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Polycyclic aromatic hydrocarbon degradation by novel bacteria isolated from burrow sediments of marine benthic macrofauna

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POLYCYCLIC AROMATIC HYDROCARBON DEGRADATION BY NOVEL BACTERIA ISOLATED FROM BURROW SEDIMENTS OF MARINE BENTHIC MACROFAUNA

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An Abstract of the Thesis Presented
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Polycyclic aromatic hydrocarbons (PAH) are significant environmental pollutants and represent a severe health threat. Many cosmopolitan areas including coastal environments are heavily impacted by PAH. This research investigates the isolation of PAH-degrading bacteria from burrow sediment of marine benthic macrofauna and their potential in bioremediation.

Macrofaunal burrow sediment is biogeochemically distinct from bulk sediment and has high microbial activities. Samples were collected from a local uncontaminated cove and PAH degradation potential was measured by incubating sediment slurries with exogenous PAH. Burrow sediments from two polychaetes and a mollusc showed significantly higher PAH degradation potential than the bulk sediment. The degradation potential was sensitive to the oxygen level during incubation. Prolonged anoxia arrested the PAH degradation in the slurries however degradation resumed when oxygen became available. Periodic anoxia only slowed the rate of degradation, These

results indicated that burrow sediments likely harbored bacteria which degraded PAH using oxygen.

The presence of PAH-degrading bacteria from burrow sediments was confirmed by enrichment culture of the burrow sediment slurries. Isolate LC8 was isolated from *Nereis* burrow sediment and isolate M4-6 was isolated from *Mya* burrow sediment. These isolates were characterized using phenotypic and phylogenetic techniques. The combined results suggested that LC8, *Lutibacterium anuloederans* (nov.gen., nov.sp.), is a novel genus most closely related to the genus *Erythrobacter*. M4-6, *Cycloclasticus spirillensus*, is a new species of *Cycloclasticus* which is an established PAH-degrading genus.

To study the potential for the two isolates to serve as bioremediation agents, LC8 and M4-6 were re-introduced into a sediment matrix and the degradation potential of the inoculated slurries was measured. Although both strains were isolated from the same locale, LC8-inoculated slurries showed much better PAH degradation potential in both uncontaminated and heavily polluted sediments. M4-6-inoculated slurries showed similar PAH degradation but the rate declined rapidly with time. Moreover, M4-6 seemed to be sensitive to other pollutants in the system.

In summary, two novel ***PAH*** degraders were isolated from marine macrofaunal burrow sediments. They degraded PAH under laboratory conditions and when re-introduced into a sediment matrix. However, M4-6 will not be a suitable bioremediation agent due to its sensitivity to other pollutants which may be present in the environment.

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POTENTIAL POLYCYCLIC AROMATIC HYDROCARBON DEGRADATION IN MACROFAUNAL BURROW SEDIMENTS

Abstract

A variety of polycyclic aromatic hydrocarbons including naphthalene, phenanthrene and dibenzothiophene were degraded with little or no lag in oxic slurries of marine sediments from burrow walls constructed by benthic macrofauna. PAH degradation potentials associated with burrow sediments of two polychaetes *(Nereis virens* and *Clymenella torquata*) and a mollusc *(Mya arenaria)* were generally greater than potentials for non-burrow sediments; relative rates of degradation varied among the burrow wall sediments depending on the PAH assayed. Comparisons of the effects of available electron acceptors (oxygen, nitrate, ferric iron, and sulfate) indicated that significant degradation of benzene, hexadecane and PAH occurred only in the presence of molecular oxygen. However, the capacity for oxic phenanthrene degradation was stable during incubations with alternating oxic and anoxic conditions, suggesting significant anoxia tolerance.

Introduction

Polycyclic aromatic hydrocarbons account for a significant fraction of crude oil and petroleum-based products such as creosote **(86).** In addition, PAH are derived from numerous natural sources, perhaps the most important of which is biomass burning **(46). As** a consequence of extensive fossil fuel use and pollution, PAH occur ubiquitously in marine systems, especially coastal systems that are subject to numerous intense point sources and less intense but widespread diffise sources of pollution. Since many PAH are mutagenic or carcinogenic (69), their fate has been the focus of extensive geochemical, biochemical, physiological and ecological analyses.

Results from early microbiological analyses suggested that PAH degradation depends on molecular oxygen to initiate ring cleavage (11, **12, 26,** 71). However, some monoaromatics, e.g. benzene and toluene, are labile under anaerobic conditions **(30, 3** 1, **40, 67). In** addition, naphthalene and phenanthrene have been degraded under sulfate-reducing conditions in chronically contaminated sediment **(24).** Nevertheless, numerous recent studies indicate that aerobic transformations result in more extensive and rapid degradation of a greater diversity of PAH, including those with high molecular weights **(18, 26, 34, 48).**

In coastal marine sediments, high rates of benthic oxygen uptake limit molecular oxygen to the upper several millimeters of sediment **(16, 32)**, thereby substantially constraining the potential for aerobic PAH degradation. Nonetheless, macrofaunal burrow ventilation with oxygenated water may facilitate sub-surface aerobic degradation, and in some cases inhibit anaerobic metabolism (10). The importance of burrows and burrow ventilation is evident from burrow surface area and ventilation rates. Kristensen

(57) has estimated that at a modest density (700 m^{-2}), *Nereis virens* burrows increased the sediment-water interface area by 150%; individual burrow ventilation rates up to 400 ml hr^{-1} have been reported (85). Ventilation with oxygenated surface water undoubtedly accounts for well documented increases in aerobic ammonia oxidation rates in the burrows of diverse macrofauna (55, 60, 61, 74, 83), and may contribute to enhanced transformations of iron, manganese, sulfur, and organic matter.

Anaerobic transformations are also enhanced in at least some burrows. This is due in part to the fact that ventilation and oxic conditions occur only intermittently (5, 20, 55, 85). In the case of denitrification, enhanced activity is due to increased nitrate availability from nitrification during oxic periods and close coupling between these processes (17). Patterns of sulfate reduction along radial transects of burrow walls vary more than those of nitrification and denitrification, with enhanced activity near burrows in some instances and apparent inhibition in others (39, 43). In this case, parameters other than ventilation and oxygen, e.g., organic matter supply, may be important. Regardless, burrows may be sites of enhanced anaerobic aromatic degradation by sulfate-reducing bacteria (13, 14, 24, 30, 75) or denitrifiers (7, 19).

We report here PAH degradation potentials for burrow sediments from polychaetes (*Clymenella torquata* and *N. virens*) and a mollusc (*Mya arenaria*). Radiotracers were used to compare degradation for benzene and hexadecane under oxic and anoxic conditions: Stable substrates were used to assay oxic PAH degradation and to determine responses to periodic changes in oxygen availability.

Materials and Methods

Sediment collection

Burrow and non-burrow sediments were collected from the intertidal zone of Lowes Cove, Maine. This site has been described previously ***(6*, 43,** 52, 78) and it is not significantly contaminated. C. *torquata* burrows (semi-rigid tubes penetrating about 8-10 cm into the sediment surface) were collected intact since they consist of sediment cemented into a stable structure. C. *torquata* tubes were later homogenized for use in assays other than sulfate reduction. *M. arenaria* burrow sediments were collected from upper Lowes Cove after exposing the animals with a clam fork. *M. arenaria* was typically found at a depth of 15-20 cm. Burrows were formed by extension of the animal's siphons to the sediment surface. Only the innermost (1-2 mm thick) oxidized, light brown layer of sediment from 5-10 cm depth surrounding the siphons was scraped off with a spatula. Burrow sediments of *N. virens* were collected similarly. In all cases, burrow identification was based on both animal occupants and burrow morphologies. Burrow sediments from multiple individual were collected and pooled for triplicate slurries preparation.

^{14}C-hexadecane and ^{14}C-benzene degradation

n-[1-^{14}C]hexadecane (2.15 GBq mmol $^{-1}$) was obtained from Amersham Life Science. [UL-^{14}C] benzene (2.15 GBq mmol^{-1}) was obtained from Sigma Chemical Company. For aerobic degradation experiments, slurries were prepared under oxic conditions using a 1:10 dilution of burrow (C. *torquata* and *M. arenaria)* from multiple

animals or non-burrow sediments in artificial seawater **(ASW)** containing (per liter): 22.79 g of NaCl; 3.98 g of Na_2SO_4; 0.72 g of KCI; 83.0 mg of NaBr; 31.0 mg of $NaHCO_3$; 27.0 mg of H_3BO_3; 0.27 g of NH_4Cl; 89.0 mg of $Na_2HPO_4.7H_2O$; 3.15 g of Tris-HCl; 11.18 g of $MgCl_2.6H_2O$ and 1.46 g of $CaCl_2.2H_2O$. The pH was adjusted to 7.6 with NaOH (92). Ten cubic centimeters of slurry were transferred to each of two sets of 160 cm' polyethylene serum bottles containing a headspace of air. Approximately 37 kBq of ^{14}C-hexadecane (final slurry concentration, 2.4 nmol cm^{-3}) or ^{14}C-benzene (final slurry concentration, 2.4 nmol cm^{-3}) were added to replicate bottles for each set. All bottles were sealed with rubber stoppers fitted with a center well containing 0.2 ml of 0.5 N KOH to trap evolved $^{14}CO_2$. The bottles were incubated with shaking (– 150 rpm) at room temperature in the dark. Center wells were removed from triplicates in each set at intervals; the well contents were added to scintillation vials containing 10 ml of Scintiverse (Fisher Scientific, Inc.) and radioactivity was measured by scintillation counting.

For anaerobic degradation assays, slurries were prepared under anoxic conditions in a glovebag containing nitrogen. Five cubic centimeters of anoxic slurry were incubated in modified 5-cc syringes (luer tips removed) using ASW without sulfate. Radiolabelled substrates were added as above to sealed syringes but at **4.8**nmol cm^{-3}. Nitrate, sulfate or ferric iron at final concentrations of 20 µmol cm^{-3} were added to the slurries, except for the unamended controls. Ferric iron was used as a freshly precipitated oxyhydroxide. The syringes were incubated at room temperature in the dark in a reciprocating shaker (– 100 rpm) under an atmosphere of nitrogen. $^{14}CO_2$ production was measured at intervals by expressing subsamples into scintillation vials

that were subsequently sealed with rubber stoppers fitted with center wells containing 0.2 ml of 0.5 N KOH. The sub-samples were acidified by injecting 2 ml of 1 N HCl. Radioactivity from $^{14}CO_2$ trapped in KOH was measured as above.

Aerobic PAH degradation

Stock solutions (0.11 mM) of naphthalene, 1,4-dimethylnaphthalene, acenaphthene, dibenzothiophene and benz[a]-anthracene were prepared individually in dichloromethane (DCM). These PAH are representative of commonly observed pollutants in coastal sediments. Individual stocks (9 µl) were transferred into 40 cm^3 glass serum bottles, each containing a small amount (0.1 g) of autoclaved bulk sediment as a carrier. The stock solution was mixed thoroughly into the sediment, and DCM was evaporated for several minutes prior to the addition of 10 cm^3 of burrow (*C. torquata* and *M. arenaria)* or non-burrow sediment slurries prepared as described above. The final PAH concentrations in the slurries were approximately 100 nmol cm^{-3}. The bottles were sealed with Teflon-lined stoppers and aluminum-crimp seals, and incubated at room temperature with shaking (–150 rpm) in the dark. The triplicate bottles were flushed with air at regular intervals (2-3 d). At defined intervals, triplicate bottles were sacrificed, and PAH were extracted from the slurries.

Phenanthrene degradation under varied oxygen regimes

Sediment slurries (10% wt/vol) were prepared using *N. virens* burrow wall sediments and artificial seawater. Ten cubic centimeter volumes were transferred to 40 cm^3 glass serum bottles and subsequently sealed with Teflon-lined stoppers. About 50 µg of phenanthrene in 9 µl of DCM were added to each bottle. The serum bottles were

flushed with either air or nitrogen and incubated in darkness at ambient temperature with shaking (150 rpm). The bottle headspaces were flushed daily with air or nitrogen to maintain oxic or anoxic conditions in two sets of replicates; in a third set, oxic and anoxic conditions were alternated on a daily basis. Triplicate bottles from each set were sacrificed at defined intervals, the contents acidified, and phenanthrene extracted with hexane for analysis as described below. In an additional experiment, slurries were prepared anaerobically as above, except that **ASW** without sulfate was used as a sediment diluent. Three sets were amended with nitrate, manganic oxide or ferric oxyhydroxide (both freshly produced) to final concentrations of 20 μmol cm^{-3} of slurry. The fourth set of bottles was not amended and was used as a control. These slurries were incubated under anoxic conditions as above prior to flushing the headspaces with air for oxic incubations. **A** set of autoclaved sediments was used to control for abiological losses.

PAH extraction and quantification

Six milliliters of DCM:methanol (1:2) containing 1,8-dimethylnaphthalene as an internal standard were added to 10 cm^3 of slurry in serum bottles. The bottle contents were shaken for 2 min and aqueous and organic phases were separated by centrifugation after transfer to 20 ml glass screw-cap test tubes sealed with Teflon-lined caps. PAH in the organic phase were analyzed using a Varian model 3400 gas chromatograph equipped with a flame ionization detector and an SPB5 capillary column (15 m x 0.53 mm ID, 1.5 μm film thickness; Supelco). Peak areas from each sample were normalized for the internal standard and PAH concentrations were estimated from standard curves.

Extraction efficiencies were > 95%. Some samples were also analyzed by gas chromatography-mass spectrometry using a Hewlett Packard 6890 GC equipped with an HP5972A mass selective detector and an HP-5MS column (30 m x 0.25 mm; 0.25 μm film thickness). The detection limit of the method is 200 ng (g sediment dry weight)-'.

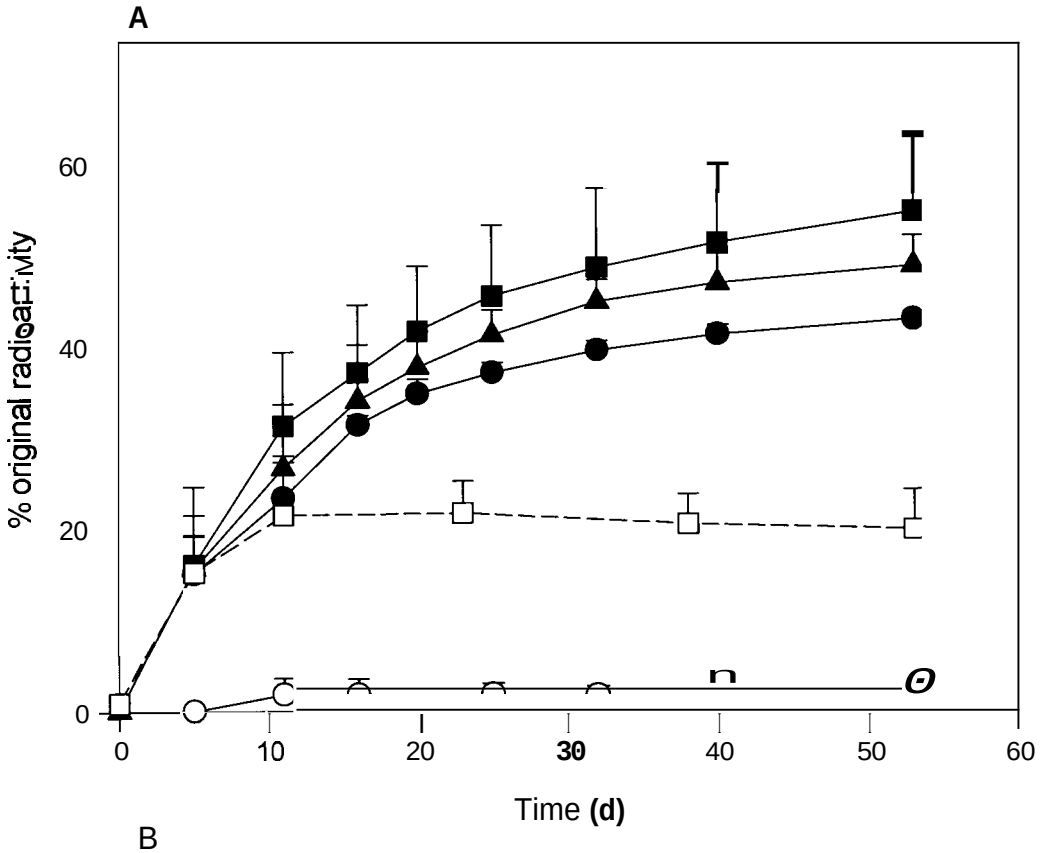
Results

^{14}C-hexane and ^{14}C-benzene degradation

Over 53 d, $^{14}CO_2$ accumulated in a pattern consistent with exponentially decreasing degradation for added ^{14}C-hexadecane and ^{14}C-benzene (Fig. 1.1). Total $^{14}CO_2$ accumulated from ^{14}C-hexadecane was consistently greater for oxic *M. arenaria* than *C. torquata* burrow sediment, although the differences were not statistically significant (Fig. 1.1A). $^{14}CO_2$ production was greater for burrow than non-burrow sediment where maximum $^{14}CO_2$ accumulation reached approximately 50% of the total added during oxic incubations. In non-burrow sediments incubated anaerobically with nitrate as the terminal electron acceptor, less than 20% of the added ^{14}C was recovered as $^{14}CO_2$. Results for other alternate electron acceptors were similar (data not shown). Oxidation in autoclaved controls was minimal (1-2%).

A similar pattern was observed for aerobic ^{14}C-benzene degradation: burrow sediments showed significantly higher $^{14}CO_2$ accumulation than did non-burrow sediment (Fig. 1.1B). Degradation patterns for *M. arenaria* and *C. torquata* sediments were almost identical with about 66% of the added radiolabel recovered as $^{14}CO_2$. Only

Figure 1.1. Mineralization of radiolabelled PAH by burrow sediments. A. Time course of $^{14}CO_2$ production from n-[1-^{14}C]hexadecane by oxic burrow wall sediment slurries of *C. torquata* (▲) or *M. arenaria* (■), oxic non-burrow sediment (●), anoxic non-burrow sediment with nitrate (□) or autoclaved controls (○). B. Time course of $^{14}CO_2$ production from [UL-^{14}C]benzene; symbols as for A. All data are means of triplicate measurements ± 1 S.E. Error bars not shown are smaller than the symbol size.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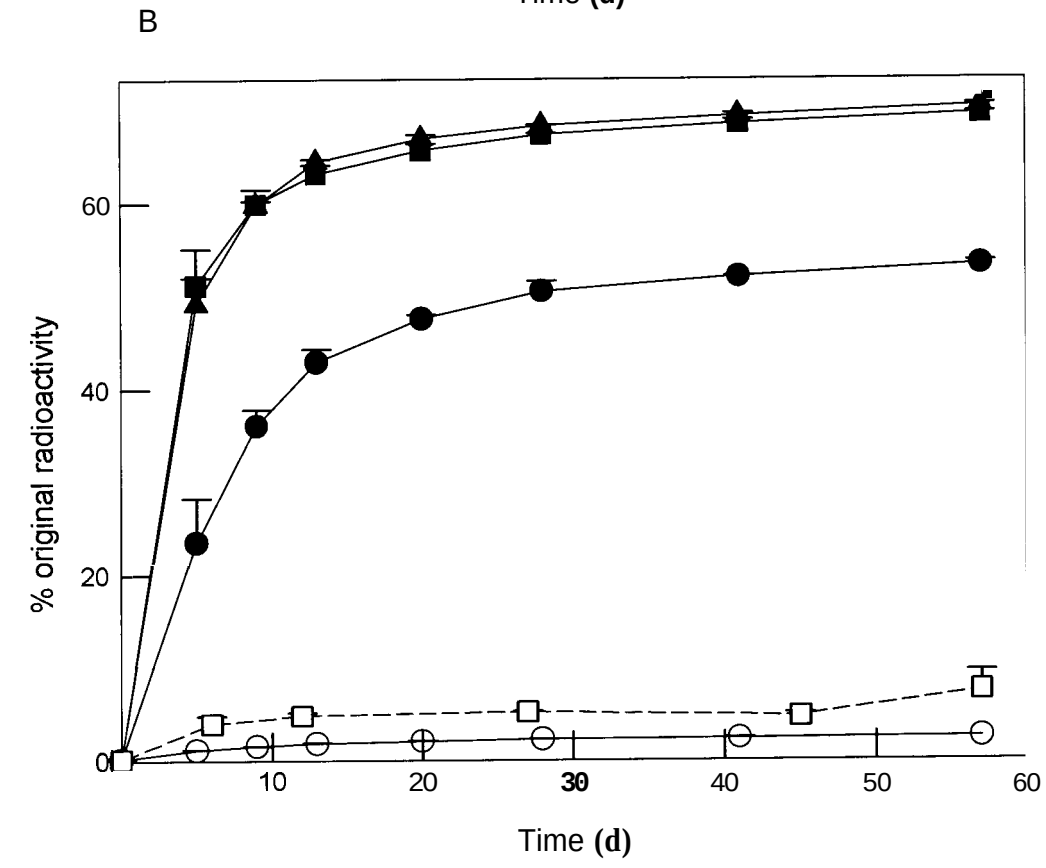
Time (d)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Time (d)

50% of the added benzene was mineralized by non-burrow sediment. In contrast to results for ^{14}C-hexadecane, anaerobic ^{14}C-benzene mineralization was very limited (< 4%) with nitrate as the final electron acceptor. Results for other electron acceptors and non-burrow sediments were similar (not shown).

PAH degradation

Naphthalene degradation proceeded rapidly without a lag in all oxic burrow and non-burrow sediments (Fig. 1.2A). For burrow sediments, complete loss of naphthalene occurred within 5 d with an average loss rate of about 17% d^{-1}. Degradation rates in non-burrow sediments were about the same. Much slower naphthalene losses, about 3% d^{-1}, were observed for autoclaved control sediments, probably as a result of the high volatility of naphthalene.

Degradation of 1,4-dimethylnaphthalene (1,4-DMN) was similar among all sediment types assayed except for the autoclaved control (Fig. 1.2C). **A** gradual but steady decrease in 1,4-DMN concentration was observed. **As** in the naphthalene treatments, degradation occurred without any apparent lag period. The loss rate was about 5% d^{-1} for burrow sediments, and slightly lower **(3%** d^{-1}) for non-burrow sediment. At the end of 19 d, about 90% and 95% of the added 1,4-DMN were removed from C. *torquata* and *M. arenaria* sediments, respectively.

Oxic C. *torquata* burrow sediments and non-burrow surface sediments rapidly degraded dibenzothiophene (DBTP) after initial lag periods of about 12 d and 7 d, respectively (Fig. 1.2D). The average loss rate was about 12% d^{-1} for both sediment types. Little degradation was observed in *M. arenaria* and anoxic sediments compared

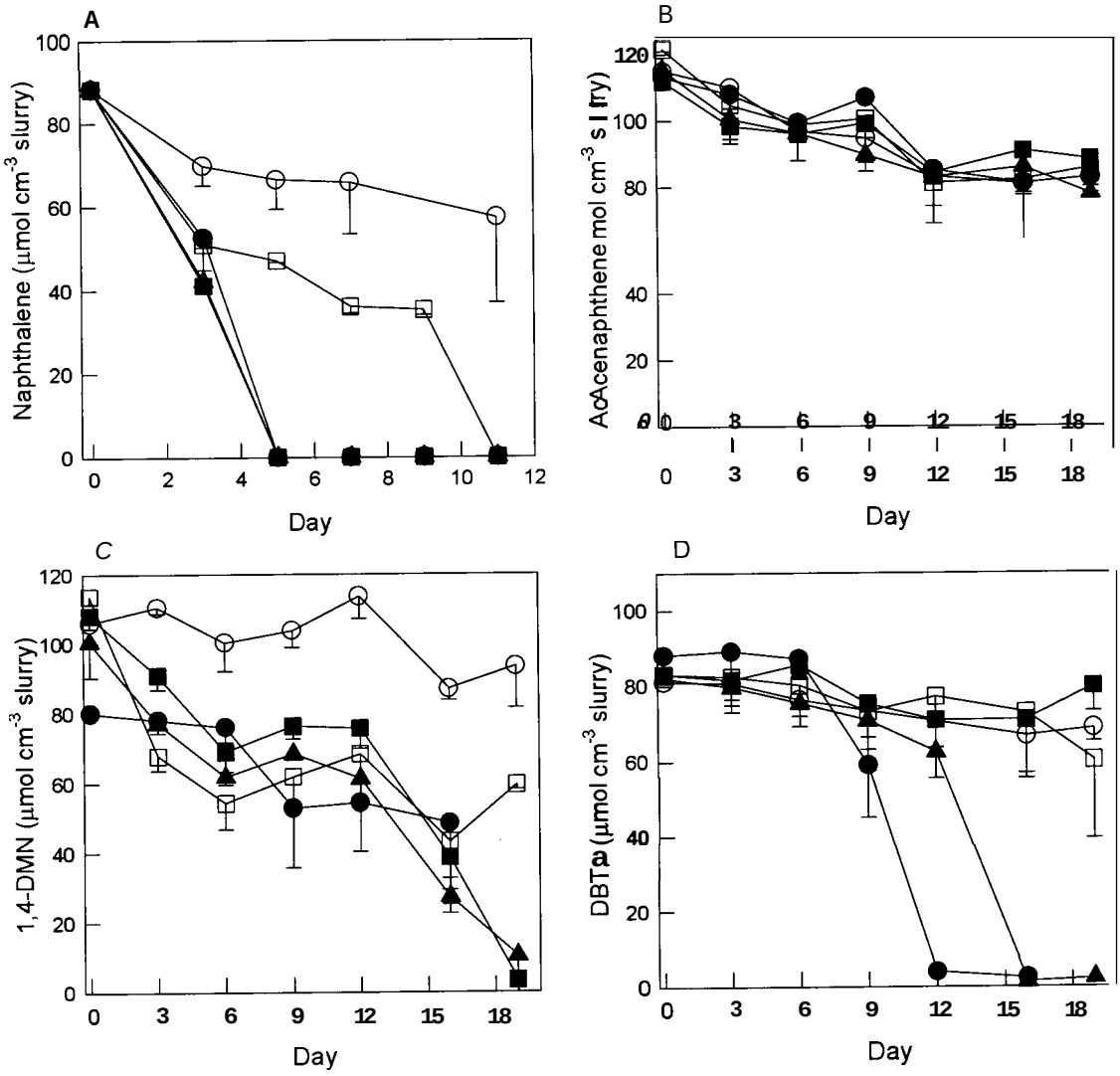


Figure 1.2. Degradation of PAH by burrow sediments. **A.** Time course of naphthalene degradation in slurries of oxic non-burrow surface sediments (●), anoxic surface sediments (□), autoclaved controls (○), or oxic burrow wall sediments from *C. torquata* (▲) or *M. arenaria* (■). B. Time course of acenaphthene degradation; symbols as in **A. C.** Time course of 1,4-dimethylnaphthalene degradation; symbols as in **A.** D. Time course of dibenzothiophene degradation; symbols as in **A.** All data are means of triplicate measurements ± 1 S.E. Error bars not shown are smaller than the symbol size.

to the control. GC-MS analysis revealed a DBTP metabolite, dibenzothiophene sulfoxide, in organic extracts from C. *torquata* and surface sediment samples but not extracts of other sediments (data not shown). In contrast to DBTP and the other PAH, neither acenaphthene (Fig. 1.2B) nor benz[a]anthracene (data not shown) were degraded relative to sterile controls during the time course of the assays (20 d).

Phenanthrene degradation

Phenanthrene degradation occurred rapidly after a lag of typically 3-7 d (Figs. **1.3,** 1.4) in *N.* ***virens*** burrow sediment slurries incubated under oxic conditions. Complete degradation of 5 μg of phenanthrene cm^{-3} of slurry was observed within about 48 h after the initial lag. Phenanthrene degradation did not differ from anoxic controls for slurries initially incubated under oxic conditions and subsequently switched to anoxic conditions (Fig. 1.3). Loss of phenanthrene under anoxic condition with nitrate was similar to autoclaved control. Phenanthrene losses in these controls as in others appeared to arise as a result of changes in adsorption and extraction efficiencies over time.

Phenanthrene was also degraded in slurries incubated under alternating oxic and anoxic conditions **(24** h cycle) after an initial **9** d lag that was equivalent to the lag for slurries incubated under continuously oxic conditions (Fig. 1.4). However, degradation was markedly slower under the alternating regime (substrate level dropped to below detection level after approximately 13 d versus **3** d after initial lag for complete degradation). In contrast, pre-incubation under anoxic conditions for 2 wk did not result in an extended lag period for degradation, or reduce the rates of phenanthrene

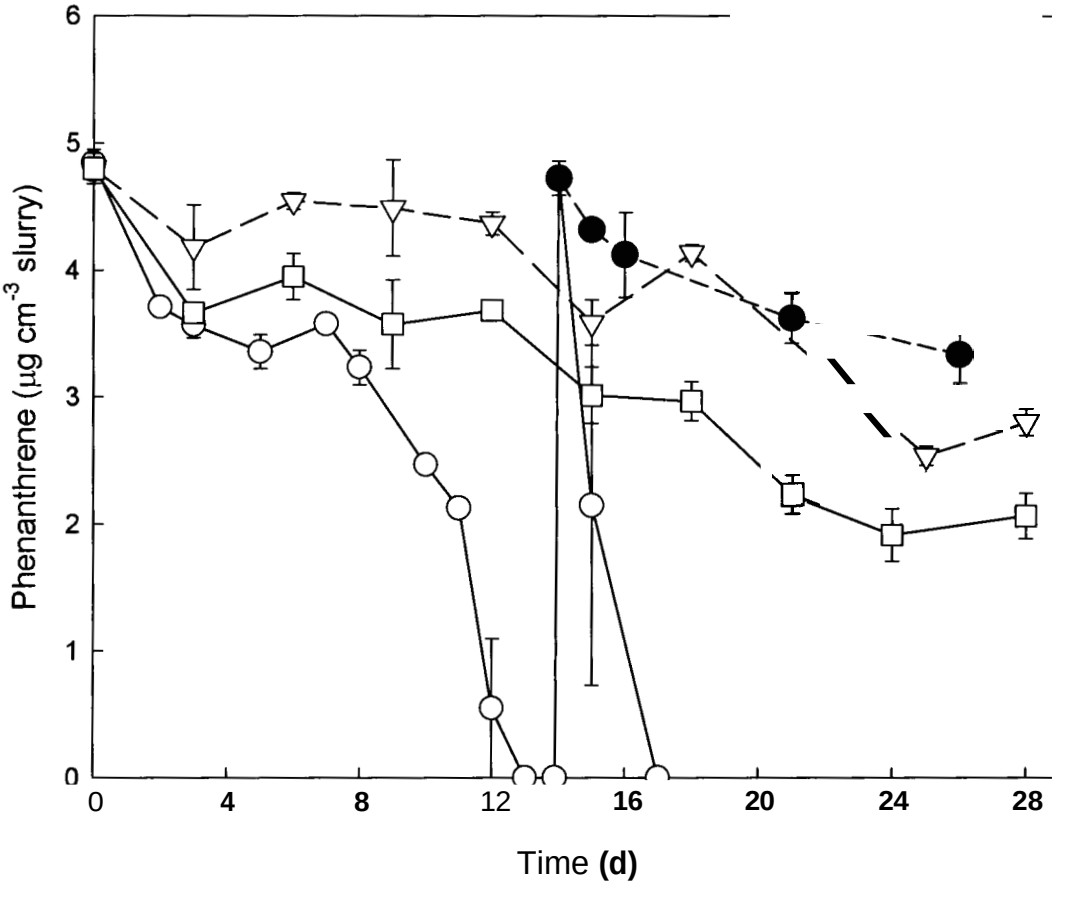


Figure 1.3. Effect of oxygen on phenanthrene degradation. Time course of phenanthrene degradation in slurries of ***N. virens*** burrow wall sediment incubated continuously under oxic (O) or anoxic (O) conditions and after switching from oxic to anoxic conditions (O). Loss of phenanthrene under anoxic conditions is similar to the autoclaved control (V). Arrow indicates addition of phenanthrene (5 µg cm^{-3} slurry). All data are means of triplicate measurements ± 1 **S.E.**

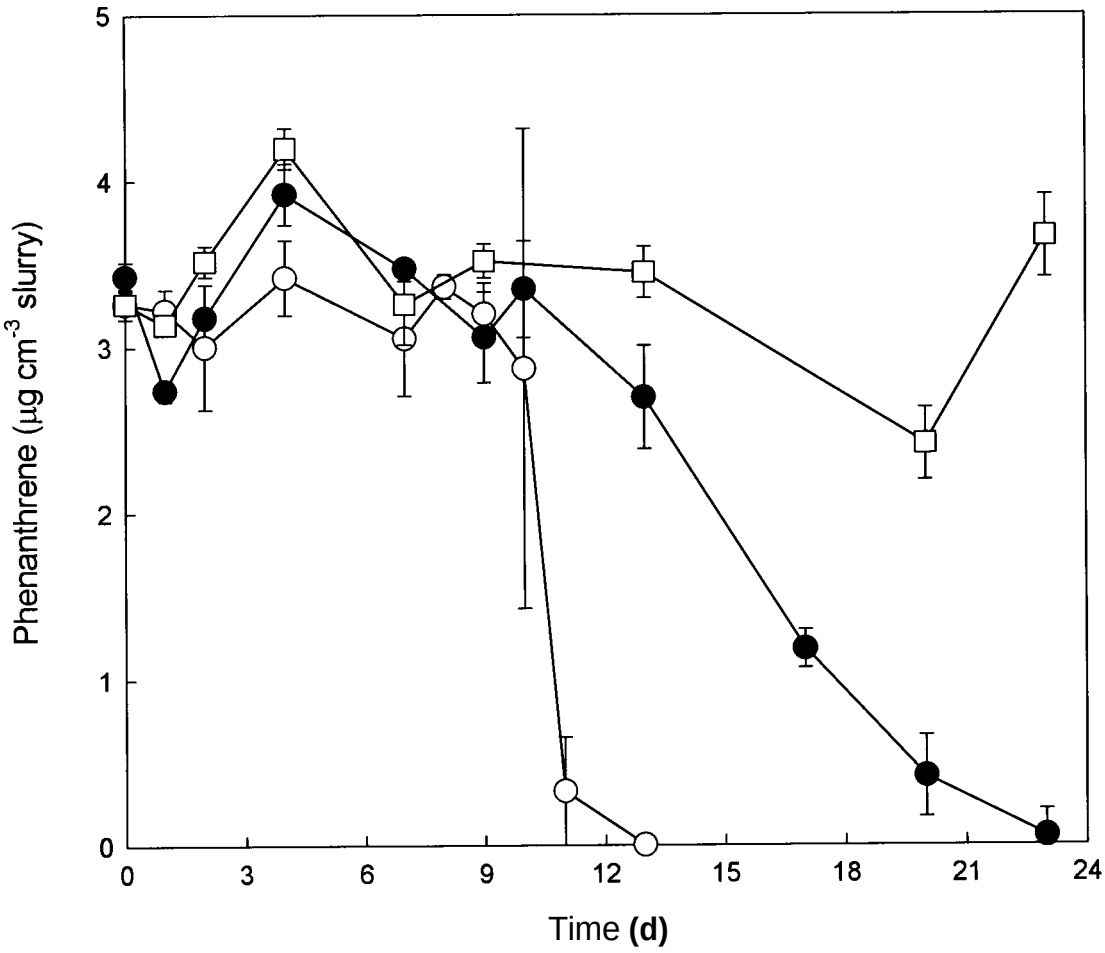


Figure 1.4. Degradation of phenanthrene under alternating oxic-anoxic condition. Time course of phenanthrene degradation in slurries of *N. **vzrens*** burrow wall sediment incubated continuously under oxic (○) or anoxic (□) conditions or with alternating oxic and anoxic conditions on a 24 h cycle (●). All data are means of triplicate measurements ± 1 **S.E.**

degradation subsequent to oxygen introduction (Fig. 1.5). Addition of nitrate or other terminal electron acceptors (manganese, iron) did not alter the outcome of assays with an anoxic pre-incubation (data not shown). Apparent losses in the presence of nitrate were comparable to those of sterile controls in similar assays.

Discussion

Because benthic microbial communities and their processes are governed in large part by local oxygen regimes (32), burrows and burrow ventilation can have a substantial impact on the distribution and activity of various bacterial functional groups. Ammonia oxidizers, which depend strictly on molecular oxygen, are thus active along burrow walls in addition to oxic surface sediments (57). Likewise, sulfidogens are active in intermittently anoxic burrow sediments (77) as well as in anoxic sub-surface sediments and anoxic microzones in oxic sediments. The distribution and rates of these and other processes vary among burrow types, which is not surprising since burrowing and ventilating behavior vary considerably among macrofauna, even for congeneric taxa (56, 58, 59).

Chung and King (22) reported marked variations in inner burrow wall sulfate reduction rates, potential denitrification rates as well as potential ammonia oxidation rates that in some cases do not correlate obviously with burrow ventilation or behavioral characteristics (e.g., motility, feeding mode). The specific mechanisms that account for these biogeochernical patterns are uncertain but it is likely that they affect a wide range of microbial activities, including PAH degradation. While some recent evidence indicates that several aromatics can be degraded anaerobically **(24,** 30, 40, 67), data from

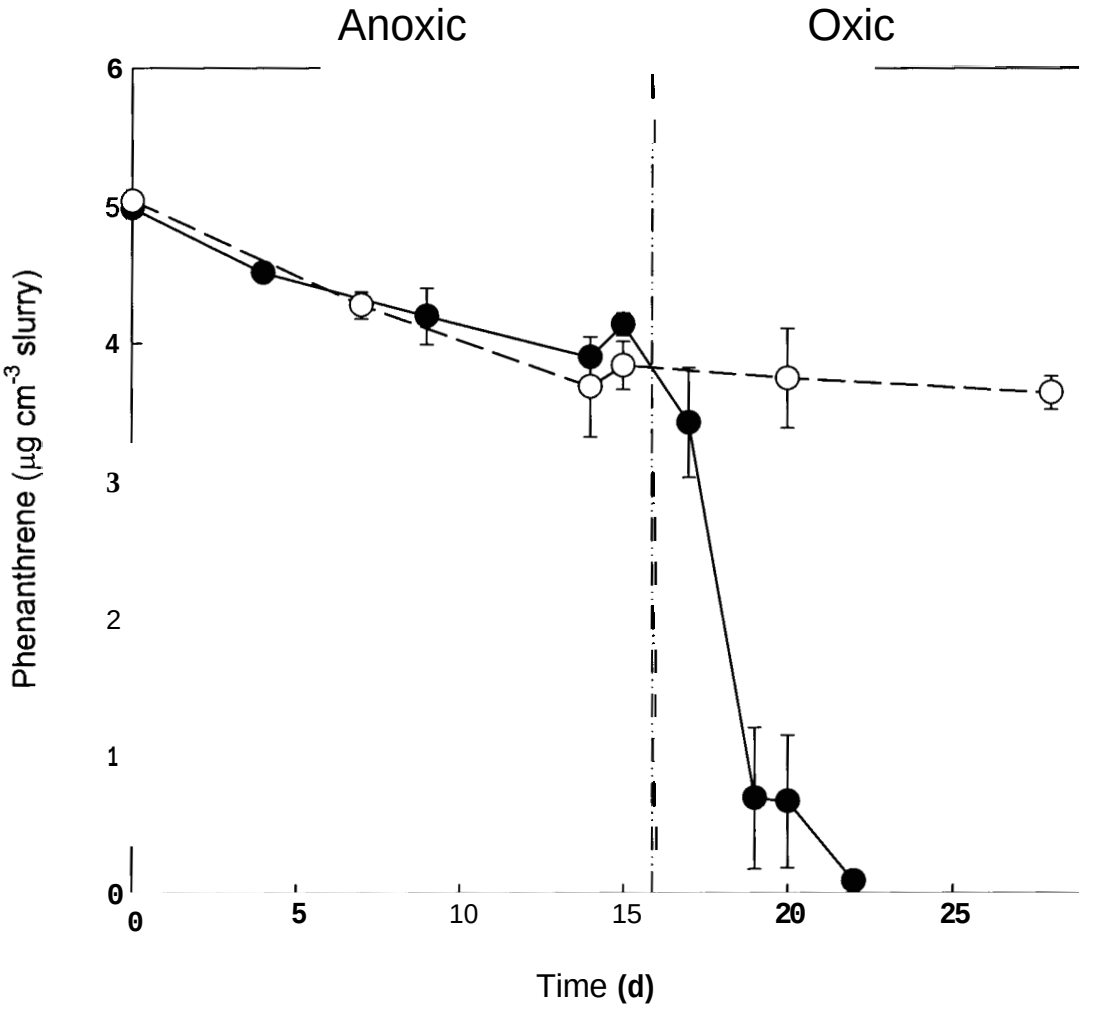


Figure 1.5. Effect of anoxic pre-incubation on phenanthrene degradation. Time course of phenanthrene degradation in slurries of *N. **virens*** burrow wall sediment incubated initially under anoxic conditions with added nitrate (●); dashed-dotted line indicates switch to oxic conditions; (O) represents autoclaved control. All data are means of triplicate measurements ± 1 S.E.

this study suggest that oxic degradation dominates in largely uncontaminated sediments. For example, aerobic benzene degradation occurs rapidly in burrow and non-burrow sediments, but is minimal under anoxic conditions. Likewise, even though hexadecane appears more labile than benzene under anoxic conditions, oxic degradation still dominates total activity (Fig. 1.1). In the case of phenanthrene, minimal degradation (comparable to autoclaved control) occurs under anoxic conditions (Figs. 1.3-1.5), while aerobic degradation is rapid after a short lag phase. The observed lag phase most likely represents the time required for induction and synthesis of dioxygenases suitable for PAH degradation. The concentration of other micronutrients in the sediments may also limit how fast the PAH degraders (as well as other non PAH-degrading indigenous microbes which are competing for the same micronutrients) can proliferate, and thus affect the length of the lag period.

In contrast to oxic surface sediments, oxygen is available in burrow sediments only intermittently, since burrow ventilation is a periodic process (56, 58, 59, ***85).*** The nature of ventilation is not well described, but appears to vary substantially among the macrobenthos. ***Nereis virens*** typically ventilates its burrows for short intervals (about ***5*** min) interspersed among longer resting periods (about 30 min). In addition, much longer periods of quiescence (hours) occur with no ventilation (85). During quiescent periods, rapid oxygen uptake by burrow sediments and animal tissues can lead to cessation of aerobic microbial metabolism, initiation of anaerobic metabolism, excretion of organic acid metabolites by the burrow host and decreases in burrow water pH ***(85).*** Experimental evidence suggests that such periodic oscillations can stimulate bulk organic matter degradation (4).

Data presented here indicate that periodic variations in oxygen levels do not preclude aerobic phenanthrene degradation by *N.* ***virens*** sediment slurries when oxygen is available. After initiation under oxic conditions, phenanthrene degradation ceases upon oxygen removal, but resumes without lag after reintroduction of oxygen (Figs. **1.3,** 1.4). Further, complete loss of exogenous phenanthrene occurs under alternating oxic-anoxic conditions, albeit at a slower rate than under continuously oxic conditions. Repeated oxic-anoxic shifts appear to have little cumulative effect since degradation rates remain approximately constant during oxic phases (Fig. 1.4). Thus, short-term anoxia (24 h) has little deleterious effect on phenanthrene degradation. Likewise, long-term (2 wk) anoxia has little deleterious effect. Lag times for and rates of phenanthrene degradation after introduction of oxygen are comparable for sediments incubated initially for 2 wk under anaerobic conditions or incubated continuously under oxic conditions (Fig. 1.5).

In this context, phenanthrene degraders share traits with other oxygen-dependent functional groups. Methane-oxidizing bacteria also depend on molecular oxygen, but tolerate extended periods of anoxia well (80). Thus, survival under anaerobic conditions may be a relatively wide-spread phenomenon among aerobic sediment bacteria that promotes their persistence and activity in intermittently oxic environments. Although the ability to respire anaerobically (e.g., with nitrate) may account for anoxia tolerance of some bacterial groups, it is not clear to what extent this is important for PAH degraders.

The results in this study indicate that burrow sediments cannot be considered a simple extension of the sediment surface. Others looking at different taxa and processes have reached similar conclusions **(3,** 4, 61). While naphthalene and 1,4-DMN

degradation (Figs. 1.2A, B) occur similarly in each of the various burrow and non-burrow sediments, DBTP degradation is sediment-specific, occurring only for C. *torquata* and surface sediments (Fig. 1.2C). This pattern differs notably from those for sulfate reduction, denitrification and ammonia oxidation, variations in which may be attributed to differences in population sizes of otherwise similar microbial communities. In contrast, variability in PAH degradation suggests that different burrow sediments may harbor phylogenetically and functionally diverse assemblages of PAH degraders. The specific nature of these ensembles might reflect variations in physical regimes resulting from diverse ventilation patterns. Alternatively, different macrofaunal taxa may excrete metabolites that select for or inhibit certain populations with specific metabolic capacities. In support of these notions, **2** morphologically and physiologically distinct isolates have been obtained from burrows of *N. virens* and *M. arenaria* **(23).**

Berardesco et al. (15) have documented very diverse communities of phenanthrene-degrading bacteria isolated from surficial sediments of Boston Harbor. Spatial (but not temporal) variations in the diversity of this community on the centimeter scale appear minimal. While small-scale variations in the diversity of PAH degraders in Lowes Cove surface sediments might also be limited, results reported here indicate that significant sub-surface variation may occur as a consequence of macrofaunal diversity. The extent of such variation may ultimately contribute to differential fates for the various components of PAH found *in situ.*

Benthic macrofauna can also affect the fate of PAH through other mechanisms. Sediment reworking by deposit-feeding and burrowing re-distributes PAH to the water column and oxic sediment surfaces, thereby enhancing the potential for aerobic

degradation (53, 68). In addition, macrofauna can assimilate and metabolize some PAH directly (33, 49, 68). Although the relative significance of these processes and burrow wall "mediated" degradation remain uncertain, it seems evident that manipulation of benthic macrofaunal populations and their associated microbiota represents a viable approach for *in situ* bioremediation of contaminated marine sediments.

Chapter 2

ISOLATION AND CHARACTERIZATION OF NOVEL POLYCYCLIC AROMATIC HYDROCARBON DEGRADERS FROM MACROFAUNAL BURROW SEDIMENTS

Abstract

Two marine bacteria were isolated from the burrow sediments of benthic macrofauna by enrichment culture. Strain LC8 (from a polychaete) and M4-6 (from a mollusc), are aerobic, Gram-negative and require sodium chloride (>1%) for growth. They can use 2- and 3-ring PAH as sole carbon and energy sources. Strain LC8 can also co-metabolize pyrene to phenanthrene dicarboxylic acids and pyrene-diol in the presence of phenanthrene. Both strains utilize various sugars, organic acids and amino acids as energy and carbon sources. Based on the PAH metabolites they form, both strains possess an enzyme system similar to the well-characterized naphthalene dioxygenase. Physiological and phylogenetic analyses based on 16S rRNA gene sequences suggest that M4-6 is a new spirillum belonging to the genus *Cycloclasticus* (an established marine PAH degrader) while LC8 appears to belong to a new genus most closely related to *Eythrobacter*.

Introduction

Polycyclic aromatic hydrocarbons are ubiquitous pollutants. They are primarily released to the environment through incomplete organic matter combustion (e.g. fossil fuel use, biomass burning). Many high-molecular-weight PAH elicit toxic and mutagenic responses from a wide variety of organisms (66). PAH are very hydrophobic, resulting in strong adsorption to organic phases in soils and sediments. This decreases availability for remediation but does not eliminate toxicity for benthic organisms. Nonetheless, many microorganisms degrade PAH in culture, and PAH can be degraded in the complex matrices of soil and sediments.

Most known bacterial PAH degraders have been isolated from heavily contaminated terrestrial environments **(8,** 25, 91). These include various strains of *Pseudomonas, Comamonas, Acinetobacter* and *Sphingomonas.* However, PAH pollution is not constrained to sites impacted by fuel spills or locally high levels of fossil fuel use. PAH pollution occurs ubiquitously, even in apparently pristine environments with relatively little fossil fuel use. Although there has been little emphasis on PAH degraders in such environments, the ability of microbes to degrade PAH in systems exposed to low chronic levels may determine the extent of long-term PAH accumulation.

As is the case for terrestrial systems, marine environments include both heavily impacted sites and far more numerous sites exposed to low levels of PAH input. Several novel marine PAH degraders, e.g., *Cycloclasticus* sp., have been isolated from contaminated sediments (29). Some of these isolates also occur in largely pristine systems (37). The potentially ubiquitous distribution of marine PAH degraders may indicate that the capacity for PAH degradation in polluted systems depends on the

diversity and characteristics of naturally-occurring populations, rather than the introduction of new taxa or selective modification of existing ones.

Although naphthalene and phenanthrene degradation have been reported under anoxic conditions for heavily contaminated sites, in general, efficient and rapid PAH degradation depends on molecular oxygen availability. Oxygen is often limited to the top several millimeters of surface sediment (76), severely constraining the potential for PAH degradation. The extent of oxygen limitation may be offset to some degree by the activities of benthic macrofauna, which physically mix sediments and introduce oxygen into sub-surface sediments by burrow ventilation **(3).**

In addition to serving as conduits for oxygenated water, macrofaunal burrows are sites of intense microbial activity. Many biogeochemically important processes such as ammonification and sulfate reduction are elevated in burrow sediments compared to bulk sediment (43, *55,* 61). The unique environment of macrofaunal burrows may also provide enhanced PAH degradation. This proposal is supported by observations based on slurries prepared from burrow sediments of different macrofauna. These slurries exhibited enhanced PAH degradation potentials relative to slurries of non-burrow sediment (22).

We report here results of efforts to enrich, isolate and characterize PAH degraders from macrofaunal burrow sediments. We describe the isolation of two aerobic, obligately marine PAH-degrading bacteria from burrows of a polychaete (*Nereis virens*) and a mollusc (*Mya* arenaria) in the intertidal mudflat of Lowes Cove, Maine. Both strains use naphthalene and phenanthrene aerobically as sole carbon sources, probably through a *nah*-like dioxygenase system (naphthalene dioxygenase). 16S rDNA

phylogenetic analysis and phenotypic characterization suggest that strain LC8 belongs to a new genus and that M4-6 is a new *Cycloclasticus* species.

Materials and Methods

Sampling and slurry preparation

Animal burrow sediments were collected from the intertidal zone of Lowes Cove, Maine, during summer 1998 by using a sterile spatula during low tide. This site and burrow sediment collections have been described in detail previously (6, 22, 43, *52)* and the collection site is not known to be contaminated with PAH. Sediment samples were transferred to the laboratory within 1 h and processed immediately. Surface sediment (top **1-3** mm) and bulk sediment (10-15 cm) were also collected and processed similarly. *M. arenaria* burrow sediments were collected after exposing them with a clam fork. Only the oxidized light-brown layer lining the burrow (1-2 mm thick) was removed with a spatula. Burrow sediments of *N.* ***virens*** were collected similarly. In all cases, burrow identification was based on the presence of animals and distinctive burrow morphologies. Burrow sediment slurries were prepared with artificial seawater **(ASW),** (92). Unless stated otherwise, 10% (w/v) slurries were used in the following experiments. For abiological controls, bulk sediments were autoclaved at 121 °C, then cooled; this cycle was repeated three times over a period of 72 h.

Enrichment and isolation of phenanthrene-degrading bacteria

Two hundred fifty milliliters of a 10% sediment slurry was prepared in a 1-L flask. **A** 2% phenanthrene stock solution was prepared in acetone, and added to the slurry at an initial concentration of 10 ppm. Additional phenanthrene was added when

less than 0.5 ppm of phenanthrene remained in the slurry. The phenanthrene concentration in the slurry was slowly increased to about 100 ppm over a period of 2 **wk,** and maintained at this level for the duration of the enrichment. After 4 wk of enrichment, subsamples from the flasks were serially diluted with a mineral salts medium ONR7a (29). Spread plates were prepared with ONR7a solidified with 1.5% agar and then sprayed with a 2% phenanthrene solution in acetone. The plates were incubated at room temperature (about 23 "C) for up to **3** wk. Colonies showing a clearing zone on the crystalline phenanthrene layer were picked and streaked onto new minimal agar plates with phenanthrene. The process was repeated until pure cultures were obtained. Two isolates with distinctive colony and cell morphologies and relatively high growth rates were chosen for further studies.

Phenotypic characterization

Cell morphologies were examined with phase contrast microscopy. Cell motility was tested with motility agar. Routine microbiological tests including Gram reaction, nitrate reduction, oxidase, catalase, gelatinase, lipase, phosphatase, and glucose fermentation were performed according to standard methods (90). Sodium ion requirements were tested by substituting sodium salts in the medium for the corresponding potassium salts. Salinities were adjusted by varying the concentrations of inorganic salts in ONR7a to final values of 3.5-, 10-, 35-, and 70 ppt. Cultures were incubated at 24 "C with shaking (120 rpm). Growth of isolates was tested at the following temperatures: 4-, 10-, 15-, 20-, 25-, 30-, 37-, and 42 "C. Poly-β-hydroxybutyrate (PHB) inclusions were visualized with Sudan Black.

For bacteriochlorophyll detection, a 50-ml culture of strain LC8 was grown to late log phase on pyruvate. The culture was centrifbged and washed with ONR7a twice and collected by centrifugation at about 6500 x ***g***. The cell pellet was extracted by vortexing with 5 ml of an acetone:dimethyl sulfoxide mixture (9:1) for 2 min. Absorbance of the organic layer was scanned from 300 nm to 800 nm using a Beckman model DU-600 spectrophotometer.

The following compounds were tested as sole carbon sources for growth of the isolates using ONR7a (for M4-6) or ONR7y (ONR7a + 0.05% yeast extract, required for growth of LC8): arabinose, fructose, galactose, glucose, lactose, mannose, ribose, sucrose, alanine, aspartate, glycine, phenylalanine, proline, serine, valine, sodium citrate, sodium fbmurate, gluconic acid, glucuronic acid, sodium glycolate, malic, sodium malonate, sodium acetate, sodium propionate, sodium pyruvate, sodium succinate, sodium tartarate, betaine, ethanol, glycerol, mannitol and hexadecane. The above compounds were added to minimal ONR7a medium at 15-20 mM. The following aromatics (about 1 g in 100 ml of medium) were also tested as sole carbon sources for growth: phthalate, salicylate, biphenyl, naphthalene, 1,4-dimethyl naphthalene, 1,8-dimethyl naphthalene, acenaphthene, fluorene, anthracene, phenanthrene, dibenzothiophene, benz[a]anthracene, pyrene, chrysene and fluoranthene. Isolate growth was scored positive or negative by comparing the turbidity of the medium after 72 h of incubation at room temperature with shaking (120 rpm) to that of a control receiving no carbon source. Whole-cell fatty analyses of LC8 and M4-6 were performed by Microbial ID, Inc (Newark, DE) by acid hydrolysis and gas liquid chromatography.

PAH and potential metabolites analysis

Loss of PAH from culture media and slurries was measured by gas chromatographic-mass selective detection after organic solvent extraction. Briefly, slurries or cultures were acidified to pH < 2.0 with 5N HCl and mixed with one-half volume of n-hexane in a Teflon-lined screw cap tube. Dibenzothiophene was added as internal standard. The tubes were shaken for 2 min, and aqueous and organic phases were separated by centrifugation at about 2400 x *g*. The organic layer was concentrated under a stream of nitrogen, and PAH were reconstituted in n-hexane. The samples were then injected into an HP 6890 gas chromatograph with a 5972A mass selective detector equipped with an HP-5MS capillary column (30 m x 0.25 mm x 0.25 μm film thickness) using helium as a carrier gas. Peaks were identified based on retention times and mass spectra. PAH recoveries were in the range of 85-90 % for slurries extraction and about 95% for culture extraction. For PAH metabolites, the reconstituted extracts were methylated with Methyl-**8** (N,N-Dimethylformamide dimethylacetal) or silylated with MSTFA (*N*-Methyl-*N*-(trimethylsilyl)trifluoro-acetamide) according to manufacturer directions before assay. Gas chromatograph operating conditions were as follows: inlet temperature, 250 "C; auxiliary (interface), 280 "C; helium flow, constant at 1 ml min^{-1}; GC temperature program, 70 "C hold 2 min, ramp to 250 "C at 10 "C min^{-1}, hold 5 min at 250 °C. The detection limit of the method is 200 ng (g sediment dry weight)$^{-1}$.

Aqueous hydroxynaphthoic acid (HNA) production in culture media was measured with a Gilson HPLC system equipped with a reverse phase **C18** column (Alltech Prosphere 300 PAH 5u, 250 mm x 4.6 mm). The mobile phase was 75% methanol (acidified to pH 2.75 with phosphoric acid) with a flow rate of 1 ml min^{-1}.

HNA was detected by a Kratos UV absorbance detector at 254 nm with a detection limit of 0.5 μM.

Microscopy

Early log-phase cells grown on ONR7y (for LC8) or ONR7a (for M4-6) with pyruvate were centrifuged and washed with appropriate growth media. For scanning electron microscopy, cells were transferred to poly-L-lysine-coated cover slips. The cells were first fixed with vapor phase glutaraldehyde for 10 min and then pre-fixed with a 2.5% glutaraldehyde solution for 10 min followed by post-fixation with 1% osmium tetroxide solution for **30** min. The cells were then dehydrated through an alcohol wash series (50%, 75%, 85%, 95% and 100% [twice], 5 min each). The cells were then dried with a critical point drier and sputter-coated with gold (250 Å). Cells were viewed with an Amray model 1000 scanning electron microscope. For visualization of flagella, similarly grown cells were concentrated by centrifugation and washed gently in ONR7a. **A** drop of cell suspension was transferred to a Formvar/carbon coated copper grid and allowed to settle for 2 min. Excess liquid was blotted dry and the preparation was stained with 1% uranyl acetate solution (95). Cells were viewed with a Philips model EM201 transmission electron microscope.

16S rDNA sequencing and phylogenetic analysis

Total genomic DNA was extracted from *3* **ml** of late-log-phase cells growing on ONR7a with phenanthrene as sole carbon source using a standard sodium dodecylsulfate-proteinase K lysis procedure (**81**). Polysaccharides were removed by incubating the lysate with 50 μl of 10% hexadecyltrimethylammonium bromide (CTAB)

at 65 "C for 10 min. Proteins and cell debris were removed by phenol-chloroform-isoamyl alcohol (25:24:1) extraction and the DNA was precipitated with isopropyl alcohol.

PCR amplification of the 16S rDNA gene was accomplished using the following universal bacterial primers: 27f (5'-AGA GTT TGA TCA TGG CTC AG-3') and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3') (63). PCR reactions were performed in a 100 µl volume containing: approximately 100 ng of DNA, dNTPs (200 µM of each), 200 nM of each primer and 10 µl of PCR assay buffer. The final concentration of magnesium ion was 1.5 mM. The following temperature program was used: 5 min at 95 "C, followed by 30 cycles consisting of 1 min at 94 "C, 1 min at 55 "C and 2 min at 72 "C. A final 10 min extension at 72 "C was included at the end of the program. PCR products were separated on a 0.8% agarose gel. A 1.5 kb DNA band from the gel was excised and purified using a Qiagen QIAquick gel extraction kit. Both strands of the purified DNA were then sequenced by the DNA Sequencing facility of University of Maine using an automatic DNA sequencer (ABI) with the PCR primers and 2 sets of internal primers, 926E 5′-AAA CTY AAA KGA ATT GRC GG-3'; 907r: 5'-CCG TCA ATT CMT TTR AGT TT-3'; 357f: 5′-CTA CGG GRS **GCA** GCA G-3' and 519r: 5'-**GWA** TTA CCG CGG CKG CTG-3') (63).

The unambiguous consensus sequences were submitted to GenBank for a BLAST search and results were used as a guide for phylogenetic tree construction. Additional related 16S rDNA sequences identified from BLAST were retrieved from GenBank. Sequences were aligned using the Sequence Alignment and Modeling

Software System (University of California, Santa Cruz) and Clustalw (European Bioinformatics Institute). The sequences were further manually edited to remove gaps and then analyzed using computer-based phylogenetic packages. All phylogenetic inferences (including distance matrix calculation, maximum likelihood and maximum parsimony analysis, neighbor-joining analysis and bootstrap data set generation) were performed using PHYLIP95 including DNADIST.exe; DNAML.exe; DNAPARS.exe; NEIGHBOR.exe; SEQBOOT.exe using the default parameters. For DNADIST.exe, Kimura 2-parameter algorithm was used; the transition/transversion ratio was set at 2.00 with one substitution rate. For DNAML.exe, the transition/transversion ratio was set at 2.00 with one substitution rate; empirical base frequencies were used; and the analyses were done without global rearrangement nor input order randomization. DNAPARS.exe was performed without input order randomization using ordinary parsimony. NEIGHBOR.exe was performed without input order randomization nor subreplicates. Tree construction was performed with TREEVIEW (Win32) version 1.5.2(73).

Nucleotide seauence accession number

The 16S rDNA sequence of strains LC8 and M4-6 have been deposited in the GenBank database under the accession numbers AY026916 and AY026915, respectively.

Results

Enrichment culture and strain isolation

Slurries of macrofaunal burrow sediments showed significant phenanthrene degradation after a 2-wk incubation (Fig. 2.1). The phenanthrene degradation rate for

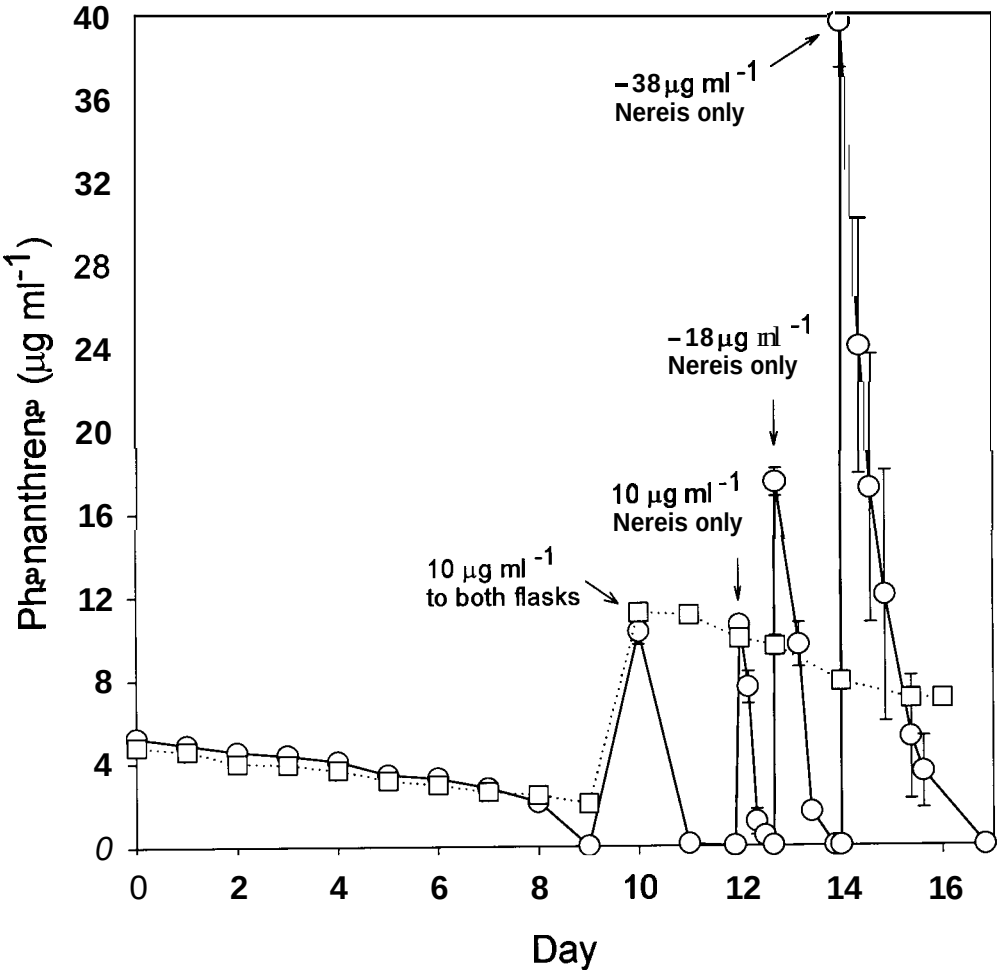


Figure 2.1. Enrichment for phenanthrene degraders. Enrichment for phenanthrene-degradation in ***Nereis*** burrow sediment slurries. Arrows indicate addition of additional phenanthrene. (O) ***Nereis*** sediment, (□) autoclaved sediment. Data are means of triplicate ± 1 S.E.

the *Nereis* enrichment flask was about 15 μg ml^{-1} d^{-1} after 2 wk. Degradation was detected after a lag period of about **8** d. Results from *Mya* burrow sediment were similar with a lag period of about 10 d. From **4** isolation plates **(2** from *Nereis* burrow and *2* from ***Mya*** burrow), **4** distinctive phenanthrene degrading colonies were identified. Pure cultures of the PAH degraders were obtained after **4** to 5 transfers to new isolation plates. Isolate LC8 from *Nereis* sediments, and isolate **M4-6** from ***Mya*** sediment were chosen for further studies based on their unique cell and colony morphology, and large zone of clearing on phenanthrene-sprayed agar plates. Both LC8 and **M4-6** grew very slowly on minimal agar plates with phenanthrene as a sole carbon source. Zone of clearing were visible after approximately 1 wk.

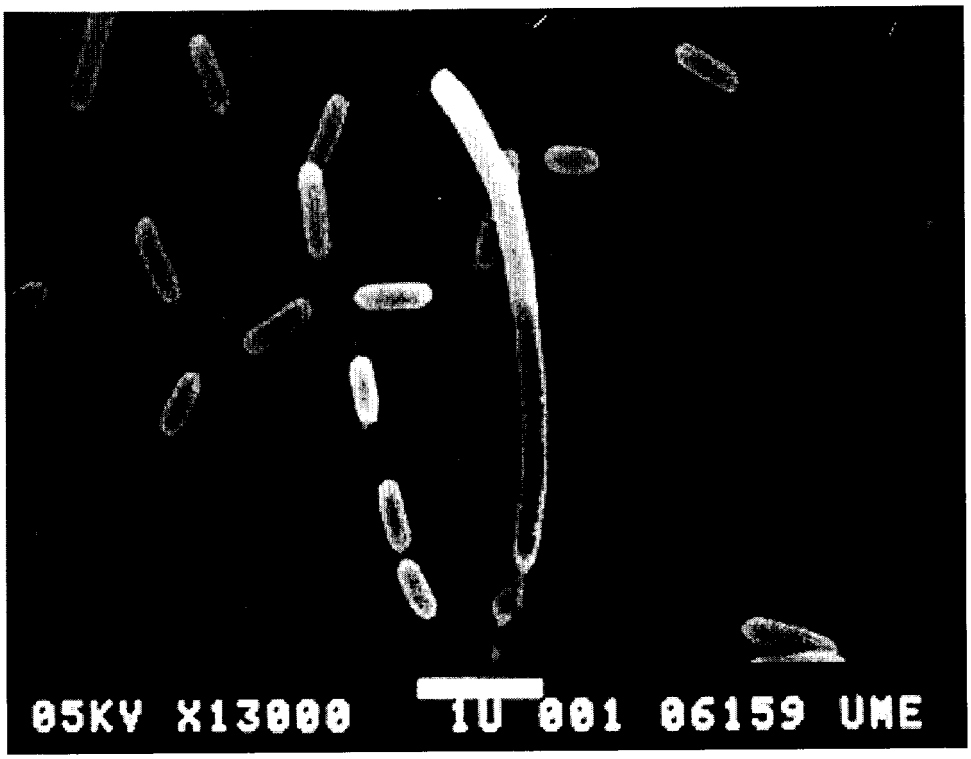
Phenotypic characteristics

Both LC8 and **M4-6** are aerobic obligate marine bacteria requiring sodium chloride for growth. Potassium salts cannot be substituted for sodium salt. Both isolates tolerate salinities up to 70 ppt.

LC8 is a Gram-negative, catalase-positive, oxidase-positive, non-motile rod about 0.5 by 1.5 to 2.0 μm (Fig. 2.2 **A).** LC8 forms pale yellow colonies on minimal agar plates with phenanthrene or other non hydrocarbon as carbon source. The following compounds are utilized by LC8 as sole carbon sources in minimal ONR7y media: arabinose, fructose, galactose, glucose, mannose, ribose, sucrose, citrate, fumurate, glucuronic acid, gluconic acid, acetate, pyruvate, tartarate, glycerol, mannitol and hexadecane. LC8 also utilizes naphthalene and phenanthrene as sole carbon and energy sources, transforms dibenzothiophene to dibenzothiophene sulfoxide (Tables 2.1 and

Figure 2.2. SEM and TEM of LC8. Scanning electron micrograph at 13000X (A), and transmission electron micrograph (B) of negatively stained LC8 at 15500X, showing cell morphology, variable cell length and presence of a single polar flagellum.

A



B

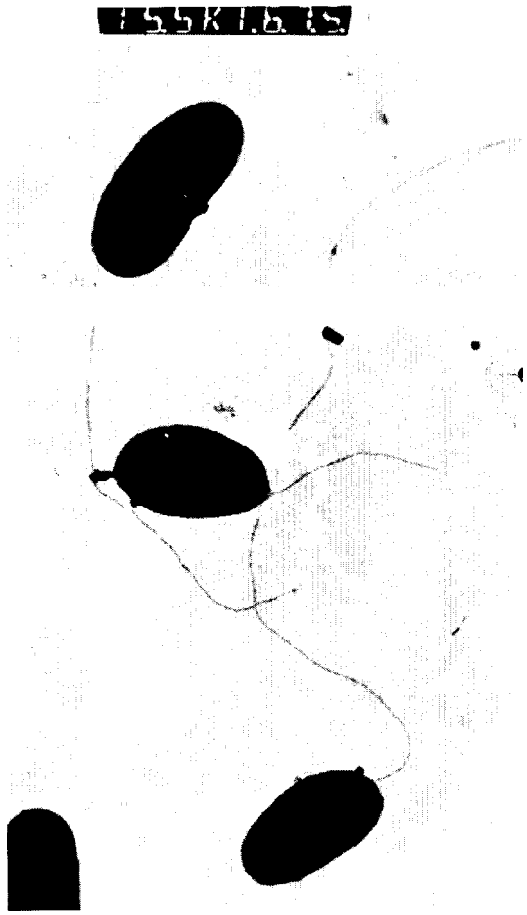


Table 2.1. Phenotypic comparison among LC8, **M4-6** and ***Cycloclasticuspugetii.***

	LC8[a]	M4-6"	*Cycloclasticus pugetii*[b]
Source	*Nereis* burrow	*Mya* burrow	Puget Sound
Enriched with	Phenanthrene	Phenanthrene	biphenyl
Cell shape	short rod	spirilla (polymorphic)	short rod
Motility	–	+	+
Flagella arrangement	Single polar	Single polar	single polar
Cell diameter (um)	1.5-2.0~0.3-0.5	1.5-2.0x0.5	0.5x1.O-2.0
Gram reaction	Gram negative	Gram negative	Gram negative
Colony morphology	yellowish, round, opaque	white, round, translucent	small, round, no pigment
PHB accumulation	–	+	–
Na^+ requirement	+	+	+
NO_3 reducted to NO_2	+	–	+
Oxidase	+	+	+
Catalase	+	+	+
Amylase	nd	nd	–
Gelatinase	–	–	–
Lipase	–	–	+
Phosphatase	+	+	
Acid from carbohydrates	nd	nd	nd
Ferment carbohydrates	–	+	nd
Lysis in SDS (2%)	–	–	nd
Salinity tolerance (ppt)	10-70	10-70	10-70
Growth temp/optimum	15-30/20	15-37/30	4-2%

nd: not determined

a: this study
b: Dyksterhouse et al, 1995

2.2) and co-metabolizes pyrene in the presence of phenanthrene (Fig. 2.3). LC8 also reduces nitrate and exhibits phosphatase activity, but does not hydrolyze gelatin, produce extracellular lipase, ferment glucose, or accumulate poly-β-hydroxybutyrate (PHB). LC8 grows at temperatures ranging from 15 to 30 °C. Though non-motile under routine growth conditions, negatively stained cells possess a single polar flagellum as revealed by transmission electron microscopy (Fig. 2.2 B). Although LC8 has a light yellowish pigment, an acetone:DMSO (9:1) extract of cells revealed no absorption maxima between 400 and 800 nm, characteristic of bacteriochlorophylls. Whole-cell fatty acid analysis (Table 2.3) from cells grown on rich marine media revealed that the major fatty acid is C18:1 ω7c (56.7%). Other significant constituents are C16:1 ω7c (17.2%), C16:0 (6.4%) and C17:1 ω7c (3.0%).

M4-6 is a Gram-negative, catalase-positive, oxidase-positive, phosphatase-positive, motile spirillum about 0.5 μm by 1.5 to 2.0 μm (Fig. 2.4A). M4-6 does not exhibit gelatinase and lipase activities, but does ferment glucose and accumulate PHB. M4-6 grows at temperatures ranging from 15 to 37 °C. Transmission electron microscopy revealed a single polar flagellum (Fig. 2.4B). The following compounds were utilized by M4-6 as sole carbon sources in minimal ONR7a media: arabinose, fructose, galactose, glucose, mannose, ribose, citrate, fumurate, glucuronic acid, gluconic acid, malonate, acetate, propionate, pyruvate and mannitol (Table 2.2). M4-6 also utilized naphthalene and phenanthrene as carbon and energy sources, but did not transform dibenzothiophene or co-metabolize pyrene (Table 2.1). Whole-cell fatty acid

Table 2.2. Summary of carbon utilization by LC8 and **M4-6.**

Carbon Source	LC8	M4-6
arabinose	+	+
fructose	+	+
galactose	+	+
glucose	+	+
lactose	–	–
mannose	+	+
ribose	+	+
sucrose	+	–
citrate	+	+
fumurate	+	+
gluconic acid	+	+
glucuronic acid	+	+
glycolate	–	–
malic	–	–
malonate	–	+
NaOAc	+	+
propionate	–	+
pyruvate	+	+
succinate	–	–
tartarate	+	–
alanine	–	+
aspartate	–	+
glycine	–	–
phenylalanine	+	–
proline	+	+
serine	–	–
valine	–	–
betaine	–	–
ethanol	–	–
glycerol	+	–
mannitol	+	+
Hexadecane	+	–
phthalate	–	–
salicylate	+	+
biphenyl	–	–
naphthalene	+	+
1,4-DMN	–	–
1,8-DMN	–	–
acenaphthene	–	–
fluorene	–	–
anthracene	–	–
phenanthrene	+	+
dibenzothiophene	–,t	–
benz[a]anthracene	–	–
pyrene	–,c	–
chrysene	–	–
fluoranthene	–	–
SDS	–	+
Triton X100	–	+,d
Tween80	+ i	+ i

t: transform
t: transform
c: co-metabolism
i: growth increase
d: growth decrease

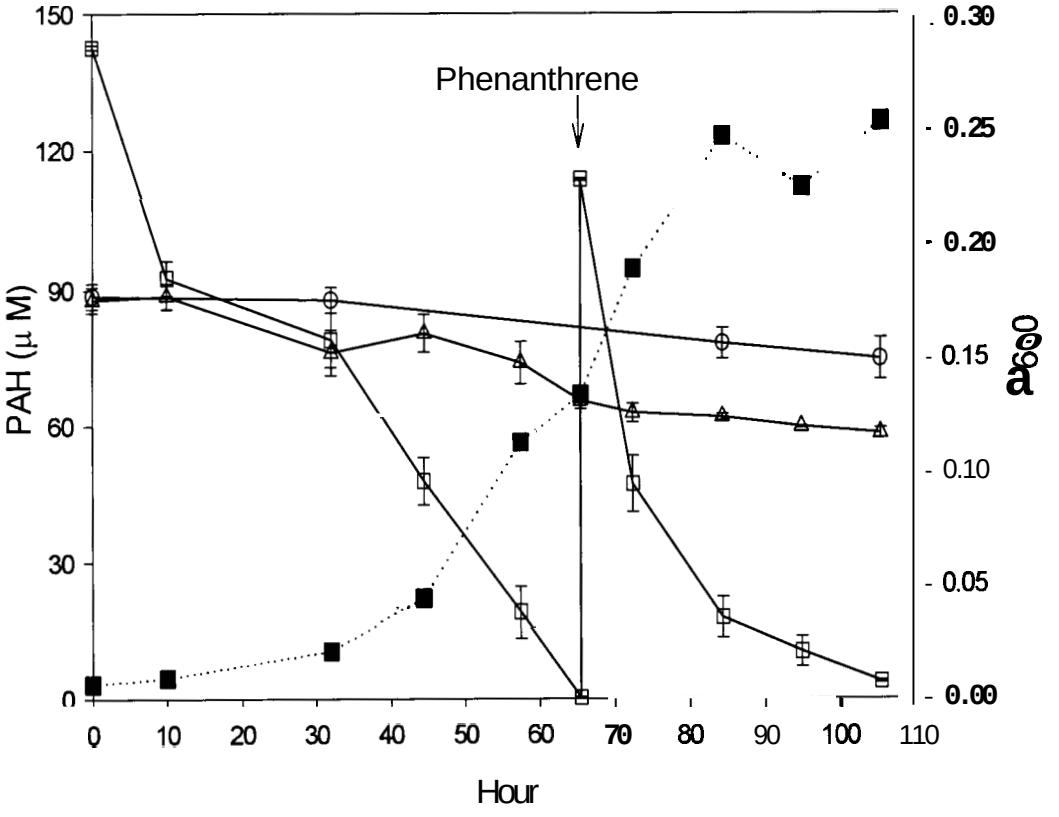


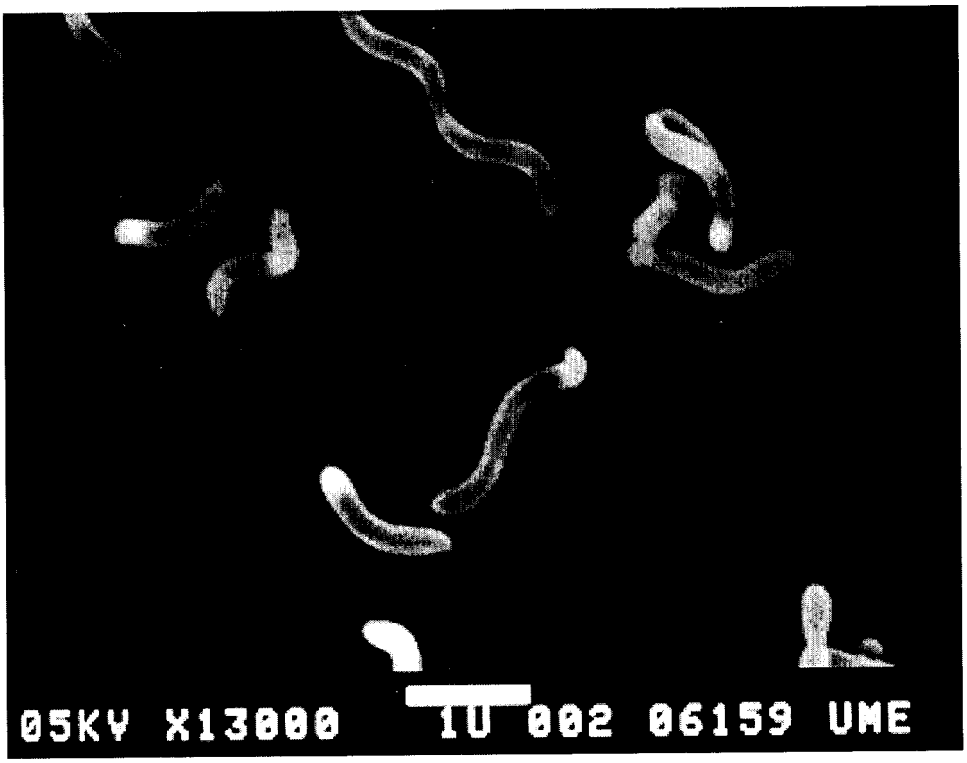
Figure 2.3. Pyrene co-metabolism by LC8. Phenanthrene degradation and pyrene co-metabolism by LC8. Pyrene loss was only apparent when the culture was supplied with phenanthrene (A). LC8 growth (■) was measured as A_{600}. Pyrene loss in the medium without phenanthrene (O) was minimal. Phenanthrene (O) was replenished at day 67. Data are means of triplicate ± 1 standard error.

Table 2.3. Fatty acid composition of whole-cell extract of the isolates grown on rich marine media.

Fatty acid	% of total fatty acids in: LC8	M4-6
11:0 ISO		3.08
11:0 ISO 30H		1.15
12:0 30H		2.57
14:0 ISO		0.79
14:0	1.78	3.68
14:0 20H	2.21	
13:0 ISO 30H		2.60
16:1 ω7c +	17.20	12.23
15:0 ISO 20H		
15:0 ISO		33.50
15:0 ANTEISO		12.37
16:0 ISO		1.14
16:1 ω9c		3.48
16:1 ω5c	1.62	
16:0	6.44	7.19
ISO 17:1 ω9c		4.66
17:0 ISO		2.90
17:1 ω7c	3.02	
17:1 ω6c	2.09	
16:0 20H	2.69	
18:1 ω9c	0.72	1.52
18:1 ω7c	56.73	0.80
18:1 ω5c	1.69	
18:0		0.15
11 methyl 18:1 ω7c	2.35	
18:1 20H	1.48	

Figure 2.4. SEM and TEM of M4-6. Scanning electron micrograph at 13000X (A), and transmission electron micrograph (B) of negatively stained M4-6 at 15500X, showing spirillum morphology and presence of a single polar flagellum.

A



B

analysis of cells grown on rich marine media (Table 2.3) revealed *3* major fatty acids in M4-6: iso C15:0 (33.5%), anteiso C15:0 (12.4%) and C16:1 ω7c (12.2%).

PAH degradation

LC8 and M4-6 used phenanthrene similarly in liquid culture (Figs. 2.5A and B). In both strains, hydroxynaphthoic acid (HNA) was produced as phenanthrene was utilized. Growth occurred linearly while phenanthrene was present and all HNA was eventually metabolized by both strains. Also, as stated above, LC8 co-metabolized pyrene in the presence of phenanthrene (Fig. 2.3) while M4-6 did not. GC-MSD analysis revealed 2 potential pyrene metabolites (Figs. 2.6A and B): pyrene-diol and phenanthrene dicarboxylic acid (PDA).

Various detergents were tested for their ability to improve PAH utilization. Sodium dodecylsulfate and Triton X-100 at 1% inhibited growth of LC8 but did not affect growth of M4-6. In contrast, both LC8 and M4-6 showed enhanced growth in the presence of Tween 80, however, none of the detergents tested improved phenanthrene degradation (Table 2.2).

Phylogenetic analysis

PCR amplification of 16S rDNA from LC8 and M4-6 resulted in 1452 bp and 1496 bp of sequence respectively (Table 2.4 and 2.5). **A** Blast search from GenBank revealed that the closest matches to LC8 were ***Erythrobacter*** sp. JP13.1. (GeneBank accession number AY007680, with a nucleotide identity of 96%); ***Erythromicrobium ramosum*** (GeneBank accession number AB013355, with a nucleotide identity of 94%);

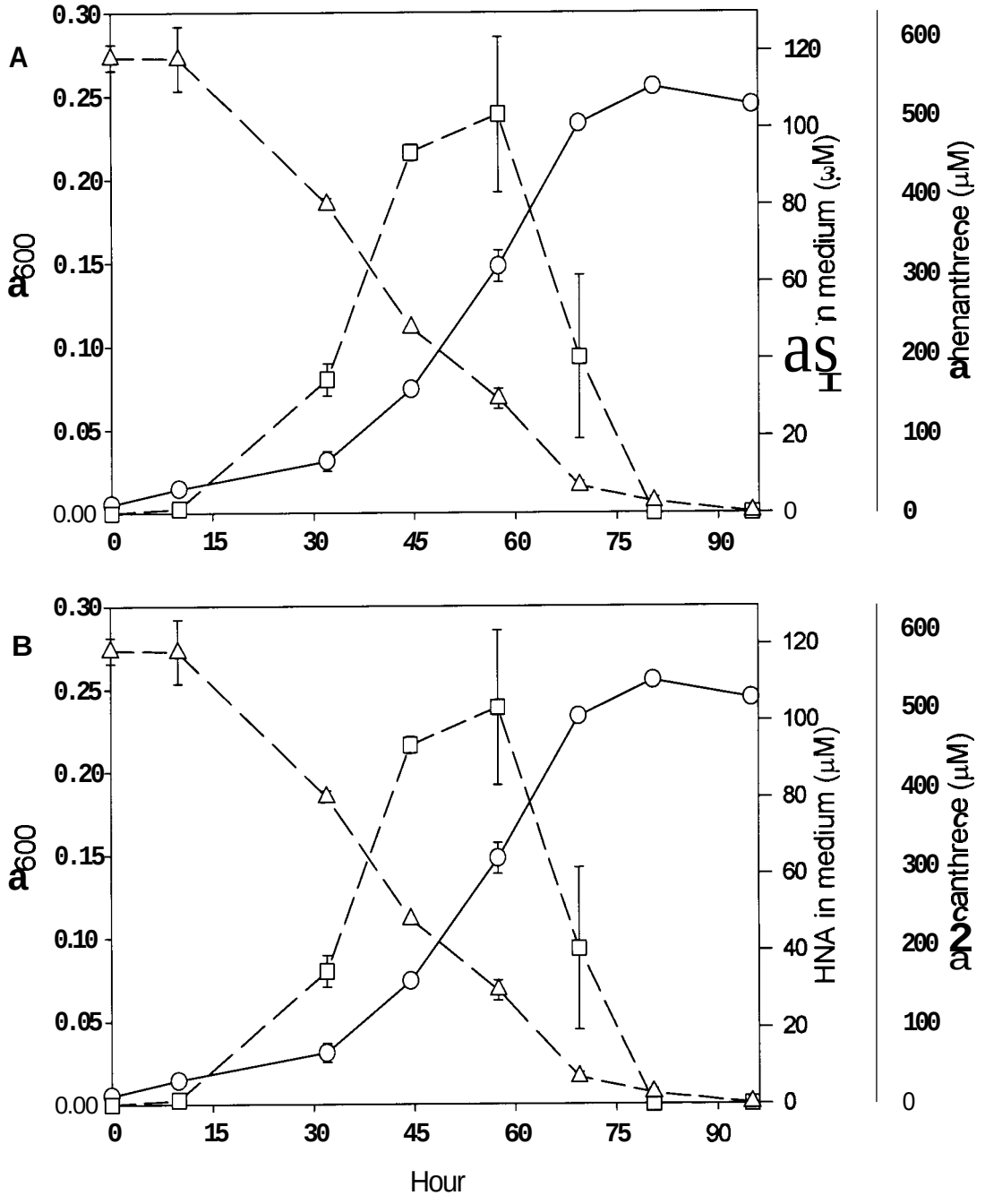
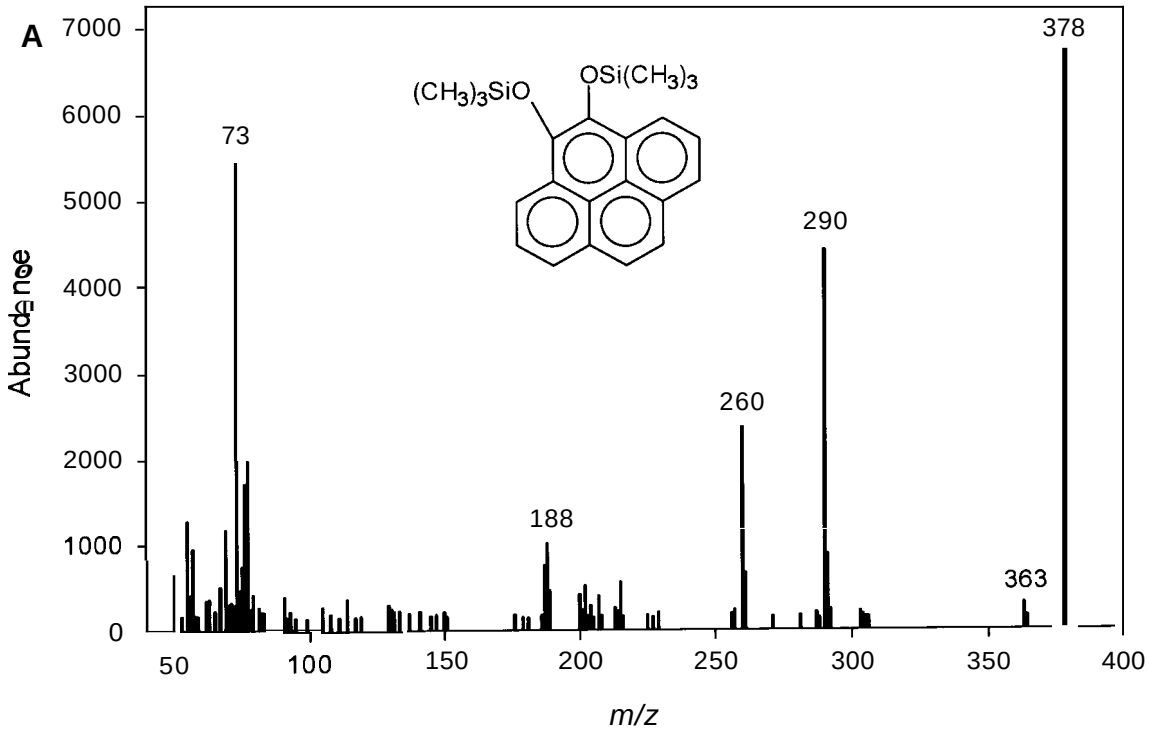


Figure *2.5.* Growth curves of LC8 and M4-6. Growth curves of LC8 (A) and M4-6 (B) on phenanthrene, showing growth (O) and concomitant loss of phenanthrene (A) and the changes in **HNA** concentration (O) in the medium. Data are means of triplicate ± 1 standard error.

Figure 2.6. Mass spectra of pyrene metabolite. Pyrene metabolites produced by LC8 when co-metabolized with phenanthrene: Silylated pyrene-4,5-diol (A) and methylated phenanthrene-4,5-dicarboxylic acid (B).


A
Abundance
7000
6000
5000
4000
3000
2000
1000
0
50
100
150
200
250
300
350
400
m/z
73
188
260
290
363
378
(CH3)3SiO
OSi(CH3)3

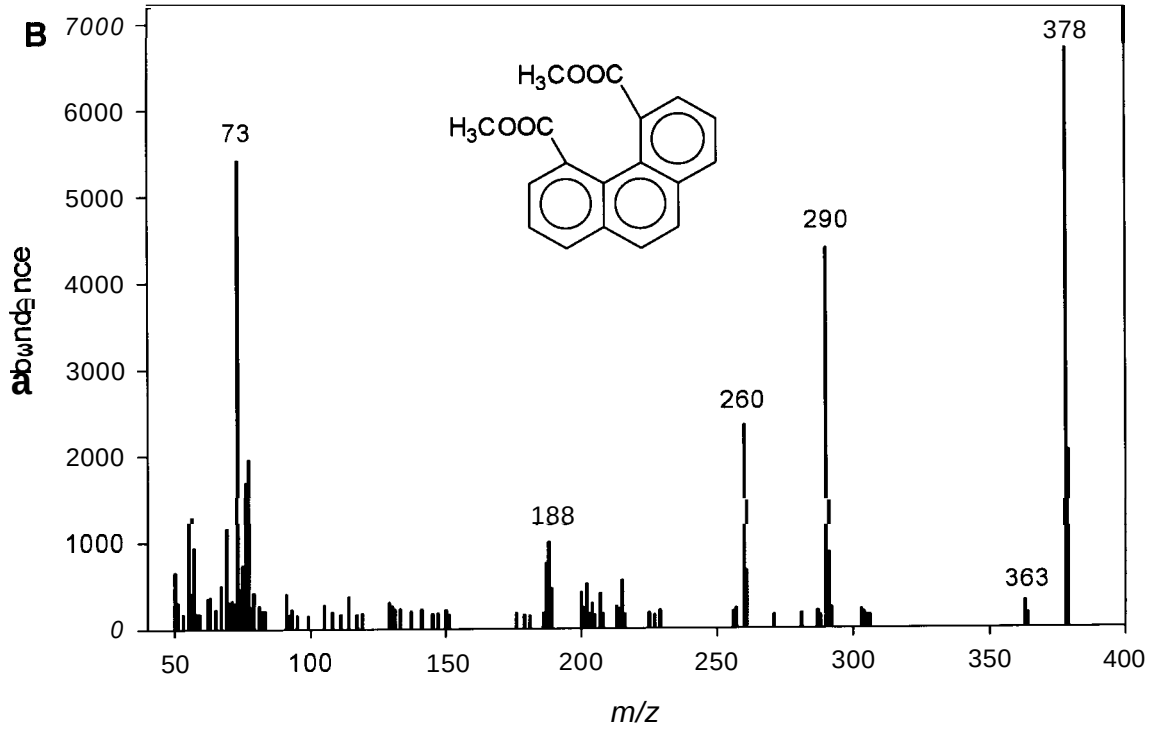

B
Abundance
7000
6000
5000
4000
3000
2000
1000
0
50
100
150
200
250
300
350
400
m/z
73
188
260
290
363
378
H3COOC
H3COOC

Table 2.4. Full length 16S rDNA sequence (1452 **bp)** of LC8

```
   1 TAGAGTTTGA TCATGGCTCA GAACGAACGC TGGCGGCATG CCTAACACAT
  51 GCAAGTCGAA CGAACCCTTC GGGGTGAGTG GCGCACGGGT GCGTAACGCG
 101 TGGGAACCTG CCTTTAGGTT TGGAATAACA GTGAGAAATC GCTGCTAATA
 151 CCAAATAATG TCTTCGGACC AAAGATTTAT CGCCTTTAGA TGGGCCCGCG
 201 TTGGATTAGC TAGTTGGTGG GGTAAAGGCT CACCAAGGCG ACGATCCATA
 251 GCTGGTCTTA GAGGATGATC AGCCACACTG GGACTGAGAC ACGGCCCAGA
 301 CTCCTACGGG AGGCAGCAGT GGGGAATATT GGACAATGGG CGAAAGCCTG
 351 ATCCAGCAAT GCCGCGTGAG TGATGAAGGC CTTAGGGTTG TAAAGCTCTT
 401 TTACCAGGGA TGATAATGAC AGTACCTGGA GAATAAGCTC CGGCTAACTC
 451 CGTGCCAGCA GCCGCGGTAA TACGGAGGGA GCTAGCGTTG TTCGGAAATA
 501 CTGGGCGTAA AGCGAACGTA GGCGGTCTAT TAAGTCAGGG GTGAAATCCC
 551 GGAGCTCAAC TCCGGAACTG CCCTTGAAAC TGCTAGACTA GAATCCTGGA
 601 GAGGTCAGTG GAATTCCGAG TGTAGAGGTG AAATTCGTAG ATATTCGGAA
 651 GAACACCAGT GGCGAAGGCG ACTGACTGGA CAGGTATTGA CGCTGAGGTT
 701 CGAAAGCGTG GGGAGCAAAC AGGATTAGAT ACCCTGGTAG TCCACGCCGT
 751 AAACGATGAT AACTAGCTGT CCGGGCTCAT AGAGCTTGGG TGGCGCAGCT
 801 AACGCATTAA GTTATCCGCC TGGGGAGTAC GGTCGCAAGA TTAAAACTCA
 851 AAGGAATTGA CGGGGGCCTG CACAAGCGGT GGAGCATGTG GTTTAATTCG
 901 AAGCAACGCG CAGAACCTTA CCAGCCTTTG ACATCCTTCG ACGGTTTCTG
 951 GAGACAGATT CCTTCCTTCG GGACGAAGTG ACAGGTGCTG CATGGCTGTC
1001 GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA GTCCCGCAAC GAGCGCAACC
1051 CTCATCCTTA GTTGCCATCA TTTAGTTGGG CACTTTAAGG AAACTGCCGG
1101 TGATAAGCCG GAGGAAGGTG GGGATGACGT CAAGTCCTCA TGGCCCTTAC
1151 AGGCTGGGCT ACACACGTGC TACAATGGCA TCTACAGTGA GCAGCGATCC
1201 CGCGAGGGTT AGCTAATCTC CAAAAGATGT CTCAGTTCGG ATTGTTCTCT
1251 GCAACTCGAG AGCATGAAGG CGGAATCGCT AGTAATCGCG GATCAGCATG
1301 CCGCGGTGAA TACGTTCCCA GGCCTTGTAC ACACCGCCCG TCACGCCATG
1351 GGAGTTGGTT TCACCCGAAG GTGGTGCGCT AACCGGTTTA CCGGAGGCAG
1401 CCAACCACGG TGGGATCAGC GACTGGGGTG AAGTCGTAAC AAGGTAACCG
1451 TA
```

Table 2.5. Full length 16S rDNA sequence (1496 bp) of M4-6

```
   1 GATCATGGCT CAGATTGAAC GCTGGCGGCA TGCCTAACAC ATGCAAGTCG
  51 AACGGAAACG ATGCTAGCTT GCTAGCAGGC GTCGAGTGGC GGACGGGTGA
 101 GTAATGCATA GGAATCTACC TAATAGTGGG GGACAACCTG GTGAAAACCA
 151 GGCTAATACC GCATAATCCC TACGGGGCAA AGCAGGGGAC CTTCGGGCCT
 201 TGCGCTAATA GATGAGCCTA TGTCGGATTA GCTAGTTGGT GAGGTAATGG
 251 CTCACCAAGG CAACGATCCG TAGCTGGTTT GAGAGGATGA TCAGCCACAC
 301 TGGGACTGAG ACACGGCCCA GACTCCTACG GGAGGCAGCA GTGGGGAATA
 351 TTGCACAATG GAGGAAACTC TGATGCAGCA ATGCCGCGTG TGTGAAGAAG
 401 GCCTTAGGGT TGTAAAGCAC TTTCAGTAGG GAGGAAAAGT TTAAGGTTAA
 451 TAACCTTAGG CCCTGACGTT ACCTACAGAA GAAGCACCGG CTAACTCCGT
 501 GCCAGCAGCC GCGGTAATAC GGAGGGTGCA AGCGTTAATC GGAATTACTG
 551 GGCGTAAAGC GCGCGTAGGC GGTTAAACAA GTCAGATGTG AAAGCCCCGG
 601 GCTCAACCTG GGAACTGCAT TTGAAACTGT TTAGCTAGAG TGTGGTAGAG
 651 GAGAGTGGAA TTTCAGGTGT AGCGGTGAAA TGCGTAGATA TCTGAAGGAA
 701 CACCAGTGGC GAAGGCGGCT CTCTGGACCA ACACTGACGC TGAGGTGCGA
 751 AAGCGTGGGT AGCAAACGGG ATTAGATACC CCGGTAGTCC ACGCCGTAAA
 801 CGATGTCAAC TAACTGTTGG GCGGGTTTCC GCTTAGTGGT GCAGCTAACG
 851 CAATAAGTTG ACCGCCTGGG GAGTACGGCC GCAAGGCTAA AACTCAAATG
 901 AATTGACGGG GGCCCGCACA AGCGGTGGAG CATGTGGTTT AATTCGATGC
 951 AACGCGAAGA ACCTTACCTA CCCTTGACAT ACAGAGAACT TTCTAGAGAT
1001 AGATTGGTGC CTTCGGGAAC TCTGATACAG GTGCTGCATG GCTGTCGTCA
1051 GCTCGTGTCG TGAGATGTTG GGTTAAGTCC CGTAACGAGC GCAACCCTTA
1101 TCCTTAGTTG CTACCATTTA GTTGGGCACT CTAAGGAGAC TGCCGGTGAT
1151 AAACCGGAGG AAGGTGGGGA CGACGTCAAG TCATCATGGC CCTTATGGGT
1201 AGGGCTACAC ACGTGCTACA ATGGCCGGTA CAGAGGGCCG CAAACTCGCG
1251 AGAGTAAGCT AATCCCTTAA AGCCGGTCCT AGTCCGGATT GCAGTCTGCA
1301 ACTCGACTGC ATGAAGCTGG AATCGCTAGT AATCGCGGAT CAGAATGCCG
1351 CGGTGAATTC GTTCCCGGGC CTTGTACACA CCGCCCGTCA CACCATGGGA
1401 GTGGGTTGCA AAAGAAGTGG GTAGGCTAAC CTTCGGGAGG CCGCTCACCA
1451 CTTTGTGATT CATGACTGGG GTGAAGTCGT AACAAGGTAA CCCGTA
```

Porphyrobacter sp. KK351 (GeneBank accession number AB033326 with a nucleotide identity of 94%) and *Sphingomonas* sp. (GeneBank accession number AF181571 with a nucleotide identity of 92%). These sequences and other related sequences were used to construct a best tree based on maximum-likelihood phylogenetic analysis (100 bootstrap samples). The results indicated that LC8 was phylogenetically closest to *Erythrobacter* with an *Erythrobacter*-LC8 branch that is distinct from other sphingomonads (Fig. 2.7). *Porphyrobacter* and *Erythromicrobium* formed another clade that is distinct from the *Erythrobacter*-LC8 branch and the rest of the sphingomonads. The trees generated from maximum parsimony and neighbor-joining methods shared the same overall topography with the maximum likelihood tree, and presented the same relationship among LC8, *Erythrobacter* and the sphingomonads (data not shown).

A similar analysis of M4-6 16S rDNA sequence data revealed a very high nucleotide sequence identity with *Cycloclasticus* sp. E (99.7%) as well as with several other *Cycloclasticus* species. The results strongly suggest that M4-6 belongs to the genus *Cycloclasticus.* ***An*** unrooted best tree from maximum likelihood analysis revealed that M4-6 and all the selected *Cycloclasticus* species were in the same close cluster (Fig. 2.8). Results from maximum parsimony and neighbor-joining methods were equivalent (data not shown).

Discussion

Macrofaunal burrows enhance a number of microbial activities. For example, elevated levels of nitrification (61) and sulfate reduction (43) have been documented for

Figure 2.7. 16s rDNA based phylogenetic tree of LC8. Best tree based on Maximum likelihood analysis of the 16S rDNA sequence data of **LC8** and selected members of Sphingomonadaceae and alpha-proteobacter. Scale bar represents 10% nucleotide divergence. Bootstrap values greater than 50 are reported based on 100 resamplings for maximum likelihood.

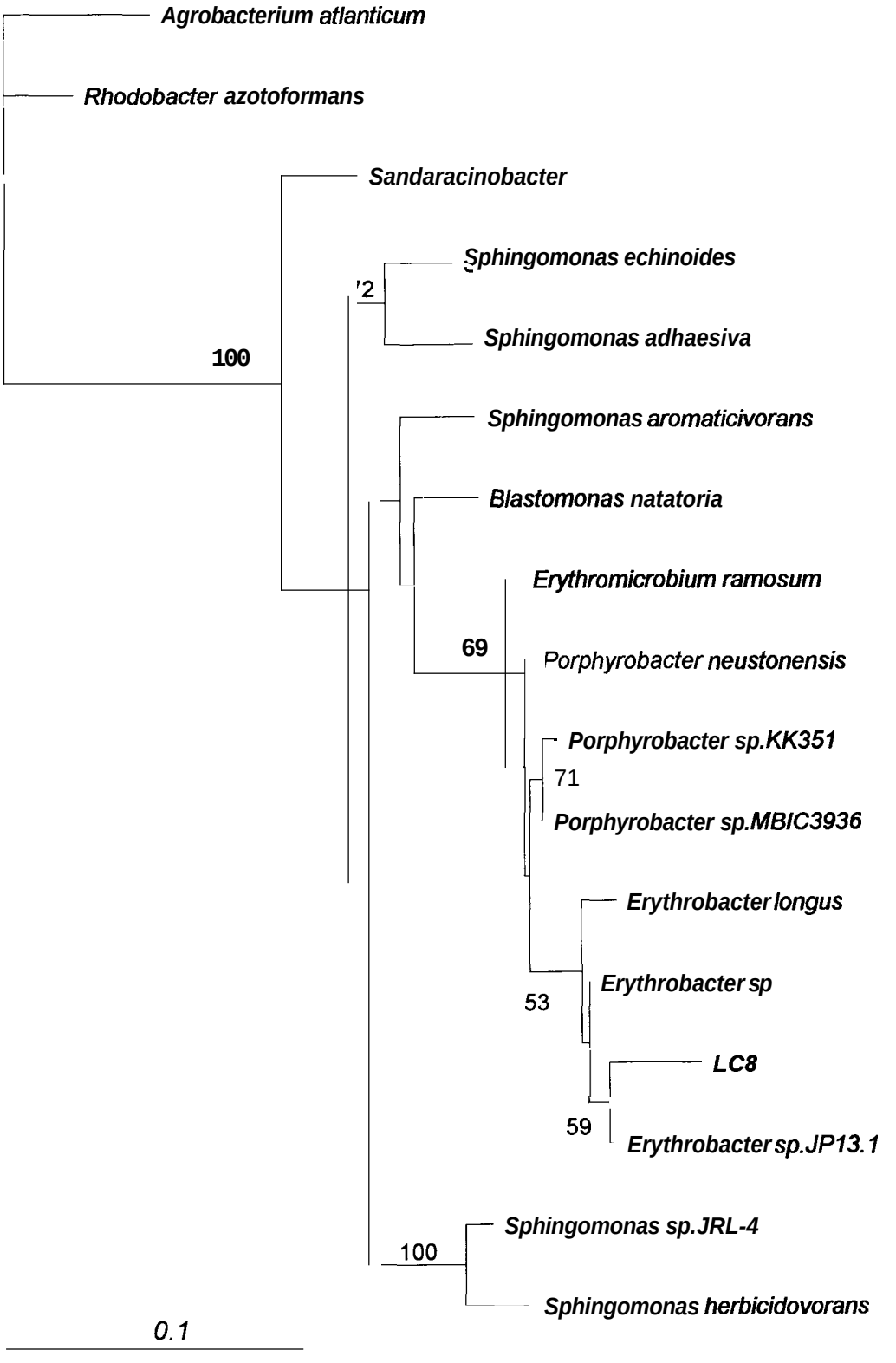
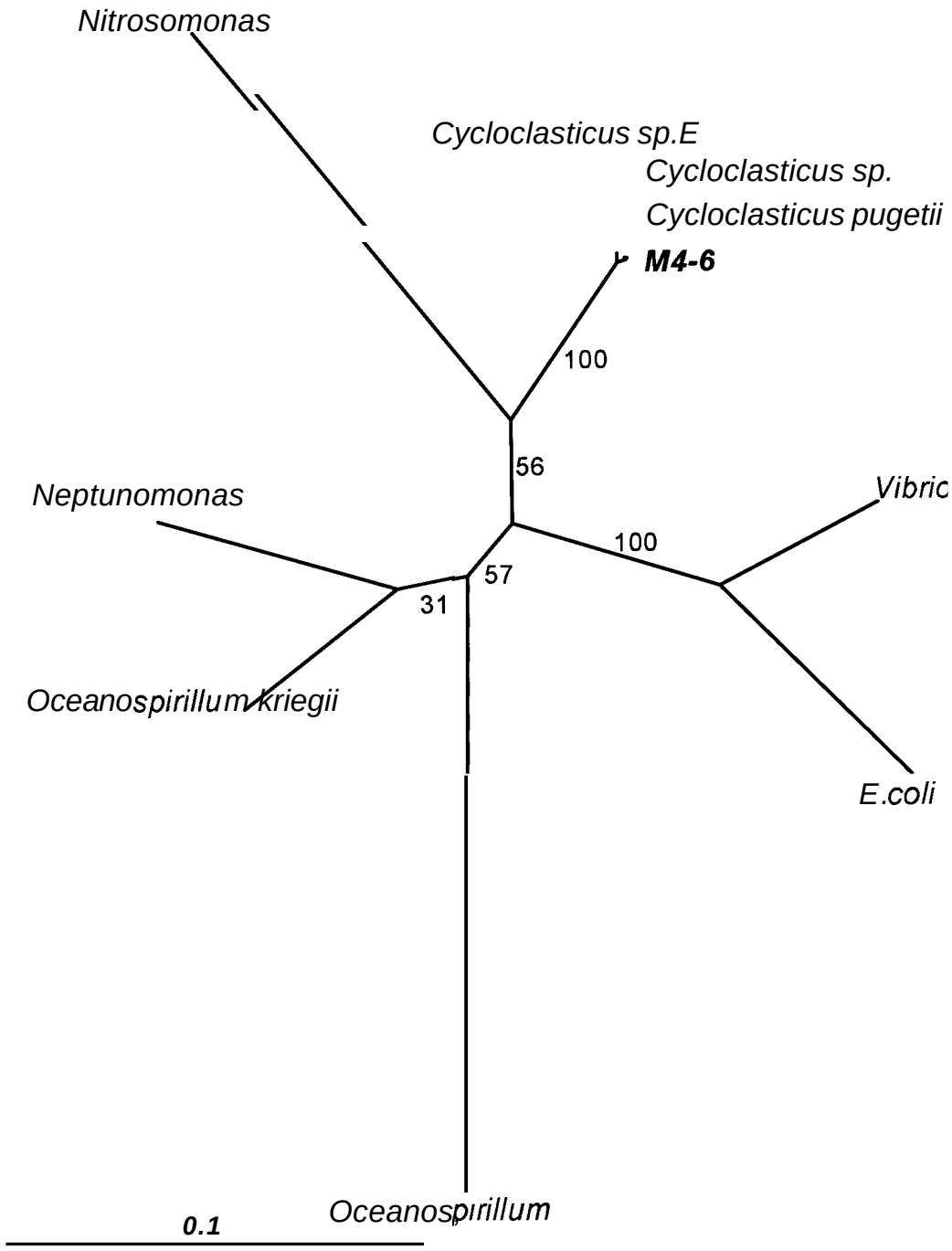

Agrobacterium atlanticum
Rhodobacter azotoformans
Sandaracinobacter
Sphingomonas echinoides
'2
Sphingomonas adhaesiva
100
Sphingomonas aromaticivorans
Blastomonas natatoria
Erythromicrobium ramosum
69
Porphyrobacter neustonensis
Porphyrobacter sp.KK351
71
Porphyrobacter sp.MBIC3936
Erythrobacter longus
Erythrobacter sp
53
LC8
59
Erythrobacter sp.JP13.1
Sphingomonas sp.JRL-4
100
Sphingomonas herbicidovorans
0.1

Figure 2.8. 16s rDNA based phylogenetic tree of **M4-6.** 16S rDNA phylogenetic analysis of M4-6 and with selected members of Proteobacteria. Scale bar represents 10% nucleotide divergence. Numbers are bootstrap values based on 100 resamplings for maximum likelihood.


Nitrosomonas
Cycloclasticus sp.E
Cycloclasticus sp.
Cycloclasticus pugetii
M4-6
100
56
Neptunomonas
Vibric
100
57
31
Oceanospirillum kriegii
E.coli
Oceanospirillum
0.1

macrofaunal burrow sediments. Burrows also support higher levels of microbial biomass than bulk sediments (39, 51). In addition, *N. **virens*** and *M. **arenaria*** burrow sediments harbor PAH-degrading bacteria (22). Since Lowes Cove is not believed to be contaminated with PAH, it is possible that burrows provide an environment suitable for maintenance of some PAH-degrading taxa. Thus, burrows in pristine systems may provide a source of organisms that can respond to chronic or acute PAH pollution, and perhaps serve as a resource for neighboring heavily contaminated areas.

Aerobic PAH degraders in burrows sediments may also promote anaerobic PAH degradation. For example, hydroxylated or carboxylated PAH metabolites excreted by aerobes often can be degraded by anaerobes more readily than the parent compound (70). Zhang and Young (104) have shown that many small PAH molecules as well as long chain hydrocarbons were carboxylated or hydroxylated prior to anaerobic degradation. The presence of both aerobic and anaerobic bacteria in close proximity in burrows along with intermittent oxygen availability may enhance degradation relative to that occurring exclusively under one condition.

Two distinct PAH degraders were isolated from Lowes Cove burrow sediments. Morphologically, LC8 appears to be a sphingomonad. Many ***Sphingomonas*** sp. have been reported to degrade a variety of PAH, which is not surprising since many of these isolates have been obtained from contaminated terrestrial systems (2, 9, 28, 50). In addition, a marine ***Sphingomonas*** capable of degrading PAH has been reported **(38).** However, unlike LC8, this marine ***Sphingomonas*** does not utilize aliphatic hydrocarbons (e.g. hexadecane). Further, LC8 appears to be non-motile, as is the case for

Sphingomonas. Nonetheless, LC8 possesses a polar flagellum (Fig. 2.2B), and thus may be motile under conditions that differ from those used in this study.

Despite a number of similarities with the genus *Sphingomonas,* 16S rDNA sequence analysis indicates that LC8 is most similar to the genus *Erythrobacter* (nucleotide sequence identity 96%). The next closest match in the GenBank database is with the genera *Erythromicrobium* (sequence identity 94%) and *Porphyrobacter* (sequence identity 94%). However, the bootstrap values do not confidently support the placement of LC8 within the ***Erythrobacter-Porphyrobacter*** branch. Thus, the possibility that LC8 is either an *Erythrobacter, Erythromicrobium* or *Porphyrobacter* is remote. LC8 does not appear to contain photosynthetic pigments. In contrast, all *Erythrobacter, Erythromicrobium* and *Porphyrobacter* are aerobic anoxygenic phototrophs containing bacteriochlorophyll (35, 102, 103).

Although *Erythrobacter* and *Sphingomonas* share few physiological traits, they are phylogenetically similar based on 16S rDNA sequence analysis. Indeed, Kosako et al. (54) have proposed a new family, Sphingomonadaceae, which consists of six genera: *Erythrobacter, Sphingomonas, Erythromicrobium, Porphyrobacter, Sandaracinobacter* and *Zymomonas.* Because LC8 is most similar to and clusters with the *Erythrobacter* clade of this family, it does not appear to be a new species of *Sphingomonas,* even though it degrades PAH as do other sphingomonads. However since it has only a 96% 16S rDNA sequence identity to *Erythrobacter* and lacks bacteriochlorophyll, it is excluded from all other genera of the Sphingomonadaceae. Accordingly, a new genus and species within the Sphingomonadaceae is proposed here – *Lutibacterium anuloederans* (nov. gen. nov. sp.).

The phylogenetic status of **M4-6** is clearer than that of *Lutibacteriurn anuloederans.* Blast search results and phylogenetic analysis of the **M4-6** 16S rDNA sequence conclusively establish it as a member of the genus *Cycloclasticus.* Maximum likelihood analysis showed that **M4-6** formed a cluster with all the *Cycloclasticus* species analyzed. However, none of the previously reported *Cycloclasticus* species has a spirillum morphology. This suggests that **M4-6** is a novel *Cycloclasticus* species. **M4-6** is further differentiated from extant *Cycloclasticus* species by phenotypic characterization, which reveals that many of the characters of previously identified species are absent from **M4-6.** For example, Dyksterhouse et al. (29) described several *Cycloclasticus* strains from Puget Sound, all of which were short rods. Also, *C. pugetii* reduces nitrate to nitrite, has lipase activity and lacks PHB accumulation, while **M4-6** shows no nitrate reduction and lipase activity but accumulates poly-β-hydroxybutyrate (Table 2.1).

Cycloclasticus has a cosmopolitan distribution. It has been isolated from Alaska, Puget Sound and the Gulf of Mexico. However, C. *spirillensus* proposed sp. nov. **(M4-6)** is the first isolate documented from the northwestern Atlantic. Like some of the previously described *Cycloclasticus* strains, C. *spirillensus* was isolated from an area not contaminated with PAH. Thus, *Cycloclasticus* appears to include a versatile and diverse group of marine PAH degraders adapted to use both low and high PAH concentrations.

Both *Lutibacterium anuloederans* and *C. spirillensus* may possess nah-like dioxygenase systems for PAH metabolism. When grown with phenanthrene, the major observable intermediate for both is hydroxynaphthoic acid, a known phenanthrene metabolite from PAH degraders employing nah-like dioxygenases (1, **21, 42).** In

addition, Lutibacterium anuloederans metabolizes dibenzothiophene to dibenzothiophene sulfoxide. Denome et al. (27) have described a soil pseudomonad carrying a nah-like enzyme system (coded for by the ***dox*** operon) performing the same conversion. The nah dioxygenase system was first described in terrestrial PAH-degrading Pseudomonas (100). However, similar systems have been described in some marine PAH degraders (36, 45).

Lutibacterium anuloederans is also one of the few reported marine bacteria that can metabolize 4-ring PAH. Pyrene is co-metabolized through the intermediates pyrene-diol and phenanthrene dicarboxylic acid. These intermediates can be produced by the same dioxygenase system acting on phenanthrene. Relaxed substrate specificity is not uncommon (84) for PAH dioxygenases.

Description of *Lutibacterium anuloederans* (Luti, L., n. lutum, mud. anulo, L., n. anulus, ring; ederans, L. edere, to eat; ring-eating mud bacterium).

Gram-negative, non-motile, short rod with a single polar flagellum. Aerobic. Requires sodium ion for growth. Catalase and oxidase positive. PAH including naphthalene and phenanthrene are used as sole or principal carbon sources for growth. In addition, strains utilize selected organic acids and amino acids, including citrate, acetate, pyruvate and phenylalanine. Nitrate is reduced to nitrite. Colonies are small, round, opaque and entire with yellow pigmentation. The principal fatty acids in whole-cells grown on rich marine media are C18:1 ω7c, C16:1 ω7c and C16:0.

Description of *Cycloclasticus spirillensus* (spirillensus, L. of or pertaining to a spiral).

Gram-negative, motile spirillum with a single polar flagellum. 1.5-2.0 μm x 0.5 pm. Aerobic. Requires sodium ion for growth. Catalase and oxidase positive. PAH

including naphthalene and phenanthrene are used as sole or principal carbon sources for growth. In addition, strains utilize selected organic acids and amino acids, including citrate, acetate, pyruvate, alanine and proline. Nitrate is not reduced to nitrite. Colonies are small, round, translucent and entire with no pigmentation. The principal fatty acids in whole-cells grown on rich marine media are: iso C15:0, anteiso C15:0 and C16:1 ω7c.

Chapter 3

PHENANTHRENE UPTAKE POTENTIAL OF SEDIMENT SLURRIES INOCULATED WITH MARINE PAH DEGRADERS

Abstract

The ability of **2** marine PAH degraders to enhance phenanthrene removal in sediments was investigated. Sediment slurries from Lowes Cove, **ME** and Little Mystic Channel, Boston, MA were inoculated with *L. anuloederans* (LC8) and *C. spirillensus* (M4-6). The inoculated slurries were maintained with or without exogenous phenanthrene and incubated under aerobic conditions. There were substantial differences in phenanthrene uptake potentials of the inoculated sediment slurries, suggesting that the potential for PAH degradation by the inoculated strains were modulated by the sediment environment. Although both isolates showed very high phenanthrene degradation rates under defined laboratory culture condition, their effectiveness as bioremediation agents is strongly influenced by other environmental factors.

Introduction

Polycyclic aromatic hydrocarbons are ubiquitous, persistent environmental pollutants. They are a natural component of coal and other petroleum products with high hydrophobicity and tend to associate with the organic fraction in the environment such as sediments and soil **(11,** 96, **101).** Despite the persistence of many PAH, especially those with high molecular mass, many microorganisms have been shown to degrade PAH in the environment. The most common microbial degradation of PAH is catalyzed by microbial dioxygenases under aerobic conditions **(45, 62, 64,** 89, **93).** Nevertheless, various reports have shown PAH oxidation under anaerobic conditions coupled to the reduction of various alternate electron acceptors such as sulfate, nitrate and carbon dioxide **(13, 24, 40, 72, 82)** though the rate is slower than aerobic degradation.

All of these environmental isolates were capable of PAH degradation under laboratory conditions. However, bioremediation of PAH contamination requires the bacteria to degrade PAH in a complex, natural sediment matrix. Bioremediation is an attractive alternative to more conventional cleanup processes such as dredging and disposal which is very costly **(44).** Instead of treating the contaminated sediment with physical or chemical means, a microbial population capable of degrading the contaminant(s) *in situ* is used.

We have isolated and characterized **2** novel marine PAH degraders, *Lutibacterium anuloederans* and *Cycloclasticus spirillensus,* from a non-contaminated area of Lowes Cove, Maine **(22).** These two new strains were capable of rapid degradation of naphthalene and phenanthrene under laboratory conditions. ***L.***

anuloederans was also capable of co-metabolizing pyrene, a four-ring carcinogenic PAH, to phenanthrene dicarboxylic acid. Both strains can tolerate a wide range of environmental conditions including salinity and temperature.

To study the possibility of using either isolate for bioremediation of PAH, we incubated ***L.*** *anuloederans* and *C. spirillensus* in natural sediments slurries and monitored the phenanthrene uptake potential of the inoculated slurries. Sediments were collected from a relatively uncontaminated intertidal mudflat and a heavily contaminated harbor (88). We showed that both ***L.*** *anuloederans* and *C. spirillensus* can both be re-introduced into natural sediments and still degrade PAH. However, the rate of PAH uptake of the inoculated sediments and the time period over which bacteria remain active in the sediment are highly affected by the source of the sediment.

The objectives of this work were first to, investigate whether the novel marine PAH degraders ***L.*** *anuloederans* (LC8) and *C. spirillensus* (M4-6) can degrade PAH in a natural sediment matrix rather than in defined culture media, and second, to examine the effect of source sediment on the PAH degradation potential of the two strains.

Materials and Methods

Sampling and slurry preparation

Uncontaminated sediments were collected from the intertidal zone of Lowes Cove, Maine, during summer 1999 at low tide. This site has been described in detail previously (6, **43,** 52) and is not known to be contaminated with PAH. Bulk sediment (10 to 15 cm in depth) was exposed using a "clam fork" and then transferred to a clean

container. Samples were transported back to the laboratory within 1 h at ambient temperature (about 25 °C) in sealed Ziploc bag and processed immediately.

For contaminated sediment, a sampling site was chosen in Little Mystic Channel of Boston, MA. It receives industrial run off from the nearby pier as well as combustion-derived pollution from heavy automobile traffic in the area. The surface of the sediment was black and showed an oily sheen. Removing the top layer of sediment revealed the black sediment below which was anaerobic as suggested by the strong smell of sulfide.

Sediment cores were collected using plastic tube and were sealed on both ends using rubber stoppers. The cores were transported back to the laboratory within 8 h. Cores were stored under flowing seawater. For abiological controls, bulk sediment was autoclaved at 121 °C and allowed to cool to room temperature. The cycle was repeated three times over a period of 3 d. Sediment slurries were prepared with artificial seawater **(ASW)** (92). Ten-percent (w/v) slurries were used in all experiments.

Bacterial cultures were grown in a mineral salt medium (ONR7a) with pyruvate as carbon source (29), to which about 100 µg of phenanthrene was added for induction of the bacterial PAH degradation enzymes. Yeast extract (0.05%) was added for growth of LC8. Bacterial cells were collected by centrifugation after the A_{600} of the culture had reached about 0.6 for LC8 and 1.0 for M4-6.

Sediment slurries were prepared in triplicate sets with **ASW** in 1-L flasks. Bacterial suspensions were added to triplicate flasks with either live or autoclaved Lowes Cove or Little Mystic Channel sediment slurries. The final bacterial concentration in the sediment slurries was about 10^8 CFU cm^{-3} estimated by plate count of the inocula. One triplicate set of slurries from each source received an initial phenanthrene spike of about

10 μg cm^{-3}. The sediment slurries were incubated in the dark at room temperature (about 22°C) with shaking (~110 rpm) except during sampling. The flasks were sealed with a rubber stopper fitted with rubber tubing containing a sterile glass wool plug.

PAH analysis and phenanthrene uptake potential

At intervals, 25 ml subsamples of the slurries were transferred to sterile serum bottles. The bottles were spiked with 250 μg of phenanthrene and sealed with a Teflon-lined stopper. The rate of phenanthrene uptake in the bottles was measured by analyzing phenanthrene concentrations in 4-ml sample at intervals (4 to 24 hrs) and the rate of phenanthrene loss (phenanthrene uptake potential) was used as a measure of the persistence of the cells to degrade phenanthrene in the sediment slurries. Additional 5 ml subsamples were collected from Little Mystic Channel sediment slurries for residual phenanthrene measurement. Phenanthrene levels in the spiked slurries were monitored and maintained at around 10 μg cm^{-3} by adding appropriate amounts of 2% phenanthrene solution (in acetone) after sampling.

PAH content and loss of PAH from slurries was measured and monitored by gas chromatographic-mass selective detector analysis after organic solvent extraction. Briefly, slurries or cultures were acidified to pH < 2.0 with 5 N HCl and mixed with one-half volume of n-hexane in a Teflon-lined screw cap tube. The tubes were shaken for 5 minutes, and phases separated by centrifugation at 4000 rpm (about 2400 x *g*). The organic extracts were incubated with copper filings for 30 min and then passed through ENVI-Florisil columns (Supelco Part No. 5-7058) to remove sulfur and polar compounds respectively. The organic layer was concentrated under a stream of

nitrogen, and the PAH extracted was reconstituted in n-hexane. The samples were analyzed with an HP 6890 gas chromatograph with a 5972A mass selective detector equipped with HP-5MS capillary column (30 m x 0.25 mm x 0.25 μm film thickness) using helium as carrier gas. Peaks were identified based on retention times and mass spectra. Operating conditions of the gas chromatograph were as follows: inlet temperature, 250 "C; auxiliary (interface), 280 "C; helium flow, constant at 1 ml per minute; temperature program, 70 "C hold 2 minutes, ramp to 250 "C at 10 "C per minute, hold 5 minutes at 250 "C. The detection limit of the method is 200 ng (g sediment dry weight)-'

Results

PAH levels in sediments

The PAH level in Lowes Cove (LC) sediment was below the detection limit of 200 ng (g sediment dry weight)-' (Table 3.1). Little Mystic Channel (LMC) sediment had a phenanthrene level of over 100 μg (g sediment dry weight)''. The combined 4-ring PAH (Pyrene, benz[a]anthracene and chrysene) in the LMC sediment was over 80 μg (g sediment dry weight)-'. These values agreed with a previous report (98) and are indicative of anthropogenic impacts received by the sampling site.

Phenanthrene uptake potential of inoculated slurries

In degradation experiments, the two strains behaved very differently in the tested conditions. When inoculated in LC sediment in the absence of exogenous phenanthrene, LC8 inoculated slurries showed an initial high phenanthrene uptake potential (about 2 $\mu g\ cm^{-3}\ hr^{-1}$). The potential dropped rapidly starting from day 7 following a sigmoidal

Table 3.1. PAH concentration in Lowes Cove (LC) and Little Mystic Channel (LMC) sediment.

	PAH concentrations [a] μg (g sediment dry wt)$^{-1}$	
	Lowes Cove, ME	Little Mystic Channel, Boston Harbor, MA
Phenanthrene	B.D.[c]	110 ± 14
4-ring PAH [b]	B.D.	**82** ± 7

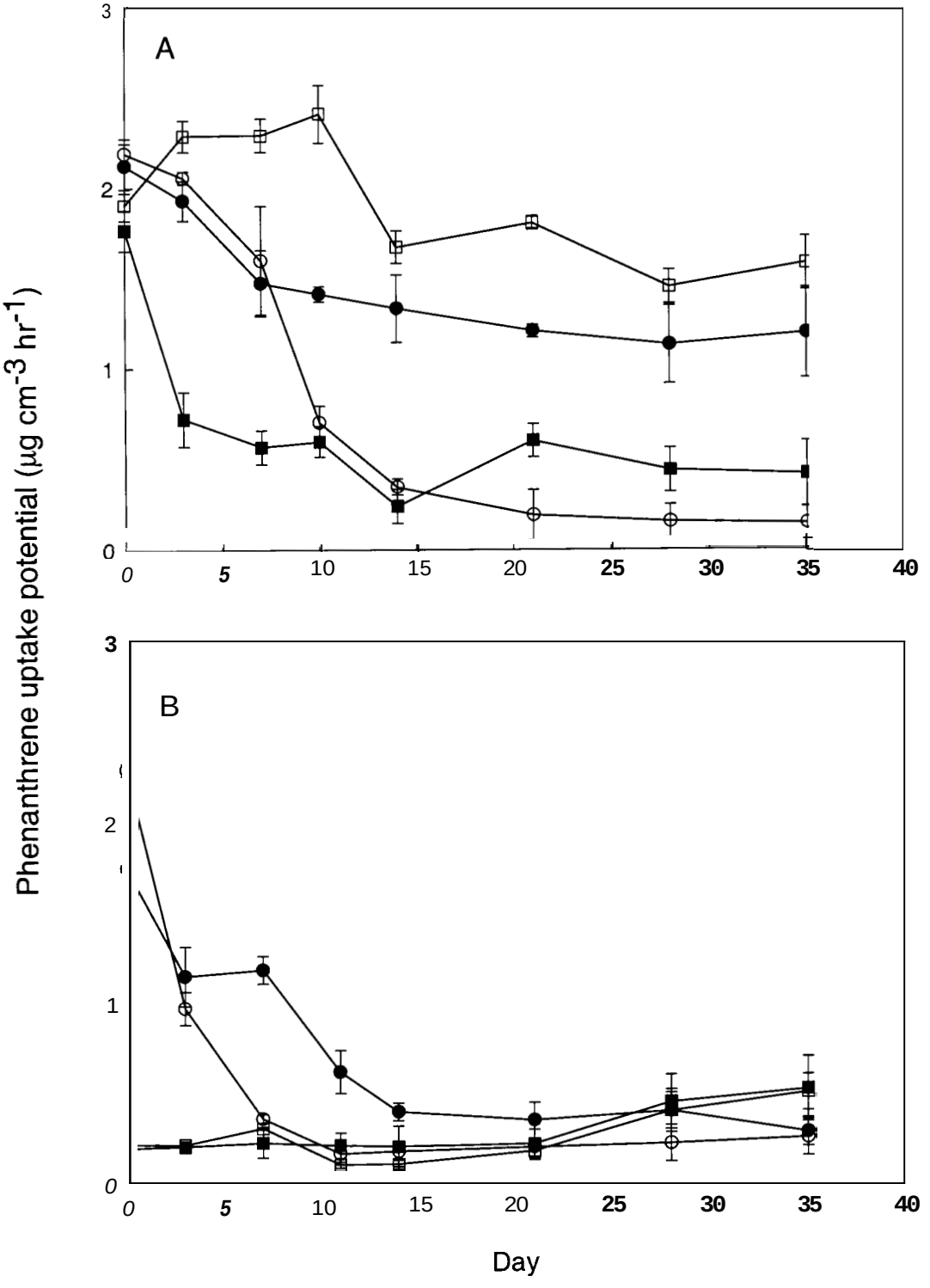
curve (Fig. 3.1A). At the end of 35 d, the uptake rate was less than 0.1 $\mu g\ cm^{-3}\ hr^{-1}$ (< 5% of the initial rate). However, with LMC sediment, phenanthrene uptake potential increased initially from 1.9 to about 2.5 $\mu g\ cm^{-3}\ hr^{-1}$ during the first 10 days and then dropped off gradually and reached 1.5 $\mu g\ cm^{-3}\ hr^{-1}$ by the end of day 35 (about 70% of the initial rate) (Fig. 3.1A). When exogenous phenanthrene was added to the slurries, initial phenanthrene uptake potential in both LC and LMC sediments were similar. However, uptake potentials in both cases dropped off rapidly in the first 5 to 7 d. In LC sediment, the decrease was about 40% in the first 14 d and then leveled off at about 1 $\mu g\ cm^{-3}\ hr^{-1}$. In LMC sediment, uptake potential seemed to level off at around 0.6 $\mu g\ cm^{-3}\ hr^{-1}$ after 11 d.

In the control treatments, autoclaved sediments from either source showed minimal phenanthrene degradation with or without exogenous phenanthrene (Figs. 3.2A, B). In slurries receiving no live cells, phenanthrene uptake potential for LC sediment remained similar to the autoclaved sediment, but in LMC sediment, uptake potential showed a slight increase after 21 d (Fig. 3.2B).

Responses of **M4-6** were similar in both LC and LMC sediments with or without exogenous phenanthrene. In LC sediment without added phenanthrene, initial uptake potential was high (around 2 $\mu g\ cm^{-3}\ hr^{-1}$) then dropped rapidly over 7 d and leveled off at around 0.2 $\mu g\ cm^{-3}\ hr^{-1}$ (Fig. 3.1B). However, uptake potential in LMC sediment remained low up to day 21 followed by a slight increase from 0.2 to around 0.5 $\mu g\ cm^{-3}\ hr^{-1}$ (Fig. 3.1B). Addition of phenanthrene in LC sediment slowed the drop in uptake

Figure 3.1. Phenanthrene uptake potential of inoculated sediment slurries. Phenanthrene uptake potential of different sediment slurries inoculated with LC8 **(A)** and M4-6 (B). (O): LC, no exogenous phenanthrene; (O): LC, with exogenous phenanthrene; (O): LMC, no exogenous phenanthrene; (■): LMC, with exogenous phenanthrene.

Phenanthrene uptake potential of sediment slurries inoculated with LC8 and M4-6



Phenanthrene uptake potential of control sediment slurries

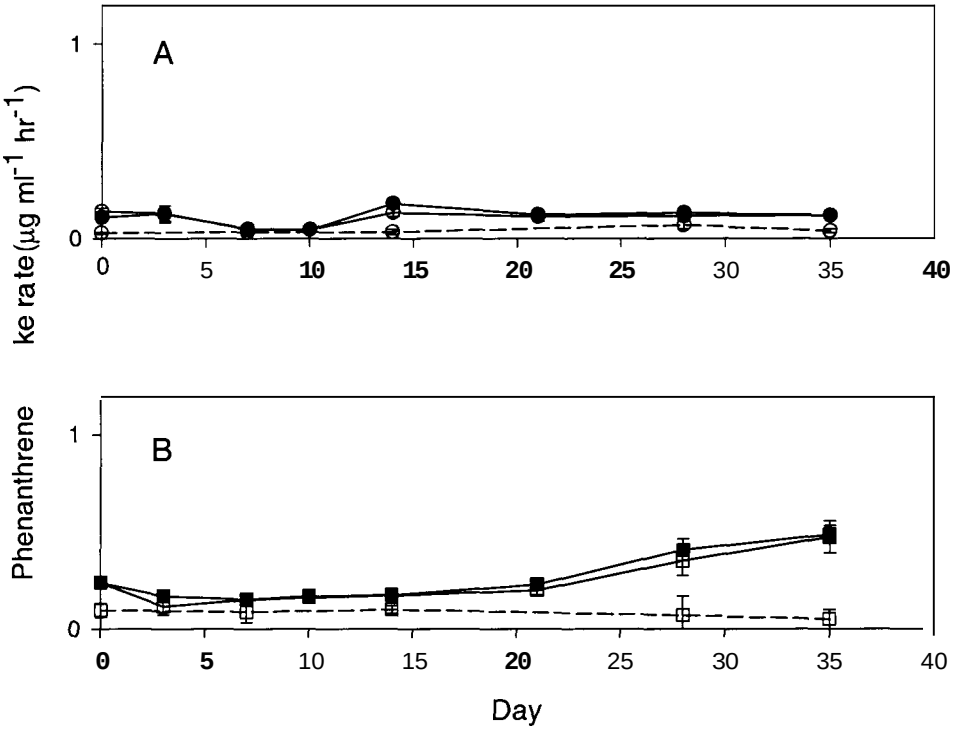


Figure 3.2. Phenanthrene uptake potential of control sediment slurries. Uninoculated and autoclaved control sediment slurries phenanthrene uptake potential of Lowes Cove **(A)** and Little Mystic Channel (B). **(O)**: Uninoculated LC, no exogenous phenanthrene; ***(O)***: Uninoculated LC, with exogenous phenanthrene; **(O)**: Uninoculated LMC, no exogenous phenanthrene; (■): Uninoculated LMC, with exogenous phenanthrene. Dashed lines represent autoclaved treatments.

potential. Uptake potential dropped from 1.8 to around approximately 0.4 $\mu g\ cm^{-3}\ hr^{-1}$ over 14 d and stayed at this level. In LMC sediment, the rate remained low but increased slightly after 21 d.

Figure 3.3 shows a typical phenanthrene uptake rate measurement of the subsamples showing significant variability among the replicates.

The residual phenanthrene level in LMC sediment without exogenous phenanthrene addition showed a very rapid loss of phenanthrene when inoculated with LC8 (Fig. 3.4). Phenanthrene levels decreased by 90% after 3 d and approached the detection limit after 7 d of incubation. In slurries inoculated with M4-6, phenanthrene loss was minimal during the first 21 d followed by a loss of *75%* over the next 14 d. The onset of the loss more or less coincided with the increase of degradation potential in LMC sediment without inoculation (Fig. 3.2B).

Discussion

Anthropogenic activities represents a major source of PAH pollution to the coastal environment, the high PAH concentration in LMC sediment demonstrates the impact (Table 3.1). Boston Harbor, especially the inner part of the harbor, is heavily polluted with a variety of contaminants including PAH, heavy metals such as mercury, lead and others such as PCBs (65, 88). Although phenanthrene, a 3-ring PAH, is relatively labile in aerobic sediments, the anaerobic condition of LMC prevented any significant degradation. When uninoculated LMC sediment was incubated in the presence of oxygen, there was evidence of phenanthrene loss after a 3-wk incubation period (Fig. 3.4). Presumably, during that period the aerobic PAH degraders were

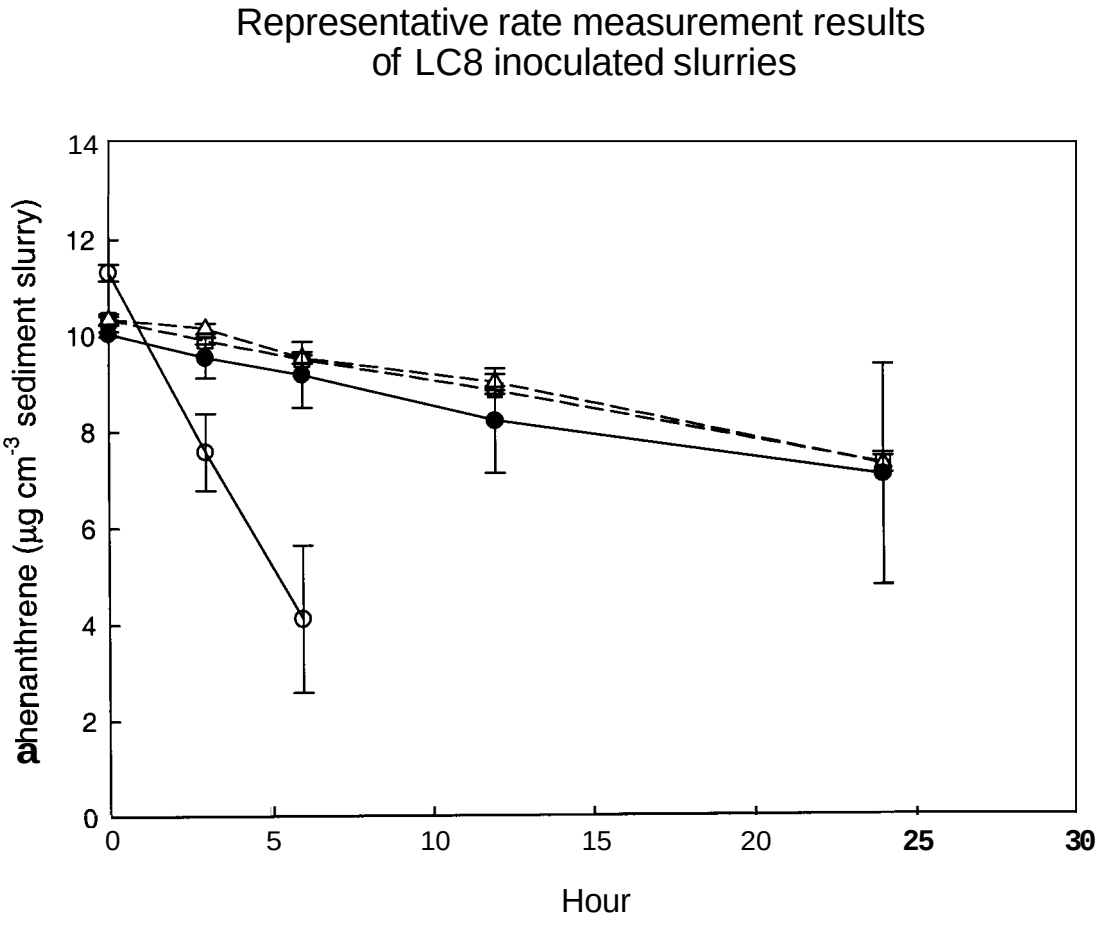


Figure 3.3. Representative rate measurement result. Measurement taken on day **28.** **(O):** autoclaved LC sediment; (A): uninoculated LC sediment; ***(O):*** inoculated LC sediment without exogenous phenanthrene; **(O):** inoculated LC sediment with exogenous phenanthrene.

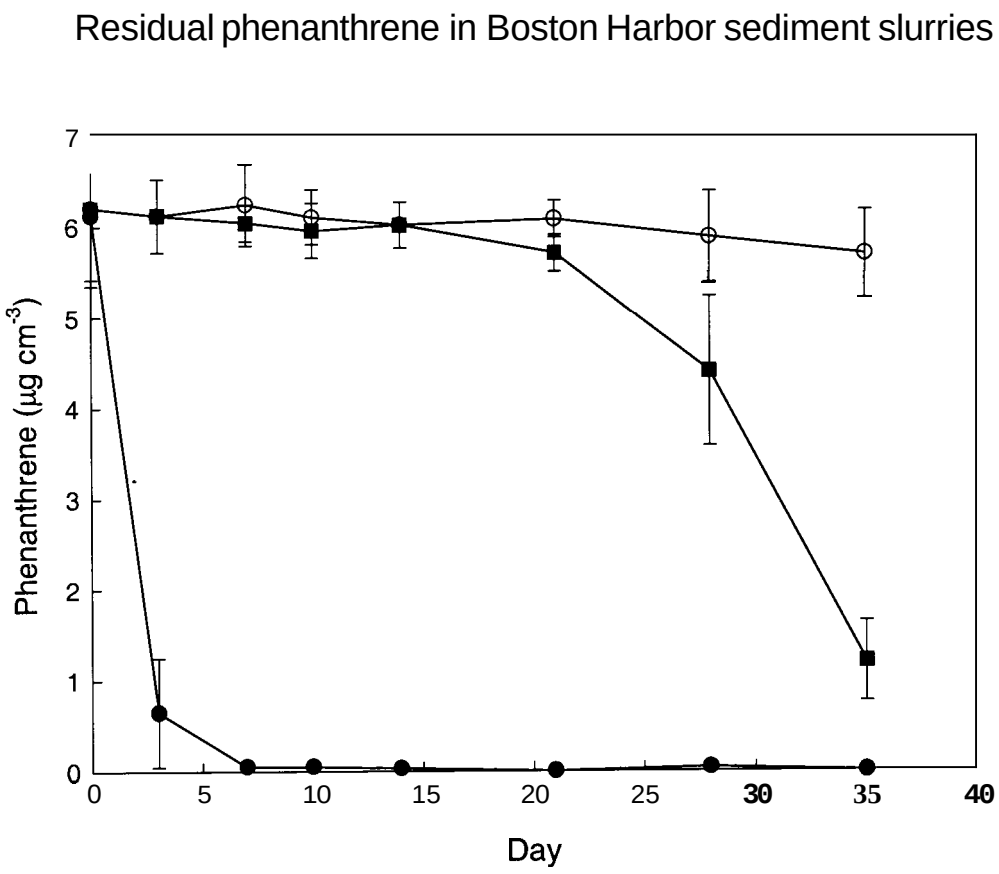


Figure 3.4. Residual phenanthrene concentration in inoculated LMC sediment. LMC sediment slurries (no exogenous phenanthrene added) were inoculated with LC8 (O); M4-6 (■). Autoclaved control (O) showed minimal loss.

enriched and resulted in phenanthrene degradation. The phenanthrene uptake potential of the slurry also increases at the end of the third week (Fig. 3.2B). Yet, the uninoculated LC sediment did not show the same response. The phenanthrene uptake rate stayed the same through the 5-week period. These results suggest that PAH degraders are more readily enriched from the contaminated sediments. Most isolated PAH degraders were isolated from contaminated areas (1, 25, 72, 87, 91, 94). However, uncontaminated burrow sediments of macrofauna from Lowes Cove, **ME** seems to be an environment suitable for some PAH-degrading taxa to thrive (23).

Most PAH degraders isolated from the environment were subsequently studied under well defined laboratory conditions (8, 25, 41, 47, 94). However, bioremediation requires the degradation to proceed in a less defined environmental setting. Both LC8 and M4-6 are capable of degrading exogenous phenanthrene when reintroduced into a sediment matrix. This suggests that both strains are likely to degrade PAH in their native environment. However, the phenanthrene uptake potential of slurries inoculated with LC8 and M4-6 differ significantly (Fig. 3.1). Besides the intrinsic difference between the 2 strains, the phenanthrene uptake potential of the inoculated slurries seems to depend primarily on two factors: the source of the sediment and the presence of a suitable carbon source.

The sediment matrix now represents a source of energy as well as contaminant. LC8 responded very differently in the two sediment sources (Fig. 3.1A). In the uncontaminated LC sediment without exogenous phenanthrene, the inoculated LC8 did not sustain phenanthrene uptake, indicating a loss of cell viability or the ability to degrade phenanthrene. This is likely a result of the lack of utilizable carbon in the

slurries. The indigenous bacteria may also compete for micronutrients with the inoculated cells and limit their proliferation and thus decrease the uptake potential. In LMC sediment inoculated with LC8, there was an initial increase in uptake potential. The uptake potential then decreased but remained at a higher level compared to the LC sediment. The uptake potential may be fueled by the extra carbon in the LMC sediment (52, 98). This includes the high level of utilizable phenanthrene present in LMC sediment. LMC sediment may also provide other essential nutrients for the growth of LC8.

The sediment matrix is also a source of inhibitory contaminants. M4-6 inoculated LMC sediment slurries have very low phenanthrene uptake potentials with or without exogenous phenanthrene (fig. 3.1B). The inhibition was immediate and very strong. The phenanthrene uptake potential was similar to the control from the day of initial inoculation. This indicates that the LMC sediment may be inhibitory to phenanthrene degradation or toxic to M4-6. It is not clear whether loss of viability of M4-6 is the major reason for the loss in phenanthrene uptake potential as non-quantitative microscopic analysis showed that cell number of M4-6 only decreased after 7 d of incubation (data not shown). There are many pollutants present in LMC sediment that are toxic, e.g. heavy metals such as silver and mercury (65). The sediment is likely to have a high level of sulfide, possibly resulting in sulfide toxicity. These various parameters may affect the survival of the added cells in the slurries as well as the degradation potential. In this respect, LC8 seems to have a higher tolerance for these contaminants than M4-6.

Additional carbon has a positive impact on the survival and maintenance of phenanthrene uptake potential of slurries inoculated with LC8. Sediment inoculated with LC8 maintained a higher uptake potential in the presence of exogenous phenanthrene and in LMC sediment (Fig. 3.1A). However, when phenanthrene was added to the LMC slurries, uptake potential diminished. The reason is not clear. It is unlikely that the addition of 10 yg of phenanthrene cm^{-3} would elevate the PAH loading in the LMC slurries to a toxic level. However, it may be possible that the extra phenanthrene enhances co-metabolism of high molecular weight PAH in the sediment and releases metabolites that are toxic. It is also possible that the continual replenishing of phenanthrene in LMC sediment during incubation may have triggered the desorption of more toxic PAH from the sediment. The small amount of acetone added may have released other toxic pollutants such as PCB in the sediments. Nevertheless, LC8 inoculated slurries showed much higher phenanthrene uptake potential over an extended period of time in both non-impacted and heavily polluted sediments.

LMC sediment seems to contain indigenous aerobic phenanthrene degraders. Degradation potential in the uninoculated sediment increased after 21 d. Conceivably, the aerobic phenanthrene degrader population increased during the incubation period.

LC8 was very efficient in removing phenanthrene from the heavily contaminated LMC sediment. Over 90% of the 6 yg of phenanthrene cm^{-3} present was removed within 3 d. In addition, the degradation potential of the inoculated slurries remained high (Fig. 3.1) after most of the phenanthrene in the slurries had been removed. This suggests that LC8 remains viable in the slurries even without phenanthrene, probably by consuming other available carbon sources.

Bacteria and other microorganisms capable of degrading PAH and other environmental pollutants are now routinely isolated from the environment. However, not all of them are suitable bioremediation agents. Both LC8 and **M4-6** were isolated under similar condition using similar enrichment methods (23). They both degrade 2-ring and 3-ring PAH. Yet, **M4-6** is not a good candidate for bioremediation. On the other hand, LC8 showed strong survival and maintenance of degradation potential even in the highly contaminated Little Mystic Channel. Some PAH degraders, including *Micromonospora,* were much more resistant to toxic contaminants such as heavy metals **(97)** and they would be better candidates for bioremediation.

M4-6, *Cycloclusticus spirillensus*, shares the same PAH degradation capability with other species of the genus *Cycloclasticus* **(29, 36, 99).** Geiselbrecht et al. **(36)** have suggested that *Cycloclusticus* is a numerically dominant species in their studied area. Our results suggest, however, that PAH degradation demonstrated under laboratory conditions does not necessarily translate to potential PAH degradation under environmental conditions.

The simple bioslurry setup employed in this study provides a rapid and easy screening of PAH degraders for use in bioremediation. Bioremediation or bioaugmentation involves capital-intensive investment such as elaborate pump- and well-systems and excavation of contaminated soil/sediments. ***An*** initial rapid screening method using small-scale laboratory setups can provide essential data for determining the feasibility of a particular bioremediation scheme **(79).**

BIBLIOGRAPHY

1. Aitken, M. D., W. T. Stringfellow, R. D. Nagel, C. Kazunga, and S. H. Chen. 1998. Characteristics of phenanthrene-degrading bacteria isolated from soils contaminated with polycyclic aromatic hydrocarbons. Can. J. Microbiol. 44:743-752.

2. Allen, C. C. R., D. R. Boyd, F. Hempenstall, M. J. Larkin, and N. D. Sharma. 1999. Contrasting effects of a nonionic surfactant on the biotransforrnation of polycyclic aromatic hydrocarbons to cis-dihydrodiols by soil bacteria. Appl. Environ. Microbiol. 65:1335-1339.

3. Aller, R. C. 1988. Benthic fauna and biogeochemical processes in marine sediments: The role of burrow structures, p. 301-338. *In* T. H. Blackburn, and J. Sorensen (eds), Nitrogen cycling in coastal marine environments. John Wiley & Sons, Chichester.

4. Aller, R. C. 1994. Bioturbation and remineralization of sedimentary organic matter: effects of redox oscillation. Chem. Geol. 114:331-345.

5. Aller, R. C. 1982. The effect of macrobenthos on chemical properties of marine sediment and overlaying water, p. 53-102. *In* P. L. McCall, and M. J. S. Tevesz (eds), Animal-sediment relations: the biogenic alterations of sediments. Plenum Publishing Company, New York.

6. Anderson, F. E., L. Black, L. E. Watling, W. Mook, and L. M. Mayer. 1981. A temporal and spatial study of mudflat erosion and deposition. J. Sed. Petrol. 51:729-736.

7. Arcangeli, J. P., and E. Arvin. 1995. Cometabolic transformation of o-xylene in a biofilm system under nitrate-reducing conditions. Biodegrad. **6:** 19-27.

8. Ashok, B. T., **S.** Saxena, and J. Musarrat. 1995. Isolation and characterization of four polycyclic aromatic hydrocarbon degrading bacteria from soil near an oil refinery. Lett. Appl. Microbiol. **21:** 246-248.

9. Balkwill, D. L., G. R. Drake, R. H. Reeves, J. K. Fredrickson, D. C. White, D. B. Ringelberg, D. P. Chandler, M. F. Romine, D. W. Kennedy, and C. M. Spadoni. 1997. Taxonomic study of aromatic-degrading bacteria from deep-terrestrial-subsurface sediments and description of *Sphingomonas aromaticivorans* sp. nov., *Sphingomonas subterranea* sp. nov., and *Sphingomonas stygia* sp. nov. Int. J. Syst. Bacteriol. **47:** 191-201.

10. Banta, G. T., M. Holmer, M. H. Jensen, and E. Kristensen. 1999. Effects of two polychaete worms, *Nereis diversicolor* and *Arenicola manna*, on aerobic and anaerobic decomposition in a sandy marine sediment. Aquat. Microb. Ecol. **19:** 189-204.

11. Bauer, J. E., and D. G. Capone. 1985. Degradation and mineralization of the polycyclic aromatic hydrocarbons anthracene and naphthalene in intertidal marine sediments. Appl. Environ. Microbiol. **50:** 81-90.

12. Bauer, J. E., and D. G. Capone. 1988. Effects of co-occurring aromatic hydrocarbons on degradation of individual polycyclic aromatic hydrocarbons in marine sediment slurries. Appl. Environ. Microbiol. 54: 1849-1865.

13. Bedessem, M. E., N. G. Swoboda-Colberg, and P. J. S. Colberg. **1997.** Naphthalene mineralization coupled to sulfate reduction aquifer-derived enrichments. FEMS Microbiol. Lett. **152:**213-218.

14. Beller, H. R., A. M. Spormann, P. K. Sjarma, J. R. Cole, and M. Reinhard. **1996.** Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Appl. Environ. Microbiol. **62:**1188-1196.

15. Berardesco, G., S. Dyhrman, E. Gallagher, and M. P. Shiaris. **1998.** Spatial and tempera1 variation of phenanthrene-degrading bacteria in intertidal sediments. Appl. Environ. Microbiol. **64:**2560-2565.

16. Blackburn, T. H., and N. D. Blackburn. **1993.** Rates of microbial processes in sediments. Phil. Trans. Royal. Soc. Lond. **344(A):**49-58.

17. Blackburn, T. H., N. D. Blackburn, K. Jensen, and N. Risgaard-Petersen. **1994.** Simulation model of the coupling between nitrification and denitrification in a freshwater sediment. Appl. Environ. Microbiol. **60:**3089-3095.

18. Boldrin, B., A. Tiehm, and C. Fritzsche. **1993.** Degradation of phenanthrene, fluoranthene, and pyrene by a *Mycobacterium* sp. Appl. Environ. Microbiol. **59: 1927-1930.**

19. Bossert, I. D., and L. Y. Young. **1986.** Anaerobic oxidation of p-cresol by a denitrifying bacterium. Appl. Environ. Microbiol. **52: 1117-1122.**

20. Bussman, I., and W. Reichardt. **1991.** Sulfate-reducing bacteria in temporarily oxic sediments with bivalves. Mar. Ecol. Prog. Ser. **78:**97-102.

21. Cerniglia, C. E. **1992.** Biodegradation of polycyclic aromatic hydrocarbons. Biodegrad. **3:**351-368.

22. Chung, W. K., and G. M. King. 1999. Biogeochemical transformations and potential polyaromatic hydrocarbon degradation in macrofaunal burrow sediments. Aquat. Microb. Ecol. **19:**285-295.

23. Chung, W. K., and G. M. King. 2001. Isolation and characterization of novel polyaromatic hydrocarbon degraders from macrofaunal burrow sediments. Appl. Environ. Microbiol. Appl. Environ. Microbiol. Accepted.

24. Coates, J. D., J. Woodward, J. Allen, P. Philp, and D. R. Lovley. 1997. Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. Appl. Environ. Microbiol. 63:3589-3593.

25. Dagher, F., E. Deziel, P. Lirette, G. Paquette, J. G. Bisaillon, and R. Villemur. 1997. Comparative study of five polycyclic aromatic hydrocarbon degrading bacterial strains isolated from contaminated soils. Can. J. Microbiol. **43:**368-377.

26. Delaune, R. D., J. Patrick, W.H., and M. E. Casselman. 1981. Effect of sediment pH and redox conditions on degradation of benz(a)pyrene. Mar. Pollut. Bull. **12:**251-253.

27. Denome, **S.** A., D. C. Stanley, E. **S.** Olson, and K. D. Young. 1993. Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* strains: complete DNA sequence of an upper naphthalene catabolic pathway. J. Bacteriol. **175:**6890-6901.

28. Dutta, T., **S.** Selifonov, and I. Gunsalus. 1998. Oxidation of methyl-substituted naphthalenes: Pathways in a versatile *Sphingomonas paucimobilis* strain. Appl. Environ. Microbiol. 64: 1884-1889.

29. Dyksterhouse, S. E., J. P. Gray, R. P. Herwig, J. C. Lara, and J. T. Staley. **1995.** *Cycloclasticus pugetii* gen. nov., sp. nov., an aromatic hydrocarbon-degrading bacterium from marine sediments. Int. J. Syst. Bacteriol. **45:**116-123.

30. Edwards, E. A., L. E. Wills, M. Reinhard, and D. Grbic-Galic. **1992.** Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate reducing conditions. Appl. Environ. Microbiol. **58:**794-800.

31. Evans, P. J., D. T. Mang, and L. Y. Young. **1991.** Degradation of toluene and m-xylene and transformation of o-xylene by denitrifying enrichment cultures. Appl. Environ. Microbiol. **57:**450-454.

32. Fenchel, T., G. M. King, and T. H. Blackburn. **1998.** Bacterial biogeochemistry: the ecophysiology of mineral cycling. Academic Press, New York.

33. Forbes, V. E., T. L. Forbes, and M. Holmer. **1996.** Inducible metabolism of fluoranthene by the opportunistic polychaete *Capitella* sp 1. Mar. Eco. Prog. Ser. **132:**63-70.

34. Fritzsche, C. **1994.** Degradation of pyrene at low oxygen concentrations by a *Mycobacterium* sp. Appl. Environ. Microbiol. **60: 1687-1689.**

35. Fuerst, J. A., J. A. Hawkins, A. Holmes, L. I. Sly, C. J. Moore, and E. Stackebrandt. **1993.** *Porphyrobacter neustonensis* gen. nov., sp. nov., an aerobic bacteriochlorophyll-synthesizing budding bacterium from fresh water. Int. J. Syst. Bacteriol. **43: 125-134.**

36. Geiselbrecht, A. D., B. P. Hedlund, M. A. Tichi, and J. T. Staley. **1998.** Isolation of marine polycyclic aromatic hydrocarbon (PAH)-degrading *Cycloclasticus* strains from the Gulf of Mexico and comparison of their PAH degradation ability

with that of Puget Sound *Cycloclasticus* strains. Appl. Environ. Microbiol. 64: 4703-4710.

37. Geiselbrecht, A. D., R. P. Herwig, J. W. Deming, and J. T. Staley. 1996. Enumeration and phylogenetic analysis of polycyclic aromatic hydrocarbon-degrading marine bacteria from Puget Sound sediments. Appl. Environ. Microbiol. **62:**3344-3349.

38. Gilewicz, M., Ni'matuzahroh, T. Nadalig, H. Budzinski, P. Doumenq, V. Michotey, and J. C. Bertrand. 1997. Isolation and characterization of a marine bacterium capable of utilizing 2-methylphenanthrene. Appl. Microbiol. Biotechnol. **48:**528-533.

39. Giray, C., and G. M. King. 1997. Effect of naturally-occurring bromophenols on sulfate reduction and ammonia oxidation in sediments from a Maine mudflat. Aquat. Microb. Ecol. **13:**295-301.

40. Grbic-Galic, D., and T. M. Vogel. 1987. Transformation of toluene and benzene by mixed methanogenic cultures. Appl. Environ. Microbiol. **53:**254-260.

41. Grifoll, M., **S.** Selifonov, C. Gatlin, and P. Chapman. 1995. Actions of a versatile fluorene-degrading bacterial isolate on polycyclic aromatic compounds. Appl. Environ. Microbiol. **61:**3711-3723.

42. Guerin, W. F., and G. E. Jones. 1988. Mineralization of phenanthrene by a *Mycobacterium* sp. Appl. Environ. Microbiol. **54:**937-944.

43. Hansen, K., G. M. King, and E. Kristensen. 1996. Impact of the soft-shell clam *Mya arenaria* on sulfate reduction in an intertidal sediment. Aquat. Microb. Ecol. **10:** 181-194.

44. Harayama, S. **1997.** Polycyclic aromatic hydrocarbon bioremediation design. Curr. Opin. Biotechnol. **8:**268-273.

45. Hedlund, B. P., A. D. Geiselbrecht, T. J. Bair, and J. T. Staley. **1999.** Polycyclic aromatic hydrocarbon degradation by a new marine bacterium, *Neptunomonas naphthovorans* gen. nov., sp. nov. Appl. Environ. Microbiol. **65:**251-259.

46. Jenkins, B. M., A. D. Jones, S. Q. Turn, and R. B. Williams. **1996.** Emission factors for polycyclic aromatic hydrocarbons from biomass burning. Environ. Sci. Technol. **30:2462-2469.**

47. Johnsen, K., S. Andersen, and C. Jacobsen. **1996.** Phenotypic and genotypic characterization of phenanthrene- degrading fluorescent *Pseudomonas biovars.* Appl. Environ. Microbiol. **62:**3818-3825.

48. Kanaly, R., R. Bartha, S. Fogel, and M. Findlay. **1997.** Biodegradation of [^{14}C]Benz[a]pyrene added in crude oil to uncontaminated soil. Appl. Environ. Microbiol. **63:**4511-4515.

49. Karlckhoff, S. W., and K. R. Morris. **1985.** Impact of tubificid oligochaetes on pollutant transport in bottom sediments. Environ. Sci. Technol. **19:**51-56.

50. Khan, A. A., R. F. Wang, W. W. Cao, W. Franklin, and C. E. Cerniglia. **1996.** Reclassification of a polycyclic aromatic hydrocarbon-metabolizing bacterium, *Beijerinckia* sp. strain B1, as *Sphingomonas yanoikuyae* by fatty acid analysis, protein pattern analysis, DNA-DNA hybridization, and 16S ribosomal DNA sequencing. Int. J. Syst. Bacteriol. **46:**466-469.

51. King, G. M. **1988.** Dehalogenation in marine sediments containing natural sources of halophenols. Appl. Environ. Microbiol. **54:**3049-3058.

52. King, G. M., M. J. Klug, and D. R. Lovley. **1983.** Metabolism of acetate, methanol, and methylated amines in intertidal sediments of Lowes Cove, Maine. Appl. Environ. Microbiol. **45:1848-1853.**

53. Koerting-Walker, C., and J. D. Buck. **1989.** The effect of bacteria and bioturbation by *Clymenella torquata* on oil removal from sediment. Water, *Air*, and Soil Pollution **43:**413-424.

54. Kosako, Y., E. Yabuuchi, T. Naka, N. Fujiwara, and K. Kobayashi. **2000.** Proposal of Sphingomonadaceae fam. nov., consisting of *Sphingomonas* Yabuuchi et al. **1990,** *Erythrobacter* Shiba and Shimidu **1982,** *Erythromicrobium* Yurkov et al. **1994,** *Porphyrobacter* Fuerst et al. **1993,** *Zymomonas* Kluyver and van Niel **1936,** and *Sandaracinobacter* Yurkov et al. **1997,** with the type genus *Sphingomonas* Yabuuchi et al. **1990.** Microbiol. Immunol. **44:**563-575.

55. Kristensen, E. **1988.** Benthic fauna and biogeochemical processes in marine sediments: Microbial activities and fluxes, p. **275-299.** *In* T. H. Blackburn, and J. Sorensen (eds), Nitrogen cycling in coastal marine environments. John Wiley & Sons, Chichester.

56. Kristensen, E. **1981.** Direct measurement of ventilation and oxygen uptake in three species of tubicolous polychaetes (*Nereis* spp). J. Comp. Physiol. **145(B):**45-50.

57. Kristensen, E. **1984.** Effect of natural concentrations on nutrient exchange between a polychaete burrow in estuarine sediment and the overlaying water. J. Exp. Mar. Biol. Ecol. **75:**171-190.

58. Kristensen, E. 1983. Ventilation and oxygen uptake by three species of *Nereis* (Annelida : Polychaeta). I. Effects of hypoxia. Mar. Ecol. Prog. Ser. **12:**289-297.

59. Kristensen, E. 1983. Ventilation and oxygen uptake by three species of *Nereis* (Annelida : Polychaeta). **11.** Effects of temperature and salinity changes. Mar. Ecol. Prog. Ser. **12:**299-306.

60. Kristensen, E., M. H. Jensen, and R. C. Aller. 1991. Direct measurement of dissolved inorganic nitrogen exchange and denitrification in individual polychaete *(Nereis virens)* burrows. J. Mar. Res. **49:**355-377.

61. Kristensen, E., M. H. Jensen, and T. K. Andersen. 1985. The impact of polychaete *(Nereis virens* Sars) burrows on nitrification and nitrate reduction in estuarine sediments. J. Exp. Mar. Biol. Ecol. **85:**75-91.

62. Kurkela, **S.,** H. Lehvaslaiho, E. T. Palva, and T. H. Teeri. 1988. Cloning, nucleotide sequence and characterization of genes encoding naphthalene dioxygenase of *Pseudomonas putida* strain NCIB98 16. Gene **73:**355-362.

63. Lane, D. J. 1991. 16S/23S rRNA Sequencing, p. 115-175. *In* E. Stackebrandt, and M. Goodfellow (eds), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York.

64. Larkin, M. J., C. C. R. Allen, and D. A. Lipscomb. 1999. Purification and Characterization of **a** Novel Naphthalene Dioxygenase from *Rhodococcus* sp. Strain NCIMB 12038. J. Bacteriol. **181:**6200-6204.

65. Long, E. R., D. D. MacDonald, **S.** L. Smith, and F. D. Calder. 1995. Incidence of Adverse Biological Effects Within Ranges of Chemical Concentrations in Marine and Estuarine Sediments. Environ. Manage. **19:**81-97.

66. Lotufo, G. R., and J. W. Fleeger. **1996.** Toxicity of Sediment-Associated Pyrene and Phenanthrene to *Limnodrilus hoffmeisteri* (Oligochaeta: Tubificidae). Environ. Toxicol. Chem. **15:**1508-1516.

67. Lovley, D. R., M. J. Baedecker, D. J. Longergan, L. M. Cozzarelli, J. P. Phillips, and D. I. Siegel. **1989.** Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature **339:**297-300.

68. McElroy, A. E., J. W. Farrington, and J. M. Teal. **1990.** Influence of mode of exposure and the presence of a tubiculous polychaete on the fate of Benz[a]anthracene in the benthos. Environ. Sci. Technol. **24: 1648-1655.**

69. Menzie, C. A., B. B. Potocki, and J. Santodonato. **1992.** Exposure to Carcinogenic PAHs in the environment. Environ. Sci. Technol. **26: 1278-1284.**

70. Meulenberg, R., H. H. M. Rijnaarts, H. J. Doddema, and J. A. Field. **1997.** Partially oxidized polycyclic aromatic hydrocarbons show an increased bioavailability and biodegradability. FEMS Microbiol. Lett. **152:**45-49.

71. Mihelcic, J. R., and R. G. Luthy. **1988.** Microbial degradation of acenaphthene and naphthalene under denitrification conditions in soil-water systems. Appl. Environ. Microbiol. **54: 1188-1198.**

72. Mikesell, M. D., J. J. Kukor, and R. H. Olsen. **1993.** Metabolic diversity of aromatic hydrocarbon-degrading bacteria from a petroleum-contaminated aquifer. Biodegrad. **4:**249-259.

73. Page, R. D. M. **1996.** TREEVIEW: An application to display phylogenetic trees on personal computers. Comp. Applic. Biosci. **12:**357-358.

74. Pelegrí, S. P., and T. H. Blackburn. **1996.** Effect of bioturbation by *Nereis* sp, *Mya arenaria* and *Cerastoderma* sp on nitrification and denitrification in estuarine sediments. Ophelia **42:**289-299.

75. Rabus, R., R. Nordhaus, W. Ludwig, and F. Widdle. **1993.** Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl. Environ. Microbiol. **59:1444-1451.**

76. Rasmussen, H., and B. B. Jorgensen. **1992.** Microelectrode studies of seasonal oxygen uptake in a coastal sediment: role of molecular diffusion. Mar. Ecol. Prog. Ser. **81:**289-303.

77. Reichardt, W. **1988.** Impact of bioturbation by *Arenicola marina* on microbiological parameters in intertidal sediments. Mar. Ecol. Prog. Ser. **44:149-158.**

78. Rice, D. L. **1986.** Early diagenesis in bioadvective sediments: relationships between the diagenesis of beryllium-7, sediment reworking rates, and the abundance of conveyor-belt deposit-feeders. J. Mar. Res. **44:149-184.**

79. Ringelberg, D. B., J. W. Talley, E. J. Perluns, S. G. Tucker, R. G. Luthy, E. J. Bouwer, and H. L. Fredrickson. **2001.** Succession of phenotypic, genotypic, and metabolic community characteristics during in vitro bioslurry treatment of polycyclic aromatic hydrocarbon-contaminated sediments. Appl. Environ. Microbiol. **67:1542-1550.**

80. Roslev, P., and G. M. King. **1994.** Survival and recovery of methanotrophic bacteria under oxic and anoxic conditions. Appl. Environ. Microbiol. **60:**2602-**2608.**

81. Sambrook, J., E. F. Fritsch, and T. Maniatis. (eds.) 1989 Molecular Cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

82. Sarcangeli, J. P., and E. Arvin. 1995. Cometabolic transformation of o-xylene in a biofilm system under nitrate reducing conditions. Biodegrad. 6: 19-27.

83. Sayama, M., and Y. Kurihara. 1983. Relationship between burrowing activity and the polychaetous annelid *Neanthes japonica* (Izuka) and nitrification-denitrification processes in the sediments. J. Exp. Mar. Biol. Ecol. **72:**233-241.

84. Schneider, J., R. Grosser, K. Jayasimhulu, W. Xue, and D. Warshawsky. 1996. Degradation of pyrene, benz[a]anthracene, and benzo[a]pyrene by *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site. Appl. Environ. Microbiol. 62: 13-19.

85. Scott, D. M. 1975. Respiration and ventilation of the polychaete *Nereis virens* sars. Ph.D. Thesis. University of Maine, Orono.

86. Sharak Genthner, B. R., G. T. Townsend, S. E. Lantz, and J. G. Mueller. 1997. Persistence of polycyclic aromatic hydrocarbon components of creosote under anaerobic enrichment conditions. Arch. Environ. Contam. Toxicol. **32:**99-105.

87. Shen, Y., L. G. Stehmeier, and G. Voordouw. 1998. Identification of Hydrocarbon-Degrading Bacteria in Soil by Reverse Sample Genome Probing. Appl. Environ. Microbiol. **64:**637-645.

88. Shiaris, M. P., and D. Jambard-Sweet. 1986. Polycyclic aromatic hydrocarbon in surfacial sediments of Boston Harbor, Massachusetts, USA. Mar. Poll. Bull. **17:**469-472.

89. Simon, M. J., T. D. Osslund, R. Saunders, B. D. Ensley, S. Suggs, A. Harcourt, W. C. Suen, D. L. Cruden, D. T. Gibson, and G. J. Zylstra. **1993.** Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB **9816-4.** Gene **127:**31-37.

90. Smibert, R. M., and N. R. Krieg. **1994.** Phenotypic characterization, p. **607-654.** *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (eds), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, DC.

91. Stringfellow, W. T., and M. D. Aitken. **1994.** Comparative physiology of phenanthrene degradation by two dissimilar pseudomonads isolated from a creosote-contaminated soil. Can. J. Microbiol. **40:**423-438.

92. Sverdrup, H. U., M. W. Johnson, and R. H. Fleming. **1942.** Chemistry of sea water, p. **165-227,** The Oceans their physics, chemistry, and general biology. Prentice-Hall, Inc., New York.

93. Takizawa, N., N. Kaida, S. Torigoe, T. Moritani, T. Sawada, S. Satoh, and H. Kiyohara. **1994.** Identification and characterization of genes encoding polycyclic aromatic hydrocarbon dioxygenase and polycyclic aromatic hydrocarbon dihydrodiol dehydrogenase in *Pseudornonas putida* OUS82. J. Bacteriol. **176:2444-2449.**

94. Tay, S., H. Hemond, M. Polz, C. Cavanaugh, I. Dejesus, and L. Krumholz. **1998.** Two new *Mycobacterium* strains and their role in toluene degradation in a contaminated stream. Appl. Environ. Microbiol. **64: 1715-1720.**

95. Van Bruggen, E. F. J., E. H. Wiebenger, and M. Gruber. 1960. Negative-staining electron microscopy of proteins at pH values below their isoelectric points. Its application to hemocyanin. Biochim. Biophys. Acta 42: 171-172.

96. Varanasi, U., W. L. Reichert, J. E. Stein, D. W. Brown, and H. R. Sanborn. 1985. Bioavailability and biotransformation of aromatic hydrocarbons in benthic organisms exposed to sediment from an urban estuary. Environ. Sci. Technol. **19:**836-841.

97. Vidal, C. M., A. A. Vitale, and A. A. Viale. 1999. Degradation of naphthalene-2-sulfonate by strains of *Micromonospora.* Rev. Argent. Microbiol. **31:**42-48.

98. Voparil, I. M., and L. M. Mayer. 2000. Dissolution of sedimentary polycyclic aromatic hydrocarbons into the Lugworm's (*Arenicola marina)* digestive fluids. Environ. Sci. Technol. **34:**1221-1228.

99. Wang, Y., P. C. Lau, and D. K. Button. 1996. A marine oligobacterium harboring genes known to be part of aromatic hydrocarbon degradation pathways of soil pseudomonads. Appl. Environ. Microbiol. **62:**2169-2173.

100. Yen, K., and C. M. Serdar. 1988. Genetics of naphthalene catabolism in Pseudomonads. CRC Crit. Rev. Microbiol. **15:**247-268.

101. Yunker, M. B., L. R. Snowdon, R. W. Macdonald, J. N. Smith, M. G. Fowler, D. N. Skibo, F. A. McLaughlin, **A.** I. Danyushevskaya, V. I. Petrova, and G. I. Ivanov. 1996. Polycyclic aromatic hydrocarbon composition and potential sources for sediment samples from the Beaufort and Barents Seas. Environ. Sci. Technol. **30:** 1310-1320.

102. Yurkov, V., E. Stackebrandt, A. Holmes, J. A. Fuerst, P. Hugenholtz, J. Golecki, N. Gad'on, V. M. Gorlenko, E. I. Kompantseva, and G. Drews. **1994.** Phylogenetic positions of novel aerobic, bacteriochlorophyll a-containing bacteria and description of *Roseococcus thiosulfatophilus* gen. nov., sp. nov., *Erythromicrobium ramosum* gen. nov., sp. nov., and *Erythrobacter litoralis* sp. nov. Int. J. Syst. Bacteriol. **44:**427-434.

103. Yurkov, V. V., and J. T. Beatty. **1998.** Aerobic anoxygenic phototrophic bacteria. Microbiol. Mol. Biol. Rev. **62:**695-724.

104. Zhang, X., and L. Young. **1997.** Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. Appl. Environ. Microbiol. **63:**4759-4764.

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