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Development of a standard bacterial consortium for laboratory efficacy testing of commercial freshwater oil spill bioremediation agents

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Six crude oil-degrading bacterial strains isolated from different soil and water environments were combined to create a defined consortium for use in standardized efficacy testing of commercial oil spill bioremediation agents (OSBA). The isolates were cryopreserved in individual aliquots at pre-determined cell densities, stored at –70°C, and thawed for use as standardized inocula as needed. Aliquots were prepared with precision (typically within 10% of the mean) ensuring reproducible inoculation. Five of the six strains displayed no appreciable loss of viability during cryopreservation exceeding 2.5 years, and five isolates demonstrated stable hydrocarbon-degrading phenotypes during inoculum preparation and storage. When resuscitated, the defined consortium reproducibly biodegraded Alberta Sweet Mixed Blend crude oil (typically ± 7% of the mean of triplicate cultures), as determined by quantitative gas chromatography–mass spectrometry of various analyte classes. Reproducible biodegradation was observed within a batch of inoculum in trials spanning 2.5 years, and among three batches of inoculum prepared more than 2 years apart. Biodegradation was comparable after incubation for 28 days at 10°C or 14 days at 22°C, illustrating the temperature tolerance of the bacterial consortium. The results support the use of the synthetic consortium as a reproducible, predictable inoculum to achieve standardized efficacy tests for evaluating commercial OSBA.

Keywords: bacterial inoculum; consortium; crude oil biodegradation; oil spill bioremediation agents; petroleum

Introduction

Biodegradation of crude oil in natural systems occurs through the actions of mixed microbial populations. In certain environments, conditions limit the presence or effectiveness of natural degradative consortia. For example, pristine environments lacking petroleum hydrocarbons typically support low levels of hydrocarbon-degrading microbes, whereas oil-contaminated environments may experience physical conditions (eg low temperature) or chemical conditions (eg low oxygen or fixed nitrogen levels), limiting the ability of their degradative consortia to biodegrade petroleum. In these cases, the practice of bioaugmentation or biostimulation may be warranted. The former term refers to the addition of exogenous organisms (eg those having oil-degradative phenotypes) to the environment to augment the natural flora, while the latter refers to the addition of chemicals such as nutrients (eg nitrogen, phosphate and/or oxygen) or surfactants to stimulate the natural flora.

Prior to the *Exxon Valdez* oil spill in 1989, few oil spill bioremediation agents (OSBA) for either biostimulation or bioaugmentation were commercially available. Since that time, and especially since the highly publicized field trial of bioremediation in Prince William Sound (reviewed in [19]), increasing numbers of OSBA have been marketed [1,20]. This has created a need for environmental regulatory agencies to evaluate the safety and efficacy of OSBA intended for release into the environment, so as to create a list of acceptable products from which oil spill responders could choose. The United States Environmental Protection Agency (US-EPA) and Environment Canada's Emergencies Science Division addressed this need contemporaneously by developing tiered quantitative laboratory tests (requiring microbial inocula) for evaluating OSBA. However, their approaches differed in that the US-EPA (through the National Environmental Technology Applications Corporation; NETAC) chose to use natural seawater microflora as their standard inoculum for Tier II laboratory tests [17]. In contrast, Environment Canada chose to construct synthetic consortia composed of bacteria isolated from geographically diverse sources representing different natural environments. Two such consortia were synthesized: one from terrestrial and freshwater sources for testing OSBA intended for continental use at moderate temperatures (10–25°C); and one derived from seawater and coastal sediments for testing OSBA intended for marine applications at cold temperatures (≤10°C), reflecting typical Canadian environments.

The consortia were developed for two purposes: (i) to serve as reproducible populations for testing the efficacy of biostimulatory OSBA; and (ii) as benchmark populations against which bioaugmentation OSBA could be reproducibly measured. Therefore, reliable oil biodegradation by the inoculum under standard conditions was extremely important in developing standardized OSBA tests for Environment Canada. In this paper, we describe the development of the oil-degrading freshwater consortium and its

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subsequent testing for replication (viability with storage), reliability (hydrocarbon-degrading phenotype stability), and validity in laboratory tests (reproducibility of degradation). Characteristics of the cold, marine consortium have been described elsewhere (Foght *et al*, in press), as have results from testing of commercial OSBA [3].

Materials and methods

Media and standard crude oil

The standard freshwater medium contains 0.5 g K_2HPO_4, 2.0 g Na_2SO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 2.0 g KCl, trace $FeSO_4 \cdot 7H_2O$ and 1.0 ml trace mineral salts solution per liter of twice-distilled water. Trace mineral salts solution contains 3.7 g $CaCl_2 \cdot 2H_2O$, 2.5 g H_3BO_3, 0.87 g $MnCl_2$, 0.65 g $FeCl_3$, 0.44 g $ZnCl_2$, 0.29 g $Na_2MoO_4 \cdot 2H_2O$, 0.01 g $CoCl_2$ and 0.0001 g $CuCl_2$ per liter of twice-distilled water. When required, a sterile nutrient solution (N,P: containing 25 g K_2HPO_4, 50 g NH_4Cl and 100 g KNO_3 per liter of twice-distilled water) was added to sterile freshwater medium at 20 ml L^{-1}. The composition of BYP agar and mineral medium agar used for inoculum enumeration and phenotype testing have been described [10].

Alberta Sweet Mixed Blend (ASMB) crude oil was selected as the reference oil for tests, as it has been characterized extensively by Environment Canada [15]. It was 'weathered' by evaporation under forced air at room temperature for 24 h to reduce volatile hydrocarbon content and improve quantitative analysis of residual oil. This resulted in mass losses of 17.3%, 18.6% and 13.8% for three different batches of oil prepared over a 4-year period. The weathered oils were stored, sealed, at 4°C.

Selection and identification of oil-degrading bacterial strains

Bacterial strains in our cryopreserved culture collection isolated from diverse environments and geographic sites were considered as candidates for the synthetic consortium. In preliminary tests, six of these isolates were confirmed as degrading either the aliphatic or the aromatic components of ASMB, using qualitative gas chromatography with a flame ionization detector (GC-FID; [7]) and the surrogate standards squalane and chrysene. Various combinations of the strains were incubated with ASMB to verify that they would be compatible in an oil-degrading consortium. Residual oil in duplicate flasks was qualitatively analyzed after fractionation and compared with parallel sterile controls to determine relative biodegradation losses.

The strains were subjected to standard biochemical tests [18] including: Gram stain and cell morphology, motility, nitrate reduction, anaerobic growth, production of fluorescent pigments, cell wall amino acid composition, presence and identity of mycolic acids, presence of xanthomonadin pigment, utilization and acid production from various carbohydrates, and gelatinase, amylase, oxidase, catalase and decarboxylase activities.

Phenotype stability

Pure cultures of the standard inoculum organisms were tested for the proportion of viable cells possessing the desired hydrocarbon-degrading phenotype as follows. For aliphatic-degrading isolates, a minimum of 100 isolated colonies were replica patched using sterile wooden toothpicks onto mineral medium spread with 30 μl of the aliphatic fraction of Prudhoe Bay crude oil (prepared as per Ref [7]) as a carbon source (test plate), and onto mineral medium without an added carbon source (negative control). Plates were inverted and incubated at 22°C for 2–3 weeks in the dark, then scored by comparing growth on the plates: growth on the test plate and no growth on the corresponding control was scored as a positive result. For phenotype testing of aromatic degraders, triplicate spread plates on BYP agar having well-isolated colonies (typically with 100–200 colonies each) were pregrown at 22°C for 5 days, then sprayed [16] with an ethereal solution of dibenzothiophene and re-incubated. Colonies which developed an orange color resulting from oxidation of the substrate [10] were scored as positive.

Preparation and testing of replicate inoculum aliquots

Isolated colonies of individual strains were used to inoculate 5 ml of tryptic soy broth (TSB; Difco) in test tubes and incubated at 22°C overnight on a tube roller. One to three milliliters of these seed cultures were used to inoculate 200 ml of TSB in 500-ml Erlenmeyer flasks incubated at *ca* 25°C for 24–48 h, as appropriate, with shaking at 200 rpm. Cultures were harvested aseptically by centrifugation at 10 000 × *g* for 15 min. The cell pellets were resuspended in 5–10 ml of cold, fresh TSB, then an equal volume of sterile 50% v/v glycerol was added. These 'bulk suspensions' were placed on ice for at least 10 min to allow penetration of the glycerol cryoprotectant. Viable cell numbers in the bulk suspensions were estimated by removing an aliquot, diluting it in 10 mM phosphate buffer (pH 7) and plating it in triplicate onto Plate Count Agar (PCA; Difco) or BYP agar for incubation at 22°C for 5 days. Meanwhile, the individual bulk suspensions were frozen at −70°C. After the colony forming units per ml (CFU ml^{-1}) in each bulk suspension had been estimated, they were thawed and held on ice, and diluted appropriately with sterile ice-cold TSB plus 50% glycerol (1:1 vol:vol) to achieve a calculated viable cell density of 2×10^9 CFU ml^{-1}. The diluted bulk suspensions were then individually dispensed in 100-μl aliquots using a repeat pipettor and 2.5 ml CombiTips (Eppendorf) into sterile 2.0-ml screw-cap Nalgene Cryogenic Vials (Nalge, Rochester, NY, USA), and frozen at −70°C. The actual CFU ml^{-1} in the aliquots was determined accurately for each isolate after freezing by thawing three randomly selected aliquots, diluting and plating each as above, and counting CFU in triplicate on PCA or BYP agar for each replicate aliquot. Purity of each inoculum preparation was also verified using these plates. After enumeration, colonies were screened for appropriate degradative phenotypes, as described above.

In later studies, the three aliphatic-degrading isolates, and similarly the three aromatic-degrading isolates, were combined into the same cryovial for use in efficacy testing. However, individual replicate aliquots of each isolate were also prepared for use in viability and phenotype quality control tests.

To test inoculum viability and phenotype stability during

long term storage at −70°C, at intervals randomly selected aliquots of individual isolates were thawed, diluted and tested for CFU ml^{-1} and phenotype as described above.

Biodegradation tests

The standard freshwater OSBA efficacy tests developed by Environment Canada ([12] Blenkinsopp *et al*, in preparation) employ the freshwater medium and ASMB oil incubated under standard conditions in various combinations with inoculum, OSBA, and N,P amendment. The residual oil recovered from replicate cultures is then subjected to quantitative GC-FID and GC with mass spectrometry (GC-MS) to determine the percentage of biodegradation that has occurred. Biostimulatory OSBA are tested in the presence of the standard inoculum, whose oil-degrading activity they must stimulate. For bioaugmentation OSBA, the test flasks are inoculated only with the commercial product, and the standard inoculum is used in separate quality control flasks to ensure that acceptable conditions are achieved during the test. This paper presents the results from duplicate and triplicate control flasks (containing standard inoculum, reference oil, and mineral medium with or without N,P) normalized to parallel sterile (uninoculated) controls, to demonstrate the hydrocarbon-degrading properties of the inoculum. Results from OSBA tests have been presented elsewhere [3].

Specifically, triplicate 500-ml Erlenmeyer flasks containing 200 ml freshwater medium with or without N,P were inoculated by thawing replicate inoculum aliquots on ice and quantitatively transferring the cells aseptically using a Pasteur pipette and a small amount of the sterile medium to rinse the cryovials. The viable cell number in each aliquot was sufficient to provide an inoculum density of *ca* 10^6 CFU ml^{-1} in the medium. In early trials, 400 μl oil was added volumetrically to each flask, and results were reported as mass loss per ml of oil. This method was refined in the final protocol by gravimetrically adding 400 mg weathered ASMB reference oil (accurately measured to 0.1 mg) to each flask using a tared sterile 5-ml glass syringe and reporting results on a % weight loss basis. Parallel uninoculated flasks served as sterile controls to account for abiotic losses. All flasks were stoppered with foam plugs capped loosely with foil, to permit gas exchange while minimizing volatility losses. Flasks were incubated in the dark on a gyratory shaker at 150 rpm (2.5 cm eccentricity) at either 10°C for 28 days or at 22°C for 14 days.

In early studies into the effect of temperature on the inoculum, replicate flasks were incubated in freshwater medium plus N,P at 4°, 10°, 15° and 22°C and sacrificed for analysis at weekly intervals up to 28 days. These tests were carried out using Prudhoe Bay crude oil added volumetrically, and analyzed as described below.

Recovery and analysis of residual crude oil for quantitative GC

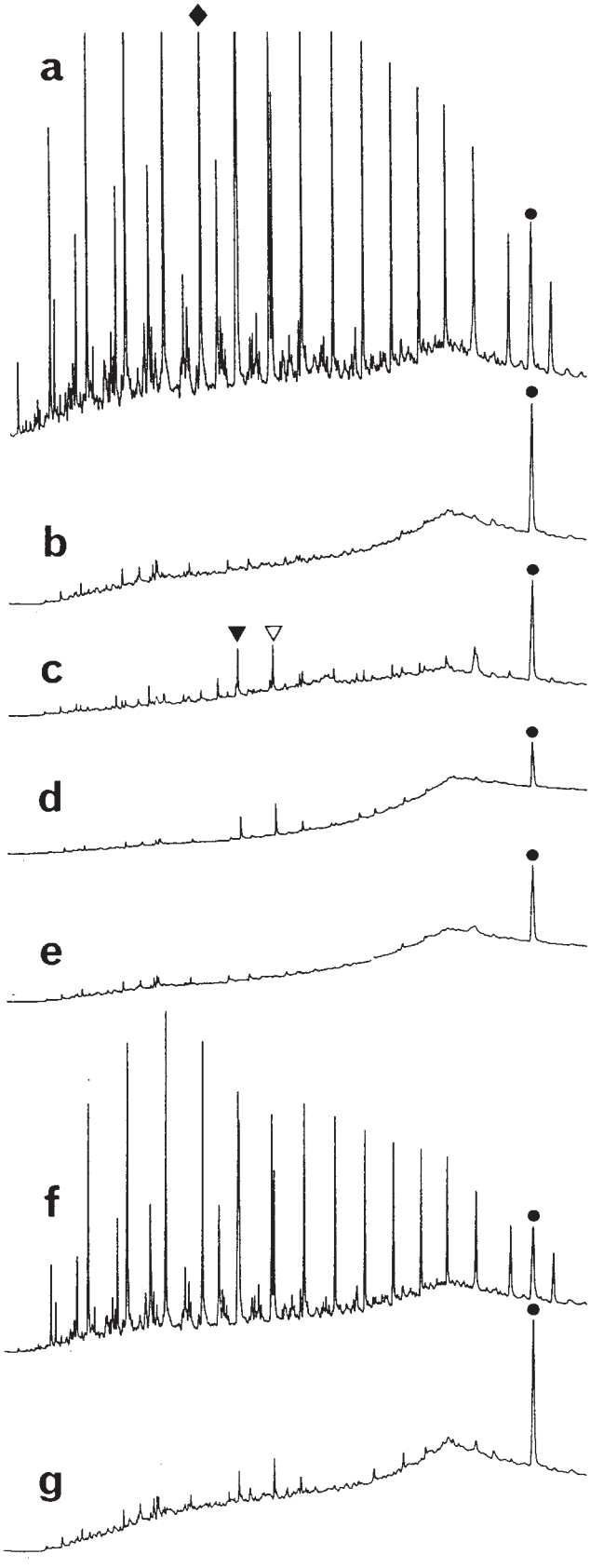
After incubation, flask contents were acidified to pH $\leq$2 with 2 ml 4 N H_2SO_4. Exactly 1.0 ml of a surrogate standard solution containing known weights of squalane (Sigma), *o*-terphenyl (99%, Aldrich) and d_{10}-phenanthrene (97 atom%, Aldrich), prepared in a volumetric flask and stored at −20°C, was added to assess the efficiency of oil extraction. Residual oil was exhaustively extracted with multiple aliquots of HPLC-grade dichloromethane (Caledon Laboratories, Georgetown, ON, Canada), dried by passage through anhydrous Na_2SO_4 and concentrated by rotary evaporation and under N_2 to less than 1 ml. The recovered oil was quantitatively transferred to 2-ml glass autosampler vials with teflon liners (Hewlett Packard).

As previously described [4, 22], a known mass of each sample was fractionated into aliphatics and aromatics by liquid column chromatography and analyzed by quantitative GC-FID and GC-MS. The following fractions were analyzed [23]: total GC-detectable petroleum hydrocarbons ('TPH', the sum of all GC-resolved and unresolved hydrocarbons), total aliphatics (the sum of all GC-resolved and unresolved hydrocarbons collected in the aliphatic fraction, including total *n*-alkanes, branched alkanes and cyclic saturates); *n*-alkane distribution (the sum of all resolved *n*-alkanes from C_8 to C_{40} plus pristane and phytane); total aromatics (calculated as TPH minus total aliphatics); five target polycyclic aromatic hydrocarbons ('PAH') and their alkyl homologues (the sum of the parent compounds and alkylated homologues of naphthalene, phenanthrene, dibenzothiophene, fluorene and chrysene); and biomarkers (eg hopanes and steranes in the aliphatic fraction). Results were expressed as mg or μg of analyte per mg residual oil, after instrument calibration and normalization to internal standards [22,23]. Percent losses of the analyte fractions were calculated relative to the recovered oil from the parallel sterile controls. In this communication, results are reported from three selected classes of analytes, namely: (i) TPH; (ii) total aliphatics; and (iii) PAH, because these

Table 1 Source and presumptive identification of the constituent strains of the freshwater inoculum

Strain	Source	Reference	Identification
Aliphatic-degrading isolates			
S+14He	freshwater pond, Alberta	[6]	*Mycobacterium* sp[a]
Husky A	oily sludge landfarming site, Saskatchewan	this study	*Mycobacterium* sp[a]
Esso AgD	refinery wastewater, Germany	[11]	*Corynebacterium* sp[b]
Aromatic-degrading isolates			
LP6a	hydrocarbon-contaminated soil, Alberta	[11, 12]	*Pseudomonas fluorescens*
EB21a	refinery wastewater, Germany	[8, 11]	*Pseudomonas alcaligenes*
S+14Ha	freshwater pond, Alberta	[6]	*Xanthomonas* sp

[a]Most similar to *Mycobacterium agri.*
[b]Most similar to *Corynebacterium callunae.*



classes respectively represent: (i) a general indicator of biodegradation; (ii) the most readily degraded fraction of the oil; and (iii) compounds of concern because of their typical environmental persistence and potential carcinogenicity.

Results

Selection and identification of inoculum strains

Six bacterial strains isolated from various environments were chosen from our cryopreserved collection of petroleum-degrading bacteria and presumptively identified by standard taxonomic methods (Table 1). We have previously observed [8] that our collection contains strains that mineralize either hexadecane or phenanthrene in the presence of oil. Therefore, we selected isolates from amongst the aliphatic- and aromatic-degrading bacteria to constitute our complete standard inoculum. Three strains of each degradative type were chosen to build redundancy into the inoculum.

In preliminary trials the compatibility of various combinations of these inocula was verified by qualitative GC-FID. Selected chromatograms from these studies presented in Figures 1 and 2 show that the aliphatic-degrading strains extensively degraded the *n*-alkanes and isoprenoids individually (Figure 1b–d), together (Figure 1e), and also when combined with the aromatic-degrading strains as a complete inoculum (Figure 1g). In contrast, the three aromatic-degraders did not significantly alter the aliphatic fraction (Figure 1f). Analysis of the aromatic fraction of the same residual oils showed that the isolates primarily degraded the lower molecular weight compounds (Figure 2b–d). The combination of three aromatic-degrading strains achieved better degradation (Figure 2e) than any individual strain (Figure 2b–d), and the best aromatic degradation was observed with the complete inoculum (Figure 2g). The aliphatic-degrading strains did not significantly degrade the aromatic fraction of ASMB (Figure 2f). Based on results from these and other combinations of the strains (data not shown), the six isolates described in Table 1 were determined to be compatible for use in the synthetic consortium.

In early experiments, biodegradation tests were conducted over a range of temperatures typical of temperate freshwater environments, to determine whether the inoculum would function under both cold and warm freshwater conditions or whether distinct consortia would be required. Figure 3 shows that comparable losses of TPH, total aliphatics and PAH were achieved by the inoculum whether incubated at 4°C, 10°C or 15°C for 28 days or at 22°C for 14 days. That is, the inoculum was capable of degrading oil hydrocarbons over a range of at least 18°C, providing that an extended incubation time was allowed at the lower tem-

Figure 1 Qualitative GC-FID analysis of the aliphatic fraction of residual ASMB oil after incubation with the following strains individually and in combinations: (a) sterile control; (b) S+14He; (c) Husky A; (d) EssoAgD; (e) combined inoculum of all three aliphatic-degrading strains; (f) combined inoculum of all three aromatic-degrading strains; (g) complete inoculum of all six strains. Peak identification: ◆, *n*-hexadecane, representing the series of normal alkanes; ▼, pristane; ∇, phytane; ●, squalane (surrogate standard).

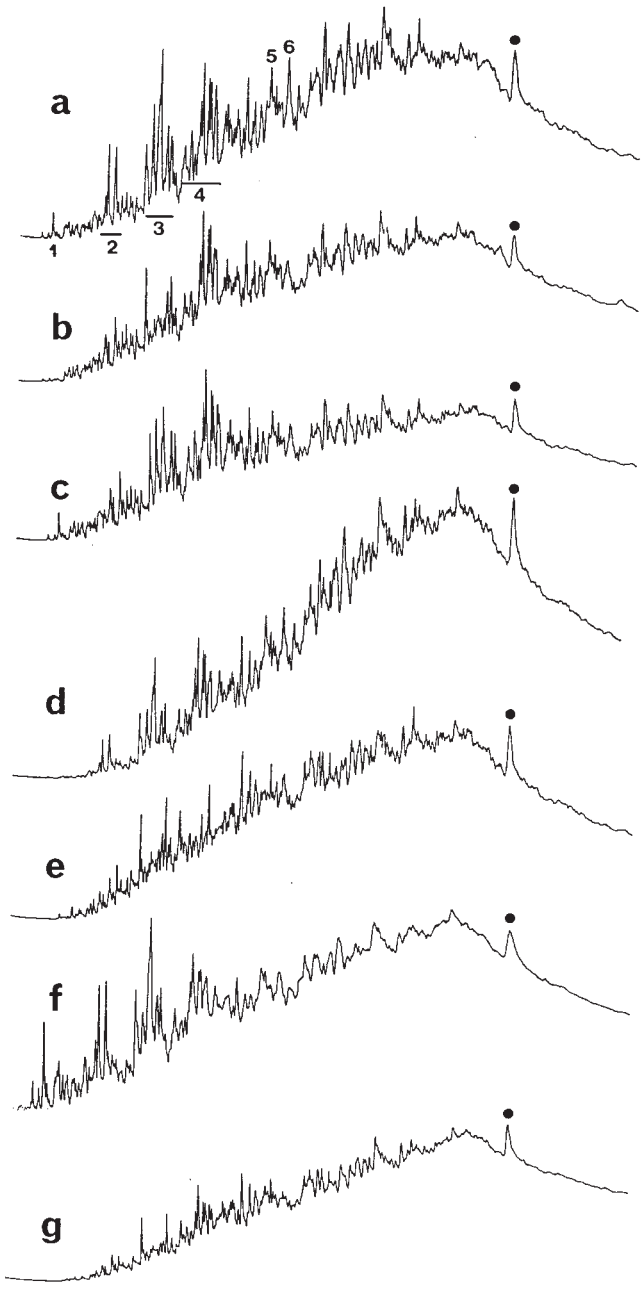


Figure 2 Qualitative GC-FID analysis of the aromatic fraction of residual ASMB oil after incubation with the following strains individually and in combinations: (a) sterile control; (b) LP6a; (c) EB21a; (d) S+14Ha; (e) combined inoculum of all three aromatic-degrading strains; (f) combined inoculum of all three aliphatic-degrading strains; (g) complete inoculum of all six strains. Identification of selected peaks and regions [7]: 1, naphthalene; 2, C_1-naphthalenes; 3, C_2-naphthalenes; 4, C_3-naphthalenes; 5, dibenzothiophene; 6, phenanthrene; ●, chrysene (surrogate standard).

peratures. In all subsequent experiments, standard incubation conditions were defined as either 10°C for 28 days (to represent 'cold, freshwater' conditions) or 22°C for 14 days (to represent 'warm, freshwater' conditions).

Replication of inoculum aliquots, phenotype stability, and long-term viability with storage

Viable cell numbers in the replicate aliquots were determined in triplicate for each of three randomly chosen aliquots. Replication was satisfactory, as shown by typical results presented in Table 2, where overall replication within triplicate analysis of three aliquots is approximately 10% of the mean.

Five of the six isolates demonstrated satisfactory phenotype stability during preparation and storage, with 100% phenotype-positive colonies in all batches and with storage time. The exception was *P. alcaligenes* EB21a, where the percentage of colonies with a positive phenotype for aromatic utilization varied among inoculum batches, ranging from 28% to 82%. However, once prepared, each inoculum batch maintained a consistent proportion of phenotype-positive colonies during storage.

Three separate batches of inoculum have been prepared and tested over a total period of more than 4 years. At intervals, three aliquots of each isolate for each batch of inoculum were thawed and their CFU ml^{-1} determined. Representative results obtained with inoculum batch 3 during storage for 2.5 years are shown in Figure 4. Equivalent results were obtained for the other two inoculum batches, with *ca* 3 years being the maximum storage time tested to date. Five of the six inoculum strains consistently maintained viability during cryopreservation at −70°C. However, *P. fluorescens* LP6a showed a characteristic decrease in viable numbers which occurred early in the storage period (by the earliest sampling time). Typically, a decrease of 60–80% of CFU was observed for each batch of LP6a prepared. The reason for this loss of viability is not known. In later inoculum preparations, we compensated for the anticipated loss by increasing the initial viable numbers of LP6a in the aliquots.

Inoculum use: reproducibility of biodegradation

Thirteen biodegradation trials have been performed over a period of 4 years using different batches of inoculum and oil. Experiments conducted with inoculum batch 1 (trials 1, 2 and 10; triplicate flasks) spanned a period of 5 months to a maximum inoculum storage time of 7 months; batch 2 experiments (trials 3–8; duplicate flasks) were conducted with freshly prepared inoculum, spanning 2 months storage time; batch 3 experiments (trials 9 and 11–13; triplicate flasks) spanned 26 months, with inoculum up to 2.5 years old (trial 9). Data for TPH, total aliphatics, and PAH losses excerpted from these trials are shown in Figure 5, to illustrate the reproducibility of biodegradation achieved by different batches of inoculum, and by the same batch of inoculum with time and under different standard incubation conditions. For each trial, the mean % loss ± 1 standard deviation is shown, calculated relative to parallel sterile controls; that is, the net losses due solely to biodegradation are presented. Although some variability in biodegradation was expected in early trials where oil was added volumetrically instead of gravimetrically, excellent replication of biodegradation within a set of duplicate or triplicate flasks (ie a trial) was observed (Figure 5). Standard deviations within a trial were typically around 7% of the mean, regardless of the batch of inoculum or the incubation con-

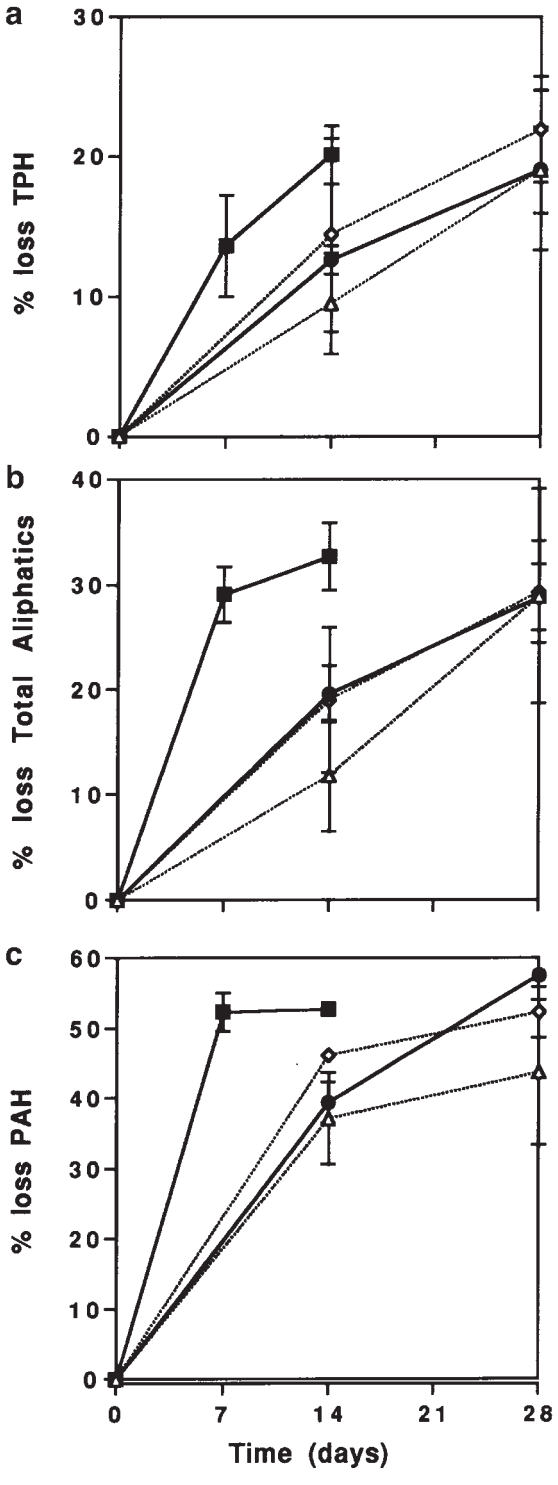


Figure 3 Biodegradation losses of (a) TPH, (b) total aliphatics and (c) PAH from Prudhoe Bay crude oil incubated under standard conditions with N,P up to 28 days at four temperatures (-—△—- 4°C; —●— 10°C; --◇-- 15°C; —■— 22°C); demonstrating activity of the inoculum over a range of 18°C. Each point is the mean of triplicate analyses; error bars (where visible) represent 1 standard deviation.

ditions used. Similar replication was observed with the aromatics (not shown), whereas loss of the readily degradable *n*-alkanes was typically ≥90% and therefore not informative regarding reproducibility. Losses for all analyte classes were consistently lower than the mean in trial 6 and higher in trial 9; trials conducted at 10°C generally yielded lower losses than those conducted at 22°C.

The pattern of biodegradation of PAH homologues in the presence of nutrients was reproducible and consistent with previous observations [7]: the % loss of a compound was related to its molecular weight and degree of alkyl substitution. That is, within an homologous series, the parent (C_0) compound was degraded more readily than its C_1, C_2, C_3 and C_4 alkyl homologues, and the naphthalene series was degraded more completely than the phenanthrene, fluorene and dibenzothiophene series (Figure 6). This shows that the synthetic consortium degraded petroleum PAH in a pattern typical of natural mixed populations [7].

Major terpane and sterane biomarkers (eg C_{23} and C_{24} tricyclic terpanes, C_{29} $\alpha\beta$- and C_{30} $\alpha\beta$-pentacyclic hopanes, C_{27} $\alpha\beta\beta$- and C_{29} $\alpha\beta\beta$-steranes, the other hopanes and steranes) were quantitatively analyzed after incubation and determined statistically to be undegraded (data not shown), as expected.

In the absence of nutrient supplementation, overall biodegradation was reduced, depicted as % loss of TPH in Figure 7 (plotted on the same scale as Figure 5 TPH for direct comparison). Similar results were seen with the aliphatic and PAH analytes (not shown). Under these suboptimal conditions, replication of triplicate cultures within a trial was still acceptable, but greater variability was observed between trials using inoculum batch 2, and also among the three inoculum batches.

Discussion

Ideological considerations

The synthetic consortium described in this study was not designed to be a 'super cocktail' of oil degraders; none of the constituent strains was considered to be an outstanding hydrocarbon degrader. Instead, the inoculum was deliberately intended to achieve moderate oil degradation, since its primary purpose was to serve as a quality control benchmark for bioaugmentation OSBA testing, demonstrating achievement of reproducible test conditions and analyses

Table 2 Typical aliquot replication for three strains (inoculum batch 3). Viable counts were determined by triplicate plate counts at time zero for each of three randomly selected aliquots

Aliquot No.	Mean CFU $ml^{-1} \times 10^{-9} \pm 1$ standard deviation in strain:		
	Husky A	EB21a	S+14Ha
1	1.09 ± 0.07	2.10 ± 0.45	3.45 ± 0.51
2	1.10 ± 0.26	2.22 ± 0.24	3.44 ± 0.30
3	1.31 ± 0.16	2.23 ± 0.35	3.65 ± 0.73
Overall mean[a]	1.17 ± 0.12	2.18 ± 0.07	3.51 ± 0.12

[a]Calculated by averaging values of triplicate sets.

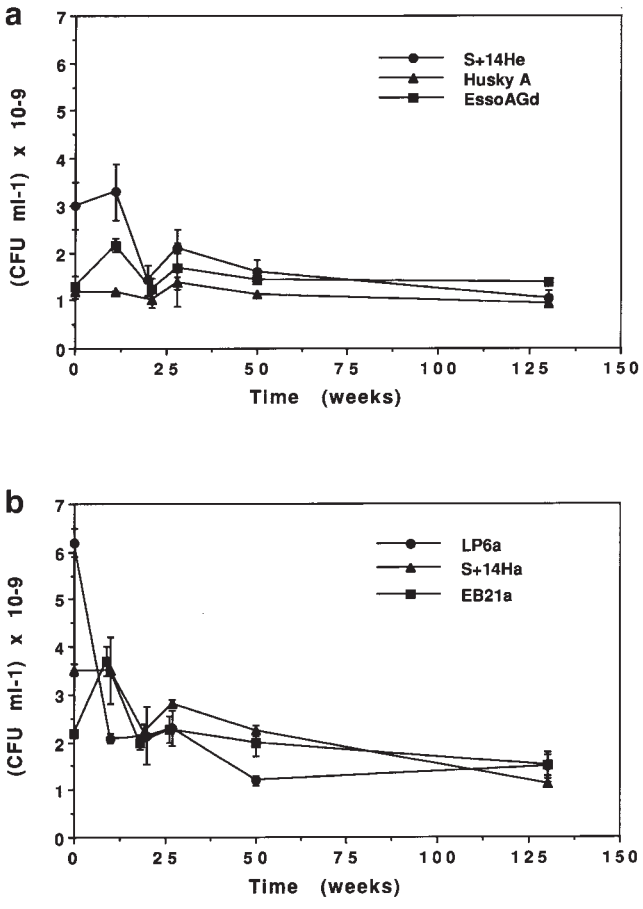


Figure 4 Viability of batch 3 standard inoculum strains during cryopreservation at −70°C. Points represent the mean of three replicates; error bars (where visible) represent 1 standard deviation. (a) Aliphatic degraders; (b) aromatic degraders.

during tests. It was not intended as an 'ideal' inoculum for commercial OSBA to surpass. Its second purpose was to provide a known, reproducible inoculum for testing the (non-biological) biostimulation OSBA, which act to enhance the degradative efficiency of oil-degrading consortia.

The use of a defined bacterial consortium to provide a standard, reproducible inoculum is the major difference between the US EPA and Environment Canada approaches to laboratory testing of OSBA efficacy. The major advantages to using a synthetic consortium are the known, predictable, and reproducible activity of the inoculum, such that OSBA efficacy test results can be compared reliably even when performed months apart. In contrast, initial trials by NETAC using indigenous oil-degrading populations present in natural seawater encountered problems of low biodegradative activity and poor reproducibility due to seasonal and geographic variability of the inoculum [21]. Aldrett *et al* [1] also conducted OSBA efficacy tests similar to the NETAC protocol, using natural seawater as the inoculum. They observed that unpredictable variability of the inoculum was a limitation to accurate and reproducible assessment of OSBA. These problems have been overcome to some degree using enrichment cultures of indigenous populations [13]. Conversely, the use of a synthetic consortium can be criticized as being too artificial compared to the natural microflora with which bioaugmentation agents must compete, and which the biostimulation agents are designed to enhance. A partial response to that criticism is that the standard inoculum is composed of different species obtained from diverse locations, and therefore could be considered a 'generic' freshwater microflora rather than representing a specific locale. Another response is that

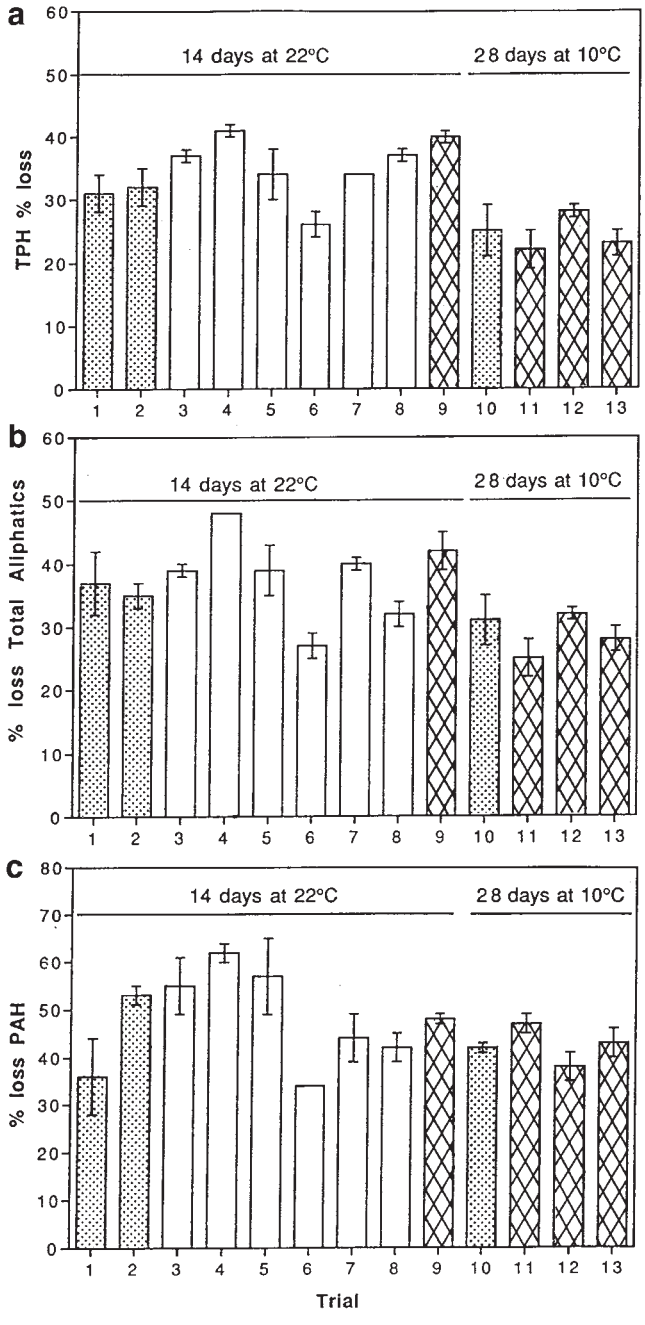


Figure 5 Biodegradation losses of (a) TPH, (b) total aliphatics and (c) PAH from ASMB using different inoculum batches incubated under standard conditions with N,P. Each trial shows the mean of duplicate or triplicate flasks; error bars (where visible) represent 1 standard deviation. Inoculum batch 1, stippled bars; batch 2, open bars; batch 3, cross-hatched bars. Overall mean % losses (± 1 standard deviation) for each analyte class: TPH = 31.5 (± 6.4); total aliphatics = 35.0 (± 6.6); PAH = 46.2 (± 8.5), calculated by averaging values from replicate sets.

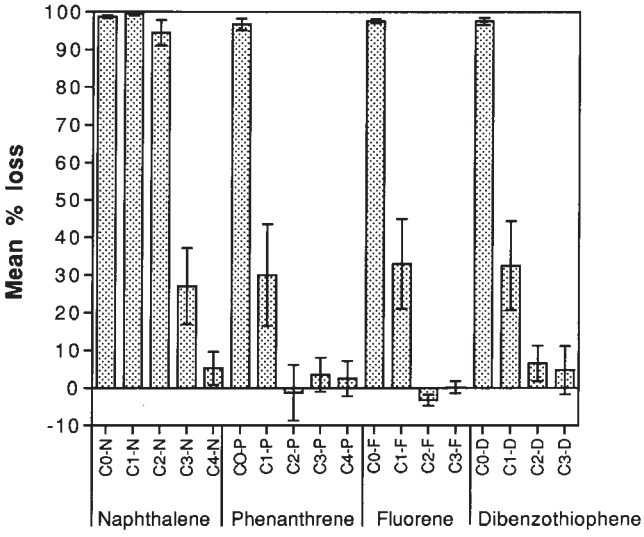


Figure 6 Biodegradation losses of homologous series of PAH from ASMB incubated under standard conditions with N,P supplementation. Each bar shows the mean % loss (n = 4 trials, ± 1 standard deviation) calculated from parallel sterile controls. The alkyl homologues are represented by C_x, where x = number of alkyl carbon substitutents.

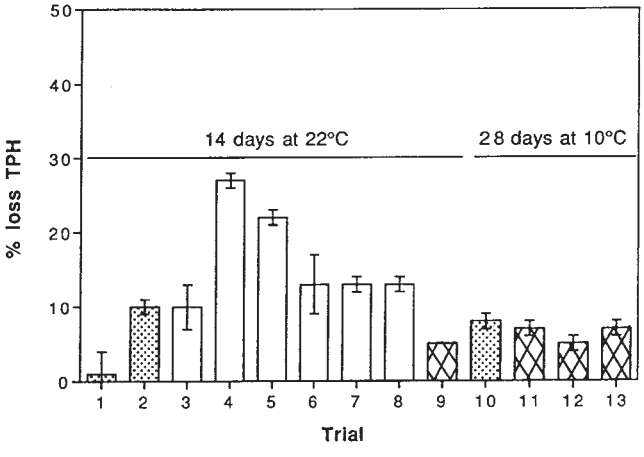


Figure 7 Biodegradation losses of TPH from ASMB using different inoculum batches incubated under standard conditions without N,P supplementation. Each trial shows the mean of duplicate or triplicate flasks; error bars (where visible) represent 1 standard deviation. Inoculum batch 1, stippled bars; batch 2, open bars; batch 3, cross-hatched bars. Overall mean % loss (± 1 standard deviation) for TPH = 10.9 (±7.1).

tailor-made consortia could be prepared to represent microflora in other environments (eg the 'cold, marine' inoculum described elsewhere [9]). The availability of such inocula would enable OSBA to be tested against consortia and under conditions representative of the environment in which they are to be used. The ideological differences between the two approaches to inoculation cannot be resolved, and in any case may be irrelevant since both tests are artificial and neither is a true surrogate for field testing.

Standard inoculum performance

Three each of the aromatic- and aliphatic-degrading bacterial strains were selected for the synthetic consortium to build in functional redundancy. In this way, it was presumed that the inoculum would be less sensitive to unintended adverse effects from handling, storage or OSBA formulations than would an inoculum comprised of only one or two components. This redundancy proved its value in the case of *P. fluorescens* LP6a, which exhibited some loss of viability with storage (Figure 4), and with EB21a which demonstrated phenotype instability. A well-designed synthetic consortium should show co-operativity in degradation of complex substrates and may overcome potential effects of toxic metabolites [5], although a theoretical disadvantage to a multi-strain inoculum is that interactions between the inoculum strains are more difficult to predict and control. This has not been an obvious problem in the freshwater inoculum to date.

Reproducibility of inoculation undoubtedly contributed to the uniform biodegradation levels achieved in the tests reported here. This reliability among tests performed over a 4-year period with inocula of different ages supports our contention that OSBA tests can be compared with confidence even if conducted several years apart. It also attests to the reliability of the standard incubation, extraction and analysis methods used in the tests. We do not know the upper limit for storage time for the inoculum, as all batches tested to date (up to *ca* 3 years) have had acceptable viability and performance.

Standard inoculum versatility

The freshwater inoculum described here was able to function reproducibly over an 18°C temperature range (Figure 3), so that this consortium can be used for both 'warm' and 'cold' incubation conditions. In addition, the inoculum biodegrades a variety of crude oils [4], demonstrating its substrate versatility. In fact, we have used the synthetic consortia (both freshwater and marine) to quantitate biodegradability of a suite of crude oils commonly transported and stored in North America [4,14].

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