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Field Application of a Genetically Engineered Microorganism for Polycyclic Aromatic Hydrocarbon Bioremediation Process Monitoring and Control

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1. INTRODUCTION

On October 30, 1996, the U.S. Environmental Protection Agency (EPA) commenced the first test release of genetically engineered microorganisms (GEMs) for use in bioremediation. This study was initiated by investigators at the University of Tennessee Center for Environmental Biotechnology (CEB) in collaboration with the Environmental Sciences Division (ESD) of Oak Ridge National Laboratory (ORNL). This test was conducted under the auspices of the U.S. Department of Energy's (DOE) Natural and Accelerated Bioremediation Research Program (NABIR). The specific objectives of the investigation were multifaceted and include 1) testing the hypothesis that a GEM can be successfully introduced and maintained in a bioremediation process, 2) testing the concept of using, at the field scale, reporter organisms for direct bioremediation process monitoring and control, and 3) acquiring data that can be used in risk assessment decision making and protocol development for future field release applications of GEMs.

The genetically engineered strain under investigation is *Pseudomonas fluorescens* strain HK44 (King *et al.*, 1990). The original *P. fluorescens* parent strain was isolated from polycyclic aromatic hydrocarbon (PAH) contaminated manufactured gas plant soil (MGP). *P. fluorescens* HK44 was created by the incorporation of a plasmid containing a salicylate inducible operon and the gene cassette for bacterial bioluminescence (*lux*) from *Vibrio fischerii*. When naphthalene is metabolized to salicylate, the *lux* transcriptional gene fusion is induced and expressed to produce enzymes responsible for a bioluminescent signal at an approximate wavelength of 490 nm. Thus, this bacterium is able to biodegrade naphthalene (as well as other substituted naphthalenes and other PAHs) and is able to function as a living bioluminescent reporter for the presence of naphthalene contamination, its bioavailability, and the functional process of biodegradation.

A unique component of this field investigation was the availability of an array of large subsurface soil lysimeters at ORNL (Figure 1). These lysimeters were originally constructed for quantifying and characterizing leachate derived from low-level radioactive wastes but were never used for this purpose. They were instead extensively modified for the GEM release experiments. The lysimeters permitted a semi-contained, controllable field test environment. In addition, they allowed accurate field replication and provision of control tests. The sheer size of each lysimeter (4 m deep by 2.5 m in

DISCLAIMER

diameter) allowed for extensive sampling, the deployment of both well-established and prototypic *in situ* sensors, and localized testing of treatments prior to global changes. Large sample cores permitted numerous assays to be performed, including procedures that determine cell enumeration data, genotypic information, hydrocarbon and naphthalene concentrations, bioluminescence, and soil dry weights. *In situ* sensors monitored temperature, bioluminescence, vapor phase volatile hydrocarbon contamination, oxygen concentration, soil moisture content, and carbon dioxide concentration. Furthermore, the lysimeters are exposed to environmental conditions over a two year period, thus providing a wealth of information on *in situ* survivability of GEMs. This article describe our experience associated with the release of a genetically modified microorganism, the lysimeter facility and its associated instrumentation, as well as representative data collected during the first eighteen months of operation.

2. METHODOLOGY

2.1. Lysimeter Design and Set-up

The lysimeter array consists of seven large lysimeters (4 m deep by 2.5 m in diameter) positioned in a circular array around a central core (6 m deep by 6 m in diameter) that houses instrumentation and plumbing for each of the lysimeters. The lysimeters and the central core were constructed of galvanized steel pipe set into concrete foundations and covered with removable stainless steel lids. Six of the lysimeters were filled with stratified beds of soil (Figure 1) and the seventh contained groundwater and was used to adjust the water level in the other lysimeters. The stratified bed consisted of a 31 cm layer of graded gravel, a 61 cm layer of coarse sand, a 92 cm layer of uncontaminated soil, a 92 cm treatment layer and a 61 cm cap of clean soil placed on top of the bed (Figure 1). The uncontaminated soil was a Huntington loam topsoil consisting of 42% sand, 40% silt, 18% clay, and 1.3% organic carbon.

Originally, a portion of this topsoil was artificially contaminated with naphthalene, anthracene and phenanthrene. A concrete mixer truck was used to combine 23 m^3 of soil with 23 kg of naphthalene and 0.23 kg each of anthracene and phenanthrene, producing approximate final average concentrations of 1000 mg/kg for naphthalene and 10 mg/kg for anthracene and phenanthrene. These concentration are representative of a PAH mixture from a typical MGP site.

The treatment zone in Lysimeters 1, 2, and 4 received the contaminated soil inoculated with HK44, Lysimeters 3 and 5 received uncontaminated soil inoculated with HK44, and Lysimeter 6 received a layer of uninoculated, contaminated soil (Table 1). Soil layers were added in approximate 10 cm increments, each of which was packed to a dry density of 1300 kg/m^3. In Lysimeters 1-5, each 10 cm increment of soil within the 92 cm treatment zone was sprayed with approximately 4 L of cell suspension using a backpack spray tank equipped with an extended nozzle. Anderson Air samplers and gravity plates were used to monitor dispersion rates of the GEMs (Ford *et al.*, 1998). One air sampler was placed 1 m from the outer edge of the lysimeter being sprayed and another was placed the same distance directly opposite on the other side. Plates were placed in concentric rings at distances of 1, 2, and 4 m from the lysimeter that was being sprayed with the HK44 culture. A total of 260 Anderson and gravity plates were used.
A plumbing system was installed to allow for liquid delivery either from the top of the

treatment zone or bottom of the lysimeter (Figure 1). The upper irrigation system used a coil of drip irrigation tubing placed at the top of the treatment zone and was used to deliver supplemental nutrient treatments. The lower plumbing arrangement was designed to collect leachate in 55-gallon drums located beneath each lysimeter. As required by the EPA, any leachate percolating out of the lysimeters was collected and tested for the presence of *P. fluorescens* HK44. When full, drums were drained into large polytanks and disinfected with hypochlorite solution. After adequate sampling to ensure no survivability of *P. fluorescens* HK44 cells, the polytanks were transported to an ORNL waste treatment plant for disposal. In addition to leachate collection, the lower plumbing system was used to adjust the water level in each lysimeter. This was accomplished by gravity feeding groundwater contained within the seventh lysimeter into the lysimeter of choice. In addition, an air distribution manifold was placed in the bottom of each lysimeter to provide aeration.

2.2. Preparation of the Original Inoculum

The *P. fluorescens* HK44 inoculum was prepared in a 500 L New Brunswick fermenter (Model IF-100) containing a medium consisting of 160 g yeast extract, 1600 g peptone, 160 g ammonium nitrate and 800 mL glycerol in 400 L water. After a 22 h growth period the culture was harvested in a continuous centrifuge and the resulting cell paste resuspended in 60 gallons of saline. This suspension was transported to the site in two 30 gallon drums

2.3. In Situ Treatment Zone Sensors

Various types of sensors were deployed to monitor parameters such as oxygen concentration, temperature, carbon dioxide concentrations, naphthalene vapor, moisture levels, and reporter bioluminescence in the treatment zone.

Oxygen and temperature sensors and data collection/storage devices (Datawrite Research Corp., Visalia, CA) were used to measure, record, and store oxygen data in Lysimeters 1, 2, 3 and 5 and temperature data in Lysimeter 2. Data were typically measured in thirty-minute intervals for a 10 day period, after which the data were retrieved with a laptop computer.

A novel biosensor was used to detect naphthalene vapors in the treatment zone of Lysimeter 1, 2 and 3. This biosensor utilizes alginate-encapsulated *P. fluorescens* HK44 cells (Stormo and Crawford, 1992) and a multi-channel fiber-optic based detection system. Encapsulated cells are placed in a well supported by a subassembly that fixes the distance between a fiber optic cable (1 mm diameter, spc1000/1150b Fiberguide Industries, Stirling, NJ) and the alginate encapsulated cells. The subassembly is then sealed within a light-tight housing constructed from a porous stainless steel tube, two end caps and a standard bulk-head tubing connector. The biosensor is lowered into one of four polyvinyl chloride pipes (PVC) buried at different depths within the treatment zones of Lysimeter 1, 2, and 3 (Figure 1). If naphthalene is present, light emitted from HK44 cells is transferred through the cable within a light-tight box. The fiber optic cable terminates into a 28-position circular fiber holder. A liquid light guide (Oriel Corp., Model 77566) attached to a rotational stage (Newport Corp., Motion-Master 2000) is positioned directly in front of the terminal end of the fiber-optic cable and collects light for 5 sec (Sayler *et al.*, 1995). The recorded counts are an average of 50 counting events,

each lasting 0.1 sec. Photons collected by the light guide are detected with a photomultiplier tube (PMT) (Model R-4632, Hammamatsu Photonics, Middlesex, NJ) mounted in a thermoelectrically-cooled housing regulated at -35 °C. The pulses from the PMT are processed by a photon counting module (Model C3866, Hammamatsu Photonics, Middlesex, NJ) and counted with a computer counter/timer board. Custom DOS-based software caches the photon count data and controls the motion of the rotational stage. Once the data is stored, the computer activates a programmed motion until light is collected from each fiber-optic probe, and then continuously repeats the cycle.

Determination of bioluminescence from soil GEMs was also monitored with the multi-channel fiber optic-based detection system. Thirteen additional fiber optic cables were buried directly into the treatment zone in Lysimeters 1, 2, and 3. Bioluminescence from soil microorganisms directly below the fiber optic cable was quantified as described above.

In addition, a portable field deployable PMT-based probe was designed and used as a process monitoring and control tool. This tool offers increased sensitivity over the fiber optic based detection system. The probe consisted of a unique light-tight and water-tight housing and a PMT module equipped with an embedded microcontroller integrating all components necessary for photon counting and a RS-232-C interface (HC135-01, Hamamatsu, Middlesex, NJ). The probe housing was attached to a 1.5-inch pipe and lowered down 3-inch internal diameter pipes (some equipped with plexiglass windows) to examine soil at the bottom of the pipes. A light-tight interface was designed to block out extraneous light. Light data were collected and stored on a laptop computer.

2.4. Soil Sampling

Soil cores were removed from each lysimeter using a Cole-Parmer soil sampler (Cole-Parmer Industries, Vernon Hills, IL). Zero-contamination sampling tubes (2.3 cm diameter and 61 cm in length) were used to prevent cross-contamination of soil cores. Typically, a site was sequentially sampled with 3 cores taken at different depths totaling 185 cm in length. The first core traversed the clean cap and was discarded. The second and third cores traversed through the 92 cm deep treatment zone and the 30 cm of soil directly beneath. Cores were stored at 4°C for transport. Once in the lab, cores were divided into 30.5 cm sections, each section thoroughly mixed, and stored separately in sterile Whirlpak bags (Fort Atkinson, WI).

2.5. Microbiological Analyses

One gram samples were removed from each Whirlpak bag and vortexed in 9 mL 0.1% sodium pyrophosphate for 1 min. Dilutions were then prepared in saline solution (0.85% NaCl) and plated in triplicate on YEPG agar plates (0.2 g yeast extract, 2.0 g polypeptone, 1.0 g glucose, 0.2 g NH_4NO_3, 17 g agar per liter, pH 7.0) to obtain total bacterial counts and on YEPSS agar plates (0.2 g NH_4NO_3, 0.2 g yeast extract, 0.5 g sodium salicylate, 2.7 g sodium succinate, 1 g polypeptone, 17 g agar, per liter pH 7.0) containing tetracycline at 14 mg/L to obtain *P. fluorescens* HK44 counts. In both cases cycloheximide was added at 50 mg/L to inhibit fungal growth. Plates were incubated in the dark at room temperature for four days. To verify the presence of the genetically

engineered plasmid, colony hybridizations were performed on presumptive *P. fluorescens* HK44 colonies growing on YEPSS plates containing tetracycline. Colonies were transferred to 82 mm diameter nylon membranes (Millipore Corp., Bedford, MA) and hybridized with a 0.3 kb *luxA* gene probe under high stringency conditions (Sambrook et al., 1989)

2.6. Global Treatments Events

To provide an additional source of inducing substrate, naphthalene and anthracene were mixed with transformer oil (Exxon Univolt 60) and added to Lysimeter 1, 2, 4, and 6. Twenty-four kilograms of naphthalene crystals and 2 kg anthracene were dissolved in 220 gallons oil that proved to be an adequate solvent while still meeting regulatory requirements that it not contain RCRA (Resource Conservation and Recovery Act) hazardous constituents. Each of Lysimeters 1, 2, 4, and 6 received 55 gallons through the irrigation tubing located directly above the treatment zone. Theoretically, this was sufficient to produce an average loading capacity of 1000 mg/kg throughout the 92 cm contamination zone. Before adding the oil, the water table in each lysimeter was raised to the sand/clean soil interface. After all the oil was added, the water table was rapidly lowered to create a slight vacuum to induce the flow of oil into the contamination zone. This process was successful in preliminary laboratory experiments.

Nutrients in the form of minimal salts were added to Lysimeter 1, 2, 4, and 6. Fifty gallons of a minimal salts (2 g $NaNO_3$, 0.75 g KH_2PO_4, 0.003 g $FeCl_3$, 0.1 g $MgSO_4$, 0.005 g $CaCl_2$, and 0.25 g Na_2HPO4 per liter) solution were added through the irrigation tubing. In microcosm studies performed in the laboratory, the nutrient solution and the supplemental addition of oil and naphthalene stimulated the growth of HK44 in lysimeter soil.

An air-pumping regime using an Inersoll-Rand 100 air compressor (Seattle, WA) began on April 11, 1997. The air-delivery scheme typically required pumping air twice a week for 4 hours at a rate of 2 SCFM.

3. RESULTS AND DISCUSSION

3.1. Risk Assessment

In July 1995, a premanufacture notification (PMN) was submitted to the EPA for proposed release of the GEM in the planned field test (Sayer, 1997). In March 1996 the University of Tennessee received an EPA Consent Order authorizing the field test under strict regulatory guidelines contained in Work Plans, Waste Management Plans, and Health and Safety Plans developed by ORNL and the CEB. Provisions in the consent order were to provide the EPA with monitoring data as well as a review of any technical changes in the work plan. This became necessary when the PMN was modified to incorporate a change in the method of soil inoculation from direct mixing to a spray application. The consent order itself was not an outright approval of field use, but rather a cautious tentative agreement of field use due to existing risk concerns. This risk assessment conclusion was made for several reasons: the recombinant genes are on a mobile genetic element, the genetic construct contains an antibiotic resistance marker, and some biovars of the species have the capacity to act as opportunistic plant pathogens, although HK44 shows no such potential based on limited analysis. Following additional

DOE Environmental Health and Safety oversights and Readiness Reviews, the field test ultimately proceeded on October 30, 1996.

3.2. Soil Storage and Initial PAH Concentrations

Prior to the loading of soil into the lysimeters, the contaminated soil and an additional 46 m^3 of uncontaminated soil were stored in separate piles on a concrete pad under plastic tarps for approximately 80 days. A moderate aging and weathering period was desired to better simulate soils from contaminated sites since PAHs have been shown to strongly sorb to soils over time (Dzombak *et al.*, 1984). Unfortunately, a delay in receiving the numerous approvals required for releasing the GEMs resulted in a total of 180 days of contaminant soil aging. Consequently, approximately 95% of the naphthalene was lost through volatilization and natural biodegradation processes. A decision was made to proceed with the loading of the lysimeters since obtaining authorization to recontaminate the soil would result in significant additional delays. Consequently, a second contamination event occurred at a later date (Day 135 of the experiment).

3.3. Inoculation of the Soil

Cell density of the initial inoculum using plate counts was determined to be 1.42×10^7 cfu/mL. Inadvertent release of GEMs during spraying was inevitable but allowable under EPA guidelines as long as adequate precautions were taken and the degree of release was documented. A total of 260 Anderson Air sample and gravity plates were used. Of these, 36 were positive for HK44 growth, usually with less than 5 colonies per plate. No plates placed beyond a 3.5 m radius contained *P. fluorescens* HK44 colonies. Initial soil concentrations are shown in Table 1.

3.4 Population Dynamics

Two types of media were used to monitor the microbial populations in the lysimeters, YEPG and YEPSS (Figure 2). The native heterotrophic culturable population is defined by microorganisms that can grow aerobically on a medium containing nutrients that can be utilized by a wide variety of microorganisms (YEPG). Although it is known that only a small percentage of bacteria in soil are culturable under any condition, this population contains bacteria that can compete with *P. fluorescens* HK44 for nutrients. The second medium is more selective for *P. fluorescens* HK44 (YEPSS). This medium contains the antibiotic tetracycline and sodium salicylate, a carbon source for *P. fluorescens* HK44. Ideally, *P. fluorescens* HK44 would be the only bacterium to grow on this selected medium. However, soils contain a great diversity of bacteria, and in the control uninoculated but chemically contaminated lysimeter (Lysimeter 6), a background bacterial population capable of growth on the selective medium was found to pre-exist at an order of magnitude lower cell density (Figure 2). To accurately determine the percentage of *P. fluorescens* HK44 present on the plates, a colony hybridization assay was performed (Figure 3). All microbiological assays were performed to monitor the population dynamics occurring within each lysimeter before and after any treatment event and for no period of time greater than 4 months. This sampling regimen was used to study not only the treatment effect but also the treatment effect under changing environmental conditions. For example, soil temperature during this 18 month period had winter minimums of 6.75°C on January 26, 1997 (Day 88) and 11°C on February 9, 1998 (Day 467) and a summer maximum of 28.5°C on July 21, 1997 (Day 264).

Temperature can have a profound effect on bioluminescence, since the enzymes responsible for bioluminescence are not stable at temperatures above 30°C (Meighen, 1991).

Several trends are apparent relative to the long-term survival of *P. fluorescens* HK44 (Figure 2). After inoculation, *P. fluorescens* HK44 colony forming units per gram dry weight decreases in all lysimeters. For those chemically contaminated lysimeters inoculated with *P. fluorescens* HK44 (Lysimeters 1, 2, and 4), there is a high level of reproducibility in population maintenance and response. For the first 135 days of the experiment, both the native total culturable heterotrophic bacterial populations and GEMs declined reproducibly. Specifically, the *P. fluorescens* HK44 populations in Lysimeter 1, 2, and 4 decreased from the initial mean value of 1.5 ($\pm$ 0.4 standard error) x 10^6 to 4.0 ($\pm$ 1.9) x 10^4 cfu/g of soil on Day 117. After the addition of the transformer oil with naphthalene and minimal salts, the mean soil population of GEM increased to approximately 3.7 ($\pm$ 3.1) x 10^6, then decreased to 1.4 ($\pm$ 0.6) x 10^5 cfu/g of soil probably due to an insufficient air supply prior to Day 154. On this date, O_2 concentration was determined to be 2%. After modification of the air supply equipment, oxygen levels increased to approximately 20%, and the GEM populations rebounded, but again declined to 7.9 ($\pm$ 3.4) x 10^4 cfu/g of soil on Day 474. Over the duration of the experiment, the percentage of tetracycline resistant bacteria in the total heterotrophic community decreased from 13% to approximately 1% on Day 474. However, these percentages overestimate the HK44 population, since the number of colonies detected on the selective medium probing positive for *lux* declined with time (Figure 3). Two plausible explanations for this finding exist. First, the nutrient augmentations used to stimulate HK44 also stimulated the native tetracycline resistant (Figure 2). Second, the plasmid containing the *lux* genes may be unstable over many generations.

Within lysimeters containing GEMS without chemical contamination (Lysimeters 3 and 5), the populations have declined from 1.8 x 10^6 to 5 x 10^3 cfu/g of dry soil in Lysimeter 3 and from 1.3 x 10^6 to 5.1 x 10^3 cfu/g of soil in Lysimeter 5, with most of the decrease detected in the first 30 days. Furthermore, the percent of HK44 cells to total culturable heterotrophic populations has decreased from 41 to 10% in Lysimeter 3 and from 29 to 2.2% in Lysimeter 5. The only difference between the treatments for these lysimeters was a groundwater flooding event that occurred on Day 233. Throughout this study, the percentage of the colonies on the selective YEPSS medium that probed positive for the *luxA* gene ranged from 57 to 96% (for both Lysimeter 3 and 5), indicating that neither the tetracycline resistant bacteria nor plasmid instability is a significant problem in these lysimeters. These data, presented in Figures 2 and 3, demonstrate that a GEM can be introduced and maintained in contaminated soils, however nutrient augmentations can stimulate a competitive population.

3.5. Detection of Bioluminescence from HK44 Cells

Another important goal of this research is the detection of bioluminescence from GEMs. Bioluminescence from HK44 was used in alginate bead biosensors to monitor volatile PAHs. Initially, the bioluminescence from microorganisms within the biosensors was minimal due to the low concentration of volatile PAHs present in the treatment zone. Light production from the biosensors dramatically increased after the addition of the

naphthalene in transformer oil, indicating that naphthalene vapor was present in the vapor phase of the treatment zone (Figure 4). The increase in the signal (Figure 4A) probably results from the induction and subsequent expression of the genes responsible for bioluminescence. In the presence of naphthalene vapor, the bioluminescent bacteria can continue to utilize nutrients and multiply, increasing the number of cells that in turn can increase bioluminescence signal. After a few days, the growth of cells on or in the alginate beads may become limited by the lack of an essential nutrient causing a decrease in the signal. In some situations, the decrease in the bioluminescent signal may also be caused by dehydration of the alginate matrix. In Figure 4B, the low level of bioluminescence detected when no naphthalene is present may result from the basal level of expression of the enzymes responsible for bioluminescence in HK44. Qualitatively, the peak height or peak area of the signal decreased with concentration. However, quantitative assessment of the signal will require information about other parameters (including oxygen concentration, temperature, and relative humidity) that can either directly or indirectly affect the bioluminescence signal. Experiments to determine the best method to quantify the bioluminescent data are on-going. Yet even with these present limitations, these biosensors have proved to be useful tools for the real-time detection of volatile PAH contamination in soil.

GEMs in the soil were also used to monitor for the presence of naphthalene contamination, its bioavailability, and the functional process of biodegradation. Although the fiber-optic based system was also used to examine directly the lysimeter soil, detection of bioluminescence directly from HK44 cells in the soil has been unsuccessful, probably due to a lower than expected HK44 biomass. To increase sensitivity, a portable photon counting PMT-based system was design to examine soil under windows located at the distal end of 3 inch pipes that terminate in the treatment zone. The probe was initially used in January of 1998, and has been used to show that much of the treatment zone is depleted of naphthalene. Localized areas examined by this detection system showed that bioluminescence from *P. fluorescens* HK44 can be stimulated if the minimal salts solution and naphthalene crystals were added to the treatment zone (Figure 5). For example, when 100 mg of naphthalene and 100 mL of minimal salts (time zero) were added to soil underneath the window, bioluminescence continued for approximately one month. Initially, the existing number of HK44 cells was probably too few to produce a measurable bioluminescent signal. After a 5 day lag, the population was large enough to produce a bioluminescent signal that continued for 26 days. Information gathered with this detection system was used to design the final perturbation involving a minimal salts addition and a water table adjustment to disperse the remaining naphthalene and oil within the treatment zone. This final test should allow for the detection of HK44 during a large scale perturbation.

4. SUMMARY.

This research has demonstrated that GEMs can be introduced and maintained in environmental soils for at least 18 months and that the population dynamics can be affected by nutrient augmentation. For example, the presence of naphthalene can be used to enrich for *P. fluorescens* HK44 in soils and the lack of naphthalene causes the GEM population to decline. Furthermore, this study has provided evidence that bioluminescence from GEMs can be used as a tool for monitoring and controlling the

bioremediation process. A GEM can bioluminesce and report conditions that are favorable for bioremediation. In the case where a GEM is present and not bioluminescing, unfavorable environmental or physiological conditions are revealed. If unfavorable conditions are detected, analytical and microbiological assays as well as localized treatments can be used to diagnose and correct the existing problem. This strategy can lead to rapid responses and eliminate the need for expensive microcosm studies that may or may not be amenable to scale-up.

5. FUTURE PLANS FOR THE LYSIMETER FACILITY

Upon completion of this two-year study, several other experiments utilizing the lysimeter arrays may be proposed to further enhance our understanding of the efficacy of bioremediative strategies in natural environments. Newly engineered bacterial strains that degrade PAHs more efficiently, generate greater light levels, and are more genetically stable are now available. Such activities can be assessed as a comparison to HK44. The use of surfactants is also being studied as a means of overcoming sorption between PAHs and soil particles, allowing the GEM to function more productively in its remediative efforts and increasing overall degradation efficiency. Other applications with engineered strains for cometabolic bioremediation of problematic contaminants such as DNAPL (Dense Non-Aqueous Phase Liquids; trichloroethylene (TCE) is a common example) are also being proposed for field research testing in the lysimeter array. Additionally, we also have plans to fill another on-site lysimeter array with soil contaminated with uranium, radionuclides, heavy metals, and mixed waste. We are especially interested in collaborations with other scientists who could benefit from studies in such a facility.

The use of large-scale field sites provides excellent scale-up for studying bacterial interactions under realistic conditions without direct release of organisms into natural ecosystems. This may be of special concern when considering the undefined risks associated with the dissemination of engineered genotypes to indigenous populations. The lysimeters provide ideal experimental sites for GEM release. They maintain a semi-contained environment with adequate safety backups and provisions for monitoring surrounding groundwater and soil to evaluate accidental GEM discharges. They are also equipped with sufficient instrumentation to monitor all relevant environmental parameters that may affect the GEM. Most importantly, though, is that they are large enough in size, both horizontally and vertically, to permit the high volume of sampling necessary to adequately assess the specific microbial interactions occurring. Results obtained from these experiments will provide valuable and essential insights into the bioremediative capabilities of GEMs, allowing for an economical and efficient means for their use in future commercial applications.

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FIGURE LEGENDS

FIGURE 1. Schematic representation of a lysimeter containing a stratified bed and *in situ* sensors.

FIGURE 2. Population dynamics of *Pseudomonas fluorescens* HK44 and response to nutrient amendments following lysimeter inoculation. Total viable counts (o) and HK44 concentrations determined by selective plating on tetracycline (●) in each of the lysimeters. Open boxes (□) represent the number of indigenous tetracycline-resistant colony forming units in Lysimeter 6. Solid arrows indicate time of oil addition; dashed arrows indicate time of minimal medium addition. The heavy dashed arrow in Lysimeter 5 represents the time at which water was added in an attempt to lower soil temperatures.

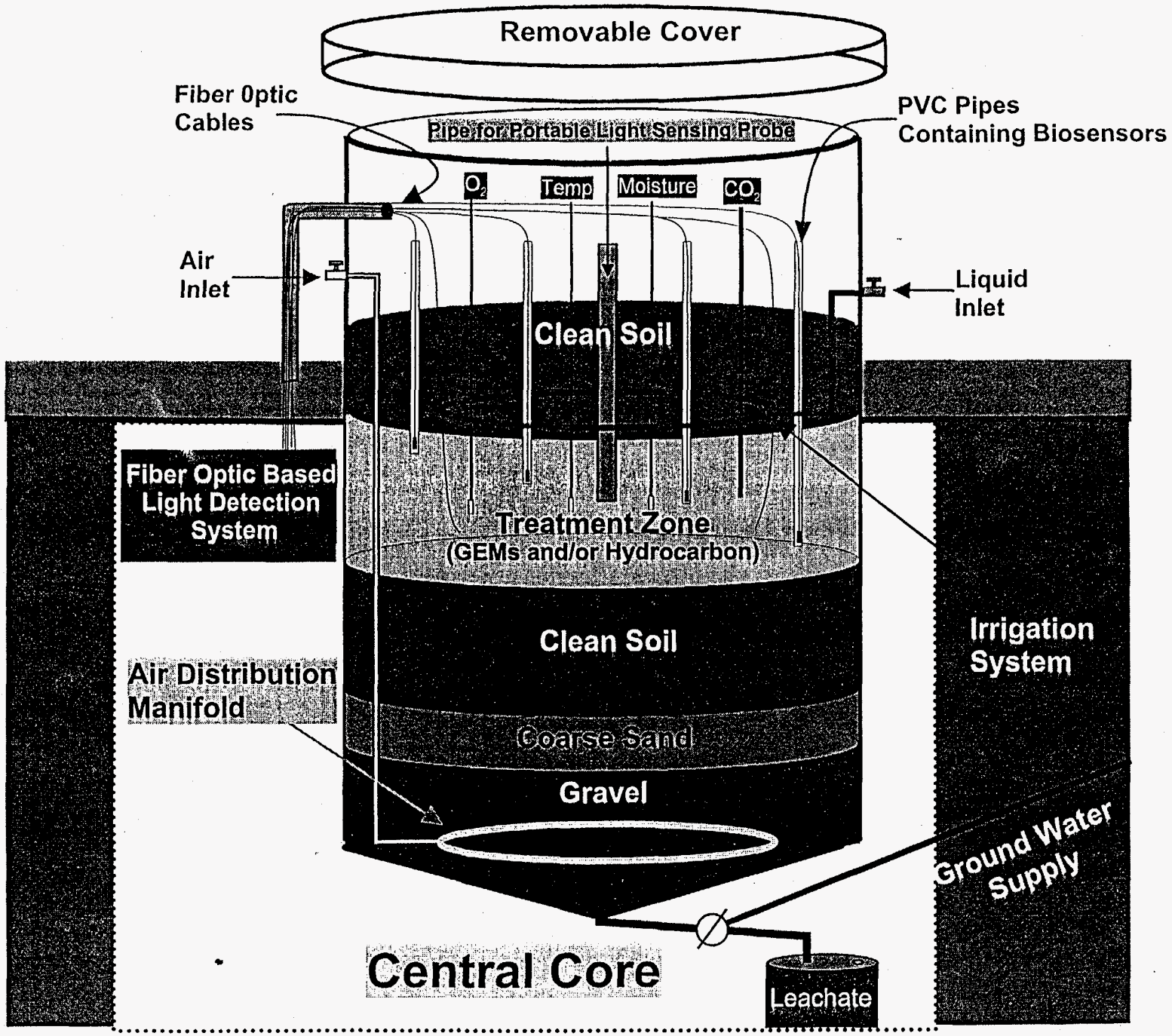
FIGURE 3. Colony hybridization data from Lysimeters 1 and 3. Results from Lysimeter 1 are typical of trends observed in Lysimeters 2 and 4 as well. The highest concentration of *P. fluorescens* HK44 cells is present in the first 31 cm of the contamination zone and gradually declines with depth. A large HK44 population was seen at the lowest depth when oil was added.

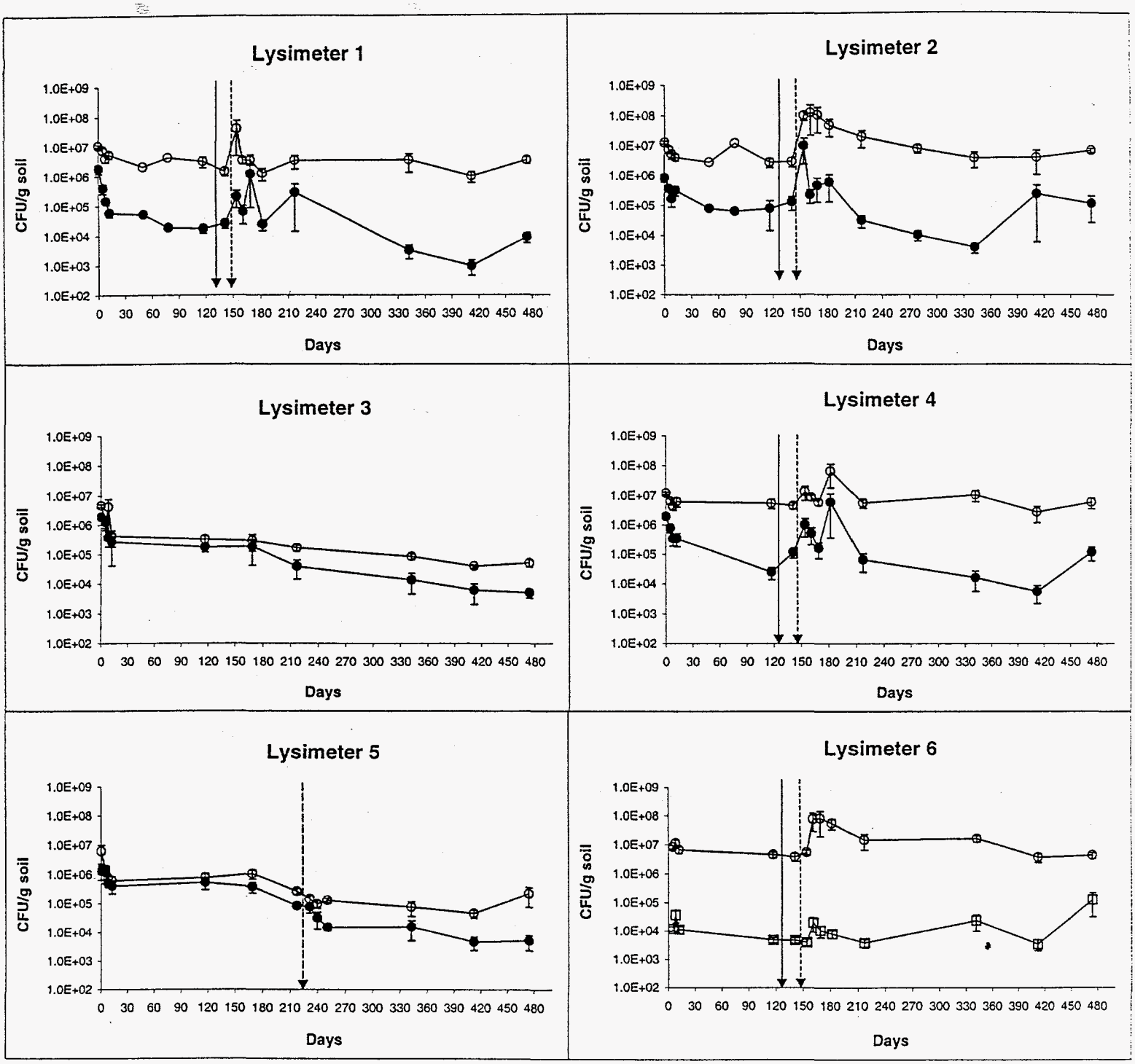
FIGURE 4. Bioluminescent response from encapsulated *P. fluorescens* HK44 cells in fiber optic biosensors at various depths in lysimeters 2 (A) and 3 (B). Lysimeter 3 contains no hydrocarbons and the low light response seen represents background levels of induction due to HK44 growth on exogenous sources of carbon in the alginate encapsulation medium. Depths originate from surface of treatment zone. Note the differences in the scale of the y-axis.

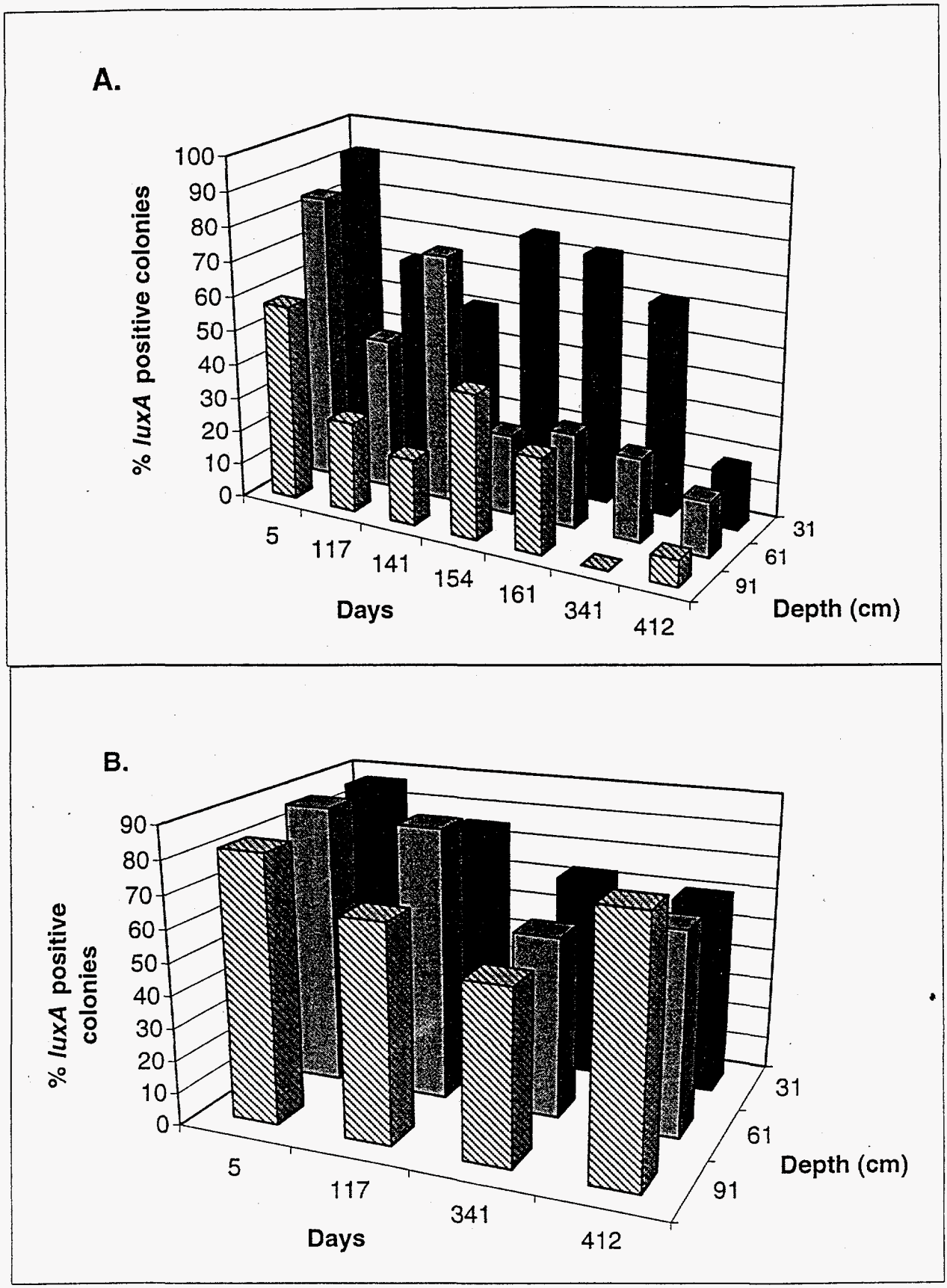
FIGURE 5. Bioluminescence from GEMs in lysimeter soil after the soil was challenged with naphthalene and a minimal salts solution. For comparison, dark counts from a soil that did not contain GEMs are presented (Lysimeter 6 soil).

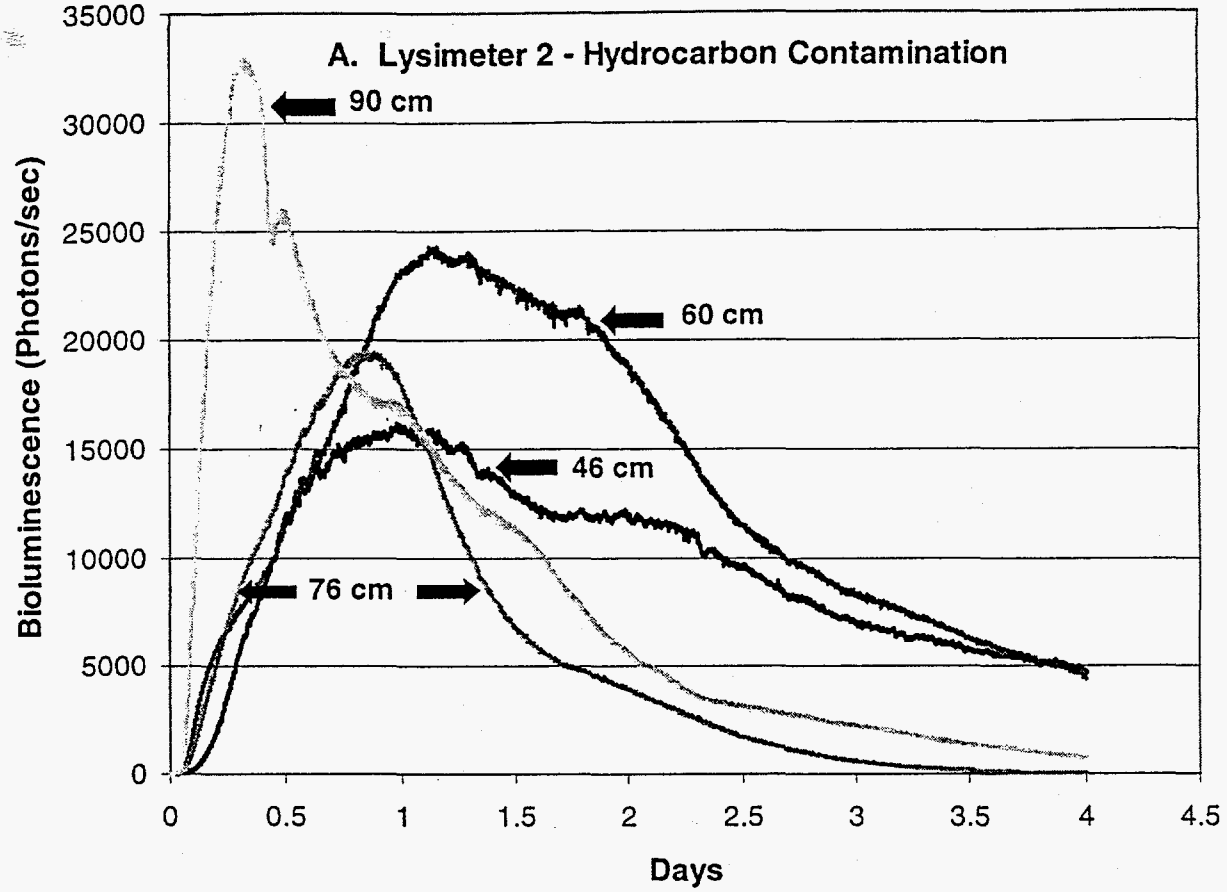
Lysimeter	Initial Concentration (CFU/g soil)[1]	Chemically Contaminated[1]	Nutrient Amendment[2]	Aeration[3]
1	$1.81 (\pm 1.02) \times 10^6$	+	+	+
2	$8.31 (\pm 3.93) \times 10^5$	+	+	+
3	$1.87 (\pm 0.09) \times 10^6$	-	-	-
4	$1.90 (\pm 0.30) \times 10^6$	+	+	+
5	$1.33 (\pm 0.99) \times 10^6$	-	-	-
6	-	+	+	+

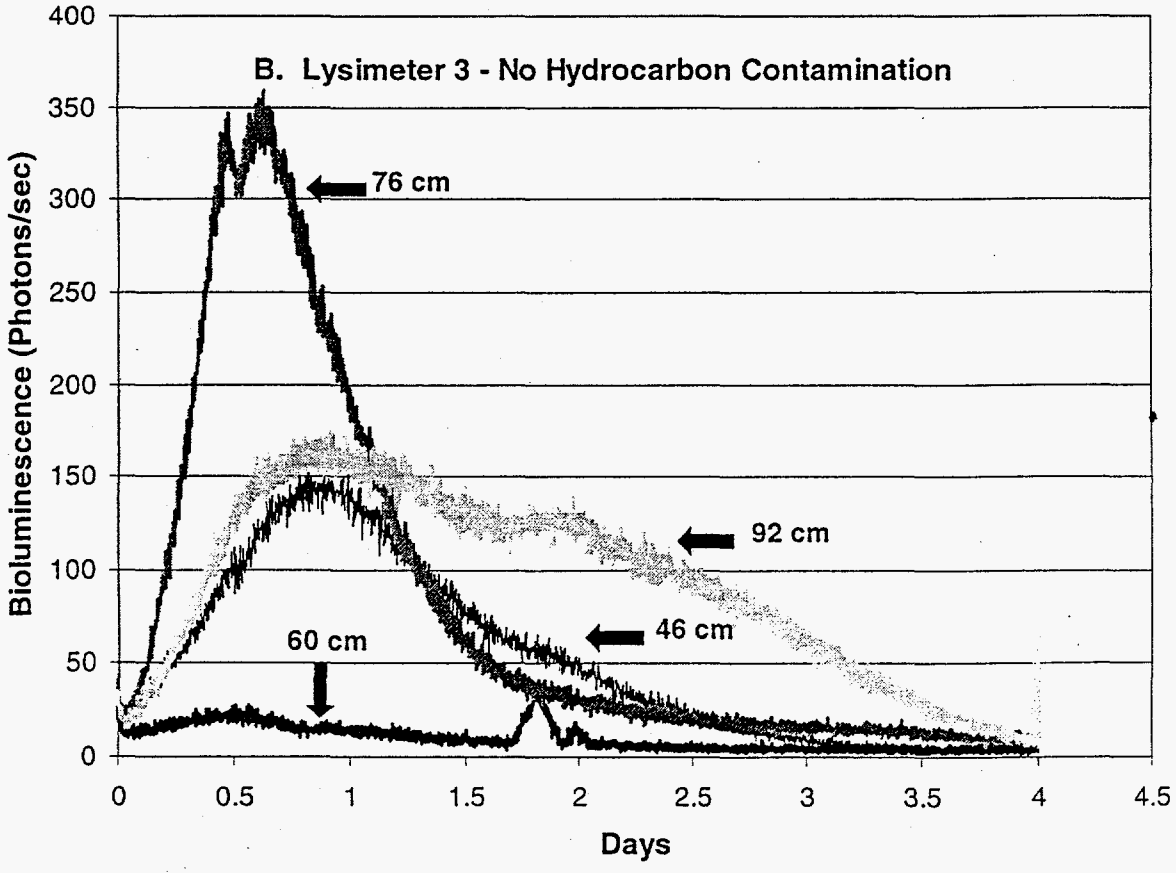
TABLE 1. Experimental treatments of each lysimeter
[1]Into each lysimeter was pumped 200 L of Exxon Univolt 60 transformer oil containing 24 g/L naphthalene and 2.4 g/L anthracene
[2]Nutrient amendment consisted of 150 L minimal salts medium per lysimeter. (minimal salts contains in g/L: $NaNO_3$, 2; KH_2PO_4, 0.75; $FeCl_3$, 0.003; $MgSO_4$, 0.1; $CaCl_2$, 0.005; Na_2HPO_4, 0.25)
[3]Lysimeters were aerated at 2 SCFM for 4 h twice weekly.

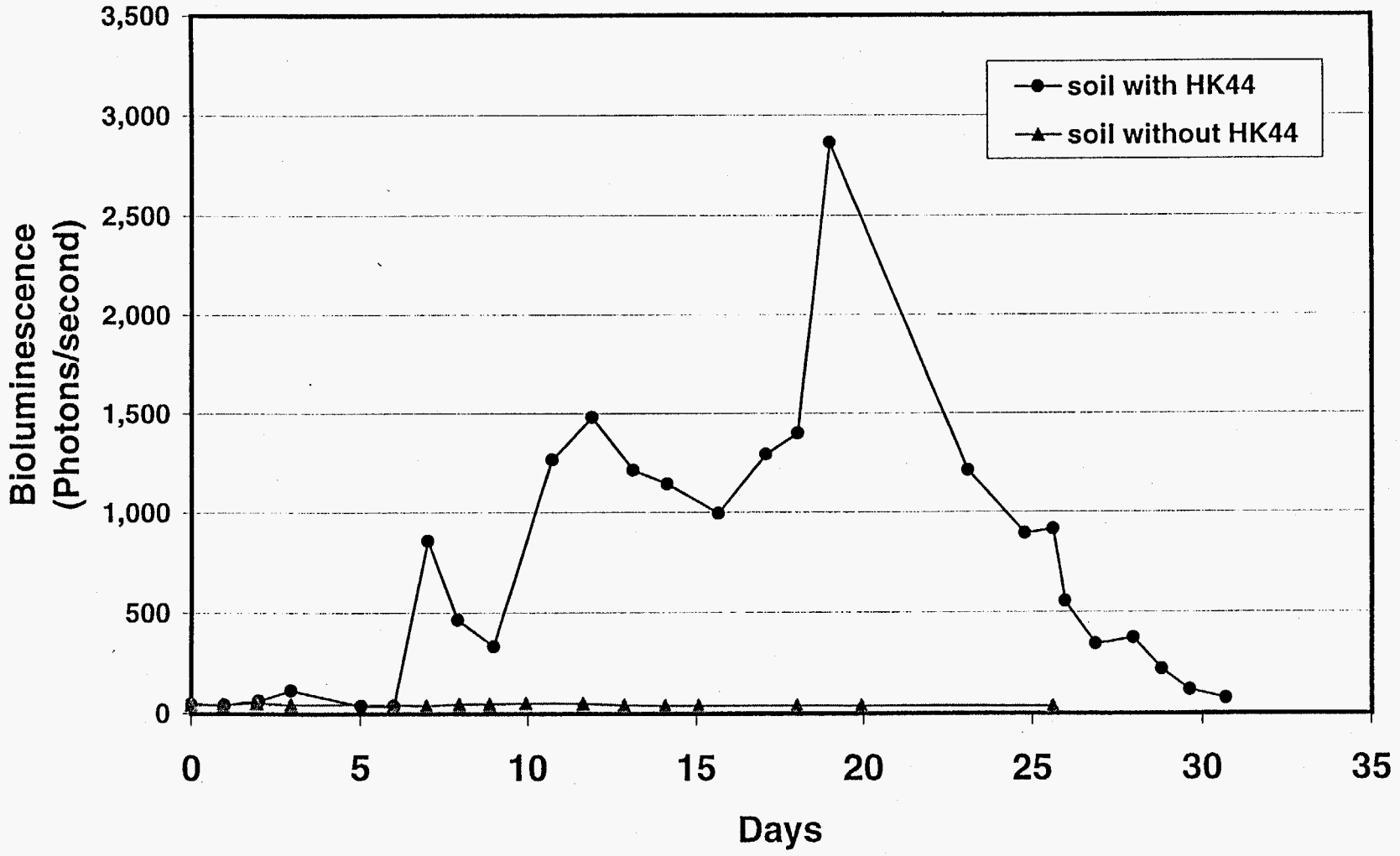

Removable Cover
Fiber 0ptic Cables
Pipe for Portable Light Sensing Probe
PVC Pipes Containing Biosensors
O_2
Temp
Moisture
CO_2
Air Inlet
Liquid Inlet
Clean Soil
Fiber Optic Based Light Detection System
Treatment Zone (GEMs and/or Hydrocarbon)
Clean Soil
Irrigation System
Air Distribution Manifold
Coarse Sand
Gravel
Ground Water Supply
Central Core
Leachate

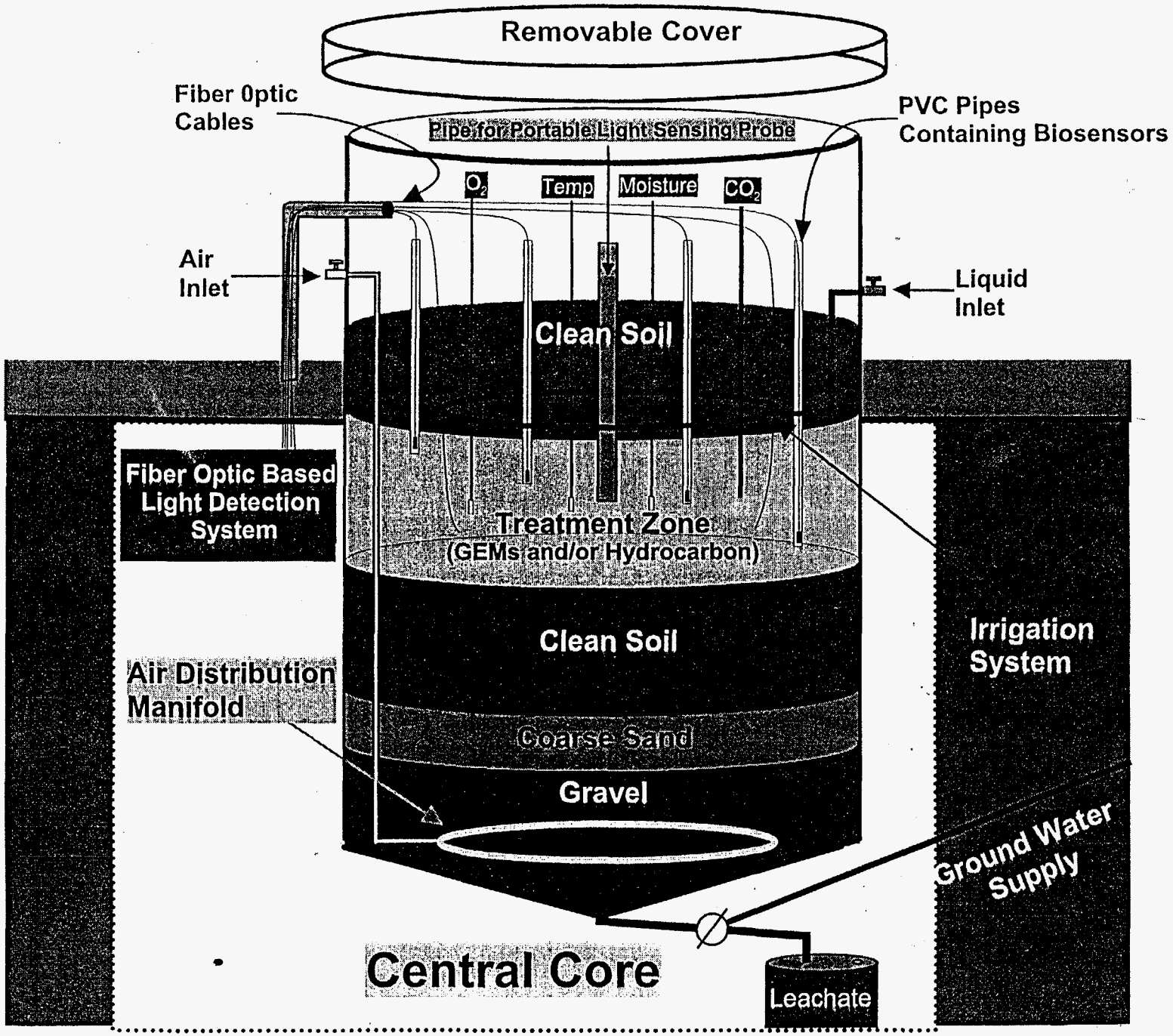

Lysimeter 1
Lysimeter 2
Lysimeter 3
Lysimeter 4
Lysimeter 5
Lysimeter 6
CFU/g soil
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
0 30 60 90 120 150 180 210 240 270 300 330 360 390 420 450 480
Days


A.
% luxA positive colonies
100
90
80
70
60
50
40
30
20
10
0
5
117
141
154
161
341
412
Days
31
61
91
Depth (cm)
B.
% luxA positive colonies
90
80
70
60
50
40
30
20
10
0
5
117
341
412
Days
31
61
91
Depth (cm)


A. Lysimeter 2 - Hydrocarbon Contamination
Bioluminescence (Photons/sec)
35000
30000
25000
20000
15000
10000
5000
0
90 cm
60 cm
46 cm
76 cm
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
Days


B. Lysimeter 3 - No Hydrocarbon Contamination
Bioluminescence (Photons/sec)
400
350
300
250
200
150
100
50
0
76 cm
92 cm
46 cm
60 cm
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
Days


3,500
3,000
2,500
2,000
1,500
1,000
500
0
Bioluminescence (Photons/second)
soil with HK44
soil without HK44
0
5
10
15
20
25
30
35
Days


Removable Cover
Fiber Optic Cables
Pipe for Portable Light Sensing Probe
PVC Pipes Containing Biosensors
O_2
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Moisture
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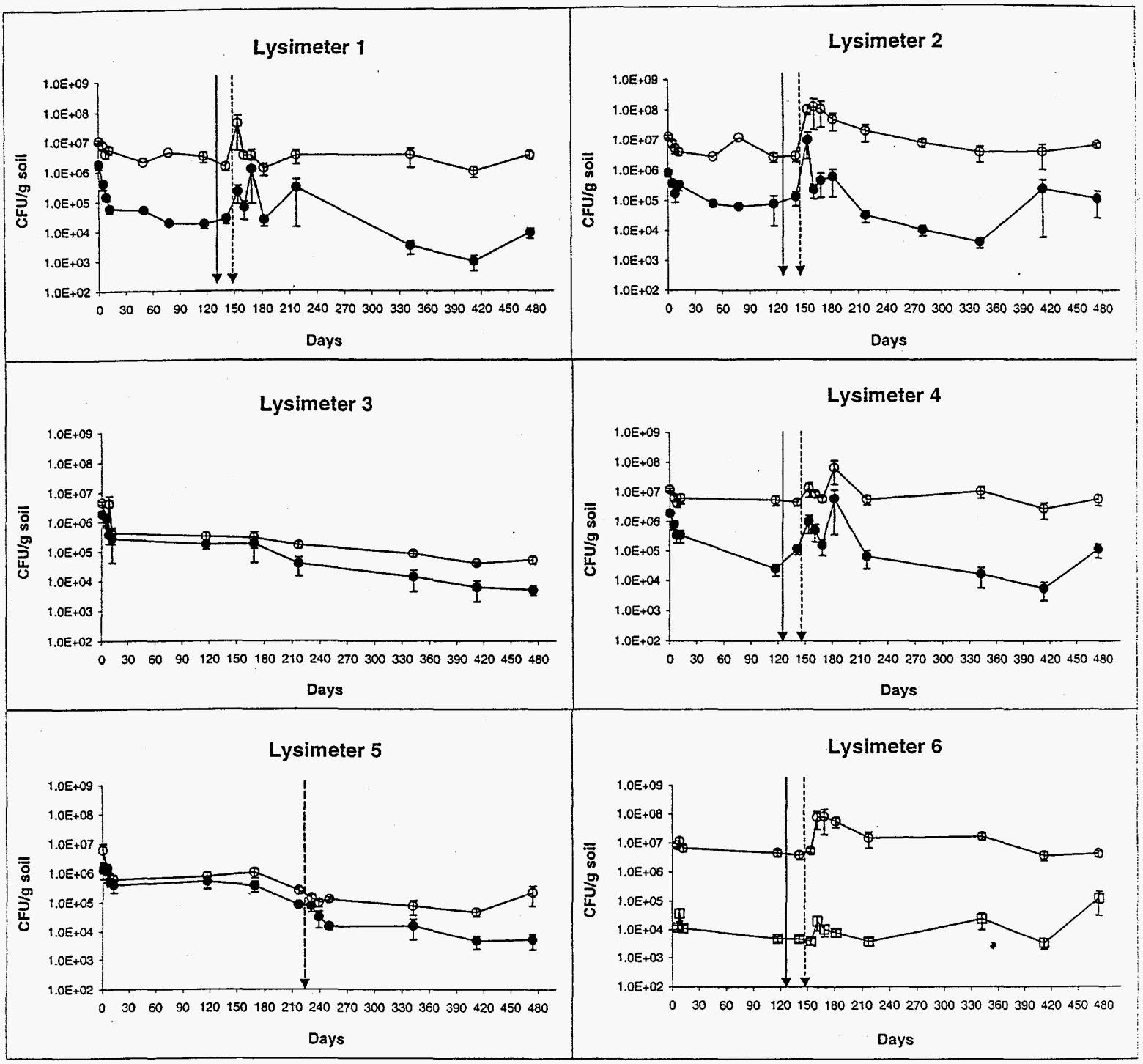
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Lysimeter 6
CFU/g soil
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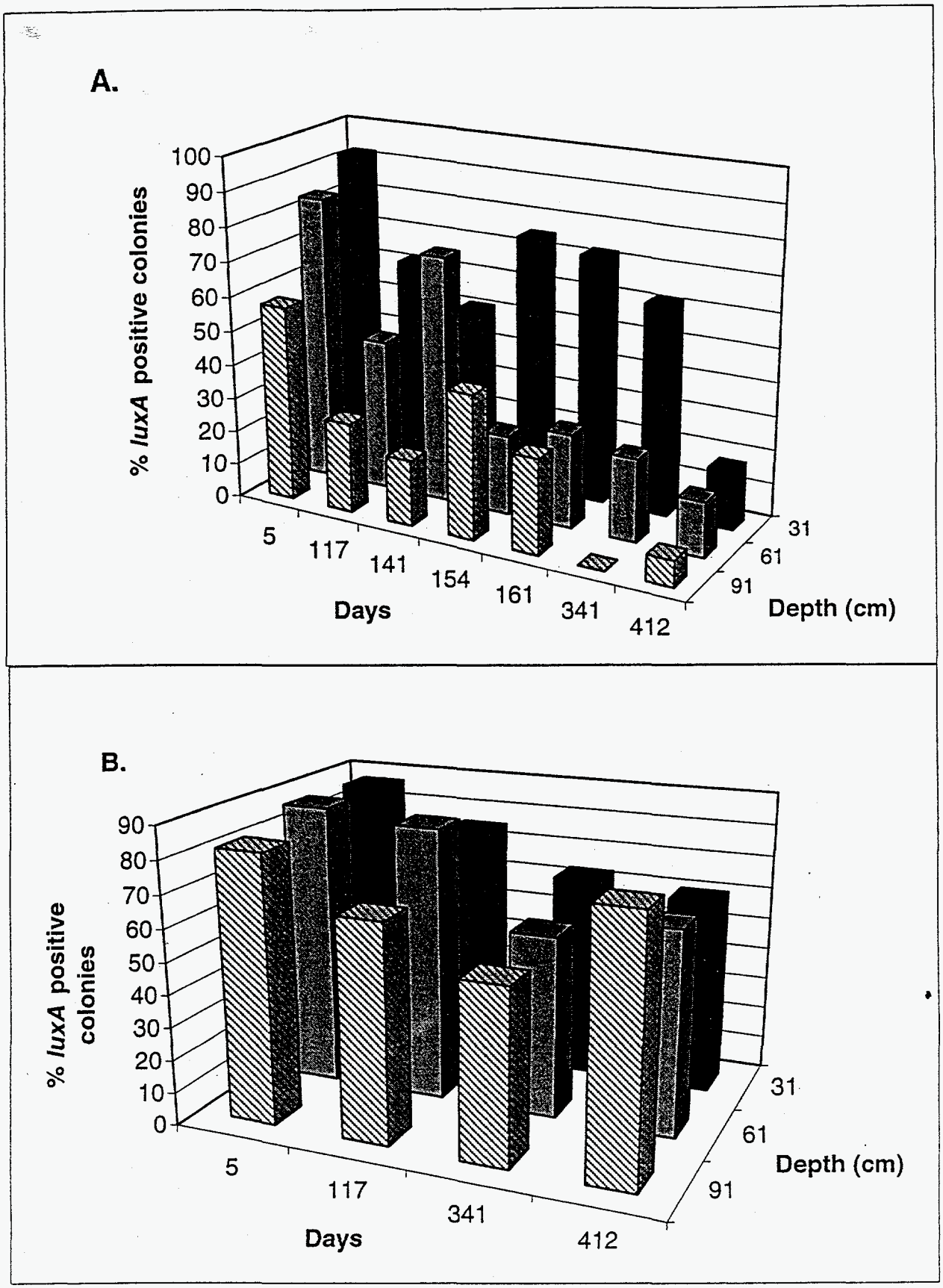
1.0E+05
1.0E+04
1.0E+03
1.0E+02
0 30 60 90 120 150 180 210 240 270 300 330 360 390 420 450 480
Days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Days
31
61
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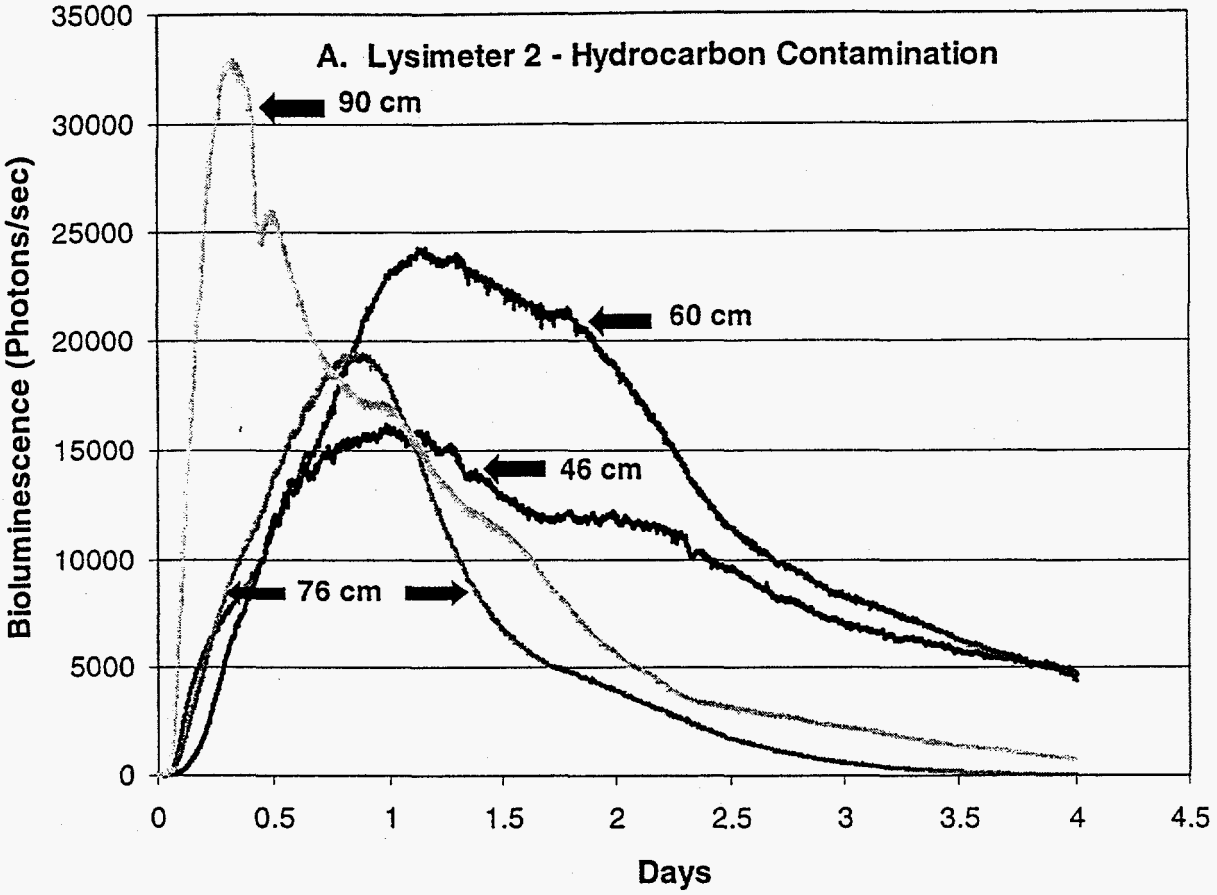
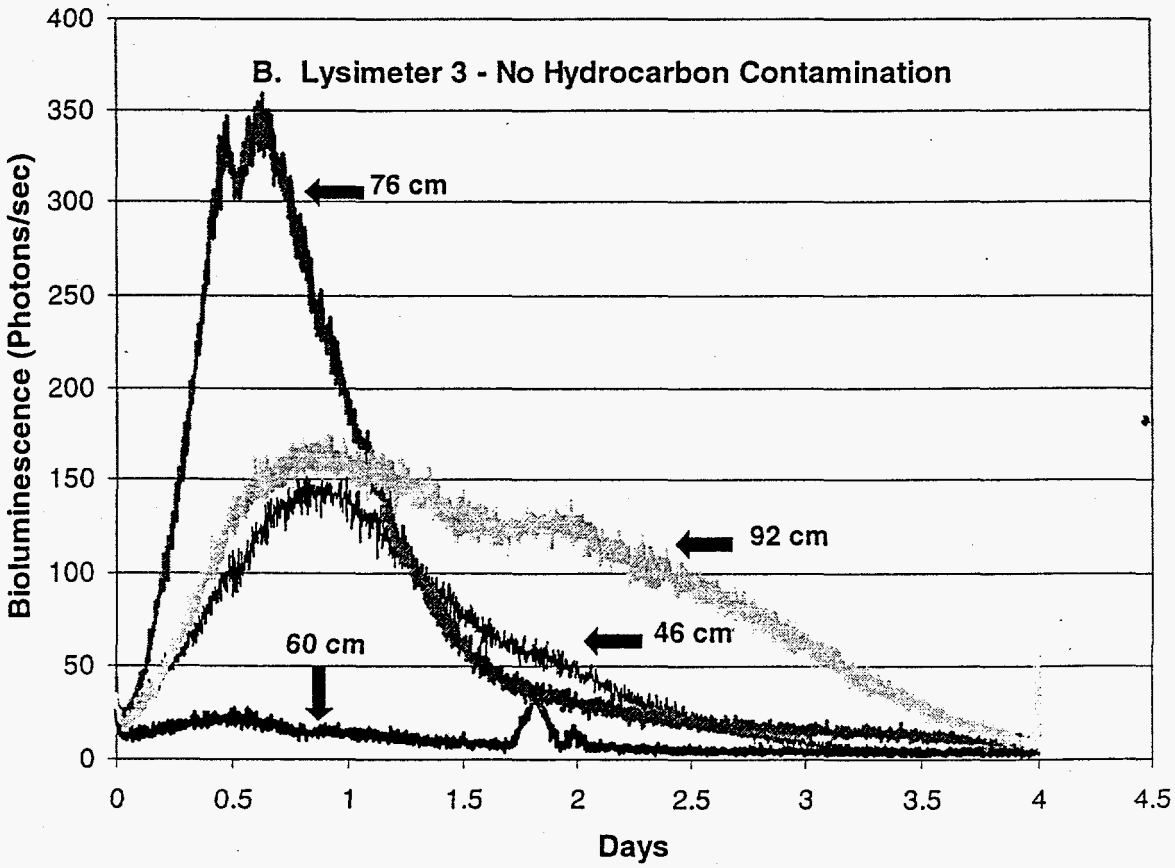
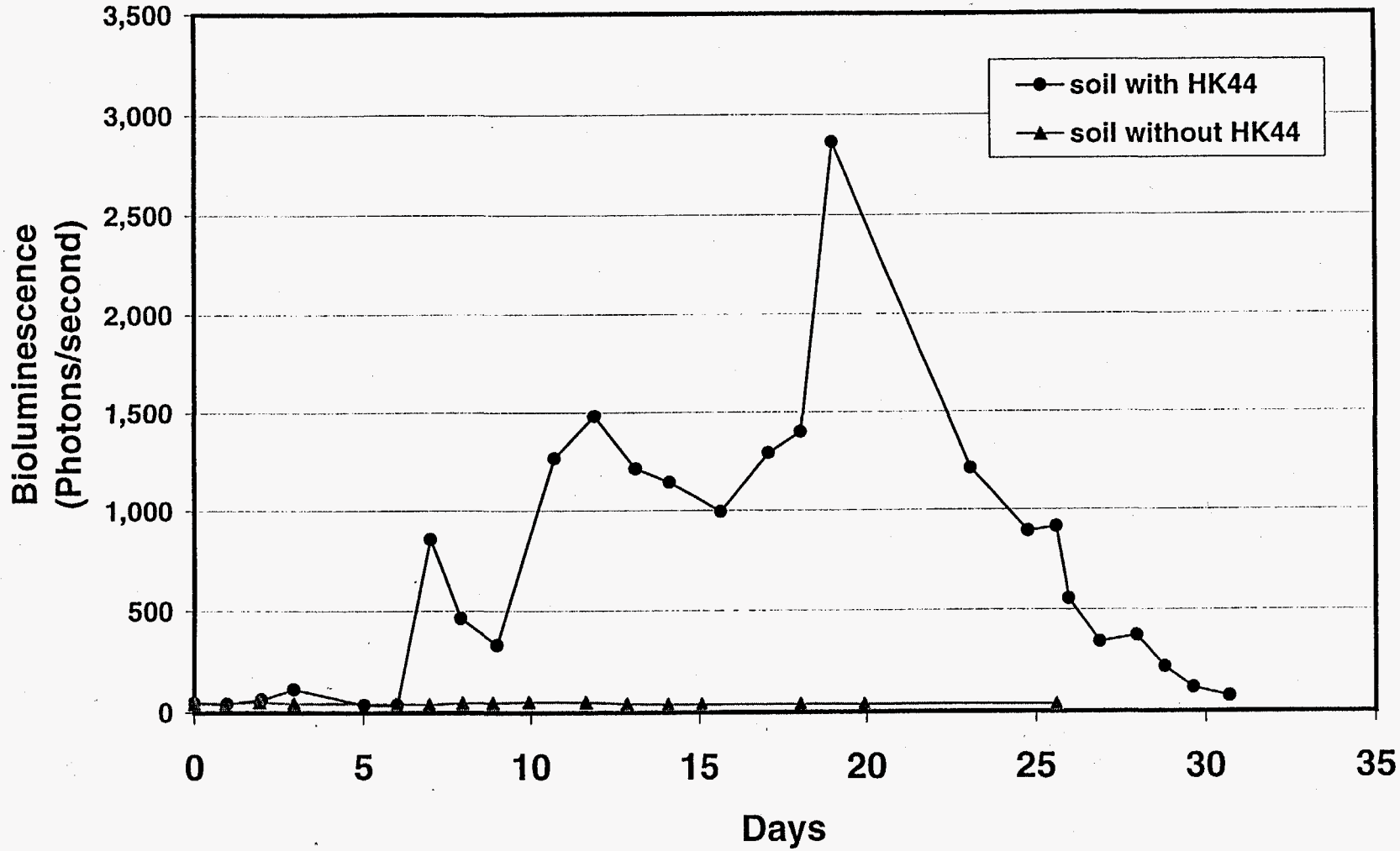
Depth (cm)
B.
% luxA positive colonies
90
80
70
60
50
40
30
20
10
0
5
117
341
412
Days
31
61
91
Depth (cm)


A. Lysimeter 2 - Hydrocarbon Contamination
90 cm
60 cm
46 cm
76 cm
Bioluminescence (Photons/sec)
35000
30000
25000
20000
15000
10000
5000
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
Days


B. Lysimeter 3 - No Hydrocarbon Contamination
76 cm
92 cm
46 cm
60 cm
Bioluminescence (Photons/sec)
400
350
300
250
200
150
100
50
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
Days


3,500
3,000
2,500
2,000
1,500
1,000
500
0
Bioluminescence (Photons/second)
soil with HK44
soil without HK44
0
5
10
15
20
25
30
35
Days