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Development of a Systems Analysis Approach for Resolving the Structure of Biodegrading Soil Systems

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ABSTRACT

An experimental and mathematical method is developed for the microbial systems analysis of polyaromatic hydrocarbon (PAH)-degrading mixed cultures in PAH-contaminated "town gas" soil systems. Frequency response is the primary experimental and data analysis tool used to probe the structure of these complicated systems. The objective is to provide a fundamental protocol for evaluating the performance of specific mixed microbial cultures on specific soil systems by elucidating the salient system variables and their interactions. Two well-described reactor systems, a constant volume stirred tank reactor (CSTR) and a plug flow differential volume reactor, are used in order to remove performance effects that are related to reactor type as opposed to system structure. These two reactor systems are well-defined systems that can be described mathematically and represent the two extremes of one potentially important system variable, macroscopic mass transfer. The experimental and mathematical structure of the protocol is described, experimental data is presented, and data analysis is demonstrated for the stripping, sorption, and biodegradation of napththalene.

Index Entries: Biodegradation kinetics; soil systems; frequency response; polyaromatic hydrocarbons; gene probes.

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NOMENCLATURE

C_A	liquid phase concentration of naphthalene	mg/L
C^B_A	concentration of naphthalene on the biomass	mg/cell
C^G_A	concentration of naphthalene in the gas phase	mg/L
C^S_A	concentration of naphthalene on the soil	mg/L
C_{in}	concentration of naphthalene in the feed	mg/L
D	dilution rate, F/A	$min.^{-1}$
F	feed flow rate	1/min.
f_L	weight fraction of lipids in the biomass	dimensionless
f_{oc}	weight fraction of organic carbon in the soil	dimensionless
H	Henry's law constant	dimensionless
K_B	partition coefficient, biomass to liquid	1/cell
K_{ow}	octanol-water partition coefficient	dimensionless
K_P	partition coefficient, soil to liquid	dimensionless
K_S	soil sorption parameter	1/g
$K_{st}a$	stripping constant, QH/F	dimensionless
Q	gas flow rate	1/min
t	time	min
R	rate of naphthalene disappearance	mg/min
V	slurry volume in reactor	L
V_l	liquid phase volume in reactor	L
W	soil concentration in reactor	g/L
X	biomass concentration	cells/L
ρ_B	biomass density	g/cell
ρ_L	lipid density	g/L
ρ_S	soil density	g/L

INTRODUCTION

Biological treatment has been the primary method for dealing with most wastewater systems. There is now an increasing need for an economic method of treating soils contaminated with toxic organics, many of which are resistant to biotransformation. Of particular interest are the thousands of manufacture gas plant (MGP) sites in the United States where the pyrolysis of coal for production of a synthetic natural gas (town gas) has resulted in soils that are contaminated with polyaromatic hydrocarbons (PAHs) to depths of over 15 feet. Bioremediation of these systems can take one of two possible forms: *in situ* treatment, or removal and subsequent detoxification in some type of soil slurry reactor. Of primary concern is the effectiveness of the cultures being used and, for evaluation and regulation purposes, a reliable estimate of the time required for remediation. Past designs of wastewater treatment facilities focused on two major system variables, oxygen demand and total biomass, and employed a simple empirical model. This approach will not work with soil systems in which the oxygen demand of the specific organics are relatively small

compared to the total oxygen demand and the biotransformation kinetics are slow relative to other abiotic fates, such as stripping and sorption.

In evaluating bioremediation of soil systems contaminated with toxic organics, the predictions for remediation time are unreliable, because the system's structure is complicated and unknown, and the correct mathematical models are not available. It has been shown (*1*) that relatively small subpopulations of degraders consisting of $<1\%$ can have a profound effect on the biotransformation of toxic xenobiotic organics (which are resistant to attack). Specific models are required for both biotransformation and population dynamics that incorporate the effects of mass transfer, degrader populations, and concentrations of specific organics.

The objective of this work is to develop a systems analysis protocol for resolving the structure of biodegrading soil systems contaminated with PAHs in order to improve the reliability of bioremediation predictions. This objective is accomplished by applying frequency response analysis to two experimental bench scale reactor systems, a CSTR and a differential volume reactor (DVR), along with the development of appropriate mathematical models for systems analysis. Three compounds (naphthalene, phenanthrene, and benzo[a]pyrene) and two soil systems (an uncontaminated soil and MGP soil) will serve as models.

Frequency Response

Frequency response was developed in chemical engineering (*2*) for resolving the dynamic structure of process-control systems and subsequently has been widely applied in many other disciplines. Applications include geophysical systems (*3,4*), human speech (*5*), biological systems (*6–8*), econometrics (*9,10*), and environmental systems (*11*). The ability of this systems analysis approach to resolve complex structures has been demonstrated by Ljung (*12*).

Several authors (*13–16*) have proposed the use of systems analysis techniques for evaluation of microbial systems. A microbial system of unknown complexity is perturbed in some manner by making an input disturbance in the concentration of some substrate, dissolved oxygen, temperature, etc., and the response of the system is monitored. The systems' response can then be used to draw inferences concerning the internal structure. The number of system unknowns can be reduced by conducting the biotransformation in a defined reactor configuration where the models for the reactor are known (fluid dynamics, mass transfer, temperature, and so on). Unexplained variations in monitored activity can then be attributed to ecological and biological effects. Figure 1 illustrates the basic concept. As an example, in a simple system with no stripping, sorption, or biological activity, the output amplitude and phase angle will be a known function of the input wave form and the time constant (residence time) of the CSTR. If, for example, stripping were present and was

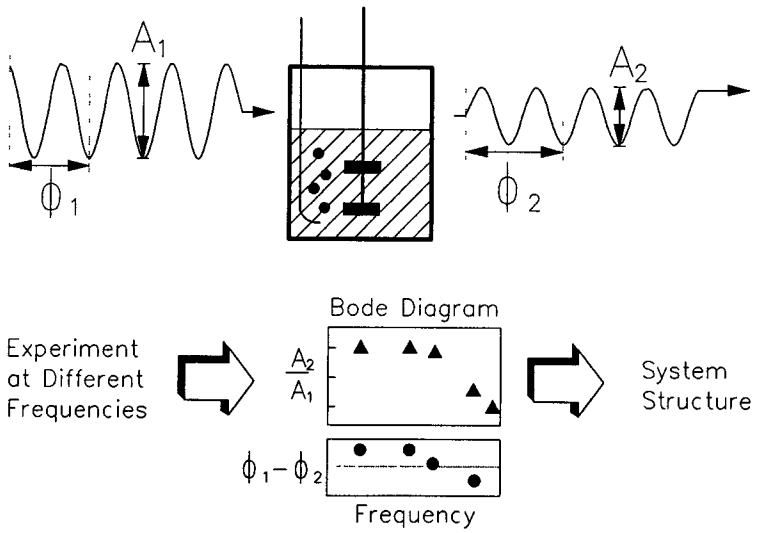

Fig. 1. Illustration of the frequency response concept for systems analysis.

accounted for by using a system model, the altered output wave form could be predicted; any discrepancy would indicate the presence of other physical or biological processes in the systems structure.

PROTOCOL DEVELOPMENT

The structure of the microbial soil slurry system is resolved into three levels: physical, ecological, and cellular. By using two well-defined reactor systems, a CSTR and a DVR, the physical transport phenomena have been resolved using simple mathematical models with the equilibrium and rate parameters for stripping and sorption determined from static and dynamic abiotic frequency response experiments. Figure 2 presents a representation of our model for the physical transport system within the CSTR bioreactor. The substrate is stripped from the liquid phase into the air and is modeled based on an equilibrium constant. The substrate is also sorbed from the liquid phase onto the surface of the soil particles and biomass, and transported into the internal structure of the soil particles. Both an equilibrium and rate constant are required to describe this process accurately.

The ecological level is determined by fitting different kinetic models (*17*) to experimental frequency response data at different cycles coupled with gene probes for discriminating degrader populations. Inferences are drawn on the cellular level by further perturbing the system after the physical and ecological effects are well defined. This method is based on

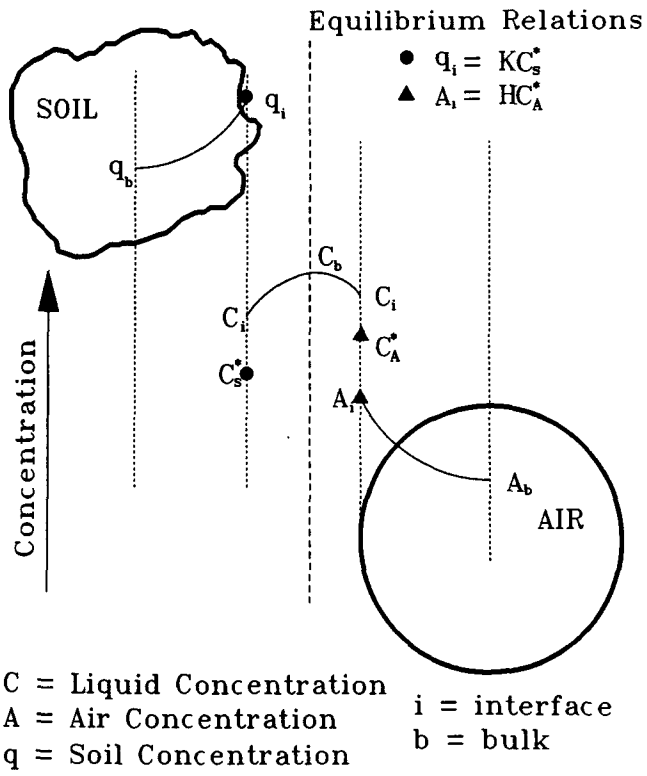

Fig. 2. The mass transport system within the CSTR.

three compounds, two soil types, and one mixed culture. Once the experimental and data-analysis procedures and the system models are well established, this method can be easily and quickly applied to any soil system, with any contaminant and any culture, for the purpose of improving the reliabilility of bioremediation predictions.

EXPERIMENTAL

The experimental equipment consisted of two different types of flow reactors: four CSTR reactors and two differential volume reactors. The physical environments were resolved experimentally using both steady-state and frequency response experiments coupled with simple mathematical expressions for stripping and sorption. Attempts were then made to quantify the structure at the ecological level, both by using biological frequency response experiments in which the PAH substrate is the control variable and employing various kinetic models for biotransformation and population dynamics.

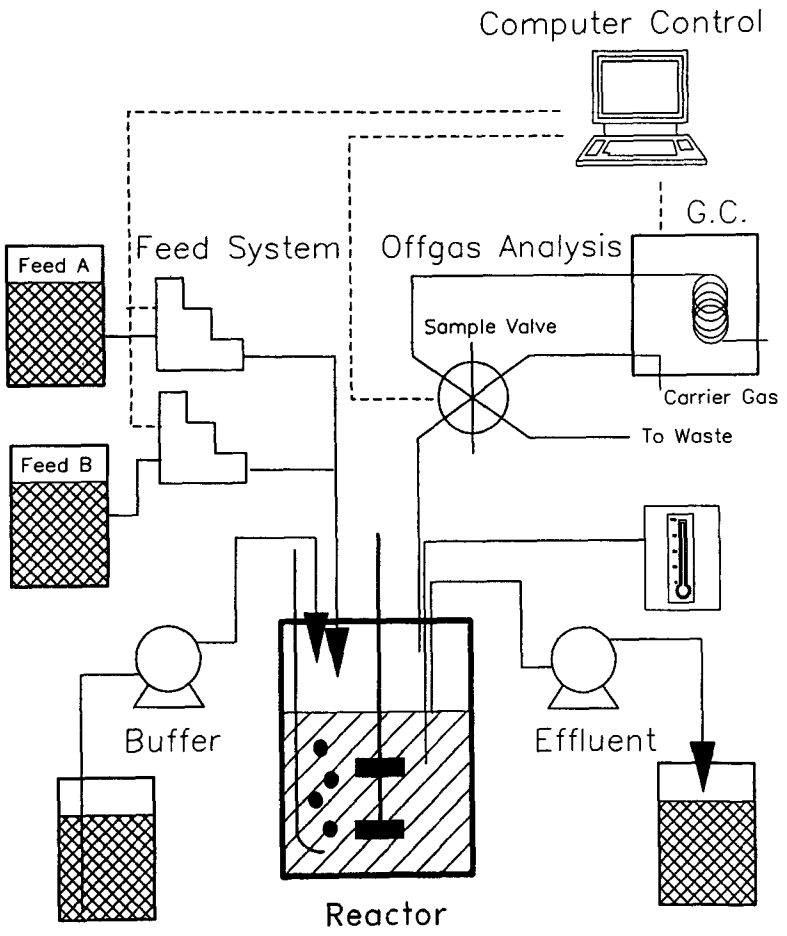

Fig. 3. Diagram of the CSTR slurry reactor system.

CSTR

The experimental CSTR reactor system is depicted in Fig. 3. This system consisted of two LH Fermentation Series 500 continuous flow bioreactors, 1 L in volume, with direct drive agitation, dissolved oxygen measurement, temperature control, and air flow control. The liquid volume in the reactor was a soil slurry, with a total volume of 0.75 L containing 400 g/L of soil. The feed was pumped continuously to the reactors using two Gilson positive displacement pumps/reactor. Each pump operated in a sinusoidal manner 180° out of phase with the other so as to maintain a constant total flow rate. One pump fed a prepared soil extract saturated with naphthalene at 4°C, the other pump fed the same material minus the naphthalene. The effluent was taken from the top of the reactor and collected using a Masterflex peristaltic pump. Effluent solids were separated once daily by centrifugation and returned to the reactor. The

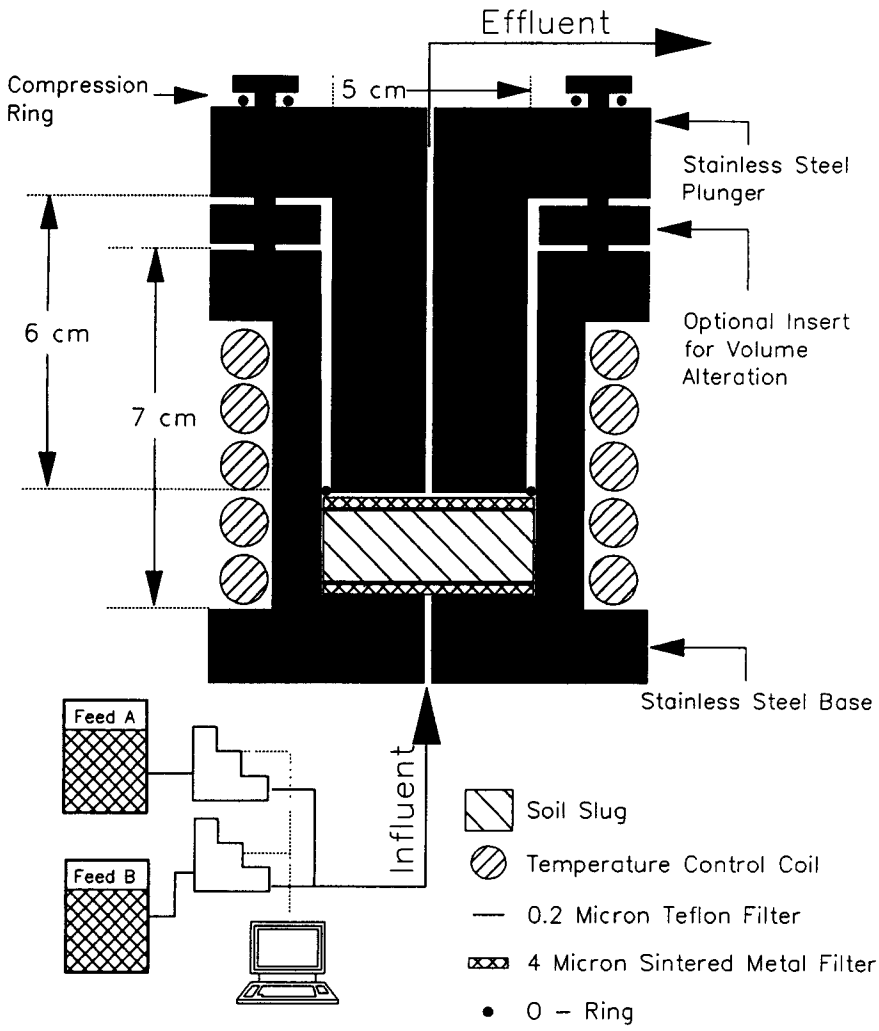

Fig. 4. Diagram of the DVR Reactor system.

reactor off-gas was automatically monitored using a Valco sampling valve, 7.0-mL sample loop, on-line Shimadzu GC-9AM gas chromatograph equipped with a flame ionization detector and a Shimadzu CR501 integrator. The frequency of the feed pumps and reactor offgas sampling were controlled by an IBM XT computer. The scheme depicted in Fig. 3 has now been used for operating a total of four reactors.

Differential Volume Reactors (DVR)

Figure 4 shows the DVR. The peripherals and mode of operation were the same as the CSTR, except for the elimination of recycling and the use of liquid-phase sampling instead of reactor offgas. The height of the soil column within the reactor was adjustable from 1/4 to 2 in, the col-

umn bed was supported by a Pall Metal porous metal filter with a porosity of 4 μm. The filter also served as a flow distributor. An identical filter was located at the outlet to prevent soil particles from leaving the reactor. The reactor was sealed with an *n*-butyl rubber O-ring capable of holding several thousand pounds of pressure. Compression rings provided control of the soil hydraulic conductivity to better simulate the undisturbed environment.

Materials and Methods

The feed solution was prepared by a hot water extraction of uncontaminated soil using an autoclave. After cooling, solids were removed by continuous centrifugation followed by pressure filtration through a 0.45-μm filter, and the material was stored at 4°C in two 55-gallon drums. Half of the soil extract was saturated with naphthalene at 50°C before cooling to 4°C. The solubility of naphthalene at 4°C is 15 mg/L; the total TOC of the soil extract is 50 mg/L. The inoculum was a mixed microbial culture prepared from a series of batch enrichments in 1/10 strength yeast extract, peptone, and glucose (YEPG) medium supplemented with 100 mg/L each of anthracene, naphthalene, and phenanthrene. MGP soil was added to the primary enrichment together with slurries from a set of PAH-exposed freshwater microcosms (*18*). The inoculum was frozen and stored at −90°C. Prior to addition to the reactors, the inoculum was pregrown from the frozen stock in YEPG medium. MGP soil was taken from a MGP site and contained a large number of PAHs.

Specific degrader populations for naphthalene were determined by using gene probe analysis. *E. coli.* BHB 2600 strain carrying the plasmid pDTG113 was used for source plasmid DNA in generating the [^{32}P] DNA probes. The strain was obtained from D.T. Gibson and contains the upper-pathway genes, coding for initial naphthalene biodegradation. Plasmid DNA was prepared by an alkaline lysis method (*19*) and purified by dye-buoyant density ultracentrifugation in cesium chloride and ethidium bromide (*19*). [^{32}P] DNA probe was prepared with a nick translation kit (Bethesda Research Laboratory, Besthesda, MD) by following the manufactures protocol. Labeled probe was purified by using a spin-column procedure (*19*). Specific activity of the labeled DNA probe was approximately 10^7–10^8 dpm/μg DNA. Samples of slurry and effluent from the reactors were added to phosphate buffers (pH 7.0) and sodium pyrophosphate (0.1% w/v), and vortexed at high speed for 2 min. Total cells were enumerated by the serial dilution technique and spread plate inoculation of YEPG agar plates, which were incubated for 4 d at 20°C. The NAH genotype population was enumerated by using DNA:DNA colony hybridization (*20,21*) using the previously described plasmid. Radiolabel gas samples were taken using 7-mL evacuated blood sample vials. The CO_2 was trapped in sodium hydroxide after using a hexane extraction to remove any volatile naphthalene from the gas sample. A scintillation counter was used to determine the CO_2 concentration (*13*).

RESULTS AND DISCUSSION

Only results from the naphthalene CSTR experiments will be reported here, since the DVR experiments have just started. Stripping from the liquid phase of the CSTR was accounted for by using a model developed by Blackburn (*22,23*) and a prediction of the Henry's law constant for naphthalene (*24*). This model was also used to predict the naphthalene liquid phase concentration from a measured value of the reactor offgas. The solubility of naphthalene in water was determined from static equilibrium experiments. The sorption/desorption of naphthalene from the soil particles was determined from frequency response experiments.

Abiotic Data

Figure 5 presents some of the experimental results. The top portion of this figure illustrates the controlled liquid phase input of naphthalene into the CSTR; the lower portion gives the measured response in the liquid phase naphthalene concentration. The soil was not saturated with naphthalene; the initial upward slope of the line results from equilibrium saturation of the soil. Figure 6 demonstrates how the sorption partition coefficients can be derived from the experimental data. A naphthalene mass balance was performed on the CSTR system using the following dynamic equation, assuming the solid/liquid-phase rate constant for naphthalene sorption was very large.

$$d(V_L \cdot C_A)/(dt) + V/\rho_s \cdot d(W \cdot C^S_A)/(dt) + V \cdot X \cdot d(C^B_A)/dt = F \cdot C_{in} - F \cdot X \cdot C^B_A - F \cdot V_L \cdot C_A/V - F \cdot W \cdot C^S_A/\rho_S + Q \cdot C^V_A - R \quad (1)$$

Variable definitions and units are given in the nomenclature. The left side of Eq. (1) represents the accumulation of substrate in the liquid, soil, and biomass, respectively. The first term on the right represents the input of substrate to the reactor, and the following four terms represent substrate removal in the biomass, liquid, soil, and vapor. The last term accounts for substrate removal by biodegradation. The three-phase equilibrium for naphthalene is described by the following expressions:

$$C^V_A = H \cdot C_A \quad (2)$$

$$C^S_A = 0.62 \cdot f_{oc} \cdot K_{ow} = K_P \cdot C_A \quad (3)$$

$$C^B_A = f_L \cdot \rho_B \cdot K_{ow} \cdot C_A/\rho_L = K_B \cdot C_A \quad (4)$$

The liquid volume, in terms of the weight of soil in the reactor, is expressed by

$$V_L = V \cdot (1.0 - W/\rho_s) \quad (5)$$

and sorption of naphthalene on the soil is related to the octanol/water partition coefficient by

$$K_S = [K_P - 1.0)/\rho_s \quad (6)$$

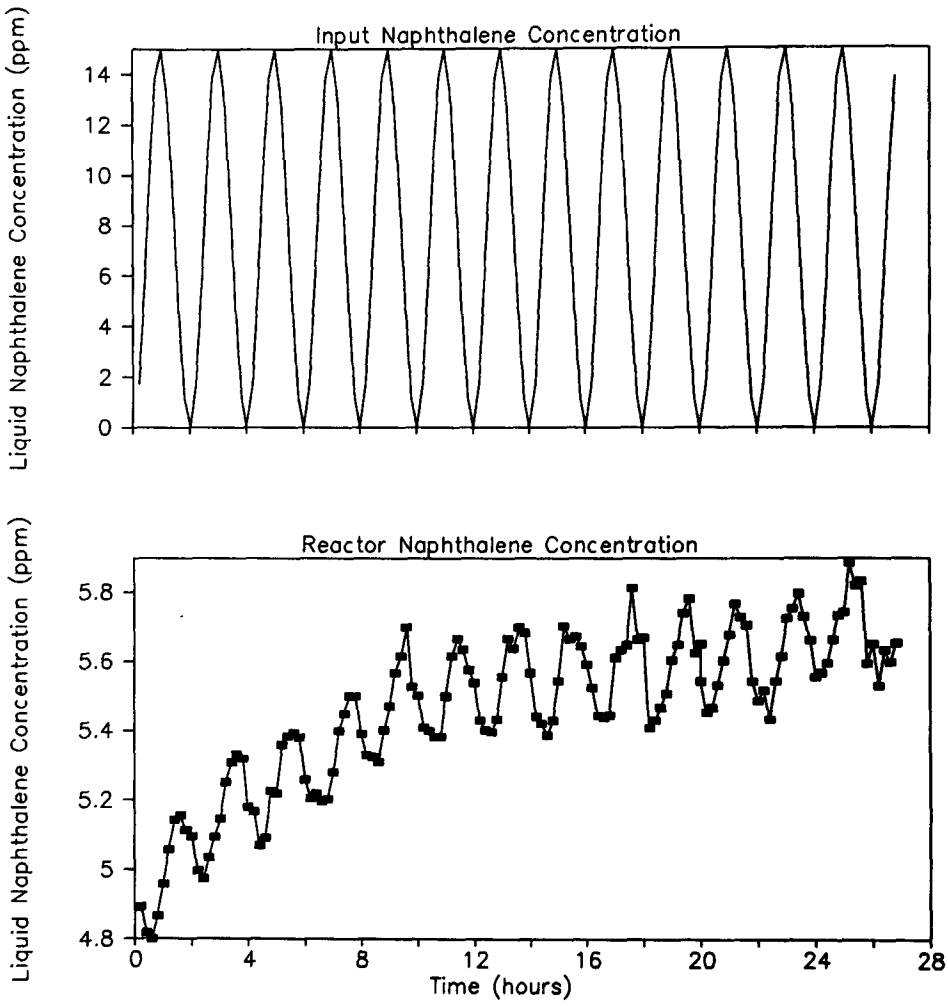

Fig. 5. Input and output data for the abiotic adsorption of naphthalene on soil in a CSTR.

Substitution of Eqs. 2-6 into Eq. 1 yields the expression used for data analysis:

$$(1.0 + K_S \cdot W + K_B \cdot X) \cdot dC_A/dt + (K_S \cdot C_A) \cdot dW/dt = D \cdot C_{in} - R/V - D \cdot C_A \cdot (1.0 - K_S \cdot W + K_B \cdot X + K_{st}a) \quad (7)$$

The value of Henry's constant, H, was determined experimentally in pure water and then in soil slurries to verify that the presence of the soil did not alter the value. These results compared favorably with literature values (24). Sorption on the biomass was modeled using the octanol/water partition coefficient (25), thus leaving K_S, the sorption/desorption equilibrium constant, as the only unknown. The present alternative for determining the value of K_S uses regression analysis or methods from Karick-

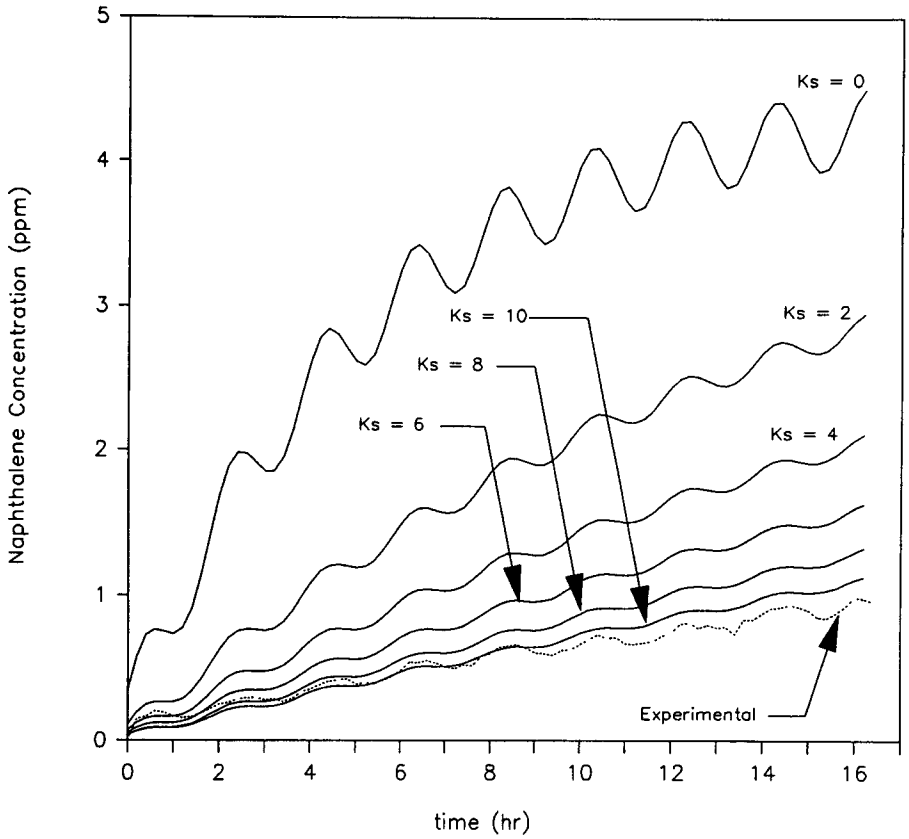

Fig. 6. Prediction of naphthalene concentration as a function of soil binding.

koff (*26,27*). The values for K_S (derived from the data presented in Fig. 5) using this simple model) decrease with increasing naphthalene concentration, indicating that the rate constant for sorption cannot be neglected. It was necessary to put the volume terms within the differential (Eq. 1), because they changed with time as soil was continuously removed from the CSTR and was replaced every 24 h. The CSTR has been redesigned to eliminate this soil loss problem.

Biological Data

Biological experiments considered the population dynamics of the CSTR system at several temperatures (Fig. 7). The total population was quite stable, with the NAH genotype population accounting for approximately 1% of the total enumerated on the YEPG plates. As can be seen from Fig. 7, the stability of the NAH subpopulation, as it related to the total population, did change slightly with temperature. The biotransformation rate constants determined from this experiments are presented in

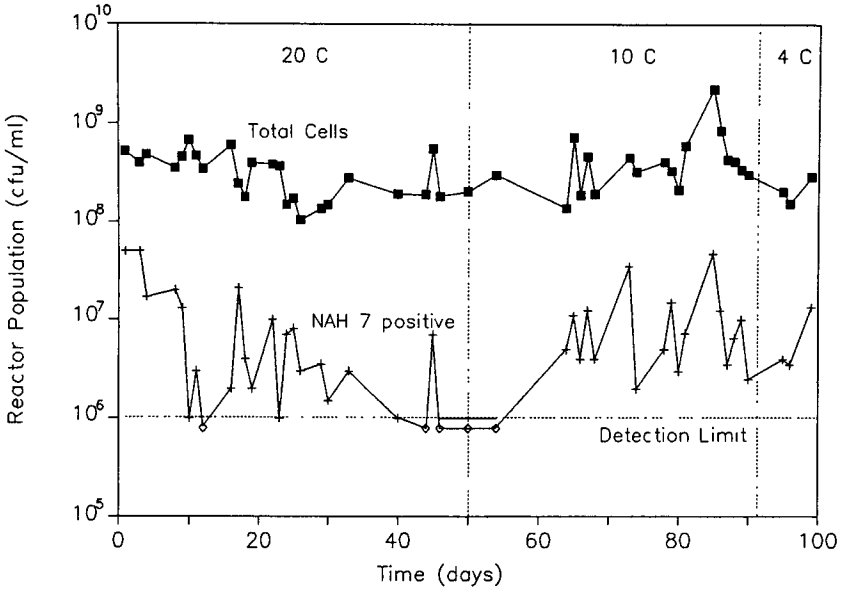

Fig. 7. Population dynamics for the total culture and the NAH subpopulation.

Table 1
Naphthalene Transformation in CSTR Slurry Reactors

Temp	Genotype Cfu/mL	1st order CSTR biotransformation rate const, h^{-1}	1st order batch rate const h^{-1} biotrans. min.		2nd order batch rate const h^{-1} biotrans. min.	
					($X10^{-9}$)	
20	10^7	>650	-	0.16	-	16
20	5×10^6	>650	0.16	0.094	32	19
10	2×10^6	50	0.076	0.044	38	22
10	5×10^6	>380	0	-	-	-
4	5×10^6	4	0.031	0.010	6.2	2.0

Table 1. These rate constants were three orders of magnitude greater than those determined from shake flask experiments under identical conditions. (*28*) This large difference indicates that mass transfer from the soil to the reactor liquid is probably the rate-limiting step in this particular system.

Radiolabel Experiments

Radiolabel experiments were conducted to gain insight into the mechanism for naphthalene biotransformation. The soil was presaturated with radiolabeled naphthalene and placed in the CSTR with a continuous input

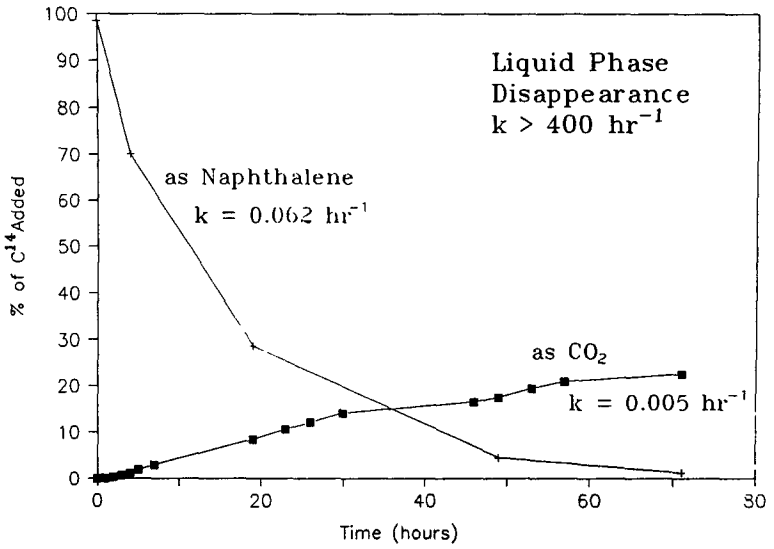

Fig. 8. Biotransformation of adsorbed vs free solution naphthalene.

flow of unlabeled naphthalene (Fig. 8). Based on reactor offgas samples, the first-order rate constant for naphthalene disappearance from the liquid phase was greater than 400/h, whereas the first-order rate constant for disappearance of the radio labeled naphthalene from the soil was between 0.005–0.062/h. This range was derived from the two limiting measurements shown in Fig. 8, the disappearance of ^{14}C-labeled naphthalene and the quantity of $^{14}CO_2$ in the reactor offgas. The discrepancy results from two sources: complete mineralization evidenced by the CO_2 measurement and our inability to acidify the liquid, which probably resulted in losing a significant amount of the $^{14}CO_2$ because of its water solubility. Coupling these results with previous experiments, which showed the naphthalene degrader population on the soil to be two orders of magnitude greater than the liquid phase population, suggests that the majority of naphthalene degraders resided in the biofilm and that they predominately utilized naphthalene from the liquid phase.

Frequency Response Experiments

Figure 9 presents (some preliminary) biological data from frequency response experiments. The top portion of the figure shows the input liquid-phase concentration, the lower portion the measured response. We know what the amplitude, frequency, and phase angle of the output wave should be without biotic or abiotic removal mechanisms. From the abiotic experiments, models were developed to account for the physical effects of stripping and sorption/desorption (the input-wave form does

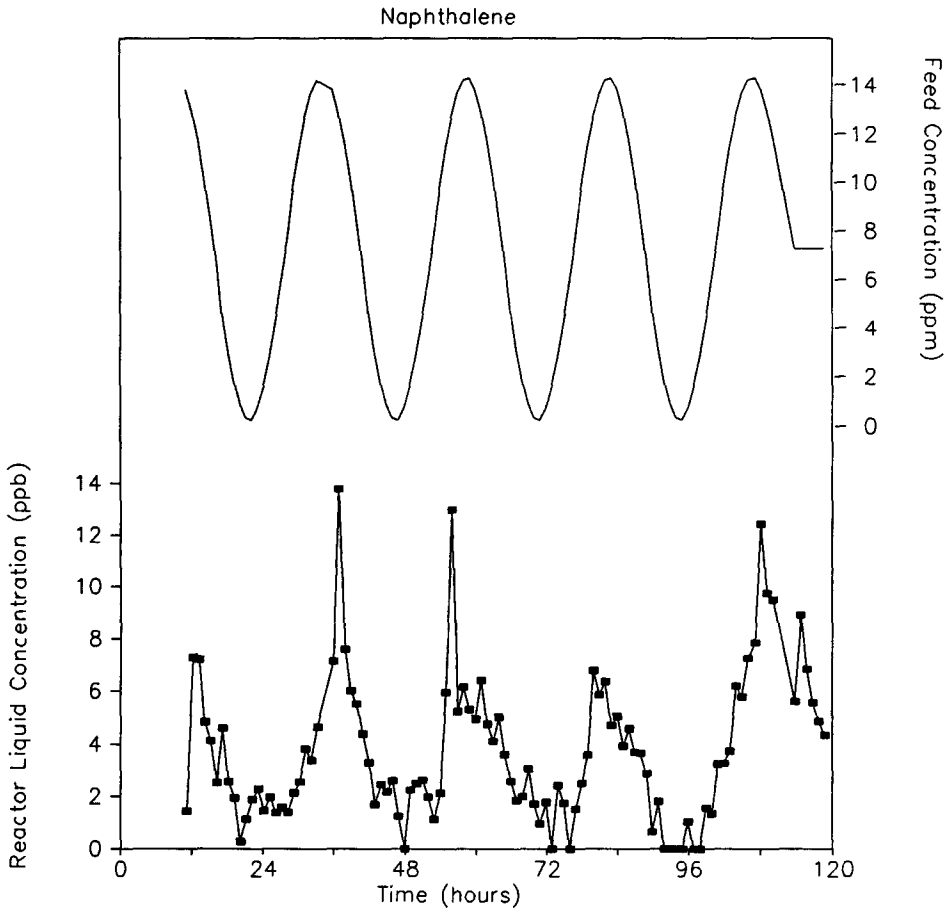

Fig. 9. Frequency response analysis of naphthalene biotransformation in a soil slurry CSTR.

not matter in this case). The biological data is analyzed by looking at the discrepancies in the output-wave form and the alteration of the output wave form produced by changing the frequency of the input-wave form. Different models for biotransformation and population dynamics are used to fit the experimental data. Those models that are able to account for the frequency, amplitude, and phase shift of the naphthalene concentration in the output, and for the dynamics of the NAH genotype population (from gene probe data) will be the ones that most accurately describe the system structure. For the biological experiments it is necessary to investigate a wide range of input frequencies in order to ensure that the range covers the time-constant values of unknown and important components of the systems structure. For example, Fig. 7 shows that the NAH genotype population was quite stable over time, but changed slightly with temperature. We would expect, if there were no other forces present, that this population would be insensitive to high-frequency pulses in the feed concentration. However, as the frequencies became slower we might

hit a critical frequency that would elicit a response from the NAH genotype population, thus enabling us to further resolve the system structure. The degree to which system structure can be resolved will depend on the system's complexity, the background noise, and the quality of existing models.

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