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A Simple Method for Quantifying Activity and Survival of Microorganisms Involved in Bioremediation Processes

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ABSTRACT

We have developed a substrate-induced growth response (SIGR) method for quantifying activity and population dynamics of microorganisms involved in bioremediation processes in soil and bioreactors. The biomass of organisms that can mineralize a given chemical can be estimated based on the concentration of that chemical needed to induce the growth of the standing population. Estimates of population size are obtained by using nonlinear regression techniques to fit a simple model of microbial population dynamics to biodegradation curves. Using this approach we obtain estimates of values for parameters such as initial population size and growth rate of organisms carrying out biodegradative processes. Our approach was validated by comparing model parameter estimates with independent estimates of the same parameters from the same bioremediation systems. Examples studied include pentachlorophenol degraders introduced into soil and 2,4-dinitrophenol degrading organisms in a bioreactor.

Index Entries: Biodegradation; bioremediation; *Sphingomonas;* 2,4-dinitrophenol; pentachlorophenol; kinetics.

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INTRODUCTION

Quantifying the biomass or activity of microorganisms involved in bioremediation processes is difficult because physical and chemical properties of environmental samples can interfere with most analytical procedures. As a result of these difficulties, indirect methods are often employed to estimate the biomass or activity of microrganisms in microbial ecosystems. This article describes a simple method for estimating the biomass and activity of microorganisms that are active in the biodegradation of specific organic chemicals. The method is similar to several indirect methods commonly used in studies of microbial ecology as well as more recent approaches that employ models to estimate parameters of microbial populations from substrate depletion or product formation kinetics.

The need for reliable estimates of both microbial biomass and the rates of microbial processes has sparked a recent flurry of modeling activity by environmental microbiologists and others (reviewed in *1* and *2*). Although this modeling has resulted in some interesting ideas and approaches, the use of models as tools for estimating microbial biomass and activity in bioreactors and contaminated soils has been limited. In order for models to be used to address specific questions in environmental science, they must contain biologically relevant parameters and be validated in the laboratory and the field (*1*).

In the present study, a substrate-induced growth response (SIGR) method was used to estimate the population density of pentachlorophenol (PCP) degraders in soil and 2,4-dinitrophenol (DNP) degrading organisms in a bioreactor. In order to validate the SIGR approach, parameter estimates from model curve fits were compared to independent estimates of microbial population densities in the two systems studied.

THEORY

The SIGR approach is based on the well-founded premise that the standing population of a given group of organisms can be estimated based on the amount of substrate (S) required to support growth of that population. In the present application, the number of PCP or DNP mineralizing organisms can be estimated by adding enough DNP or PCP to obtain growth-shaped mineralization curves, and then fitting the curves obtained with a model that contains a parameter for the initial biomass of DNP or PCP mineralizing organisms in the sample. In some cases, preliminary experiments may be needed to determine the concentration that will give the desired curve type.

Estimates of biomass and growth rate of specific microorganisms are obtained by fitting a simple model of population dynamics, expressed in terms of product accumulation or substrate depletion, to substrate

mineralization curves. Models are fit to the data using nonlinear regression analysis (*3,4*). At concentrations of substrate high enough to cause the standing biomass to grow, the initial phases of substrate mineralization kinetics can be fit to the integrated form of the logarithmic growth model expressed in terms of substrate depletion:

$$S = S_o + X_o [1 - \exp(\mu_{max} t)] \quad (1)$$

where X_o is the initial biomass of organisms in terms of the amount of substrate that would have been required to form that biomass, μ_{max} is the maximum specific growth rate, S_o is substrate concentration at time zero, and t is time. The parameter X_o has units that are the same as those for S (e.g., μg/mL) in order to make Eq. (1) as simple as possible. These units are fine for comparative purposes, say between two treatments in an experiment, but there are some cases where X_o must be converted to units commonly used in studies of biomass or population density. X_o can be converted to an estimate of the actual biomass of organisms (B_o) involved in mineralizing the substrate by multiplying X_o by a yield coefficient (Y):

$$B_o = X_o Y \quad (2)$$

In addition, in order to compare estimates from model fits to MPN estimates used in some of the experiments done in the present study, B_o can be converted to units of cells g^{-1} by multilplying by a conversion factor of 10^8 cells/mg of microbial biomass (*5*).

MATERIALS AND METHODS

Pure Culture Experiments

The organism used in the initial pure culture and soil inoculation experiments was isolated from PCP-contaminated soil from the Broderick Wood Products site near Denver, CO (*6*) and has been identified as *Sphingomonas* sp. strain RA2 (formerly in the genus *Pseudomonas*). Pure culture experiments were performed in unshaken 250-mL Erlenmeyer flasks incubated in the dark at 21 ± 2°C. Each flask contained 130 mL of a solution that consisted of 2.4 mg of Na_2HPO_4, 2.0 mg of KH_2PO_4, 0.1 mg of NH_4NO_3, 0.01 mg of $MgSO_4 \cdot 7\ H_2O$, 0.01 mg of $CaCl_2$, and 1 μL of trace element solution (*7*)/mL of deionized water.

The biodegradation of PCP was monitored using techniques similar to those of Schmidt et al. (*8*) and Hess et al. (*3*). Tracer concentrations of uniformly ^{14}C-labeled PCP was added to the culture media (approx 14,000 dpm/mL), and the final PCP concentration was adjusted by adding different amounts of unlabeled PCP. Samples (2.5 mL) were removed at regular intervals and filtered through 0.2-μm polycarbonate filters (Nucleopore, Pleasanton, CA) into test tubes containing three drops of concentrated H_2SO_4. One-milliliter aliquots of these samples were added to 4-mL

Omni vials (Wheaton Industries, Millville, NJ) and 2.5 mL of ScintiVerse II scintillation cocktail (Fisher Scientific Co., Pittsburgh, PA) were added to each vial. The samples were shaken vigorously and counted in a liquid scintillation counter (LKB Wallac, 1209 RackBeta, Turku, Finland). Mineralization of PCP was also monitored by measuring the release of chloride ions with a Model 76-17B combination electrode and a model 701A ion meter (Orion Research, Inc., Boston, MA). Uninoculated flasks were run in each experiment as controls for PCP volatilization. Bacterial cells were enumerated by direct microscopic counting in a calibrated Thoma counting chambers (0.02-mm depth, Fleischhacker KG, Schwerte, Germany) as previously described (*6*).

Soil Inoculation Experiments

The soil used in this study is the same as soil #2 described in detail by Schmidt and Gier (*9,10*). Sieved (2-mm) samples, equivalent to 44.5 g dry wt of soil, were placed in a 250-mL biometer flasks with enough deionized water to bring the soil to 50% of field capacity. Field capacity (*11*) was determined by measuring gravimetric water content of saturated soil samples that had been allowed to drain freely for 12 h. After incubating the flasks overnight, PCP and deionized water were added to achieve a PCP concentration of 300 μg/g of soil and a moisture level equivalent to 70% of field capacity. Inoculum consisted of 1 mL of a stationary-phase liquid culture of *Sphingomonas* RA2 that had been grown at a PCP concentration of 300 μg/mL. The inoculum was thoroughly mixed into the soil using a Teflon™-coated spatula. The incubation temperature was 24 ± 2°C. Evolved $^{14}CO_2$ was captured in 1.0 mL of 0.5*N* NaOH contained in the side arm of each biometer flask. The NaOH was added to 2.5 mL of scintillation cocktail in 4-mL vials, and the radioactivity was counted as described above. Curves of $^{14}CO_2$ evolution were adapted for use in the curve-fitting procedures described below according to previously described methods (*1,12,13*).

Sequencing Batch Reactor

The waste-treatment system used in this study was a sequencing batch reactor (SBR) that has been described in detail elsewhere (*3,14,15*). The SBR was operated continuously with a cycle time of 48 h and a liquid volume of 4 L. The feed water contained 10 μg of DNP and 100 μg of glucose/mL. The SBR was operated continuously for 5 mo prior to the experiments performed in this study. At the time of these studies, the SBR had reached steady-state operation as indicated by the cycle-to-cycle consistency of mineralization kinetics and microbial population dynamics (*15*). The pH of the SBR contents was 6.8, and suspended solids averaged 812 μg/mL at the time these experiments were done (*3,15*).

The biomass of DNP-mineralizing bacteria in the SBR was estimated by incubating diluted samples of SBR fluid with known concentrations of

DNP and then determining the kinetics of DNP removal in each sample. Samples (100 mL) of SBR contents were removed with a syringe and blended at high speed for 4 min with a 1-min pause after 2 min of blending. Aliquots (10 mL) of the blended material were then added to 90 mL of solution containing 0.48 mg Na_2HPO_4, 0.5 mg KH_2PO_4, 0.04 mg NH_4Cl, 0.01 mg $MgSO_4 \cdot 7H_2O$, and 0.01 mg $CaCl_2$/mL of solution in 250-mL Erlenmeyer flasks. Enough ^{14}C-labeled (14,000 dpm/mL) and unlabeled DNP were added to give final DNP concentrations of 0.16, 1.1, or 10 μg/mL. Flasks were incubated at 24 ± 2°C and sampled at regular intervals. The samples (2 mL) were immediately filtered through 0.2-μm polycarbonate filters into test tubes containing 1 drop of concentrated H_2SO_4. One-milliliter subsamples were added to 4-mL scintillation vials along with 2.5 mL of scintillation cocktail, and the radioactivity was counted as described above. To verify that DNP was mineralized, DNP was also monitored using a spectrophotometer at 260 nm and by measuring $^{14}CO_2$ accumulation and NO_2^{-1} evolution (*3,15*).

The yield coefficient (*Y*) for the DNP-mineralizing bacterium from the SBR was determined by filtering 1000 mL of early-stationary-phase cultures (10 μg of DNP/mL) through preweighed and dried 0.2-μm filters. The filters were then dried at 100°C for 12 h and reweighed to determine the dry weight of the cells. Yield was calculated by dividing the dry weight of the cells by the weight of DNP utilized by the culture. Yield determinations were done in triplicate and gave a mean *Y* of 0.48 g/g (SD = 0.01).

Microbial Counts

The most probable number (MPN) of DNP-mineralizing microorganisms in the SBR was determined using previously described methods (*3,9,10*). Samples (100 mL) of the reactor contents were blended at high speed for 4 min with a 1-min pause at the midpoint and then diluted in a series of 10-fold dilutions. Eight 1-mL aliquots of each dilution were placed in the wells of 24-well sterile tissue-culture plates (Corning Glass Works, Corning, NY), which contained 1 mL of sterile inorganic salts solution and enough DNP to give a final concentration of 5 μg/mL. The salts solution contained 500 μg of KH_2PO_4, 480 μg of Na_2HPO_4, 10 μg of $CaCl_2 \cdot 2H_2O$, 40 μg of NH_4Cl, 10 μg of $MgSO_4 \cdot 7H_2O$, and 0.5 μg of yeast extract/mL of deionized water. The tissue-culture plates were incubated in the dark for 28 d at 24 ± 2°C before DNP depletion was determined colorimetrically. An MPN table was used to calculate the population size and the 95% confidence intervals.

Chemicals

Uniformly ^{14}C-labeled PCP (11.9 mCi/mmol) and DNP (10.2 mCi/mmol) were obtained from Sigma Chemical Company (St. Louis, MO). Unlabeled reagent-grade DNP and PCP were purchased from Fluka Chemical Com-

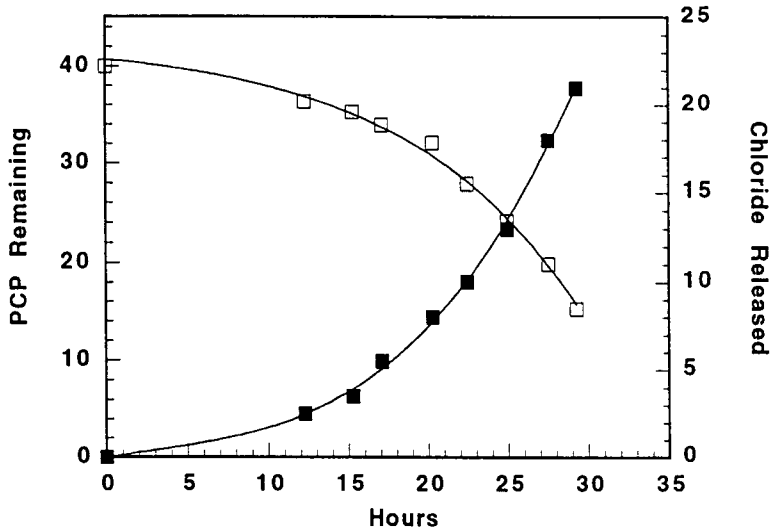

Fig. 1. Disappearance of 40 μg of PCP/mL (open symbols) and appearance of chloride (closed symbols) in a batch culture of *Sphingomonas* RA2. The symbols represent actual data, and the drawn lines represent the best fit of the integrated SIGR equation to the data (*see* Table 1).

pany (Ronkonkoma, NY), and reagent-grade glucose was obtained from Mallinckrodt, Inc. (Paris, KY). The purity of all compounds was >99%.

RESULTS AND DISCUSSION

Pure Culture Studies

The organism used in this study was previously described by Radehaus and Schmidt (*6*) and at that time was classified as a *Pseudomonas* sp. Since that initial study, further tests were performed by the German Collection of Microorganisms and Cell Cultures (DSM), and the organism has been assigned to the new genus *Sphingomonas* (*16–18*), which was previously part of the genus *Pseudomonas*. The organism is a gram-negative, catalase- and oxidase-positive rod with dimensions of approx 1 × 1.3–4.5 μm. The phospholipid fingerprint of the organism matched that of *Sphingomonas*, and the organism has a single polar flagellum. The predominant ubiquinone was ubiquinone Q_{10} and the G+C content of its DNA was 66.7 mol%. Further characteristics of this organisms are included in our initial description of the organism (*6*).

Prior to testing the SIGR method in soil or a waste-treatment system, several experiments were carried out to determine how well the SIGR method could estimate biomass levels in well-defined pure cultures. Levels of PCP, chloride, and cell population densities were simultaneously measured in batch cultures of *Sphingomonas* sp. RA2. Figure 1 shows a typical curve of PCP disappearance and chloride accumulation in a single-

Table 1
Parameter Estimates from the Fit of Eq. (1)
to the Mineralization Curves Depicted in Figure 1[a]

PCP conc., μg/mL	Substance measured	X_o, μg/mL	μ_{max}, h^{-1}
40	PCP	1.86 ± 0.53	0.091 ± 0.009
40	Cl^-	1.81 ± 0.46	0.088 ± 0.008
40	Actual inoculum	4.92	0.09

[a] Estimates for the amount of inoculum actually used were calculated based on the work of Radehaus and Schmidt (*6*).

batch culture of *Sphingomonas* sp. RA2. Equation 1 was fit to each of the curves in Fig. 1 using nonlinear regression analysis. The output from the model fits is shown in Table 1 along with independent estimates of μ_{max} and X_o.

The SIGR estimates of X_o obtained from the PCP disappearance and chloride accumulation curves were very close to one another, but were slightly lower than our independent estimate of how much inoculum was used in the experiment (Table 1). Because the SIGR method quantifies only active biomass, we interpret the lower SIGR estimate of X_o as an indication that not all of the cells added in this experiment were active. Given the inherent errors involved in any known method for measuring microbial biomass or activity, it is very encouraging that the SIGR estimates were as close to the independent estimate of X_o as they were. As an additional check on the SIGR method, estimates of μ_{max} can also be compared in Table 1. As with the estimate of X_o the SIGR estimates of μ_{max} are remarkably close to the independent estimates of μ_{max}.

Soil Inoculation Studies

An experiment was carried out to determine if the SIGR approach could be used to estimate the biomass of *Sphingomonas* sp. RA2 when a known population of the organism was added to a soil that had no previous PCP mineralizing activity. The experimental design was similar to that for the pure culture tests, except that the data from these soil incubations are $^{14}CO_2$ accumulation curves rather than PCP disappearance or chloride appearance curves. Figure 2 shows two replicate curves of PCP mineralization obtained when a small inoculum of *Sphingomonas* sp. RA2 was added to a soil containing 300 μg of PCP/g.

Parameter estimates from the two replicate curves show good agreement with each other. Estimates of μ_{max} of 0.083 and 0.093 are in agreement with the growth rates expected of *Sphingomonas* sp. RA2 growing on PCP (0.09 in Table 1), but no independent estimates of the growth rate of *Sphingomonas* sp. RA2 in soil are available for comparison. Estimates of X_o, however, can be compared with an independent estimate of X_o calculated

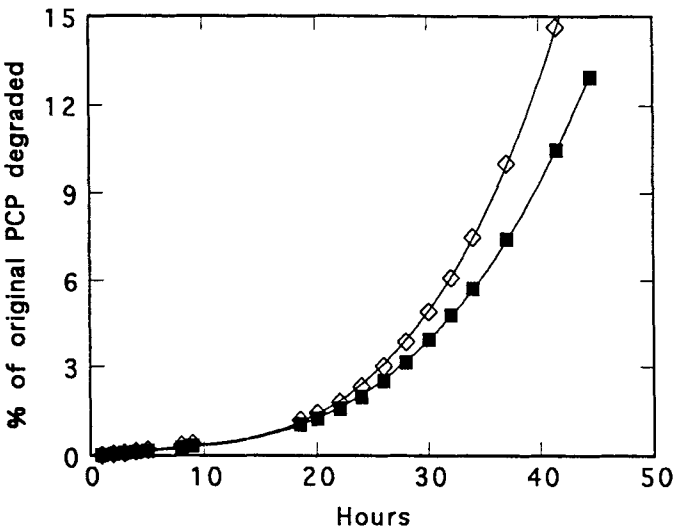

Fig. 2. Two curves of mineralization of 300 μg of PCP/g of soil by cells of *Sphingomonas* sp. RA2 added to soil. No PCP mineralization was observed in an uninoculated control soil. The symbols represent actual data, and the drawn lines represent the best fit of the integrated SIGR equation to the data.

from the amount of inoculum that was actually added to the soil. This independent estimate of 6.74 μg/g is higher than the SIGR estimates, which averaged 1.31 μg/g. As with the pure culture study, the difference between these two estimates is probably owing to the fact that the SIGR approach measures only active microorganisms. It is possible that there was a die-off of *Sphingomonas* sp. RA2 cells when the inoculum was added to the soil. *Sphingomonas* sp. RA2 is not native to the soil used in this experiment and had been cultured on laboratory media for several years prior to this attempt to reintroduce it into contaminated soil. It is also possible that even if no die-off occurred, the activity level of the added inoculum was not 100%. The inoculum used was from a stationary-phase culture, and thus, a significant fraction of the cells in the inoculum may have been dormant. Future experiments will help clarify why the SIGR method estimates a lower inoculum size than what was presumably added to the soil.

Sequencing Batch Reactor

When samples from an SBR being used to treat DNP were diluted 10-fold and incubated with various concentrations of DNP, two general shapes of DNP-consumption curves resulted (Fig. 3). At a DNP concentration of 10 μg/mL, the curve of DNP mineralization was concave downward, indicating that growth of the DNP-mineralizing population was taking place. At lower DNP concentrations (e.g., 1.1 μg/mL), the curves of DNP mineralization were concave-upward, indicating that these concentrations were not high enough to cause detecable growth of the DNP-mineralizing population. This interpretation is supported by the fact that

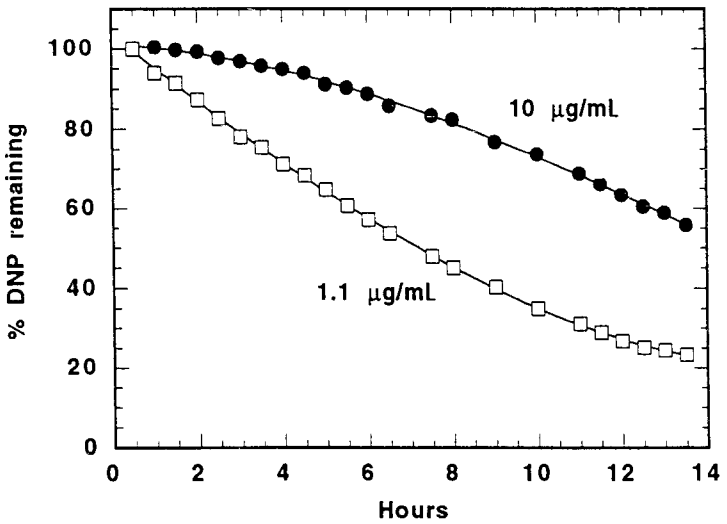

Fig. 3. Mineralization of two concentrations of DNP in diluted samples of a continuously operating SBR. Each curve represents the mean of two replicates. The symbols represent actual data, and the drawn lines represent the best fit of the integrated SIGR equation (10 μg/mL) or a nongrowth model (1.1 μg/g) to the data. This figure was redrawn using data from Schmidt (*12*).

Table 2
Parameter Estimates from the Fit of Eq. (1) to Two Curves of DNP Mineralization in Diluted Samples of a Continuously Operated SBR[a]

Test number	Pop. density, 10^5 cells/mL	μ_{max}, h^{-1}
1	13.0	0.08
2	6.0	0.11
3	4.2	0.17
4	7.3	0.14
Mean of above estimates	7.6 ± 3.8	0.13 ± 0.04
MPN estimate	11.0 ± 3.0	NA

[a]The mean of the parameter estimates (± SD) from the model fits are compared to the mean MPN estimate of cell density. The data are from Schmidt (*12*).

nongrowth models (*19*) provided the best fits (lowest residual sum of squares) to the curves of mineralization of 0.16 (data not shown) and 1.1 μg DNP/mL, whereas the SIGR model (Eq. [1]) was the model of best fit to the data from the 10 μg/mL incubations.

Estimates of X_o were obtained by fitting Eq. (1) to four curves of mineralization of 10 μg DNP/mL. The values of X_o obtained from these four curves ranged from 8.8 to 27 μg/mL of SBR contents. Values for X_o were then converted to estimates of cells/mL (Table 2) by using Eq. (2), a value

for Y of 0.48, and the conversion factor of 10^8 cells/mg of microbial biomass (*5*). This conversion was done in order to compare the model biomass estimates to independently obtained estimates of population density from the MPN technique (Table 2). The standard deviations of population estimates from both methods overlapped, indicating good agreement between the two methods given the inherent errors in any method for estimating population density of microorganisms.

The largest potential error in the comparison shown in Table 2 is in the conversion of model estimates for X_o to units of cells/mL. The conversion factor of 10^8 cells/mg of biomass is relatively conservative for aquatic systems, where it may be as much as 10-fold higher (*8*). Even if a 10-fold higher estimate were used, the population estimates from the SIGR and the MPN methods are still quite close to each other viz. $7.6 \pm 3.8 \times 10^6$ vs. $1.1 \pm 0.3 \times 10^6$ for the model estimate and the MPN estimates, respectively.

CONCLUSION

The results presented above show that the SIGR approach can be used to quantify the activity and biomass of microorganisms capable of carrying out specific biodegradative processes in both soil and a model waste-treatment system. Model estimates for biomass of degraders (X_o) agreed reasonably well with independent estimates of the same parameter. In addition, estimates for growth rate (μ_{max}) agreed very well with estimates of the same parameter from experiments with pure cultures of PCP and DNP-mineralizing bacteria.

One advantage of the SIGR approach over other methods of estimating biomass or growth is that microorganisms do not need to be extracted from soil or floc particles in order to be quantified. Extraction procedures are often difficult and are usually just the first step in quantifying the biomass of specific microbial groups. Another advantage of the SIGR approach is that estimates of biomass or activity levels can be obtained in a relatively short period of time. The incubations used in this study all lasted < 48 h compared to the 28-d incubation period used in the MPN method.

Except for our previous work (*1,9,10,12,14*), the closest analog to the SIGR method is the work of van der Werf and Verstraete (*20*). They studied the dynamics of glucose- and acetate-mineralizing organisms in soil using a curve-fitting approach and models that incorporated parameters for population density. Instead of using CO_2 production or substrate depletion kinetics, they utilized curves of O_2 consumption to obtain estimates of the active microbial biomass. In comparison, the SIGR method requires fewer parameters to model the simple curves used in the present study, thereby reducing the chance of errors related to correlations among parameters and other problems inherent in using models with more than four parameters (*1,12*).

In summary, the SIGR method employs a simple model of microbial activity and nonlinear regression analysis to obtain estimates of microbial biomass from short-term incubation experiments. The examples given in this article demonstrate the advantages of this approach for estimating the biomass of organisms capable of metabolizing a specific toxic substrate in soil and aquatic systems. The SIGR method can be easily used by scientists and technicians for monitoring the activity and biomass of organisms involved in bioremediation processes at toxic spill sites and in waste-treatment facilities.

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