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Measurement of hydrocarbon-degrading microbial populations by a 96-well plate most-probable-number procedure

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A 96-well microtiter plate most-probable-number (MPN) procedure was developed to enumerate hydrocarbon-degrading microorganisms. The performance of this method, which uses number 2 fuel oil (F2) as the selective growth substrate and reduction of iodonitrotetrazolium violet (INT) to detect positive wells, was evaluated by comparison with an established 24-well microtiter plate MPN procedure (the Sheen Screen), which uses weathered North Slope crude oil as the selective substrate and detects positive wells by emulsification or dispersion of the oil. Both procedures gave similar estimates of the hydrocarbon-degrader population densities in several oil-degrading enrichment cultures and sand samples from a variety of coastal sites. Although several oils were effective substrates for the 96-well procedure, the combination of F2 with INT was best, because the color change associated with INT reduction was more easily detected in the small wells than was disruption of the crude oil slick. The method's accuracy was evaluated by comparing hydrocarbon-degrader MPNs with heterotrophic plate counts for several pure and mixed cultures. For some organisms, it seems likely that a single cell cannot initiate sufficient growth to produce a positive result. Thus, this and other hydrocarbon-degrader MPN procedures might underestimate the hydrocarbon-degrading population, even for culturable organisms.

Keywords: MPN; hydrocarbon-degraders; crude oil; tetrazolium; INT

Introduction

Microorganisms with specific metabolic capabilities, such as oil degradation, can be enumerated based on their ability to grow on selective media. The most-probable-number (MPN) procedure [1] is particularly well suited for organisms that grow on insoluble substrates, because solid media containing a homogenous distribution of the appropriate carbon source can be difficult to prepare. Also, many agar-based solid media contain impurities that allow growth of organisms that cannot degrade the target substrate, leading to overestimation of the size of the population of interest [9,16,20].

Crude oil or refined petroleum products are usually used as the selective growth substrates when hydrocarbon degraders are enumerated by MPN [3,9,10,13,14,16,18,20]. Early methods relied on an increase in the turbidity or the protein content of the liquid medium to identify positive cultures [9,16,20], but this proved to be unreliable when a very viscous crude oil was used as the growth substrate or when a large proportion of bacteria grew in direct contact with the insoluble oil phase [2]. A radiorespirometric MPN method, in which positive dilutions are scored by evolution of $^{14}CO_2$ from radiolabelled substrates, was developed to overcome these deficiencies [13,14]. Unfortunately, this method can be tedious, and special procedures are required for handling the radioisotopically-labelled substrates and for trapping the $^{14}CO_2$ that is produced. Simpler methods that retain the sensitivity of the radiorespirometric procedure, such as reduction of resazurin [18] and emulsification of crude oil [3] have been used more recently.

In this paper we describe a simple 96-well microtiter-plate-based MPN procedure that can be used to enumerate hydrocarbon degraders in the laboratory or in the field. This method uses number 2 fuel oil (F2) as the selective substrate, and positive wells are scored by reduction of iodonitrotetrazolium violet (INT) after a 2-week incubation. The accuracy of this method was evaluated by comparing its estimates of the hydrocarbon-degrading microbial populations to those obtained from another microtiter-plate-based method for oil degraders [3]. In addition, several potential substrates and two detection methods were tested to determine the most suitable combination.

Materials and methods

MPN procedures

When 24-well microtiter plates were used for the MPN determination, 2 ml of Bushnell–Haas medium (Difco Products, Detroit, MI, USA) supplemented with 2% NaCl (BH), were added to each well. Ten-fold serial dilutions of the samples were prepared with sterile saline (2% NaCl). Eight dilutions were pipetted into two 24-well plates by addition of 100 μl of an appropriate dilution to six replicate wells. After inoculation, 20 μl of oil was added to each well as a carbon source. The plates were placed in plastic bags, sealed to reduce evaporative losses, and incubated at 20°C for 14 days.

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The 96-well plates were processed with a Beckman Biomek 1000 laboratory robot (Beckman Instruments, Fullerton, CA, USA) which filled the wells with medium, performed ten-fold serial dilutions of the sample, and added oil to the inoculated wells. The robot added 180 μl of BH to each well in 11 of the 12 rows, leaving the first row empty. It transferred 200 μl of undiluted sample to the wells in the first row, mixed their contents, and then transferred 20 μl to each well in the second row. The contents of the second row were mixed, and 20 μl was transferred to each well in the third row. This procedure of mixing and transfer was carried out for all except the last row, which served as a sterile control. Sterile pipet tips were used for each transfer. After the dilutions were completed, 2 μl of oil was added to each well as the growth substrate. The plates were sealed in plastic bags and incubated for 14 days at 20°C.

Positive wells were scored in one of two ways. When F2 was the carbon source, 50 μl of a sterile solution (3 g L^{-1}) of INT (Research Organics, Cleveland, OH, USA) was added to each well. INT competes with O_2 for electrons from the respiratory electron transport chain [11], and it is reduced to an insoluble formazan that deposits as a red precipitate in the presence of active respiring microorganisms. Red or pink wells were scored as positive. When a crude oil was used as the carbon source, a smooth oil slick developed in each well. Positive wells were scored by emulsification or dispersion of this oil slick [3]. INT cannot be used effectively with crude-oil substrates, because their dark color interferes with detection of formazan deposition.

The MPN values were calculated using a personal-computer-based program [8] that accepted more combinations of replicates and dilutions than were available in published tables [15]. This program automatically corrected the computed MPN for the bias associated with the maximum likelihood estimator [8,19].

Heterotrophic plate counts

Heterotrophic plate counts (HPC) were performed alongside the MPN determinations to obtain an estimate of the total culturable microbial population. Marine agar (Difco) spread plates were used for HPC enumeration. Serial ten-fold dilutions of the sample were prepared, and triplicate marine agar plates were inoculated with 100 μl from each of three dilutions, then immediately spread with sterile glass rods. Colonies were counted following incubation at 20°C for 6 days.

Substrate evaluation

Three oils (obtained from the American Petroleum Institute, Washington, DC, USA) were examined to determine the most effective substrate for the 96-well MPN procedure. Light Arabian (LA) is a light crude oil with a specific gravity of 0.863 g ml^{-1}. Alaskan North Slope (NS) is a medium crude that was artificially weathered by heating it under vacuum (20 mm Hg) to an equivalent temperature of 272°C (521°F), then allowed to cool. Fresh NS has a specific gravity of 0.894 g ml^{-1}. F2 is a refined product with a boiling range of between 160° and 360°C and a density of 0.856 g ml^{-1}. Its composition can vary depending on the refining process that is used, but it is usually 75–80% aliphatics and 20–25% aromatics. The aliphatic hydrocarbons are mainly in the C_9 to C_{21} range, and the aromatics are predominantly low molecular weight PAHs (eg two- and three-ring compounds).

The differences among the MPNs obtained with these substrates were analyzed by linear regression, treating the results obtained with F2 as the independent variable. Since the base 10 logarithms of the MPNs were used in this analysis, differences between the substrates were expected to affect the *y*-intercept. Therefore, a *t*-test was used to determine whether the intercepts of the best-fit lines were different from zero [4].

Because some oil components can be toxic even to hydrocarbon degraders, the effect of substrate concentration on the hydrocarbon degrader MPN was determined. Two dilutions of an oil-degrading enrichment culture (1.4×10^7 CFU ml^{-1} and 1.3×10^5 CFU ml^{-1}) were enumerated by MPN in 24-well microtiter plates with four concentrations of F2 and LA: 10, 20, 40, and 70 μl oil were used per well. The substrate effect was analyzed by linear regression of the relationship between the base 10 logarithm of the MPN estimates and the substrate concentration. A *t*-test was used to determine if the slope of the best-fit line was significantly different from zero [4].

Accuracy of the 96-well MPN method

The 96-well hydrocarbon-degrader MPN method, with F2 as the selective substrate and detection of positive wells by INT reduction, was tested by comparison to the Sheen Screen method of Brown and Braddock [3] for four oil-degrading enrichment cultures (BS48, NJ1, KN403, and SB) and four sand samples (BS48, FB, DI67, and KN110). The 96-well plates were processed with the Beckman Biomek 1000 laboratory robot as described above. The Sheen Screen method was performed in 24-well microtiter plates with NS as the selective substrate, and the samples were diluted and the plates inoculated manually, as described above. The oil-degrading enrichment cultures were analyzed directly (ie no pretreatment was required), but the hydrocarbon degraders present on the sand samples were first detached from the sand by vigorously shaking 10 g in 90 ml of a sterile 2% NaCl solution by hand for about thirty seconds. Differences between the two methods were evaluated by linear regression of the base 10 logarithm of the MPNs, treating the Sheen Screen MPN as the independent variable. If the two methods were equivalent, the *y*-intercept was expected to equal zero and the slope was expected to equal one.

The oil-degrading enrichment cultures were derived from sand or gravel samples that were collected from beaches throughout the coastal United States, and they were maintained by growth in BH with NS as the sole carbon source. The sources for all the samples that were used to evaluate the 96-well MPN procedure are given in Table 1.

The MPN method is based on the hypothesis that inoculation of a single cell into a well (or tube) will produce an observable change in the substrate or the culture medium [1]. We tested this hypothesis for the F2-MPN procedure in two ways. First, the historical average hydrocarbon-degrader MPN to HPC ratio was compared with the proportion of colonies picked from marine agar HPC plates

Table 1 Sources of hydrocarbon degraders for MPN evaluations

Culture	Type	Source
BS48	sand	Big Shell Beach, SW Texas
BS48	oil-degrading enrichment	Big Shell Beach, SW Texas
KN403	oil-degrading enrichment	Knight Island, Prince William Sound, Alaska
KN110	sand	Knight Island, Prince William Sound, Alaska
DI67	sand	Disk Island, Prince William Sound, Alaska
FB	sand	Fowler's Beach, Delaware (Delaware Bay)
SB	oil-degrading enrichment	Slaughter Beach, Delaware (Delaware Bay)
NJ1	oil-degrading enrichment	Raritan Bay, New Jersey
BS48-3	pure culture	oil-degrading enrichment culture (BS48)
Ar-1	pure culture	enrichment culture on aromatic fraction of NS crude oil
Ar-2	pure culture	enrichment culture on aromatic fraction of NS crude oil
Phe-1	pure culture	phenanthrene-degrading enrichment culture
Phe-2	pure culture	phenanthrene-degrading enrichment culture

that were capable of growth on F2 (ie the verification frequency). The historical average MPN to HPC ratio was computed from data on three oil-degrading enrichment cultures (BS48, KN403, and SB) for which many concurrent estimates of hydrocarbon-degrader and total heterotrophic microbial populations were available. Because the MPN to HPC ratios were not normally distributed, a transformed variable, θ, was used to calculate the means and 95% confidence intervals for each culture [17], where:

$$\theta = \arcsin\left(\sqrt{\frac{MPN}{HPC}}\right) \quad (1)$$

The verification frequency for hydrocarbon-degrading heterotrophs was determined for these cultures by picking every colony from several marine agar HPC plates (such that several hundred individual colonies were picked) and transferring them into BH medium containing F2 as the sole source of carbon and energy. Twenty-four-well microtiter plates were used for verification tests. After incubation for two weeks at 20°C, 0.5 ml of INT (3 g L^{-1}) was added to each well. Colonies that grew on F2 were identified by the deposition of INT-formazan.

An additional test of the hypothesis that inoculation of a single hydrocarbon degrader can produce a positive well, used pure cultures of bacteria that were selected for their ability to grow on F2. These organisms were isolated from several hydrocarbon-degrading enrichment cultures by streaking isolated colonies on marine agar. The 96-well/F2 hydrocarbon-degrader MPN assay was evaluated by performing MPNs and HPCs on subsamples from cultures that were grown on F2 in BH. All the pure cultures that were used in this evaluation grew well in BH with F2 as the sole source of carbon and energy. A *t*-test was used to determine whether the differences between the estimates provided by MPN and HPC were different at the 95% confidence level.

Results

Substrate evaluation

Because some components of crude and refined petroleum can be toxic, four different concentrations of F2 and LA were tested to determine whether substrate toxicity affected the result obtained in our hydrocarbon-degrader MPN procedure. The substrate concentration recommended for this method (20 μl $well^{-1}$ for 24-well plates or 2 μl $well^{-1}$ for 96-well plates) was within the concentration range tested. The estimated MPNs for both initial dilutions of the oil-degrading enrichment culture (Figure 1) were independent of concentration for both substrates (ie the slopes of $\log_{10}$ MPN vs substrate concentration were not significantly different from zero at the 95% confidence level). Thus, substrate concentration did not affect the MPN over the range tested. In this experiment, there were no detectable differences between F2 and LA as substrates.

The best substrate for use in the 96-well procedure was determined by testing three different oils (F2, LA, and NS) on several oil-degrading enrichment cultures and sand samples (Figure 2). The MPNs estimated with the crude oils as substrate were usually lower than those obtained when F2 was provided. This was particularly true for NS, for which the crude oil MPNs were lower than the F2 MPNs in six of seven samples. Thus, there appears to be a tendency for 96-well MPNs that use crude oil substrates to underestimate hydrocarbon degraders relative to those that use F2. Linear regression analysis, however, suggests that these differences were not statistically significant. Nevertheless, F2 is the superior substrate, because it was often difficult to detect changes in the oil sheen when crude oil was used in the small wells of the 96-well microtiter plates, whereas INT-formazan deposition was easily detected.

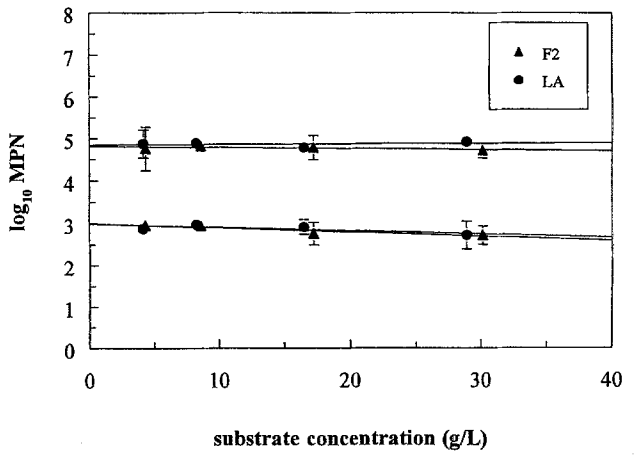

Figure 1 Effect of oil concentration on the MPN estimate of hydrocarbon-degrader population densities for two dilutions of the oil-degrading enrichment culture BS48. F2, No. 2 fuel oil; LA, Light Arabian oil

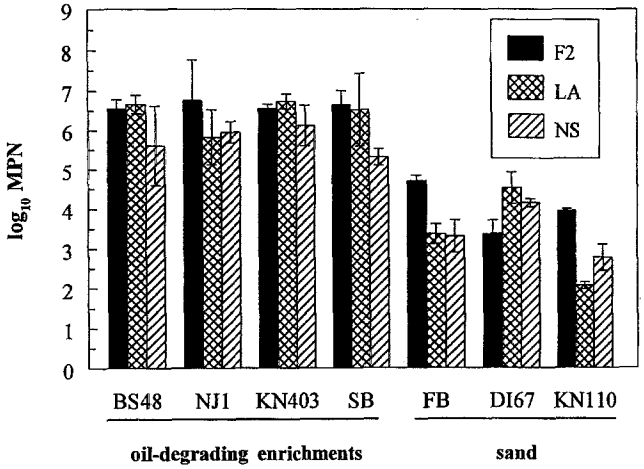

Figure 2 MPN enumeration of hydrocarbon degraders with three oils [Alaskan North Slope (NS), light Arabian (LA) and No. 2 fuel oil (F2)] in the 96-well procedure. The source of each hydrocarbon-degrading population is given in Table 1

Comparison of 96-well/F2 MPN to 24-well sheen screen

The performance of the 96-well F2 hydrocarbon-degrader MPN procedure was compared with the Sheen Screen assay by using both procedures to quantify the number of hydrocarbon degraders present in several oil-degrading enrichment cultures, and in several sand and gravel samples collected from beaches that had a history of oil input (Figure 3). For five of these eight samples, the 96-well method gave higher population estimates than did the 24-well Sheen Screen. Linear regression analysis of these data, however, indicated that the difference between the two methods was not significant at the 95% confidence level.

Accuracy of the 96-well MPN method

To obtain accurate estimates of hydrocarbon-degrader populations by MPN, a single hydrocarbon degrader must be able to grow to a sufficient density to be detected [1]. Two methods were used to test the hypothesis that inoculation of a single hydrocarbon degrader into a well will produce a positive result. First, the population estimates for several pure cultures of hydrocarbon-degrading bacteria obtained with the F2-MPN procedure were compared to the number of total heterotrophs as enumerated by plate counts on marine agar. Second, the verification frequency for hydrocarbon-degrading heterotrophs was compared to the historical average ratio of MPN to HPC for three oil-degrading enrichment cultures. The verification frequency was determined by picking colonies from marine agar HPC plates and evaluating their ability to grow on F2.

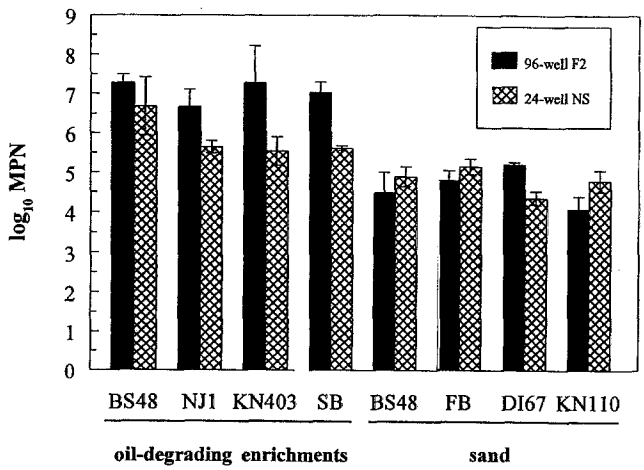

Figure 3 Determination of hydrocarbon-degrader population densities with the 24-well Sheen Screen and 96-well MPN procedures. The source of each hydrocarbon-degrading population is given in Table 1

Table 2 Comparison of hydrocarbon-degrader MPN to heterotrophic plate counts for several pure cultures of hydrocarbon-degrading bacteria

Culture	log HPC	log MPN	P^a	$\frac{HPC}{MPN}$
BS48-3	8.90 ± 0.16	8.96 ± 0.17	0.73	0.87
Ar-1	8.13 ± 0.12	7.78 ± 0.11	0.04	2.2
Ar-2	8.25 ± 0.13	8.12 ± 0.06	0.34	1.3
Phe-1	8.88 ± 0.16	7.98 ± 0.11	0.03	7.9
Phe-2	8.17 ± 0.10	7.79 ± 0.11	0.02	2.4

[a] P = the probability that the means are the same; the null hypothesis is rejected when $P < 0.05$

When pure cultures were used, the ratios of HPC to hydrocarbon-degrader MPN ranged from approximately 1 to 8, with an average of about 2.9 (Table 2). The differences between MPN and HPC were statistically significant in three of five cases, with the MPN always lower. For two of the five bacteria tested, a positive result was obtained when a single cell was inoculated into a well, but one of the cultures required inoculation of as many as eight cells per well to be scored as positive. The remaining cultures appeared to require two to three cells per well to produce a positive result. Thus, there is wide variation in the minimum number of hydrocarbon-degrading bacterial cells required to initiate growth when inoculated into mineral salts medium supplemented with F2.

The data from oil-degrading enrichment cultures are consistent with the data from pure cultures. The verification frequency for hydrocarbon-degrading heterotrophs was always greater than the historical average ratio of hydrocarbon-degrader MPN to HPC (Table 3). If inoculation of a single hydrocarbon degrader produced a positive well, these two numbers should have been the same. In one culture, as many as nine hydrocarbon degraders were required to produce a positive well. Thus, the 96-well MPN assay probably underestimates the number of hydrocarbon degraders that are present in some oil-degrading enrichment

Table 3 Comparison of the historical average MPN to HPC ratio to the verification frequency for growth on F2-degrading enrichment cultures

Culture	$\frac{MPN}{HPC}$	95% Confidence interval	Verification frequency	$\frac{vf^a * HPC}{MPN}$
KN403	0.029	0.019–0.031	0.27	9.3
BS48	0.18	0.14–0.23	0.26	1.4
SB	0.18	0.10–0.28	0.65	3.6

[a]Verification frequency

cultures. Since the 96-well method gave results that were similar to the Sheen Screen, underestimation of the hydrocarbon degrader population density is probably a general phenomenon.

Discussion

This paper describes a new most-probable-number method for enumerating hydrocarbon-degrading bacteria. Samples are diluted and incubated in 96-well microtiter plates, and F2 is used as the growth substrate. After incubation for two weeks, positive wells are scored by reduction of iodonitrotetrazolium violet. All of the required manipulations, including sample dilution and addition of F2 and INT, can be performed automatically by a laboratory robot or manually with a multichannel pipettor.

We evaluated the suitability of several potential carbon sources for this 96-well MPN method. Two crude oils, weathered Alaskan North Slope and light Arabian, and one refined product, F2, were tested. Growth on the crude oils was indicated by emulsification or dispersion of the oil slick [3], and reduction of INT was used to score wells as positive for growth on F2. Although differences between the INT-reduction and crude-oil-dispersion methods were not statistically significant, the F2 INT plates were easier to score than were the crude oil plates. INT reduction is a direct measurement of respiratory activity, and it can be observed even when the hydrocarbon-degrading bacteria grow at the oil–water interface. Thus, it is similar to the radiorespirometric procedure [2,13,14], but it is more convenient for routine application. INT reduction is more sensitive than turbidometric procedures, which is particularly important for the short path length that is characteristic of the 96-well microtiter plates. Because resazurin responds to the redox potential of the culture medium (ie it measures oxygen depletion), it also measures respiratory activity. The distinction between the red positive wells that result from INT-formazan deposition and the colorless negative wells, however, is probably simpler than distinguishing between purple, resazurin-containing negative wells and pink, resorufin-containing positive wells. Thus, INT reduction should be a more effective means of identifying positive wells in 96-well microtiter plates than either turbidity or resazurin reduction.

The accuracy of the 96-well MPN procedure was evaluated by comparing the population density estimates that it provided for several pure cultures of hydrocarbon-degrading bacteria to those obtained by heterotrophic plate counts. Both methods gave identical results for two of the five pure cultures that were tested, but statistically significant differences between the two methods were detected for the others. In all three of these cases, the MPN results were lower than the HPC, suggesting that inoculation of a single cell was not sufficient to produce a positive result. This is consistent with the results obtained using mixed cultures, where the average ratio of MPN to HPC was compared with the proportion of heterotrophic bacteria that were capable of growing on F2. Overall, it appears that between one and nine cells were required to produce a positive well. Since the 96-well procedure provided comparable estimates to the Sheen Screen method, this is probably a general phenomenon associated with enumeration of hydrocarbon degraders. Others obtained higher counts of natural microbial populations by MPN than by plating the organisms on solid media [7,9], suggesting that MPN is still the most efficient method for estimating hydrocarbon-degrader population densities, despite its systematic bias.

The underestimation reported here applies to organisms that are culturable on the selective medium used in the 96-well MPN procedure. Thus, it is distinct from the well known phenomenon in which growth-based enumeration procedures underestimate bacterial populations in environmental samples [6]. It is possible that single cells of some organisms are not capable of initiating growth on oil, because attachment of the cells to the oil–water interface is a necessary prerequisite [5,12]. There must be some stochastic component to the attachment process, such that growth does not necessarily ensue when a single hydrocarbon degrader is inoculated into medium containing oil.

This 96-well procedure is well suited for use in the laboratory and in the field. The Sheen Screen procedure, which uses 24-well plates for determination of hydrocarbon-degrader MPN, has previously been described for field use [3], but the 96-well procedure should be more suitable for field applications, because the plates are less susceptible to spilling during handling and transportation than are 24-well plates. Also, the material requirements are lower because the 96-well plates have a smaller working volume than would be required for an equivalent combination of replicates and dilutions with 24-well plates. The Sheen Screen method cannot be readily adapted to 96-well plates, however, because it is often difficult to detect emulsification in the smaller wells of these plates. INT-formazan deposition is readily detected in either 24 or 96-well plates. Furthermore, because oil emulsification and dispersion are usually attributable to products associated with alkane degradation, microbial populations that are dominated by PAH-degraders might be underestimated by the Sheen Screen method. These problems do not occur in the F2/INT method, because F2 is relatively high in PAHs. Thus, this 96-well MPN procedure is a sensitive and versatile tool that is suitable for characterizing a wide variety of hydrocarbon-degrading microbial populations in the laboratory or in the field.

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