employs the DFA technique for any of the defined bacteria, as little as a single cell per ml of sample can be visualized. This detection limit could be pushed even further given a larger sample size and a greater concentration factor, but the value of such is not apparent. DFA prepared with serospecific polyvalent antibodies against whole cells or cell walls of the bacterium are the most useful tools to date for quantification and visualization of the bacterium in natural habitats. Using polyvalent antibodies, the DFA can be made specific enough to react with single species or serogroups of any of the species without cross-reacting with nonhomologous organisms outside the serogroup as well as other species or genera of bacteria.

The DFA approach can also be used with monoclonal antibodies, although monoclonal antibodies have relatively little use in a real world situation. This lack of functionality is due to the high specificity of the antibody allowing only a very limited number of organisms in one serotype to react with the DFA prepared from monoclonal antibodies. Although other species may be present in the habitat, if it is not the one reacting with the specific monoclonal antibody, it will not be detected and thus the number of staining bacteria will be greatly underestimated. Monoclonal applications are valuable in the typing of specific groups of bacteria within the genus, species and serogroup of the homologous strain that may have come from a selected environment and caused a specific clinical infection. In these circumstances monoclonals can be used to accurately

determine the similarity of the organisms both in diagnostic and forensic investigations.

A second limitation of the DFA technique is that the DFA conjugate stains intact organisms, whether alive or dead. If the organism being detected were lysed either by natural events in their habitat or through man-made events (i.e., addition of biocides), cell wall debris caused by such events will stain and be observed microscopically. While at first blush this may seem to be a problem, in the real world it is less of a problem because bacteria are an excellent source of nutrients being at the lowest end of the food chain and thus once dead they are no longer capable of keeping the "wolves" from the door and are readily lysed and serve as food for other microbial populations.

Thus, in a nonsterile aquatic or terrestrial habitats, the bacterium, once it dies, is not readily detected because the organism does not remain intact for an extended period of time. This phenomena has been observed in sterile soils and aquatic systems. Known DFA positive organisms were killed and added back to the sterile system, and the DFA positive bacteria remained intact for extended periods of time (days). In nonsterile systems, the DFA positive organisms are made into debris within a few hours. Thus it is likely that the intact bacteria that one observes in the habitat are viable, but maybe nonculturable as Hussong *et al.*, (1987) have demonstrated for *Legionella*.

CONCLUSIONS

The data from these autecological investigations associated with the Integrated Demonstration Program on Bioremediation indicate that selected microbiological communities can be efficiently, effectively and accurately monitored using immunofluorescent techniques. It is necessary to note that while these immunofluorescent probes are specific for the microbial constituents, they may or may not be representative of the specific bacteria that are present in these subsurface environments. Only one organism, *SRL-MIIF*, was isolated from the Integrated Demonstration Site before the specific DFA probes were made. While the other bacteria were not isolated from the site, they are generic of the organisms present in the subsurface and are likely to be the bacteria associated with the various transformations particularly for the nitrogen cycling.

The use of Biolog® GN plates provides the capability of evaluating microbial systems adapted to metabolizing selected chemicals as their sole carbon and/or energy source. In adapting the Biolog® system to groundwater sampling, the goal was not to provide a constant level of microbial inoculum but rather a constant volume of groundwater. This supplies the Biolog® plates with a bacterial density of ca. 10^4-10^5 cells of a variety of species. Thus the time of incubation of the Biolog® plates needs to be standardized for the particular groundwater but it is generally a 2-week period of time at groundwater temperatures of 23°C. Several concentrations of the microorganism in the groundwater can be used simply by concentrating the sample. Based on the

results from the GN screening plates, the best metabolite or group of metabolites for growth may be chosen for further investigation or added to MT plates for further analyses (Gorden, *et al.* in press). Thus, these results suggest that Biolog® GN plates are useful tools for screening bacterial isolates and consortia to determine their ability to survive, to metabolize, to degrade selected organic chemicals, and potentially to allow one to screen groundwater systems for compounds that are useful in stimulating the microbial populations during bioremediation projects.

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TABLE 1. Sole carbon sources present in Biolog® GN microtiter plates.

Carbohydrates

N-Acetyl-D-galactosamine
N-Acetyl-D-glucosamine
Adonitol
L-Arabinose
D-Arabitol
Cellobiose
i-Erythritol
D-Fructose
L-Fucose
D-Galactose
Gentiobiose
α-D-Glucose
m-Inositol
α-Lactose
Lactulose
Maltose
D-Mannitol
D-Mannose
D-Melibiose
ß-Methylglucoside
D-Psicose
D-Raffinose
L-Rhamnose
D-Sorbitol
Sucrose
D-Trehalose
Turanose
Xylitol

Esters

Mono-methylsuccinate
Methylpyruvate

Polymers

Glycogen
α-Cyclodextrin
Dextrin
Tween 80
Tween 40

Carboxylic acids

Acetic acid
cis-Aconitic acid
Citric acid
Formic acid
D-Galactonic acid lactone
D-Galacturonic acid
D-Gluconic acid
D-Glucosaminic acid
D-Glucoronic acid
α-Hydroxybutric acid
β-Hydroxybutric acid
γ-Hydroxybutric acid
ρ-Hydroxyphenylacetic acid
Itaconic acid
α-Keto butyric acid
α-Keto glutaric acid
α-Keto valeric acid
D,L-Lactic acid
Malonic acid
Propionic acid
Quinic acid
D-Saccharic acid
Sebacic acid
Succinic acid

Alcohols

2,3-Butanediol
Glycerol

Amides

Succinamic acid
Glucuronamide
Alaninamide

Phosphorylated chemicals

D,L-α-Glycerol phosphate
Glucose-1-phosphate
Glucose-6-phosphate

Amino Acids

D-Alanine
L-Alanine
L-Alanyl-glycine
L-Asparagine
L-Aspartic acid
L-Glutamic acid
Glycyl-L-aspartic acid
Glycyl-L-glutamic acid
L-Histidine
Hydroxy-L-proline
L-Leucine
L-Ornithine
L-Phenylalanine
L-Proline
L-Pyroglutamic acid
D-Serine
L-Serine
L-Threonine
D,L-Carnitine
γ-Aminobutyric acid

Aromatic chemicals

Inosine
Urocanic acid
Thymidine
Uridine

Brominated chemicals

Bromosuccinic acid

Amines

Phenylethylamine
2-Aminoethanol
Putrescine

LIST OF FIGURE AND TABLE CAPTIONS

FIGURE 1. Location of horizontal and monitoring wells at the M-Area Integrated Demonstration Project at Savannah River Site.

FIGURE 2. Total bacterial population in MHT-2C as measured by AODC with respect to DFA for *Azotobacter chroococcum* in MHT-2C, 4C and 9C.

FIGURE 3. Total bacterial population in MHT-2C as measured by AODC with respect to DFA for *Nitrosolobus europea* and *Nitrosolobus multiformis* in MHT-2C, 4C and 9C.

FIGURE 4. Total bacterial population in MHT-2C as measured by AODC with respect to DFA for *Nitrobacter agilis* and *Nitrobacter winogradski* in MHT-2C, 4C and 9C.

FIGURE 5. Total bacterial population in MHT-2C as measured by AODC with respect to DFA for *SRL-MIIF* in MHT-2C, 4C and 9C.

FIGURE 6. Total bacterial population in MHT-2C as measured by AODC with respect to DFA for *Methanobacterium formicicum* in MHT-2C, 4C and 9C.

FIGURE 7. Total bacterial population in MHT-2C as measured by AODC with respect to DFA for *Thiobacillus ferrooxidans* in MHT-2C, 4C and 9C.

FIGURE 8. Total bacterial population in MHT-2C as measured by AODC with respect to DFA for *Legionella pneumophila* Serogroup 1 in MHT-2C, 4C and 9C.

FIGURE 9a. Activity of microbial community populations in the groundwater of MHT-2C with respect to esters.

FIGURE 9b. Activity of microbial community populations in the groundwater of MHT-2C with respect to amines.

TABLE 1. Sole carbon sources present in Biolog® GN microtiter plates.

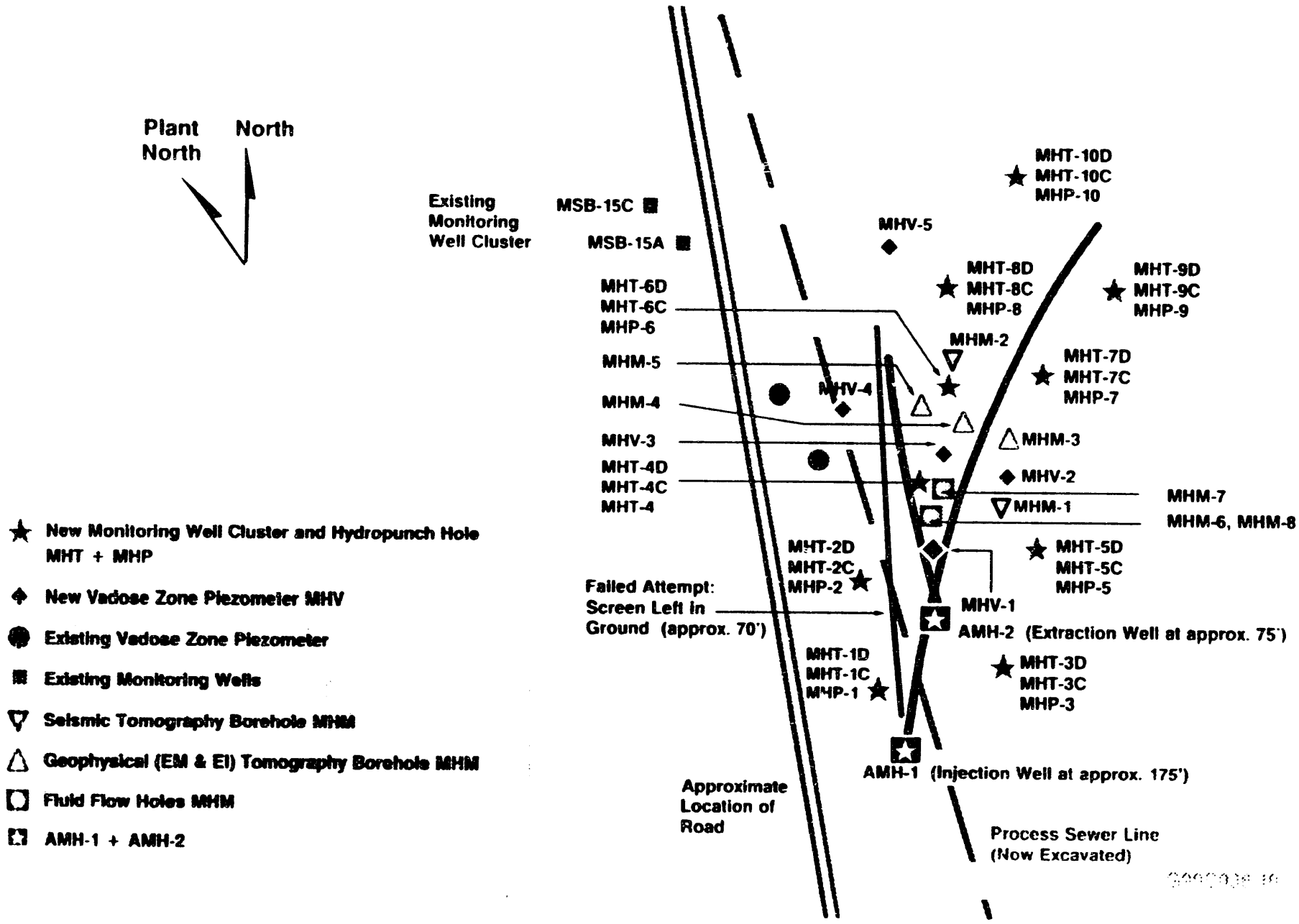

Plant North
North
Existing Monitoring Well Cluster
MSB-15C
MSB-15A
MHT-10D
MHT-10C
MHP-10
MHV-5
MHT-8D
MHT-8C
MHP-8
MHT-9D
MHT-9C
MHP-9
MHT-6D
MHT-6C
MHP-6
MHM-2
MHM-5
MHT-7D
MHT-7C
MHP-7
MHV-4
MHM-4
MHM-3
MHV-3
MHV-2
MHT-4D
MHT-4C
MHT-4
MHM-7
MHM-1
MHM-6, MHM-8
MHT-2D
MHT-2C
MHP-2
MHT-5D
MHT-5C
MHP-5
Failed Attempt: Screen Left in Ground (approx. 70')
MHV-1
AMH-2 (Extraction Well at approx. 75')
MHT-1D
MHT-1C
MHP-1
MHT-3D
MHT-3C
MHP-3
AMH-1 (Injection Well at approx. 175')
Approximate Location of Road
Process Sewer Line (Now Excavated)
New Monitoring Well Cluster and Hydropunch Hole MHT + MHP
New Vadose Zone Piezometer MHV
Existing Vadose Zone Piezometer
Existing Monitoring Wells
Seismic Tomography Borehole MHM
Geophysical (EM & EI) Tomography Borehole MHM
Fluid Flow Holes MHM
AMH-1 + AMH-2

Figure 2 - *Azotobacter chroococcum*

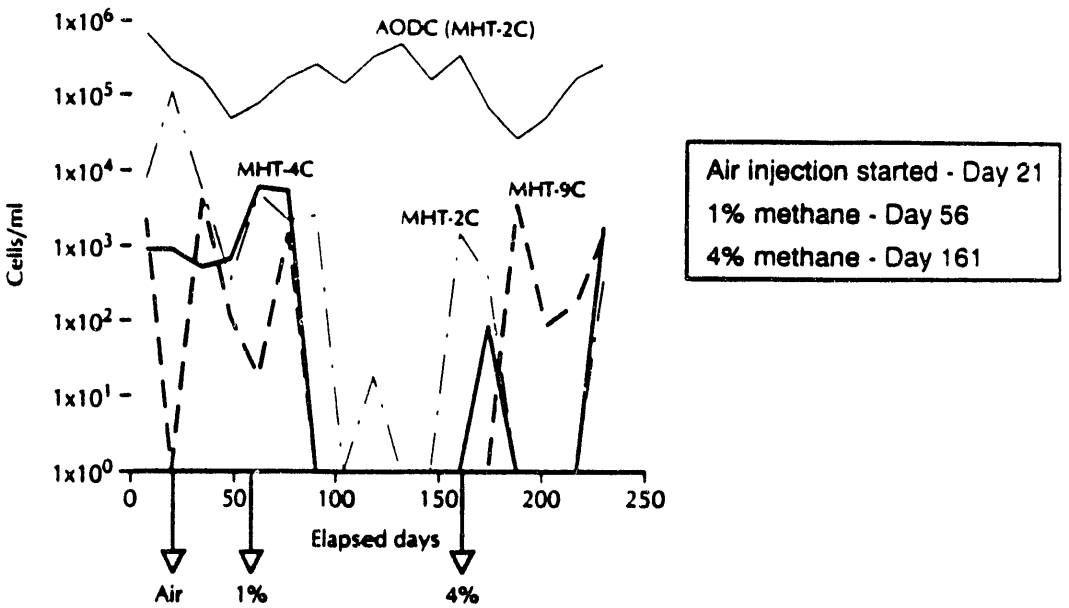


Figure 3 - *Nitrosolobus multiformis and Nitrosomonas europea*

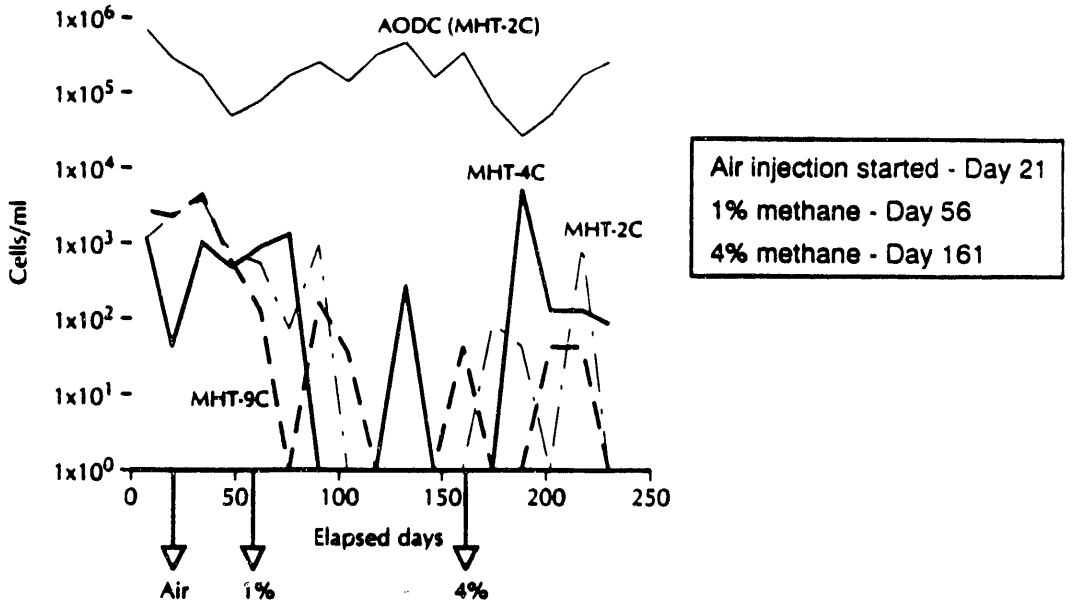


Figure 4 - *Nitrobacter agilis/winogradski*

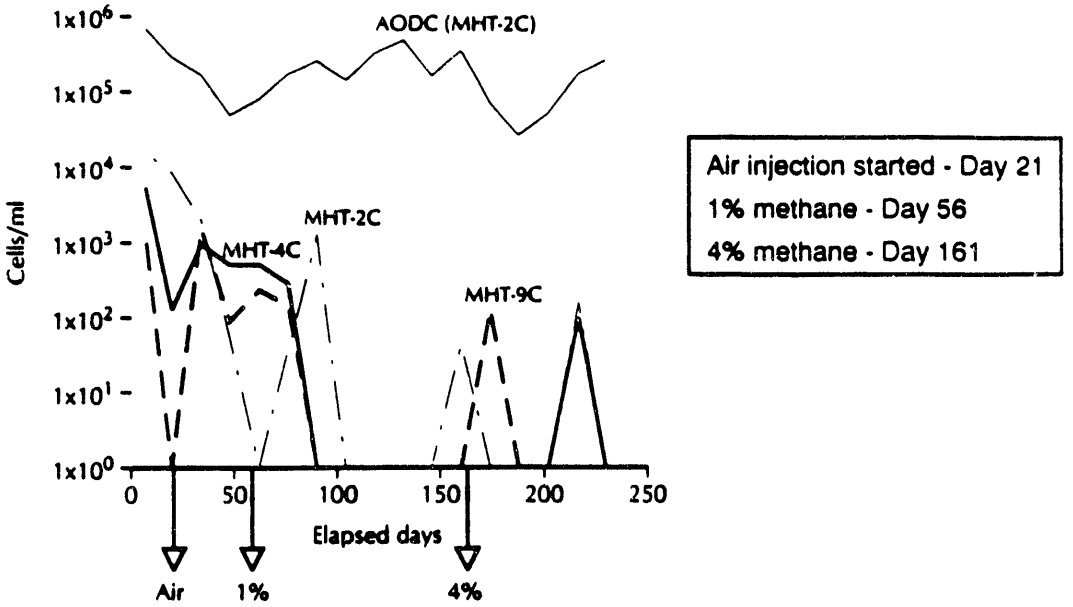


Figure 5 - *SRL-MIIF*

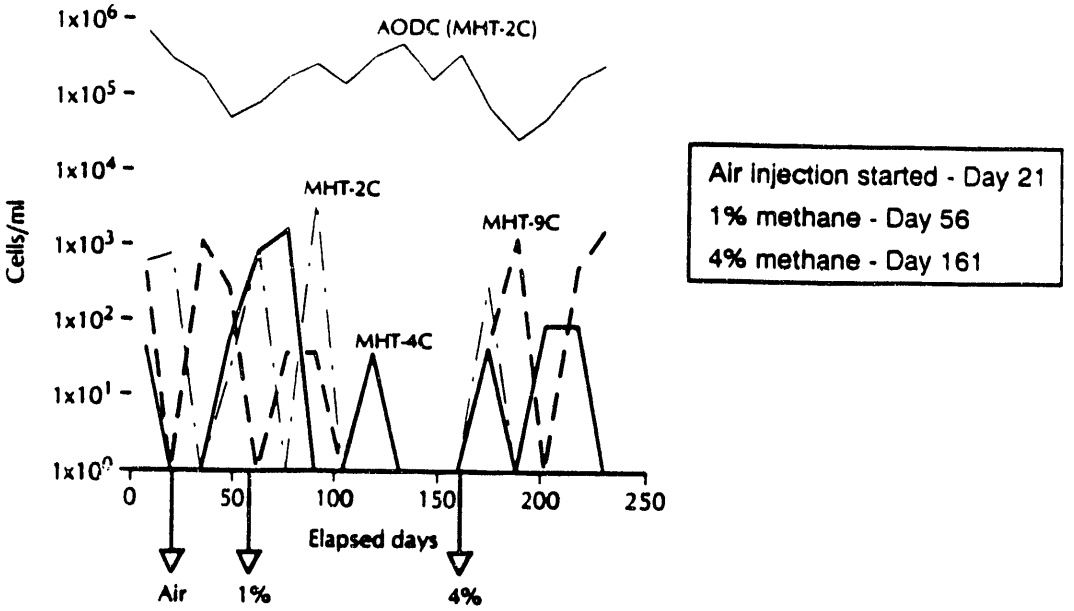


Figure 6 - *Methanobacterium formicicum*

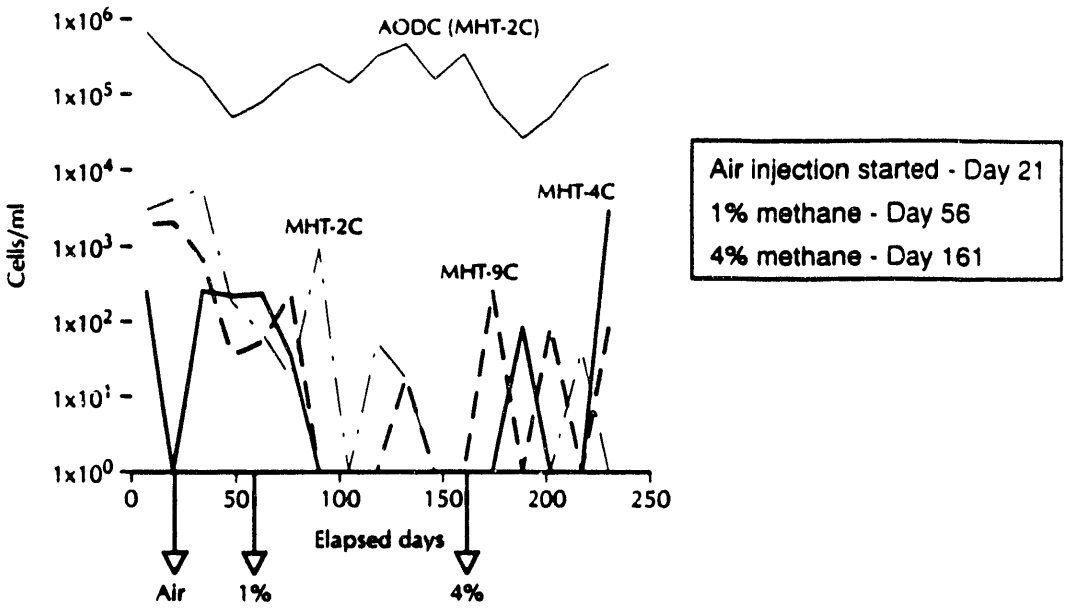


Figure 7 - *Thiobacillus ferrooxidans*

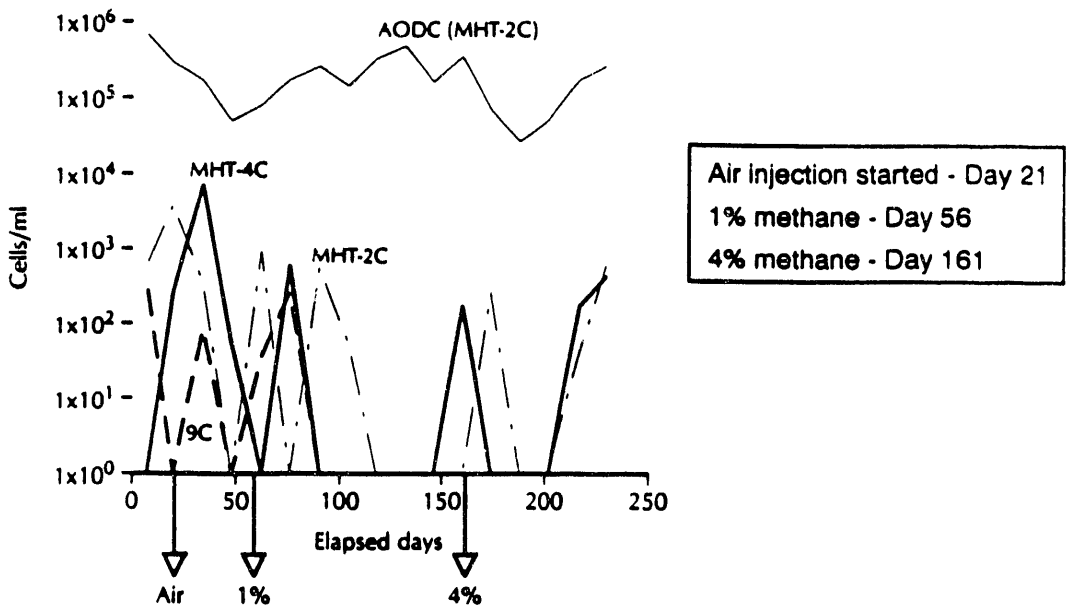


Figure 8 - *Legionella pneumophila*

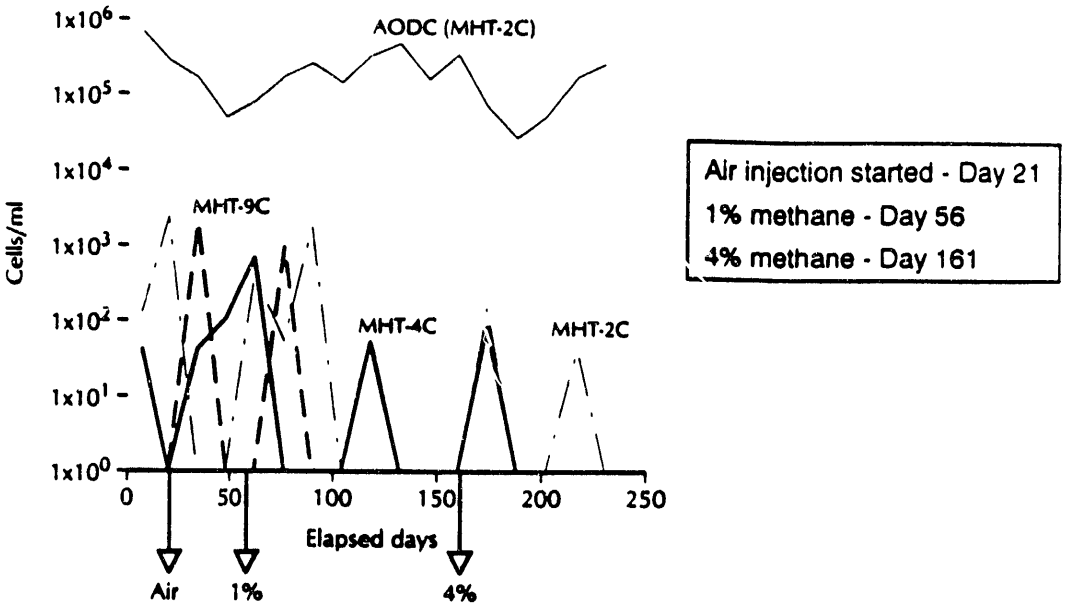


Figure 9a - MHT-2C Ester Utilization

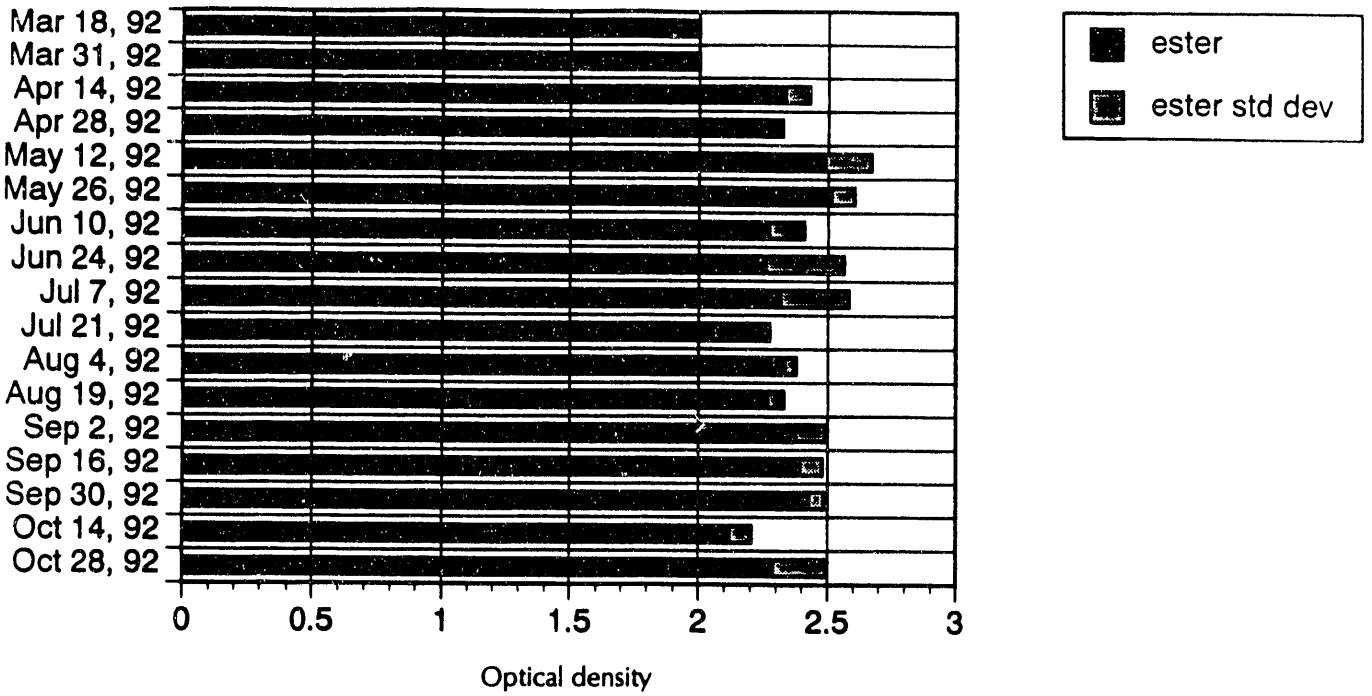
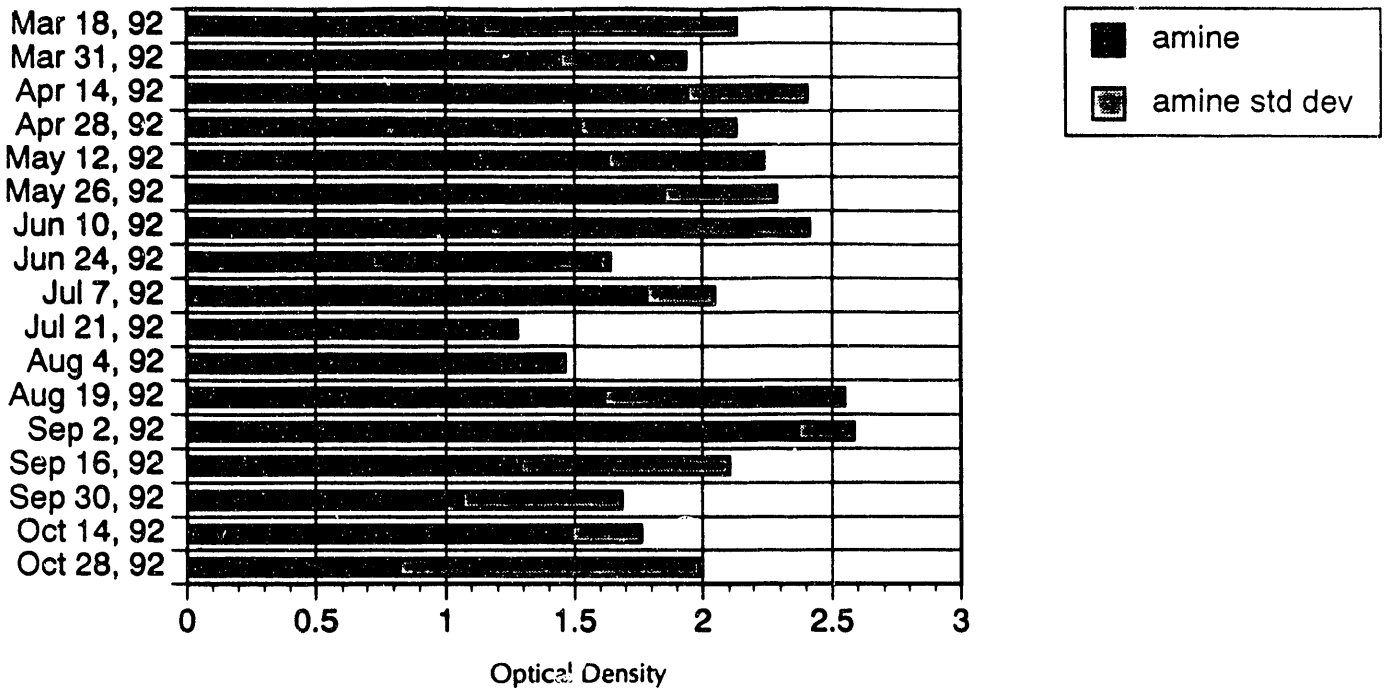


Figure 9b - MHT-2C Amine Utilization



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