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Molecular Site Assessment and Process Monitoring in Bioremediation and Natural Attenuation

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ABSTRACT

A variety of modern biotechnical approaches are available to assist in optimizing and controlling bioremediation processes. These approaches are broad-ranging, and may include genetic engineering to improve biodegradative performance, maintenance of the environment, and process monitoring and control. In addition to direct genetic engineering strategies, molecular diagnostic and monitoring technology using DNA gene probing methods and new quantitative mRNA analytical procedures allows direct analysis of degradative capacity, activity, and response under *in situ* conditions. Applications of these molecular approaches in process developments for trichloroethylene (TCE), polychlorinated biphenyls (PCB), and polynuclear aromatic hydrocarbons (PAH) bio-oxidation in soils, aquifer sediments, and ground-water treatment reactors have been demonstrated. Molecular genetic technologies permit not only the development of new processes for bioremediation, but also new process monitoring, control strategies, and molecular optimization paradigms that take full advantage of vast and diverse abilities of microorganisms to destroy problem chemicals.

Index Entries: Bioremediation; environmental biotechnology; trichloroethylene; polychlorinated biphenyls; polynuclear aromatic hydrocarbons.

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INTRODUCTION

A number of opportunities exist for utilizing genetic engineering and advanced molecular biology techniques in developing, monitoring, and controlling bioprocesses in improving remediation of environmental contamination problems. The application of these molecular technologies can be grouped into two broad categories: microbial strain development and improvement, and process monitoring and optimization.

Both of these categories represent rather traditional practices in conventional bioprocessing and biotechnology, yet they have received relatively little attention and utilization in bioremediation technology. Applications of genetic engineering technology in strain development have largely been confined to strain characterization to understand the fundamental mechanisms of biodegration of environmental contaminants. Although applications of molecular methods in process monitoring and optimization have received recent attention for environmental use, they have not yet achieved practical applications in routine *in situ* or *ex situ* processing. The objective of this article is to review briefly fundamental developments in applying genetic engineering and molecular technology for bioremediation, and to describe some recent environmentally relevant applications for important classes of environmental contaminants. Some genetic engineering strategies for strain development and improvement have focused on the following:

1. Pathway construction to expand pollutant substrate range and to avoid buildup of potentially inhibitory metabolites;
2. Modification of gene expression or gene copy number to increase biodegradative enzyme production;
3. Protein engineering to broaden substrate specificity or enhance enzyme activity; and
4. Alteration of gene regulation for process control and environmentally acceptable deployment.

These strategies have been exploited in the laboratory for biodegradation of pollutants, such as chlorobenzoates (*1*), phenol (*2*), PCB (*3*), and TCE (*4*), and their potential for improving biodegradative performance has been demonstrated. What remains to be demonstrated is the performance of such engineered or selected nonengineered strains in reactor and environmental applications. In such cases, both the organisms and the bioprocess must be able to operate effectively in a generally heterogeneous environmental matrix that hosts a variety of potentially competing or interfering microorganisms in a mixed-culture format. Added to this environmental heterogeneity is the fact that many contaminants, such as PCBs, TCE, and some PAHs, serve only as cometabolic substrates, and do not act selectively to induce or maintain biodegradative organisms by acting as a growth-supporting substrate.

In dealing with the environmental context, other genetic and molecular strategies may be brought to bear either to assist in developing organisms with greater mixed-culture competitive advantages or in developing monitoring and control strategies to assist in maximizing or optimizing system performance. The molecular technologies applied to developing new strategies for improved bioremediation of generally recalcitrant pollutants are the primary object of this article and include:

1. Developing DNA and RNA probe technology for monitoring degradative population dynamics in remediation processes;
2. The use of field application vectors to create selectively maintained strains to function effectively in remediation of cometabolically degraded pollutants;
3. Creating bioluminescent reporter gene fusions to permit on-line sensing and control of biodegradative processes; and
4. Optimizing reactor design and operating conditions to meet the molecular needs for cometabolic oxidation of pollutant substrates.

Several of these strategies have been explored for PCB, TCE, and PAH contamination problems.

PCB REMEDIATION POTENTIAL IN CONTAMINATED SOILS

Over the past decade, considerable progress has been made in the fundamental understanding of how bacteria mediate degradation of PCB (*5–7*). The structural genes for complete oxidation of biphenyl have been identified and sequenced for some bacteria. Site-specific mutagenic techniques have been used to engineer enzymes with broadened specificity (*8*). However, the majority of PCBs remain nongrowth or poor growth substrates, making it difficult to degrade them to completion, often with the accumulation of chlorobenzoates as stable intermediates. Perhaps even more important is the challenge of making bound PCB residues "available" for biodegradation. Because of the low water solubility and hydrophobic nature of PCB, the majority of the pollutant will be bound to the soil matrix making it nearly inaccessible for bacterial degradation.

In recent studies to assess the potential for remediating mineral soils contaminated by PCB at electrical substations, site assessments were carried out using molecular methods to quantify the presence of desired PCB degradative genes in soils and to evaluate treatment options to remediate soil-bound PCB residues (*8*). Preliminary studies demonstrated the apparent contamination of the Tennessee Valley Authority (TVA) Moccasin Bend Substation soil with Aroclor 1248 in concentrations ranging from 50

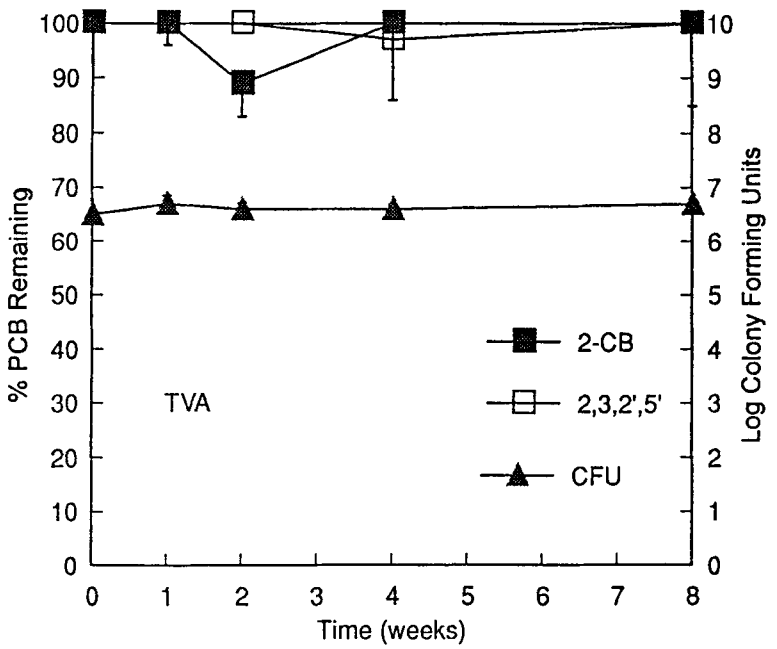

Fig. 1. The lack of PCB biodegradation in PCB-contaminated TVA substation soil. Samples for gas chromatographic analysis were taken from a soil slurry amended with 2000 ppm 2-chlorobiphenyl (2-CB). 2,3,2′,5′-Tetrachlorobiphenyl present in the soil was graphed as an indication of *in situ* PCB degradation. CFU is the number of colony forming units on nonselective agar (*10*).

to 780 ppm. Degradation of some of the lesser chlorinated congeners was apparent in analyzing GC profiles. However, lab-scale feasibility tests to promote further degradation of PCB congeners, by stimulation of the native bacterial population with 2-chlorobiphenyl as a growth substrate, indicate a poor biodegradative potential in these mineral soils (Fig. 1). In contrast, a PCB-contaminated soil from New England (200 ppm PCB) was amendable to biodegradation by stimulation of the native population with 2-chlorobiphenyl (Fig. 2). This interpretation can be reinforced using molecular probe technology to compare quantitative abundance of bacterial populations in TVA substation soil and New England soil (Table 1). In the New England soil slurries, the number of bacterial colony forming units (CFU) and the percentage of CFU hybridizing to the *bph*C gene in colony hybridization experiments increased as 2-chlorobiphenyl was degraded (Fig. 2, Table 1), suggesting a specific increase in the PCB degrading bacterial population. In the TVA soil slurries, the number of CFU did not increase over the 8-wk course of the experiment, and no *bph*C-positive colonies were detected in colony hybridization experiments (Fig. 1, Table 1).

The consequences of the above molecular diagnostic analysis are that either specific nutrients must be added to encourage the growth of PCB degraders, for example, biphenyl, or an alternative strategy for selective maintenance of PCB degraders will be required to remediate these soils.

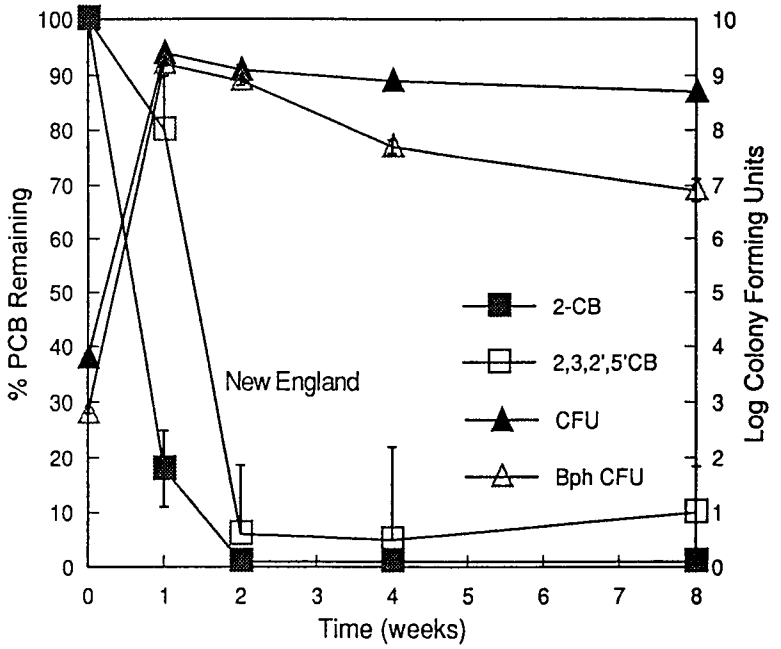

Fig. 2. PCB biodegradation and biodegradative bacterial population dynamics in PCB-contaminated New England soil. Treatment conditions were the same as Fig. 1. *Bph*CFU were those CFU that hybridized with the *bph*C gene probe (*see* Table 1).

Table 1
Total Bacterial CFU and the Percentage of Bacteria Hybridizing to the *bph*C Gene from TVA Power Plant Substation and New England Soils After the Addition of 1000 ppm 2-chlorobiphenyl

Time, wk	TVA		New England	
	CFU	%*bph*C[a]	CFU	%*bph*C[a]
0	3.5×10^6	0	7.1×10^3	8.6
1	5.5×10^6	0	2.3×10^9	69
2	4.4×10^6	0	1.4×10^9	74
4	3.6×10^6	0	1.2×10^9	5.9
8	7.0×10^6	0	4.6×10^8	1.6

[a] %*bph*C—the percentage of bacterial colonies that hybridize to the *bph*C gene (encoding 2,3-dihydroxyl biphenyl deoxygenase) from *Pseudomonas pseudoalcaligenes* KF707 (ref. *6;* the KF707 *bph*BC clone was courtesy of K. Furukawa).

One approach built around the selective maintenance concept is the development of field application vectors (FAV) (*10*). For these soils, a bacterial strain selected for growth on surfactants capable of enhancing PCB bioavailability was genetically modified by the inclusion of genes encoding enzymes required for phenyl oxidation. The resulting strain IPL5 can thus selectively grow on the surfactant used to "solubilize" PCB and simultaneously cometabolize PCB residues via the constitutive expression of the biphenyl operon (Fig. 3, Table 2) (*11*).

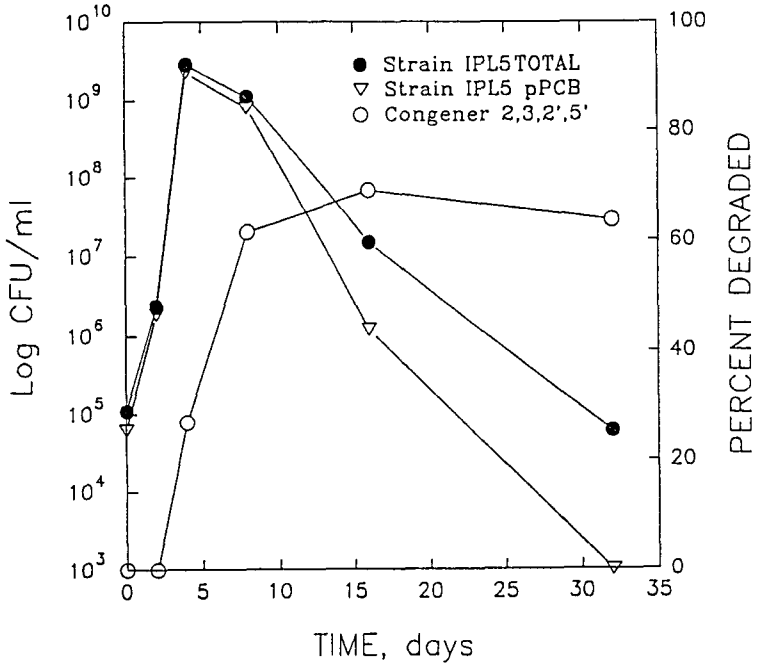

Fig. 3. Degradation of PCBs in electric power substation soil. Soil slurries consisted of 1 g of soil and 5 mL of minimal medium amended with the surfactant Igepal CO—720 (1% w/v) and inoculated with the recombinant surfactant degrading strain *P. putida* IPL5 containing a plasmid (pPCB) conferring tetracycline resistance and PCB degradative ability. Degradation of PBC congener 2,3,2′,5′-tetrachlorobiphenyl (Congener 2,3,2′,5′) and time-course concentrations of strain IPL5 (IPL5 TOTAL) and the same strain maintaining tetracyline resistance (Tet^r) and PCB degradative ability (*bph*C^+) are indicated. No PCB degradation was observed in uninoculated soil slurries or slurries inoculated with strain IPL5 without the inserted PCB degradative plasmid (data not presented). This figure is reproduced from *Applied & Environmental Microbiology* (*25*).

The two preceding examples illustrate the molecular approaches that can be applied (1) in assessing the ability of organisms within a contaminated site to promote the degradation of the pollutant of concern, and (2) in developing a new treatment strategy that integrates the biodegradative potential of the microorganisms with existing surfactant soil-washing technology currently available for the physical removal of hydrophobic contaminants from soil. However, since this latter approach using FAV does utilize a genetically modified organism, its environmental deployment would still be subject to some regulatory oversight.

OPTIMIZATION AND MONITORING TCE COMETABOLISM

Extensive evidence exists indicating that many chlorinated alkenes and alkanes can be biodegraded both aerobically and anaerobically. Tri-

Table 2
PCB Degradation[a] in TVA Power Plant Substation Soil

Peak number	PCB congener identification	Percent degraded
5	2,4,4′	25.8
6	2′,3,4/2,5,2′,6′	57.1
7	2,3,4′/2,4,2′,6′	51.7
8	2,3,6,2′	22.0
10	2,5,2′,5′	55.2
11	2,4,2′,5′	51.6
13	2,4,5,2′	>90.0
14	2,3,2′,5′	68.3
15	3,4,4′/2,3,2′,4′	33.3
17	2,3,2′,3′	69.1
19	2,5,3′,4′	42.7

[a]Percent degradation of selected PCB congeners in soil slurries (1 g soil/5 mL PAS medium) amended with 1.0% (w/v) surfactant (Igepal CO-720) and inoculated with FAV strain IPL5 (surfactant degrading *P. putida* strain IPL5 containing the cloned PCB degradative genes).

chloroethylene (TCE) has received the most attention in this area because of the magnitude and distribution of TCE ground-water contamination problems. Knowledge that anaerobically TCE can be dechlorinated to a carcinogenic intermediate, vinyl chloride (*12*), has prompted many intensive investigations into aerobic, oxygenase-mediated cometabolism of TCE (*13*). It is quite clear that many mono-oxygenases as well as dioxygenases, such as toluene dioxygenase, can act nonspecifically to incorporate oxygen into TCE, creating an unstable epoxide that is rapidly broken down.

Soluble methane mono-oxygenase (sMMO), produced by certain methanotrophs, including *Methylosinus trichosporium* OB3b, exhibits by far the highest specific activity for TCE degradation, but this enzyme is repressed by micromolar levels of copper ion, with preferential selection of particulate methane mono-oxygenase that has a much lower (perhaps 1000-fold) rate of TCE degradation. In addition, TCE binding to sMMO can inhibit enzyme activity with concurrent potential for cell inactivation.

Genetic modification of methanotrophic bacteria capable of synthesis of sMMO in the presence of copper has been achieved through strain improvement by classical chemical mutagenesis (*14*). Stability of sMMO synthesis in the presence of copper is achieved at the expense of growth rate for *M. trichosporium* OB3b. To overcome growth rate limitations, sMMO synthesis, and TCE cometabolic inactivation, a new bioreactor system has been developed to optimize sMMO maintenance and activity, and TCE biotreatment for contaminated ground water (*15*). This reactor system consists of three essential components:

1. A highly controlled zero-headspace CSTR (continuous stirred-tank reactor) with O_2 and methane mass flow control to opti-

mize sMMO production and cell growth, while limiting methane as competitive substrate for TCE oxidation;
2. A cascade of plug flow TCE-contacting columns for achieving TCE oxidation separate from sMMO production in the CSTR; and
3. A dewatering recirculation loop to return spent cells to the CSTR to maximize biomass retention and minimize methane consumption while producing sMMO.

The system as designed also includes a crossflow manifold assembly to introduce fresh cells from the CSTR to the top of any one of four plug flow TCE-contacting columns. This added feature was included to ensure that if TCE inactivation did occur in the TCE-contacting columns, fresh sMMO could be maintained independently in any of the columns in the cascade.

The abiotic operating characteristics of this reactor system are described in Fig. 4. As indicated by this figure, TCE entering the top of the first TCE-contacting column is initially diluted with the entire system coming to equilibrium within 4 d. The overall TCE mass balance indicates recovery in excess of 95%. In biotic operation, three TCE influent concentrations were employed in separate experiments: 3.0, 1.0, and 0.2 mg/L. TCE removal based on methanotrophic sMMO-mediated cometabolism for these three different experiments is described in Fig. 5. As indicated by this figure, rapid TCE removal was achieved at all three test TCE contamination levels in a single-pass mode with no crossflow. Each retention time (minutes) represented by the plot in Fig. 5 is cumulative for the TCE-contacting columns in series and for the return to the CSTR. All TCE was degraded in the 0.2 mg/L test, whereas 95 and 86%, respectively, were degaded at the 1 and 3 mg/L TCE concentration. Since TCE was not totally eliminated and sMMO activity remained relatively constant throughout the reactor and during the test run, it appeared likely that the sMMO-TCE degradative capacity of the bioreactor was exceeded at the higher TCE concentrations.

This new approach of designing and operating a reactor system around the biology of the organisms responsible for TCE degradation represents a molecular optimization strategy for improving performance of a bioremediation system. A second strategy for developing improved process monitoring and control uses a genetic engineering approach to create bioluminescent sensors for remote and on-line analysis of TCE cometabolism.

Recent developments in reporter gene fusion methods have been used to create bacteria that are induced to make bioluminiscent light when the organisms are exposed to specific chemical contaminants. This technology was first demonstrated environmentally for bacteria capable of biodegradation of polyaromatic hydrocarbons, such as naphthalene (*16,17*), and more recently for mercury transformation (*18*). This technology permits

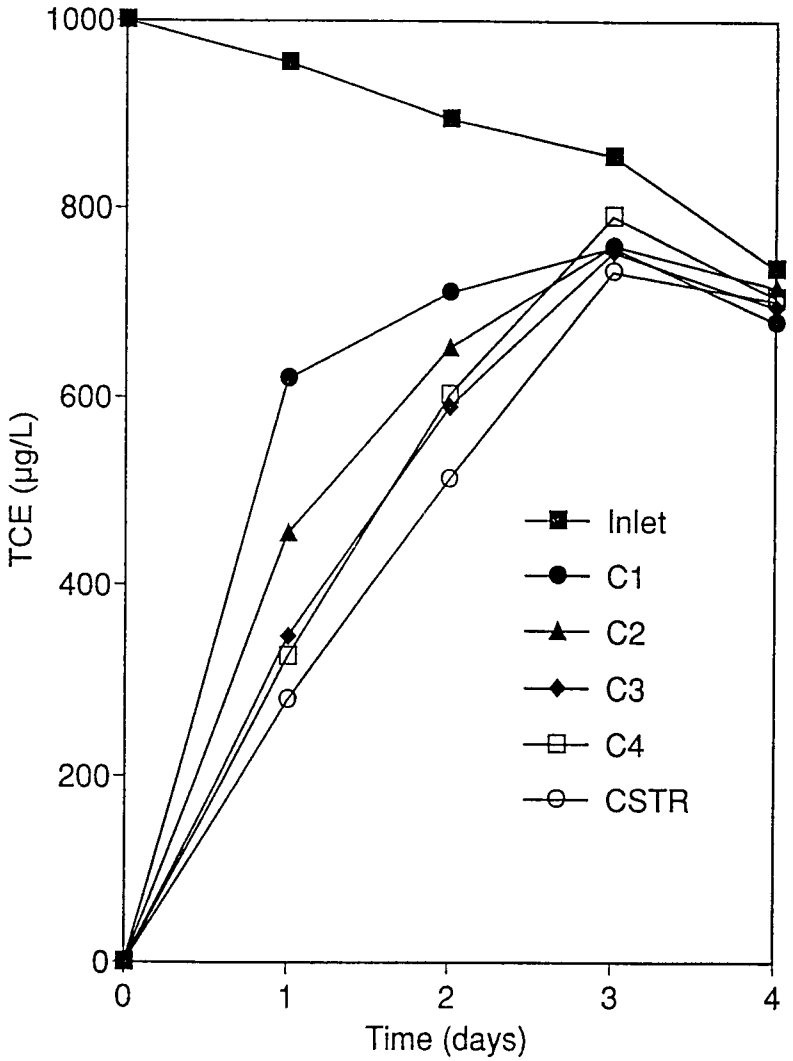

Fig. 4. Time-course for reaching abiotic steady state in a dual-stage pressured mathanotrophic TCE treatment reactor. Equilibrium was reached after 4 d, C1-C4 represent the effluent TCE concentrations from the series of four plug-flow TCE containing chambers. CSTR (continuous stirred-tank reactor).

not only sensing the presence of specific chemicals quantitatively in the environment, but also on-line monitoring of biodegradation processes during environmental remediation.

This reporter-gene technology has been extended to TCE cometabolism using the toluene dioxygenease (*tod*) chromosomal encoded pathway for toluene metabolism in *Pseudomonas putida* F1. A bioluminescent gene cassette derived from *Vibrio fisheri* containing the lux genes CDABE was utilized in creating a *tod-lux* fusion plasmid (*19*) in which a *tod*F gene fragment containing the promoter region was used to activate the lux genes

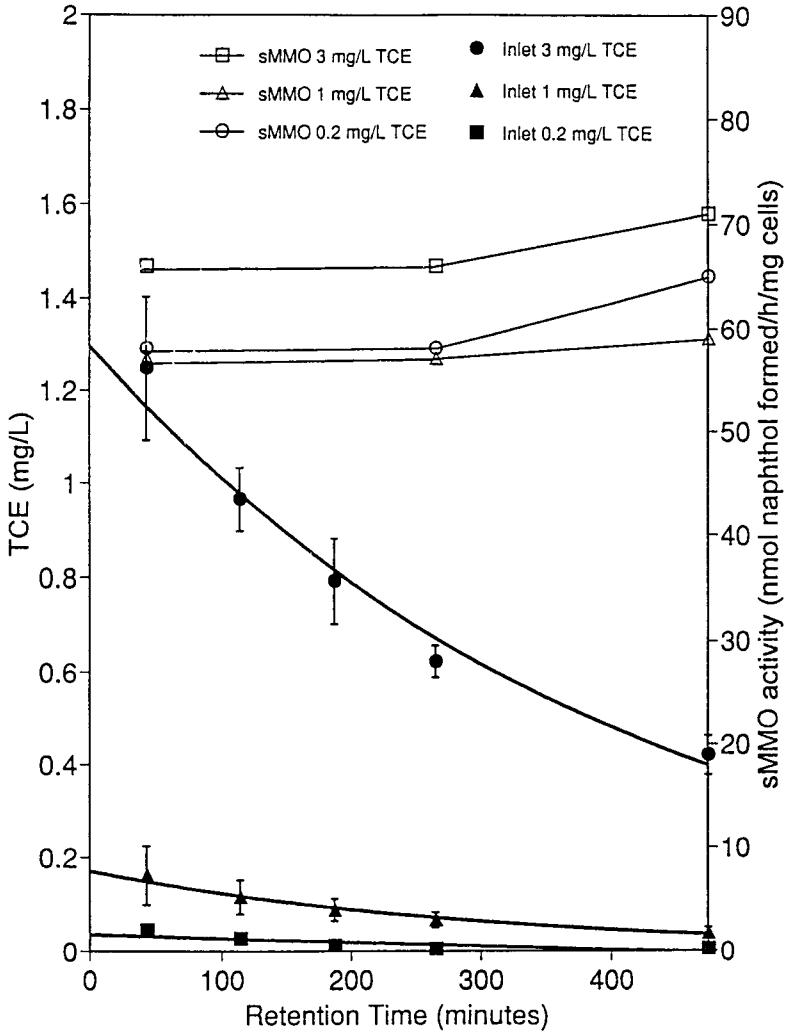

Fig. 5. Comparative TCE removal and maintenance of sMMO activity in a dual-stage pressured methanotrophic TCE treatment reactor. Three separate experimental test runs at TCE inlet feed concentrations of 0.2, 1.0, and 3.0 mg/L were performed. Each data point is the mean of five observations during periods of both steady-state and nonsteady-state operation.

transcriptionally. This plasmid, when introduced into a compatible toluene degradative host strain, produces light in response to induction by toluene.

As indicated in Table 3, bioluminescent light response is correlated with both toluene removal and TCE cometabolisms in a packed-bed treatment reactor simulation. For this example, the *P. putida* strain B2 containing the *tod-lux* fusion plasmid was immobilized in calcium alginate beads. These beads were used for the packed bed of a differential volume reactor (*20*) to simulate a subsurface aquifer environment. Light measurements

Table 3
Comparision of Strain B2 Bioluminescent
Response to TCE Removal During Perturbations
of Toluene Influent Concentration in a Packed-Bed Reactor System

Time, min	Biolum. response, nA	Toluene, m/L		TCE, mg/L		% TCE removal
		Influent	Effluent	Influent	Effluent	
0	2.5	10	10	20	20	0
5	4.6	10	6	20	16	20
15	22	0	0	20	19	5
25	43	10	6	20	16	20
35	20	0	0	20	18	10
40	10	0	0	20	20	0

were recorded on-line using a light pipe and photomultiplier, as the organisms were exposed to dynamic changes in toluene in a simulated toluene-TCE-contaminated waste stream. The light responses clearly demonstrate induction of the toluene degradation system and, by correlation, the simultaneous nonspecific removal of 20% of the TCE in the influent waste stream during toluene induction.

In the two proceeding examples of developing methodology for TCE cometabolism, both methods are undergoing further refinements and optimization. In the first example of the methanotropic reactor system, further work is needed to optimize TCE degradation at higher TCE concentrations using a crossflow format. It is also desirable to develop copper-tolerant strains with better growth characteristics further, especially in mixed-culture reactor applications. In the *tod–lux* example, there is a need to define the competitive inhibition between toluene biodegradation and TCE cometabolisms. A further improvement in this system could also include alteration of *tod* induction to avoid the use of toluene as inducing substrate. Such approaches have been previously demonstrated for the use of *Pseudomonas cepacia* strain G4 and the toluene nonoxygenase-mediated cometabolism of TCE (*21,22*).

MESSENGER RNA ACTIVITY IN PAH BIODEGRADATION

The use of reporter gene technology, such as the lux system, is an attempt to develop insight into the *in situ* "activity" of biodegradative processes in bioremediation. This issue of activity, not just presence, of degradative genes or bacteria is critical to understanding the dynamics of degradation processes and the effectiveness of engineering practices in

Table 4
Comparison of *nah*-Positive Bacterial Populations, [1-^{14}C]Naphthalene Mineralization Rates and *In Situ* Quantitation from MGP and Creosote-Contaminated Soils[a]

Soil	*nah*A Colony hybridization CFU/g	[^{14}C]Naphthalene mineralization rate ng/g of soil/h	Soluble naphthalene mg/mL	*nah*A mRNA pg/g
C	3.7 ± 0.9 × 10^6	0.51	79	24[c], 37[b]
C/i				20[b]
D	1.2 ± 0.6 × 10^6	0.032	2.0	2[b]
D/i				3[b]
G	1.2 ± 0.3 × 10^8	78	290	62[b], 75[c]
G/i				52[c]

[a]Soils subjected to salicylate are indicated by "i" following the soil letter designation. Soil bacterial populations were enumerated by the serial dilution technique and spread plate inoculation on selective media. The *nah*A population was determined by colony hybridization using an antisense RNA probe. Because final mRNA quantitation depends both on the percent recovery and a ribonuclease protection assay value, results are tabulated as individual observations.

[b]Final soil bacterial RNA purification by CsCl centrifugation.

[c]Final soil bacterial RNA purification by anion-exchange chromatography.

promoting bioremediation. To gain this understanding, molecular methods have been developed to quantify the expression of degradative genes (activity) as messenger RNA (mRNA) under field conditions. The hypothesis of this line of investigation is that quantification of mRNA is a direct measure of activity, in that transcription of DNA to mRNA is the prelude to synthesis and activity of degradative enzymes in a contaminated environment. A method of mRNA extraction and quantification has been developed and applied for polyaromatic hydrocarbon-contaminated soil of manufactured gas plant sites (*23,24*). This model system uses naphthalene-degradative genes and mRNA as an indicator for PAH biodegradation, at least for naphthalene, phenanthrene, and anthracene (*24*).

As indicated by Table 4, mRNA levels in contaminated soils are directly correlated (r^2 = 0.919) to the relative degree of contamination as measured by either naphthalene or by total PAH content taking into consideration that only five data points were used. The increase of extractable mRNA may be a reflection of increased abundance of naphthalene-degradative genes in the soil bacterial populations, which are also directly correlated with naphthalene concentration. An attempt to induce *nah* mRNA further by the addition of the specific naphthalene pathway inducer salicylate did not result in higher extractable mRNA levels (Table 4). These results suggest that either the *nah*-positive bacteria in these soils were fully induced by the naphthalene already present in the soil or that other soil constituents are inhibiting further induction. Along this line,

the fact that the mineralization rate of soil D was several orders of magnitude lower than might be predicted on the basis of *nah*A mRNA levels or soluble naphthalene, might also suggest inhibition of catabolic enzyme activity by soil constituents. The use of molecular-based techniques may therefore have limitations for the inference of actual degradative ability based on transcript-level determinations. Ongoing research is exploring alternative approaches for quantifying and identifying changes in *in situ* gene expression in soils undergoing bioremediation.

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