DOE/ER/60848--1

DE90 009956

Aquifer Microcosms and In Situ Methods to Test the Fate and Function of Pollutant-Degrading Microorganisms.

Mary Lou Krumme[3], Suzanne M. Thiem[1], Richard L. Smith[2], Daryl Dwyer[3], and James M. Tiedje[1]

[1]Michigan State University, [2]U.S. Geological Survey, [3]German Institute of Biotechnology

Little information is available on groundwater microorganism ecology, and specifically on the distribution and biochemical diversity of pollution degrading microorganisms in the aquifer. The lower cost of using microorganisms for in situ remediation is a driving force to develop this concept into a reliable technology. While the introduction of nutrients and electron acceptors may stimulate natural populations to degrade certain pollutants, low levels of pollutants and complex mixtures of pollutants may require the modification of natural populations through selective pressure or by means of genetic engineering. To determine the appropriate bioremediation strategy requires knowledge of how native organisms function in the aquifer as well as the fate (ie. dispersal, survival, and gene stability) and function (ie. gene expression and competitiveness) of introduced organisms.

This study was designed to address these issues by examining three populations of substituted aromatic compound-degraders: an indigenous population, an introduced degrader, and a genetically engineered microorganism (GEM) in the environmental conditions of a sand and gravel aquifer. The goals of this study are: 1) To gain field experience on the fate and function of pollutant-degrading organisms in the aquifer. 2) To evaluate column microcosms and survival chambers as tools for predicting the fate and function of selected and modified bacterial strains as appropriate aquifer bioremediation agents. To meet these goals, the study utilizes the combined expertise of the German Institute of Biotechnology GBF (well characterized pollutant-degrading parent and GEMs), Michigan State University (environmental probe technology), and the U. S. Geological Survey (well studied field site already instrumented with

---

a large number of observation wells and field experience with introduced microorganisms).

The study site, a sand and gravel aquifer on Cape Cod, Massachusetts, contains a plume of sewage contamination. The hydrology of the the aquifer and the contaminant plume is well documented and it is possible to obtain both water and sediment samples, as well as drill wells appropriate to measure field dispersal. The model pollutant degrading-organisms used for the study are *Pseudomonas sp.* B13, its genetically engineered derivative, *P. sp.* B13 FR1 p(FRC20p) (FR120) and *P. putida* (TOL). *P. sp.* B13 degrades 3-chlorobenzoate (3CB) while the substrate range of the GEM, FR120, has been extended to include 4-chlorophenol, 4-methylbenzoate (4MB), 4-methylphenol, and 4-chlorobenzoate. *P. putida* (TOL) degrades toluene through a catabolic pathway encoded on the genetically well-characterized TOL plasmid.

The experimental protocol for the development of aquifer microcosms is shown in Figure 1. In phase I we screened microorganisms in the Cape Cod aquifer sediment for their ability to survive and degrade specific substituted benzoates. In order to screen as many organisms as possible in three different types of aquifer material along with appropriate controls, we used a simple batch microcosm. The microorganisms we screened included *P. putida* (TOL), *P. sp.* B13 and *P. sp.* FR120. Aquifer sediment and water were collected from three different depths chosen to represent three different geochemical conditions within the containment plume. Depth 1 was located above the contaminant plume where oxygen was 300-400 μM, depth 2 was located inside the oxygenated portion (50-100 μM) of the contaminant plume, while depth 3 was located inside the anoxic portion of the contaminant plume.

The microcosms consisted of aquifer sediment (20 g), mixed with microorganisms, plus groundwater (2 ml) obtained from the same depth as the sediment and placed into 100 ml serum bottles. Either 3CB, 4MB or a mixture of both (total 50 nmol/g sediment in each case) was added to some microcosms. Autoclaved controls were also included. Oxygen was provided to all microcosms by the large air-filled headspace in the serum bottles. The microcosms were

incubated at either 12° C or 2° C in the dark. At appropriate time points, three bottles of each treatment were sacrificed and the microorganisms were enumerated by selective plating methods.

Typical results are shown in Figure 2. Survival of the added microorganisms varied with the source of the aquifer material. The number of microorganisms decreased with time to undetectable levels with depth 1 aquifer material but remained at or near the initial extractable levels after 10 weeks incubation with the depth 2 or 3 aquifer material. In the microcosms containing depth 2 aquifer material the number of microorganisms actually increased during incubation when the corresponding substrate was also present. This occurred with a corresponding decrease in the amount of substituted benzoate detected.

Medium containing 4MB as the sole carbon source allowed for growth not only of *P. putida* (TOL) but also of indigenous microorganisms in the three different aquifer depths. The number of microorganisms enumerated from the microcosms to which *P. putida* (TOL) was added was always higher than the corresponding unspiked microcosms. In contrast, medium containing 3CB was selective for only *P.* sp. B13. Indigenous microorganisms failed to grow on this media.

P. sp. B13 was able to survive for long periods of time when added to microcosms containing depth 2 and 3 aquifer materials. However in only one case was the added substituted benzoate degraded (Figure 3). This occurred with the material from depth 2 that was amended with both the xenobiotic and the exogenous Pseudomonas strain.

In Phase IIA (Figure 1) we used two different types of microcosms --modified membrane survival chambers and flow-through column microcosms-- to predict the survival of introduced microorganisms and their ability to degrade substituted benzoates.

The membrane survival chamber (Figure 4) was designed and tested as a field site tool since it allows for the containment of microorganisms. Containment of microorganisms inside the chamber would allow for addition of GEMs without the need for environmental monitoring. (These microcosms were only used in the Cape Cod

laboratory, to avoid regulatory uncertainties about the definition of containment.) The middle chamber of the microcosm contained sediment from the Cape Cod aquifer mixed with microorganisms. Groundwater obtained from the same depth as the sediment was collected in oxygen-impermeable hospital infusion bags to maintain in situ oxygen concentrations within the respective aquifer intervals. The water inside the infusion bag was pumped through the outside chambers of the microcosms. For some treatments the groundwater was spiked with substituted benzoates (200 μM). The microcosms were sacrificed at 4 weeks to determine the number of surviving microorganisms.

The second type of microcosm was a flow-through column microcosm (Figure 5). This microcosm was used to simulate the aquifer environment including the temperature of the aquifer, the oxygen concentration and the flow of water. The microcosm consisted of a plexiglass column containing aquifer sediment mixed with microorganisms. Groundwater, obtained from the aquifer and placed in hospital infusion bags was pumped into the bottom of the column at a flow rate (30 cm/day) similar to the actual aquifer groundwater flow rate. For some microcosms, substituted benzoates (200 μM) were added to the groundwater and the effluent was collected for determination of substrate concentration by use of HPLC. At appropriate time points, duplicate columns were sacrificed, the sediment was extracted and the microorganisms were enumerated by selective plating methods.

Typical results for both types of microcosms are shown in Figure 6. The microorganisms survived in the flow-through column microcosms for up to 10 weeks. The substrate 3-chlorobenzoate was not degraded in the microcosms during the 10 week incubation.

In Phase IIB (current research, Figure 1) we are continuing to work with flow-through column microcosms. We will try to: (1) determine some of the ecological factors that effect survival of the added microorganisms and (2) validate the utility of microcosms as predictive tools for determining fate and function of GEMs as bioremediation agents. Validation will be done by comparing certain parameters from microcosms and in situ. Parameters include a

determination of the number of protozoa, total direct counts of metabolically active and non-active microorganisms, survival of added microorganisms by enumeration on selective media, heterotrophic uptake potential, dissolved oxygen, and determining the number of microorganisms in certain populations (eg. fluorescent Pseudomonads, nitrate reducers, etc.).

Phase III of our research plan (1990) will be focused on the development of aquifer model ecosystems used to determine the factors that influence the fate and function of introduced microorganisms. These factors may be the amount of oxygen, concentration of pollutants, concentration of microorganisms, and temperature.

In phase IV we plan to introduce the parent strain, *P. sp.*B13 into the Cape Cod aquifer to follow its fate and transport. These results will be compared to the microcosm results to determine if our microcosms can be used to make predictions in an actual environment.

We have developed two methods of isolating DNA from soil samples: a direct lysis procedure and a procedure in which microorganisms are isolated prior to lysis. The direct lysis method yields greater quantities of DNA that is suitable for dot or slot blots. The prior isolation method yields a higher quality of DNA (larger fragments and less environmental contamination) better suited to Southern blot or Polymerase Chain reaction (PCR) analysis. Since DNA has not previously been extracted from low biomass sediments, we are optimizing these techniques for use on aquifer samples. Based on 6 fg DNA/bacterium we expect yields of between 6×10^{-10} and 6×10^{-8} g DNA/ g sediment from aquifers and have so far been able to achieve yields of approximately 1×10^{-8}. These techniques will be used to extract DNA from samples taken from the microcosms and the aquifer during Phase III and IV. The DNA will then be analyzed by DNA/DNA hybridization on Southern, colony or slot blots and by Polymerase Chain Reaction (PCR).

We have probes for the TOL plasmid and have designed probes and PCR-primers for both *P. sp.* B13 and its engineered derivative, strain FR120. DNA hybridization techniques using these probes allow

us to confirm the identities of colonies enumerated on selective media and detect strains from aquifer samples down to 10^3 to 10^4 organisms per gram of sediment. By using PCR amplification of the aquifer DNA we hope to be able to increase the sensitivity of detection to approximately one organism per gram. In addition, the PCR reaction will allow us to examine the stability of the engineered gene by monitoring any changes in the size of the PCR-product from samples taken from microcosms over time.

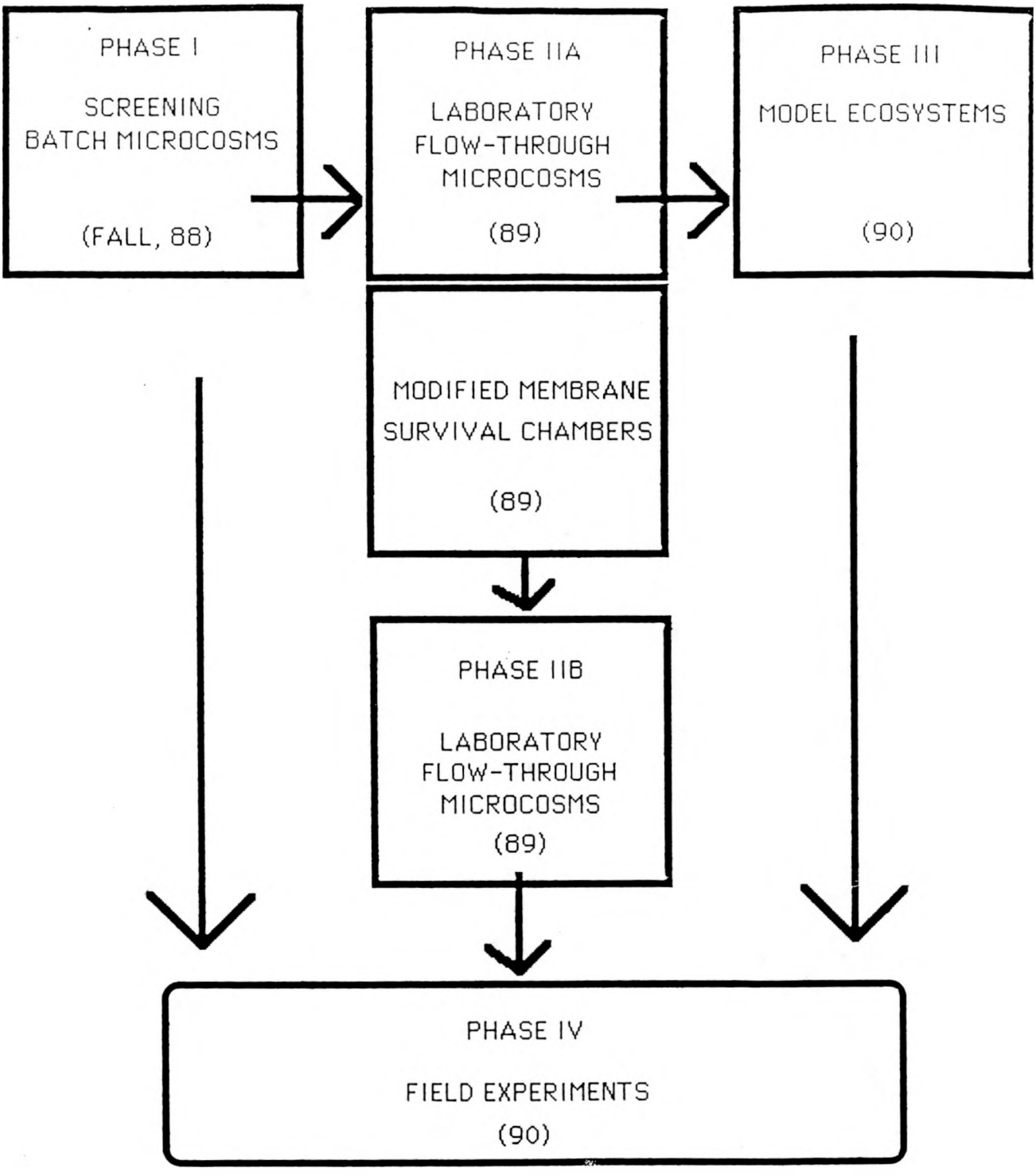

PHASE I
SCREENING
BATCH MICROCOSMS
(FALL, 88)
PHASE IIA
LABORATORY
FLOW-THROUGH
MICROCOSMS
(89)
PHASE III
MODEL ECOSYSTEMS
(90)
MODIFIED MEMBRANE
SURVIVAL CHAMBERS
(89)
PHASE IIB
LABORATORY
FLOW-THROUGH
MICROCOSMS
(89)
PHASE IV
FIELD EXPERIMENTS
(90)

Figure 1

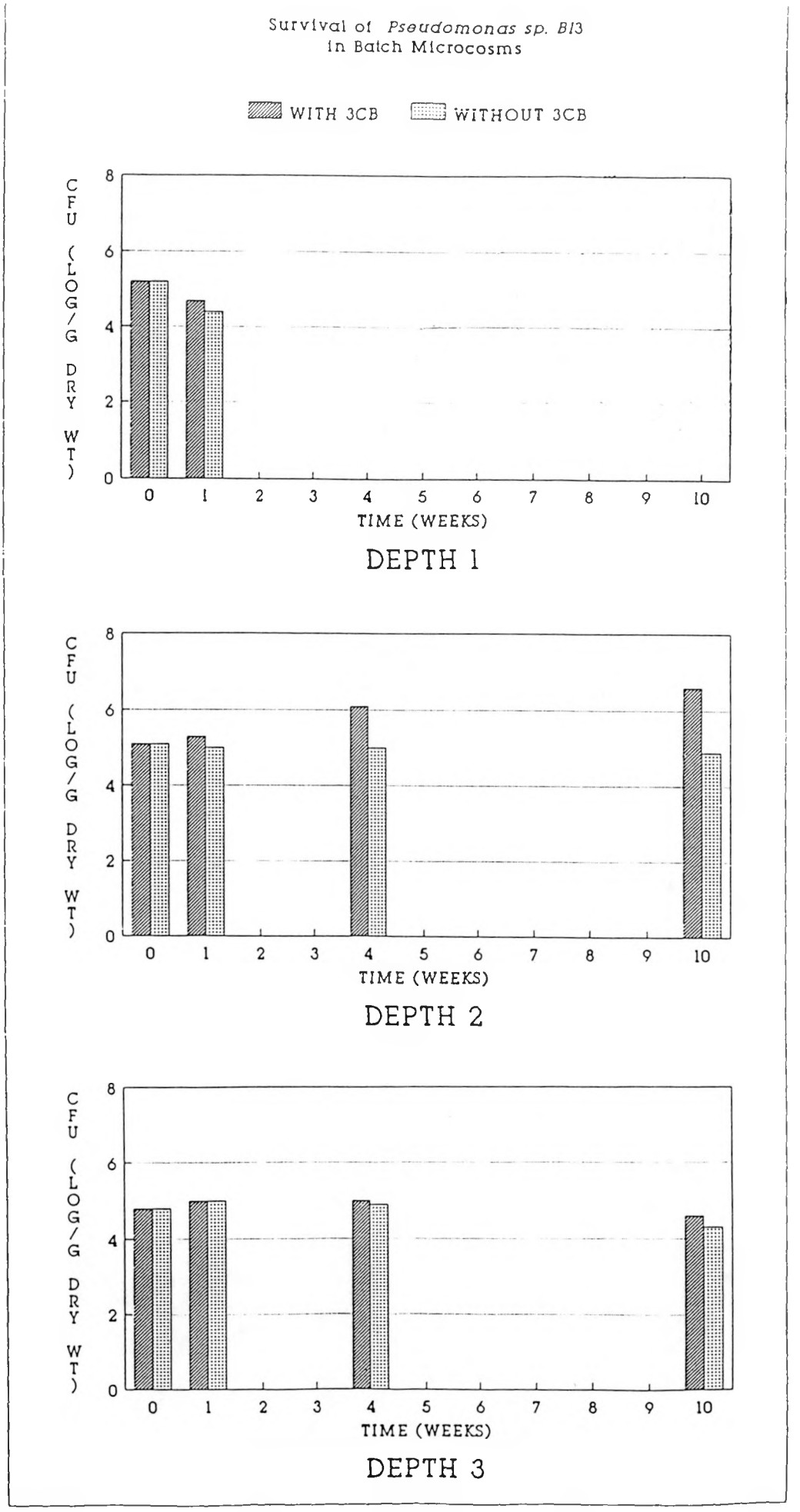

Survival of Pseudomonas sp. B13
in Batch Microcosms
WITH 3CB
WITHOUT 3CB
CFU (LOG/G DRY WT)
0
2
4
6
8
0 1 2 3 4 5 6 7 8 9 10
TIME (WEEKS)
DEPTH 1
CFU (LOG/G DRY WT)
0
2
4
6
8
0 1 2 3 4 5 6 7 8 9 10
TIME (WEEKS)
DEPTH 2
CFU (LOG/G DRY WT)
0
2
4
6
8
0 1 2 3 4 5 6 7 8 9 10
TIME (WEEKS)
DEPTH 3

Figure 2

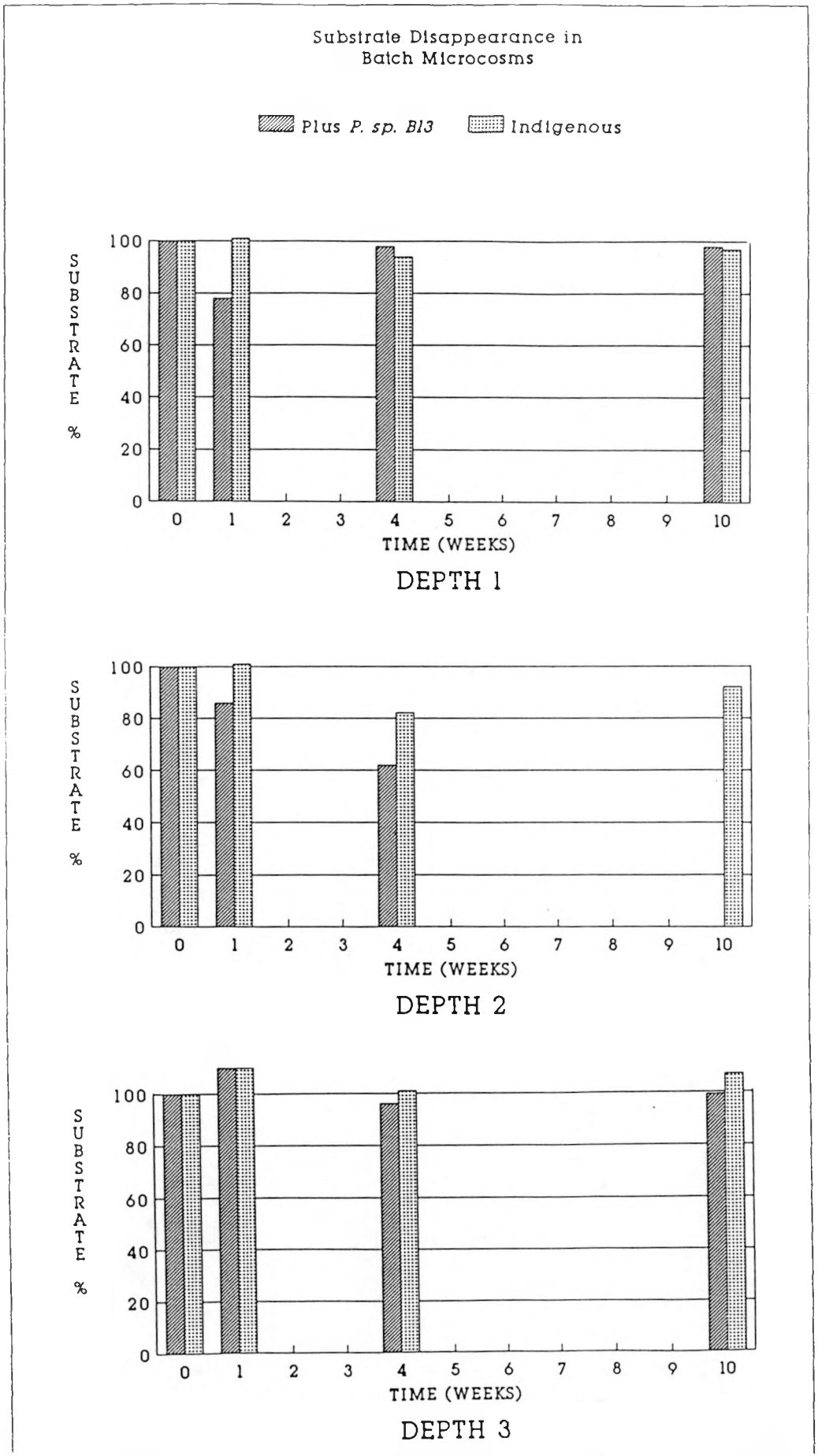

Substrate Disappearance in
Batch Microcosms
Plus P. sp. B13
Indigenous
SUBSTRATE %
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
TIME (WEEKS)
DEPTH 1
SUBSTRATE %
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
TIME (WEEKS)
DEPTH 2
SUBSTRATE %
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
TIME (WEEKS)
DEPTH 3

Figure 3

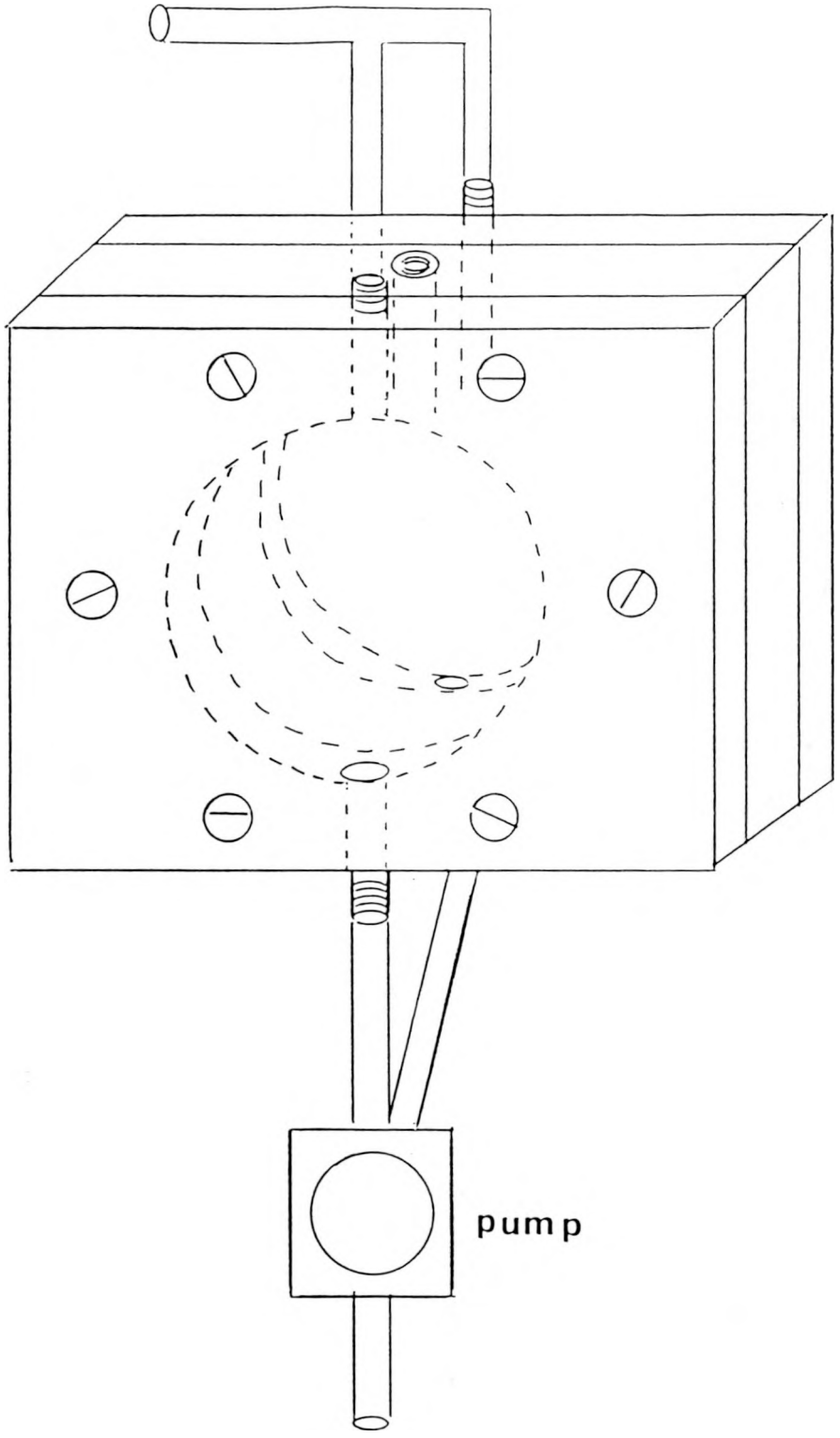


Figure 4

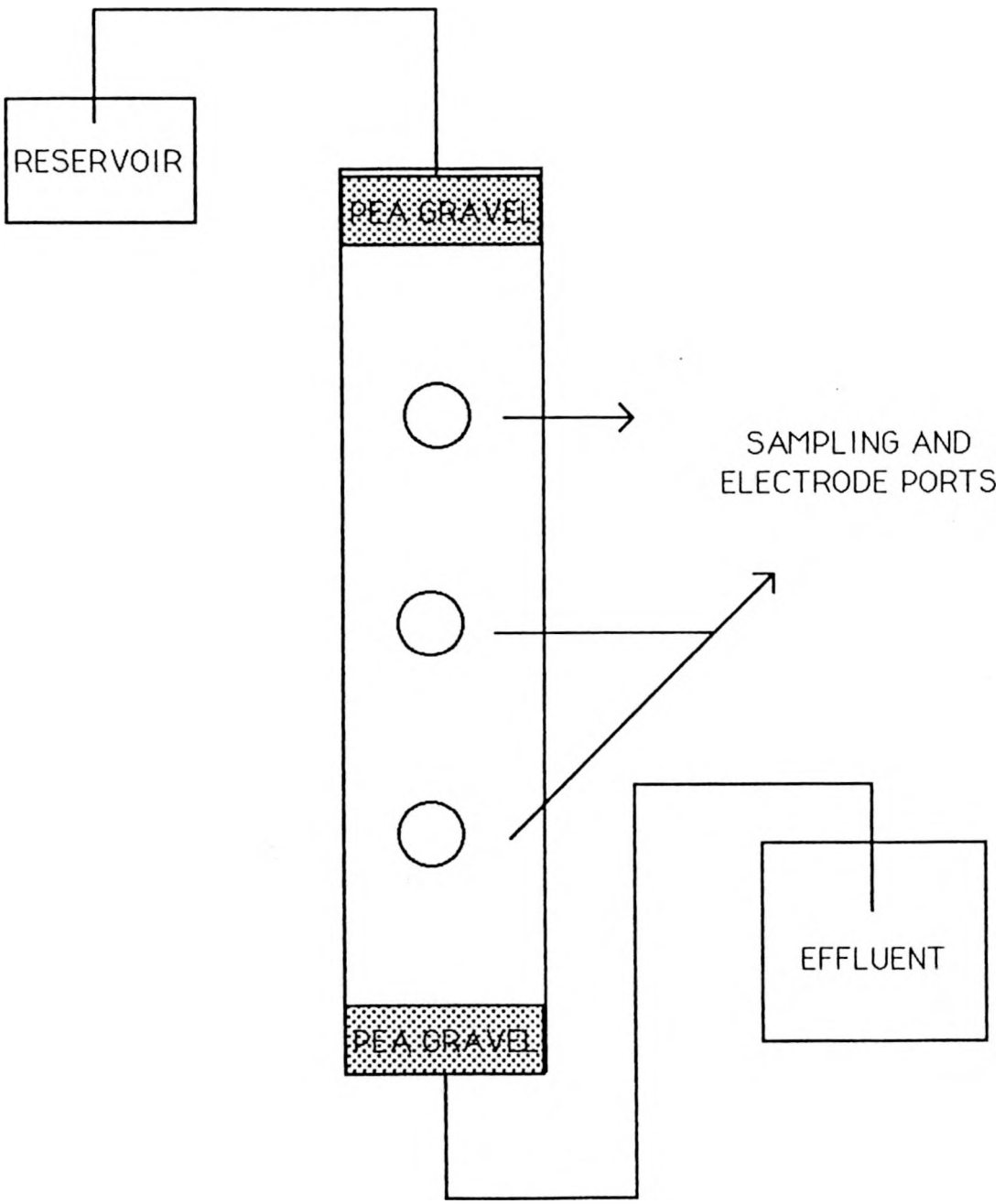
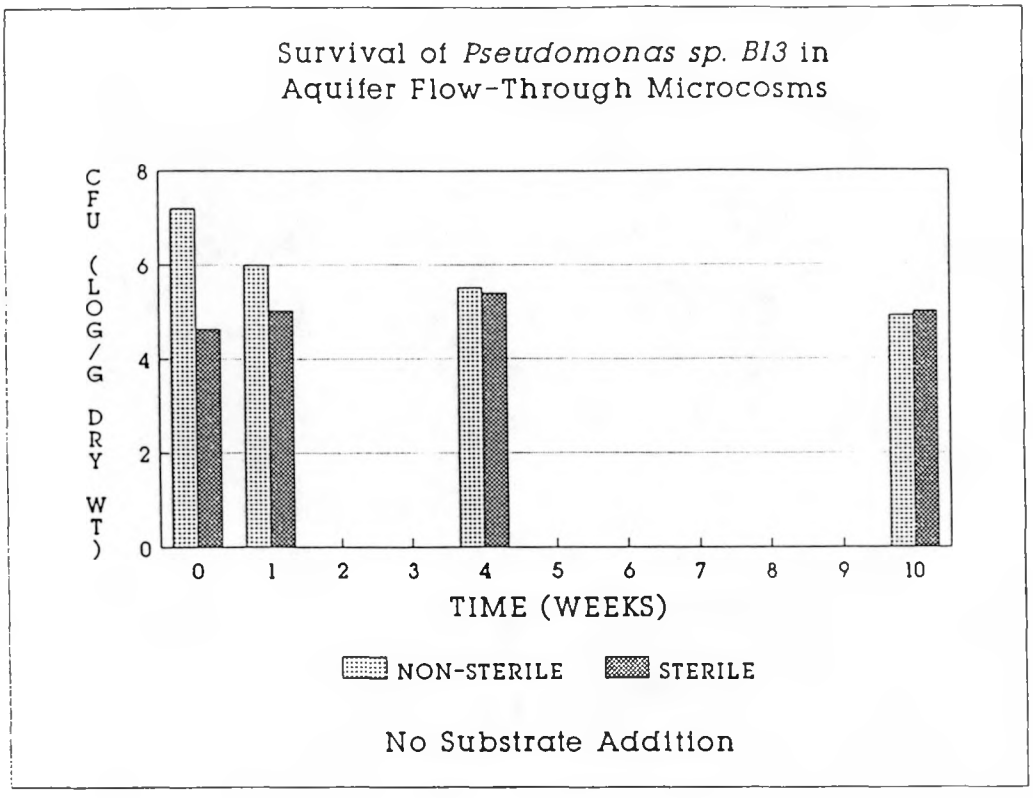

RESERVOIR
PEA GRAVEL
SAMPLING AND
ELECTRODE PORTS
EFFLUENT
PEA GRAVEL

Figure 5


Survival of *Pseudomonas sp. B13* in
Aquifer Flow-Through Microcosms
CFU (LOG/G DRY WT)
0
2
4
6
8
0 1 2 3 4 5 6 7 8 9 10
TIME (WEEKS)
NON-STERILE
STERILE
No Substrate Addition

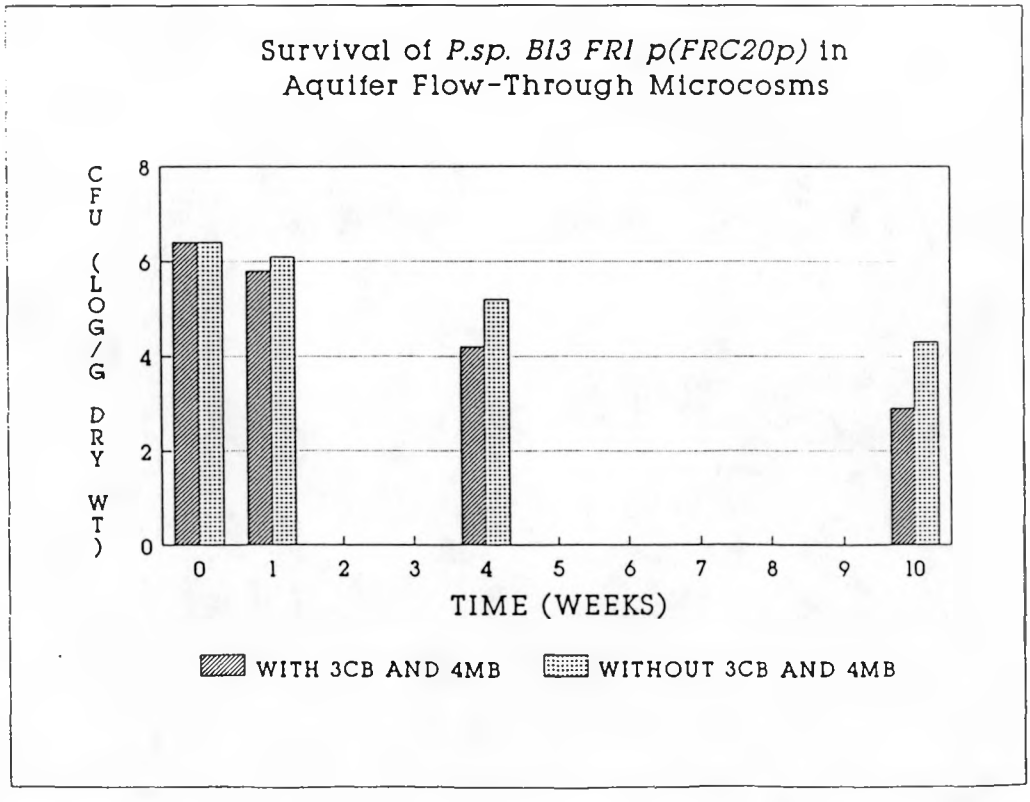

Survival of *P.sp. B13 FR1 p(FRC20p)* in
Aquifer Flow-Through Microcosms
CFU (LOG/G DRY WT)
0
2
4
6
8
0 1 2 3 4 5 6 7 8 9 10
TIME (WEEKS)
WITH 3CB AND 4MB
WITHOUT 3CB AND 4MB

Figure 6