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APPLICATION OF CHEMICALLY ACCELERATED BIOTREATMENT TO REDUCE RISK IN OIL-IMPACTED SOILS

SEMI-ANNUAL REPORT

(NOVEMBER 2001 THRU APRIL 2002)

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ALL TASKS

For

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DISCLAIMER

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EXECUTIVE SUMMARY

The results presented within are a summary of research activities from November 2001 until the end of April 2002 between the Gas Technology Institute (GTI), formerly known as Institute of Gas Technology, Department of Energy (DOE), and Federal Energy Regulatory Commission (FERC) funds managed by the Gas Research Institute (now a component of GTI). This report incorporates the data presented not in the previous semiannual reports. Data continues to indicate that Chemically Accelerated Biotreatment (CAB) processes are effective for the restoration of Manufactured Gas Plant (MGP) site wastes and petroleum/natural gas exploration and production (E&P) sites. We developed and applied a number of modifications of bioavailability screens procedure to evaluate the application and efficiency of CAB using risk-based analysis, along with traditional analytical chemistry methods (SW 846, for example), for endpoint determinations. These results will be applied to the development of environmentally acceptable endpoints (EAE) as the indicator of CAB treatment effectiveness. CAB tests focused on issues associated with the *in-situ* application mode.

During this first 6 months of the third project year, research was conducted in six major focus areas:

1. Evaluation of the process using 6 test soils with full chemical and physical characteristics to determine controlling factors for biodegradation and chemical oxidation
2. Determination of the sequestration time on chemical treatment suspectability
3. Risk factors, i.e. toxicity after chemical and biological treatment
4. Impact of chemical treatment (Fenton's Reagent) on the agents of biodegradation
5. Description of a new genus and its type species that degrades hydrocarbons
6. Intermediates generate from Fenton's reagent treatment of various polynuclear aromatic hydrocarbons.

RESEARCH SUMMARY

Title	Application of chemically accelerated biotreatment to reduce risk in oil-impacted soils
Contractors	U.S. Department of Energy DOE Contract No. DE-AC26-99BC15223 Gas Technology Institute/Gas Research Institute GRI Contract No. 8054
Principal Investigator	J. Robert Paterek
Report Period	November 2001 through April 2002
Objective	The overall program objective is to develop and evaluate integrated biological/physical/chemical co-treatment strategies for the remediation of wastes associated with the exploration and production of fossil energy. The specific objectives of this project are: chemical accelerated biotreatment (CAB) technology development for enhanced site remediation, application of the risk based analyses to define and support the rationale for environmental acceptable endpoints (EAE) for exploration and production wastes, and evaluate both the technological technologies in conjugation for effective remediation of hydrocarbon contaminated soils from E&P sites in the USA.
Technical Approach	This project is part of the program of GTI/GRI in an effort to develop and enhance the microbiological approach to the degradation of wastes present at natural gas/petroleum production and exploration sites. It is of primary importance to this program to develop the capability to biologically degrade all the mobile or available fraction of the toxic or hazardous hydrocarbons present at these sites, including the "recalcitrant" aromatic moieties, which include those compounds containing elements other than carbon in the rings. To achieve this goal, research efforts focuses on enhancing the growth of microbial cultures with capabilities for biodegrading these compounds, aiding microbial activities by sequential or concurrent chemical oxidation of recalcitrant hydrocarbon moieties, determining the mobility and toxicity of the various hydrocarbon components in E&P wastes, in the conducting parametric studies to maximize detoxification of soils to meet "environmentally acceptable endpoints," and providing scientific and engineering supports for future application of *in-situ*, landfarming, and bioslurry reactor treatment technologies. Research efforts under the GRI project focused on evaluating the performance of selected cultures under a variety of bioremediation conditions in simulated "real world" and field conditions found at representative E&P sites.

EXPERIMENTAL

Roles of soil physicochemical parameters on contaminant sequestration and effect of sequestration on biodegradability and chemical treatability are listed :

- Six model soils collected from grass- and woodland areas in N. Illinois
- Extensive characterization for various physicochemical parameters
- Soils gamma-sterilized, spiked individually with model contaminants (hexadecane, phenanthrene, pyrene) or coal tar
- Following various periods of sequestration (0-120 days), biodegradability of each individual compound was assessed in each soil using bacterial strains known to degrade each
- After same periods, abiotic chemical treatments (standard Fentons's reaction with H_2O_2 and "Fenton-like" with CaO_2) were conducted on slurries of coal tar-contaminated soils, and removal of each individual (priority pollutant) PAH was determined

Physicochemical Characterization of Model Soils

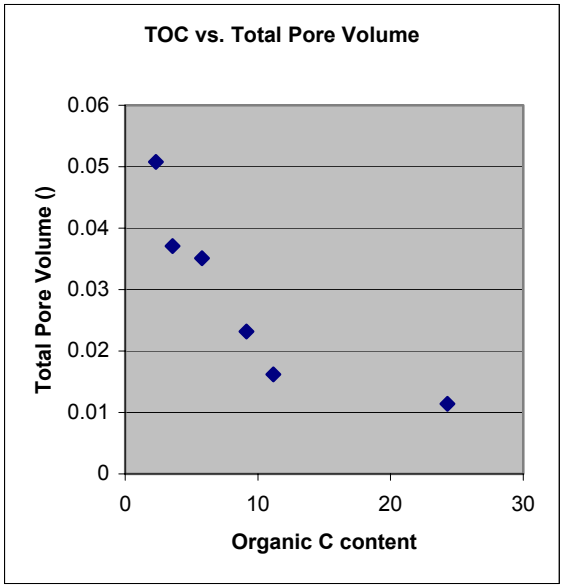
Total organic carbon was determined in two ways. The first of these, conducted by STAT Environmental, used the low-temperature (*ca.* 400 ºC) combustion method. We also used ashing at 900 ºC in a biological oxidizer. Results were as follows:

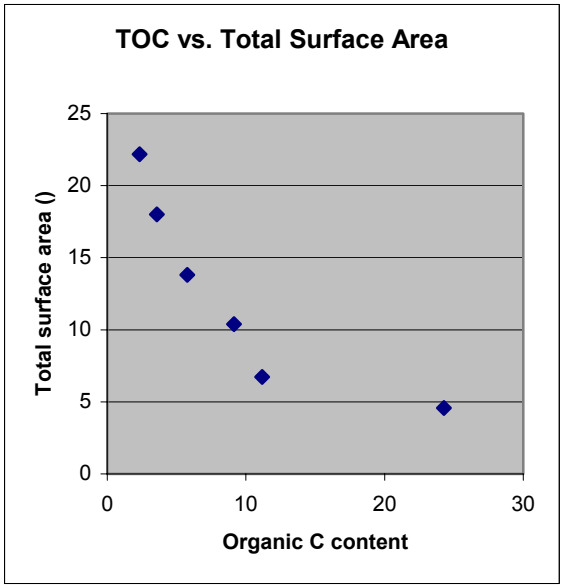
	Soil #					
	1	*2*	*3*	*4*	*5*	*6*
Low-temp combustion	**2.32**	**5.78**	**11.16**	**24.28**	**3.58**	**9.13**
High-temp combustion	**15.8**	**11.4**	**23.8**	**48.6**	**13.2**	**22.6**

Total micropore content, total surface area, and pore size distribution were conducted by the Pennsylvania State University Center for Innovative Sintered Products facility. Results for surface area and total pore volume showed a wide range of both of these parameters among the soils:

	Soil #					
	1	*2*	*3*	*4*	*5*	*6*
"Total" pore vol	0.0508	0.0351	0.0162	0.0114	0.0371	0.0232
"BJH total" pore vol	0.04473	0.02823	0.01754	0.01221	0.03354	0.02125
Total Surface Area	22.18	13.82	6.72	4.58	18.01	10.4

One thing that became clear from this data was that the total surface areas of the soil samples, as well as their total pore volumes (which would be expected to be related quantities) were both a strong function of soil organic matter content for five of the six soils under study. The one exception, soil #4, had a considerably higher total pore volume (and, hence, a higher surface area) than would be expected based on its TOC value, in comparison with the other soils:





With regard to pore size distribution, very little significant differences were seen among the six soils, with the exception of the fact that soils 1, 3, and 4 had a slightly higher content of large (> 80 μm) pores, as shown below:

Size	1	2	3	4	5	6
< 6 μm (%)	6.8	9.06	13.73	10.36	10.22	15.39
6 - 8 μm (%)	5.27	8.69	6.64	6.86	8.34	7.98
8 - 10 μm (%)	6.81	5.29	3.79	4.08	5.04	4.93
10 - 12 μm (%)	6.96	6.52	4.54	5.16	6.43	6.24
12 - 16 μm (%)	8.83	8.15	6.56	6.41	8.25	7.99
16 - 20 μm (%)	6.92	9.17	7.16	7.28	9.54	8.71
20 - 80 μm (%)	37.23	39.78	33.34	38.75	40.73	38.83
>80 μm (%)	21.19	13.33	24.24	21.1	11.44	9.93

Calculations were also performed which indicated that the actual geometries of the micropores in the various soils were also very similar (data not shown).

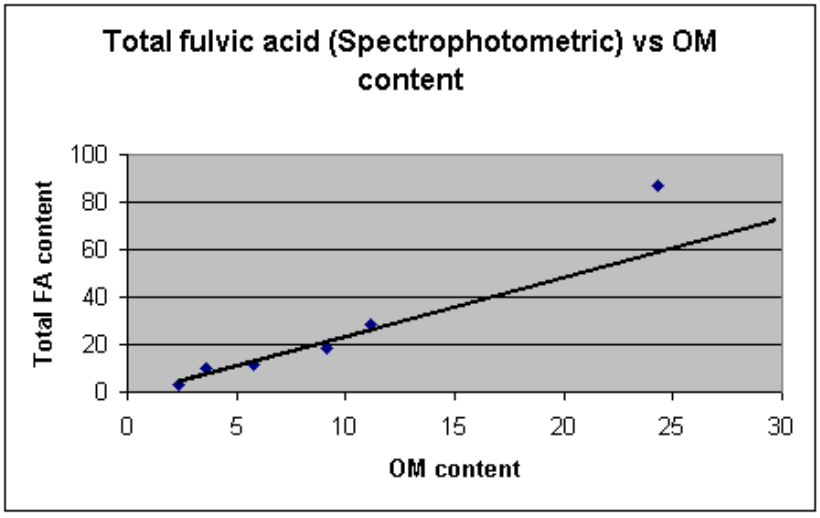
Particle size distributions were determined using the hydrometer method, and gave the following results:

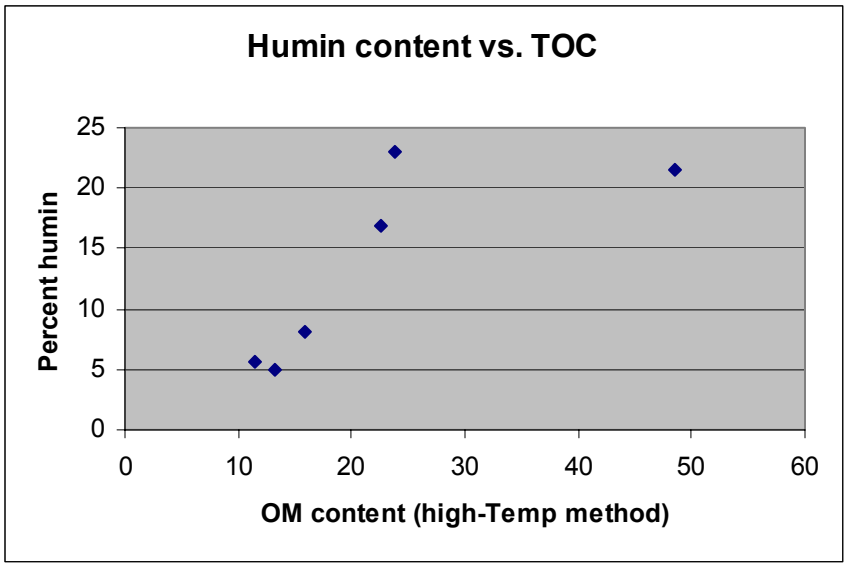
Soil #	% Sand	% Silt	% Clay	Textural classification
1	**25.4**	**51.1**	**27.5**	**Clay loam-Loam**
2	**64.0**	**25.3**	**10.7**	**Sandy loam**
3	**70.8**	**18.1**	**11.1**	**Sandy loam**
4	**73.5**	**10.6**	**15.9**	**Sandy loam**
5	**40.6**	**33.0**	**26.4**	**Loam**
6	**51.8**	**26.3**	**21.9**	**Sandy clay loam**

Humic acid quantitation was done according to methods put forth by the International Humic Substance Society. Fulvic acid was extracted as per the same methods, but was

not strictly quantified, as we had no access to a lyophilizer. Instead, relative amounts of fulvic acid extracted from each soil were determined spectrophotometrically (by absorbance at 400 nm), and the relative aromatic contents of the extracted fulvic acid fractions were measured in the same manner (ratio of A_{280} to A_{400}). Relative humin contents of the various soils were determined by ashing the residue remaining after extractions of humic and fulvic acids. Humic acid data showed that soil #2 was very rich in humic acids, and that this fraction in fact made up nearly all of the organic matter in this soil. In contrast, soil #1 showed almost no HA; the other soils were intermediate in value. Five of the six soils showed fulvic acid levels which were strongly a function of their overall organic matter content; however, soil #4, based on these results, had 30-50% more fulvic acid than would be expected. A similar patter was seen for humin content, with the inverse conclusion, namely, that soil #4 had a considerably lower humin/TOC ratio than the other five soils.

	Soil #					
	1	2	3	4	5	6
Humic acid content (%)	0.1	15.2	6.83	5.9	1.4	10.2





Data on the functional groups present in each soil's organic matter (*i.e*. aromatic vs. aliphatic carbon, oxygen content and relative polarity) was collected by solid-state ^{13}C-nuclear magnetic resonance at the Western Research Institute. Because of the low organic C present in soils 1 and 5, no meaningful data could be collected on these two

soils. Results for the other four soils showed, as follows, that there were only slight differences in the chemical makeup of the organic matter in the different soils (values given are percent of the total integrated area from the ^{13}C-NMR signal which fell into each of the functional group ranges given):

ppm	Functional group(s)	2	3	4	6
0-40	Branched & Straight-chain Aliphatics	22	17	20	18
40-90	Ethers	41	38	34	37
90-120	Ethers	13	15	11	11
120-140	Aromatic (Various ring C)	8	9	9	9
140-165	Phenolic	5	9	9	7
165-190	Carboxylic acid/Ester	11	8	10	12
190-250	Carbonyl/Ketone	0	4	7	6

Because of the incompleteness of the NMR data, pyrolysis GC-MS will be conducted on all soils in order to better address the question of chemical composition of the various organic matter fractions.

Biodegradability of various hydrocarbon contaminants in soils and impact of increasing sequestration over time:

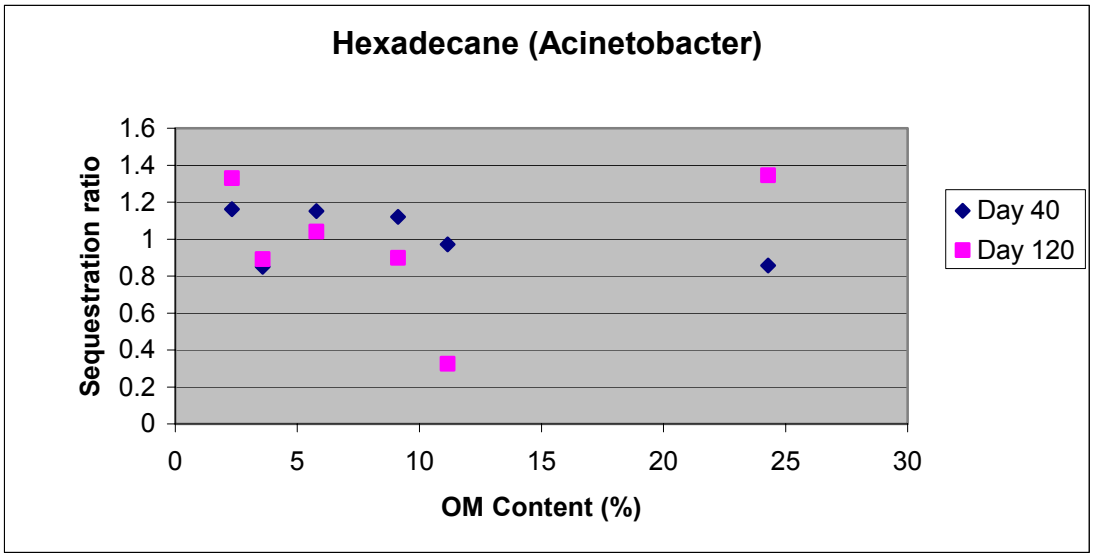
- Mineralization of each contaminant measured (using appropriate bacteria) after 0, 40, or 120 days of contact time
- "Sequestration ratio" defined as ratio of mineralization after contact to that in systems inoculated immediately after contaminant addition (in reality, there was a delay of ca. 3 days between spiking and inoculation, due to time needed to gamma-sterilize the soil microcosms)
- Degradability of hexadecane by an isolate of *Acinetobacter* was not appreciably affected by 40 days of contact with any of the model soils - as shown below, sequestration ratio remained near 1. This implies that hexadecane is not appreciably

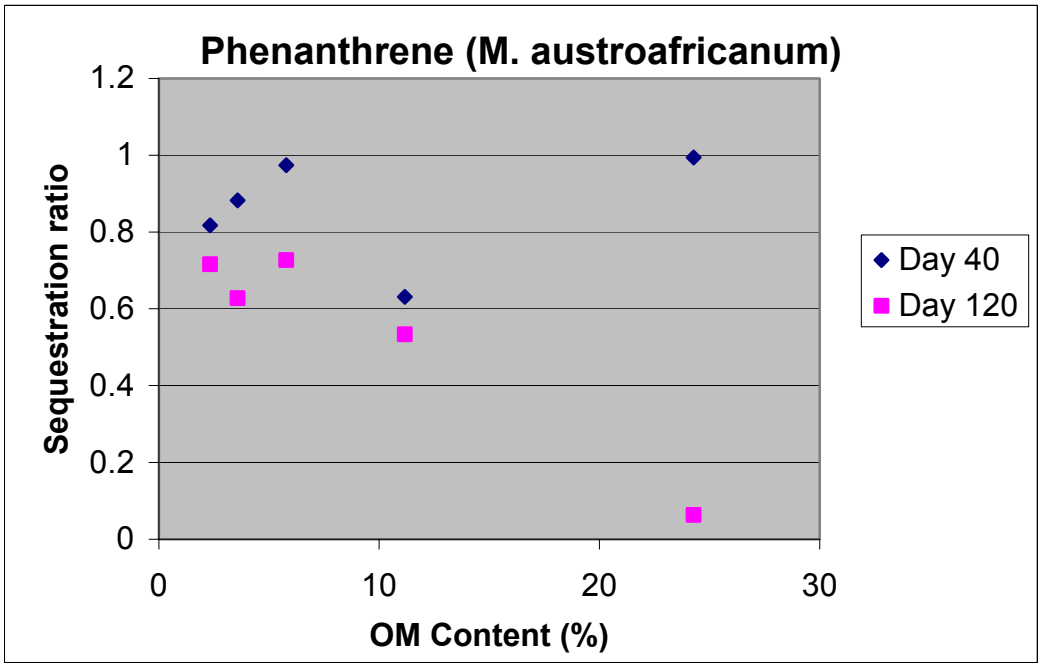
sequestered in any soil over this time frame. However, there did appear to be an effect after 120 days of contact: With 5 of the 6 soils, sequestration was strongly dependent on TOC; the exception was soil #4, in which the "sequestration ratio" value was higher than expected, meaning that biodegradation was higher than expected at this timepoint. Thus, there is some physicochemical parameter (or combination of multiple parameters) that causes soil 4 to exhibit much less sequestration of hexadecane than the other 5 soils.

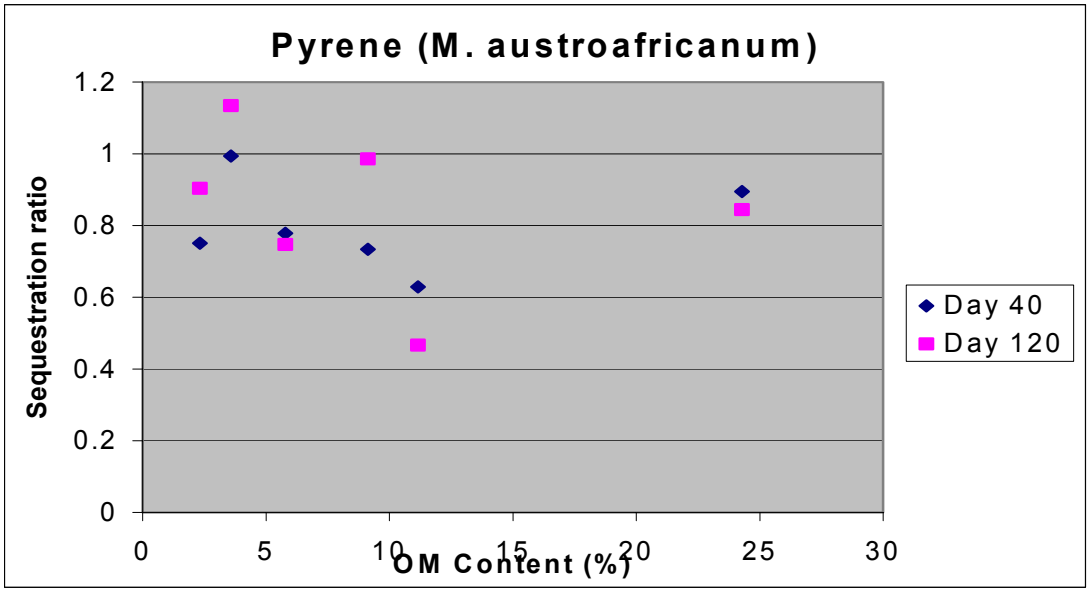
Mineralization of phenanthrene (model low-molecular weight PAH) by *Mycobacterium austroafricanum* showed a very similar pattern. Again, there was no notable sequestration effect within 40 days, but a very significant sequestration after 120 days, which was, in the case of phenanthrene, strongly TOC-dependent across all five soils examined (Soil #6 could not be included in this experiment).

- Pyrene (model HMW PAH) mineralization by *M. austroafricanum* showed some definite trends toward TOC-dependent sequestration, both at 40 and 120 days. However, in both of these cases, soil #4 was, as with hexadecane (above) considerably less prone to sequestration than were the other five model soils.
- Both hexadecane and pyrene were less sequestered (more bio-available) in soil #4 than expected based on the relationships seen in the other soils. Soils #4is similar to all of the other model soils in many respects (*i.e*. pore-size distribution, particle-size distribution, organic matter chemical composition); however, it does have higher-than expected surface are and pore volume, and also displays considerably lower-than-expected humin content and higher-than-expected fulvic acid content. Other workers have shown that soluble organic matter (*i.e.* fulvic acids) can increase water "solubility" of PAH in contaminated soils by an order of magnitude or more; thus, we are determining if this is occurring in this system, and planning and conducting experiments on possible fulvic acid uptake by *M. austroafricanum.*
- Taken together, these results demonstrate significant reductions in bioavailability of PAH in (especially in soils with high organic carbon content) over relatively short periods (*e.g*. 120 days), even when "receptor" organisms are used which are well-

adapted to the uptake of PAH. Conversely, results show that biotreatment (alone or as a component of chemical-biological approaches will be progressively less effective in these cases (if strict removal of PAH is the sole criterion).







Susceptibility to chemical oxidation of coal tar hydrocarbons in soils and impact of increasing sequestration over time:

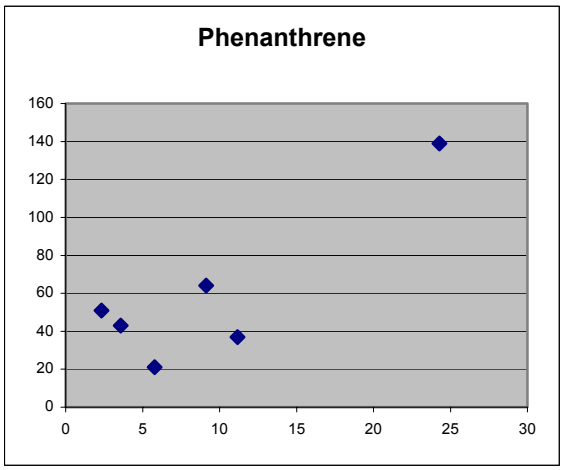
- Model soils spiked with coal tar (1000 ppm total tar concentration, resulting in total PAH content of ~250 ppm)
- Degradability of priority pollutant tar PAHs determined in H_2O_2/Fe(II) and CaO_2/Fe(II) reactions (abiotic) after 0, 40, and 80 days contact time (120-day time point will also be conducted)
- Time-zero data showed that, for all six of the six soils examined, TOC was the major determinant governing PAH degradability. This is most likely due to two factors, namely sorption of PAH onto SOM retarding its oxidation and the fact that the soils organic matter consumes some portion of the oxidizing equivalents (·OH) generated by the reaction.
- As hydrophobicity of individual PAH increases, the linearity of this function increases TOC (or some other factor which it represents) becomes the only governing factor over PAH degradability:
- Experiment conducted at day 40 showed this relationship still held for 4 of the 6 soils, but that notable deviations were occurring, particularly for in soils 1 and 5. Both of these soils showed considerably poorer removal of PAH than expected based on TOC

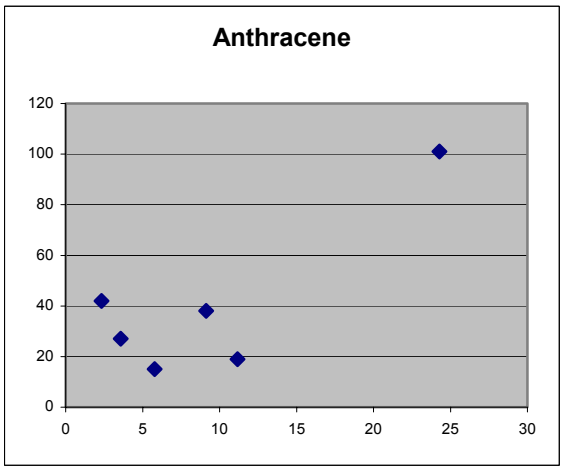
content alone. Deviations at Day 40 appeared to be larger for low-molecular-weight PAHs. Note that these two soils also have the highest pore content (see above) of the six.

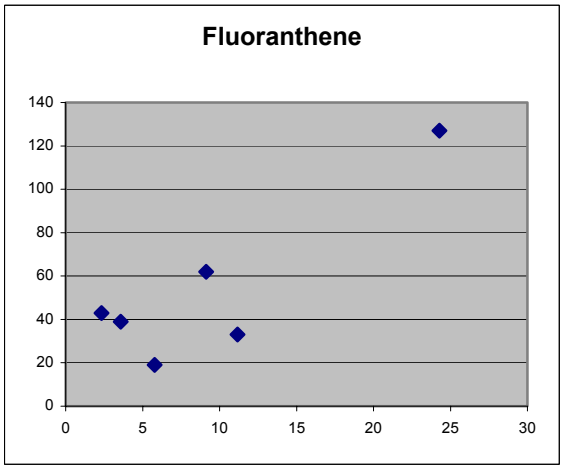
- Day 80 degradability data is similar in pattern to that at day 40, but the slope of the line formed by soils 1 and 5 (i.e. the deviation from the line based on TOC) appears to have increased for most PAHs - this is apparently indicative of increasing migration of PAH into the pore spaces in these soils, which further retards the accessibility of the PAH to the chemical oxidant.
- Following completion of the day-120 experiment, all data from this experiment will be subjected to further mathematical modeling to generate a manuscript on the effects of PAH sequestration on their chemical oxidizability.

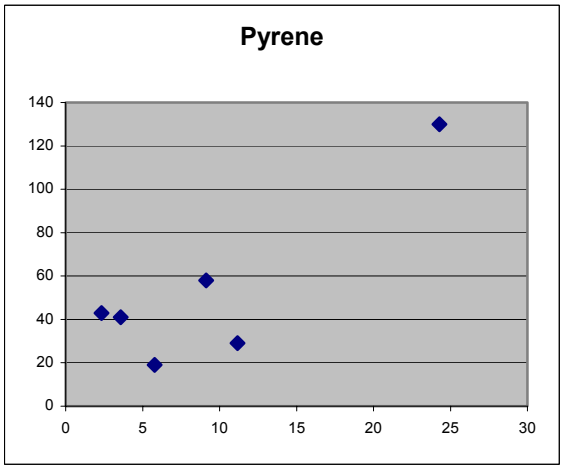
PAH recovered (% of no-oxidant control – "y" axis) vs. TOC.

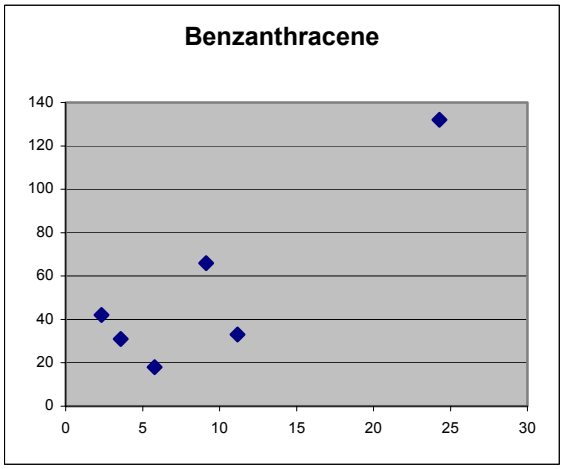
Timepoint #1 – "0" Days sequestration

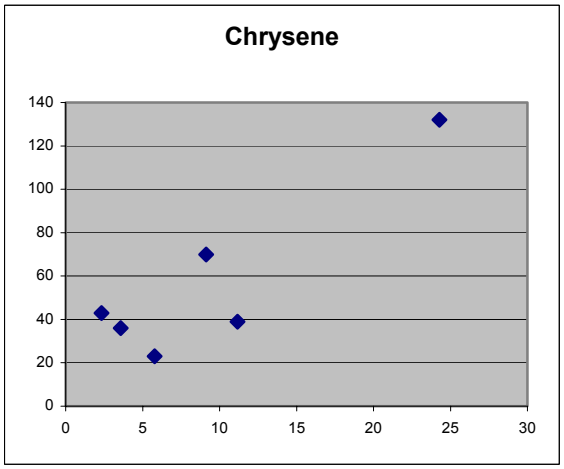












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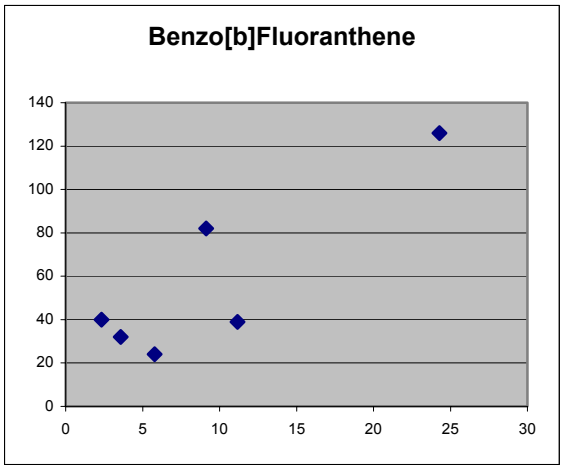
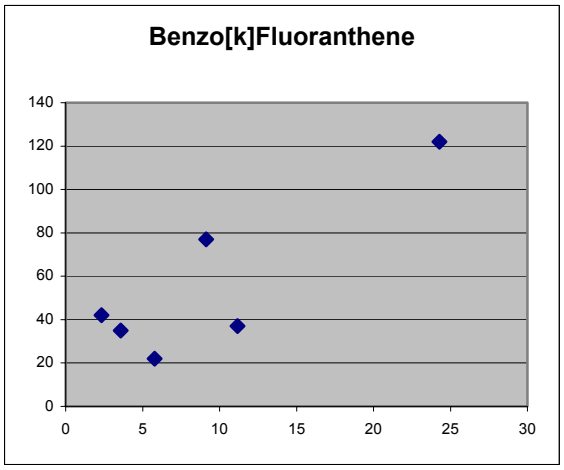
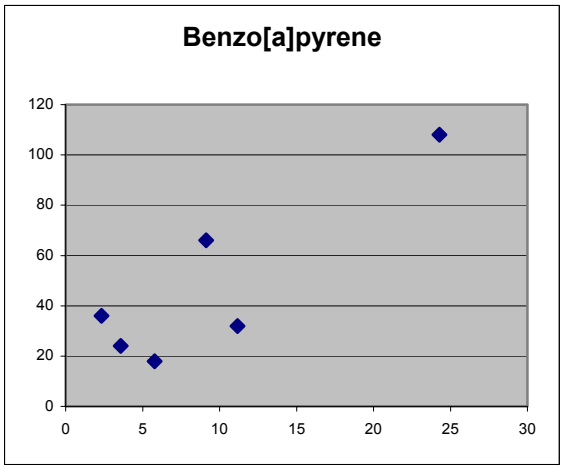
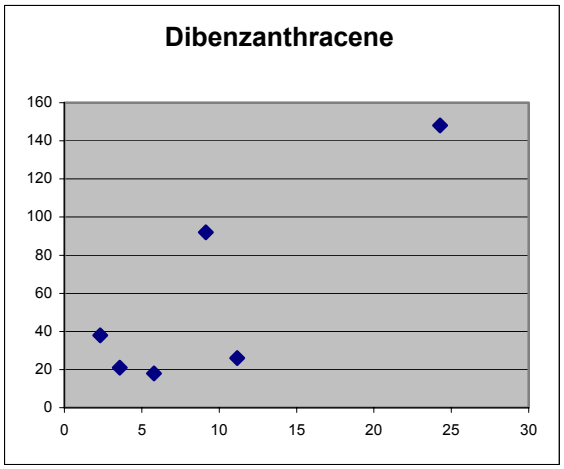
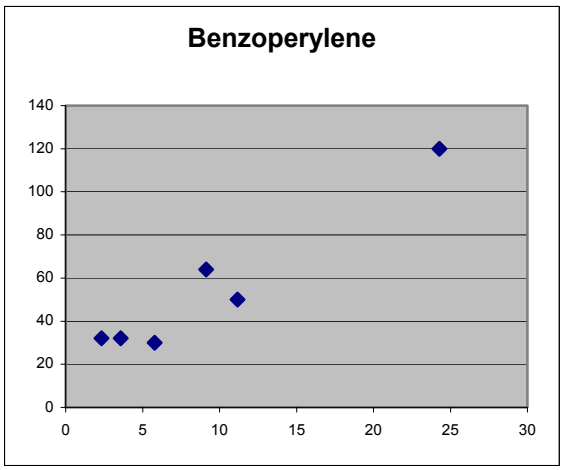
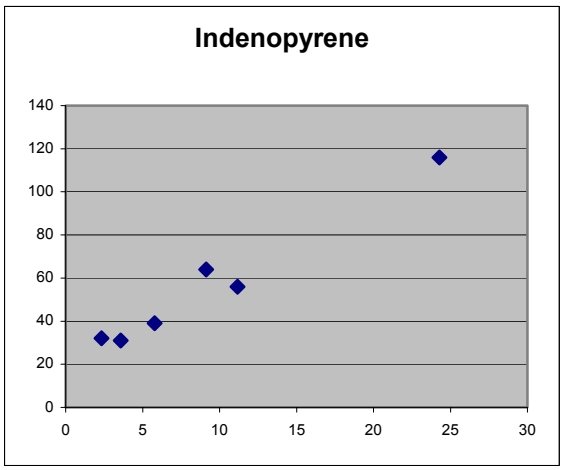




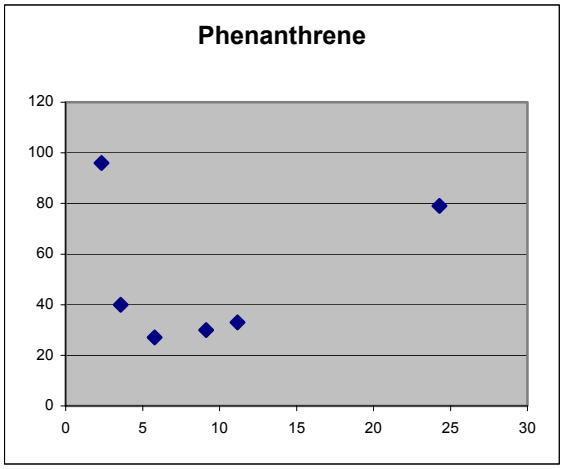


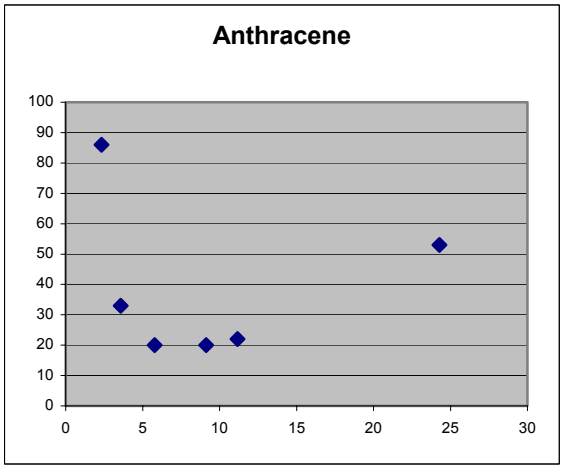


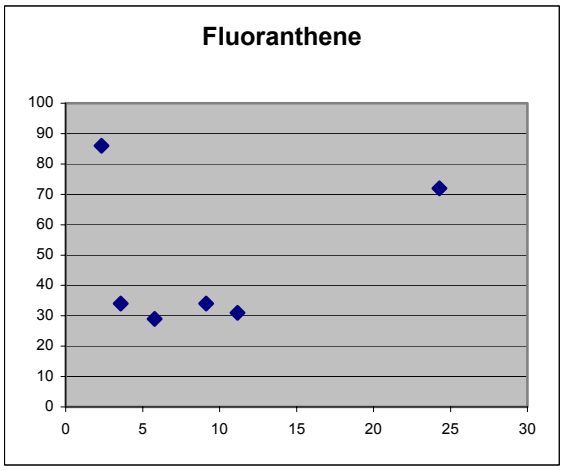


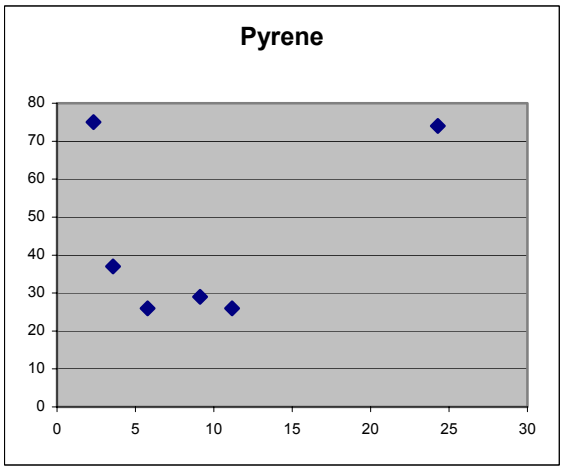


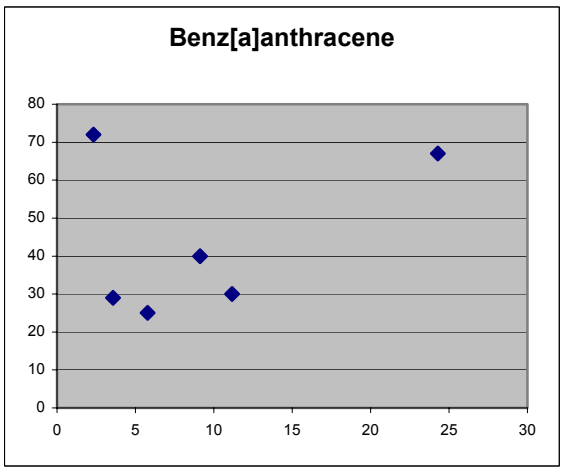
PAH recovered (% of no-oxidant control – "y" axis) vs. TOC.
TIMEPOINT #2 – 40 DAYS SEQUESTRATION

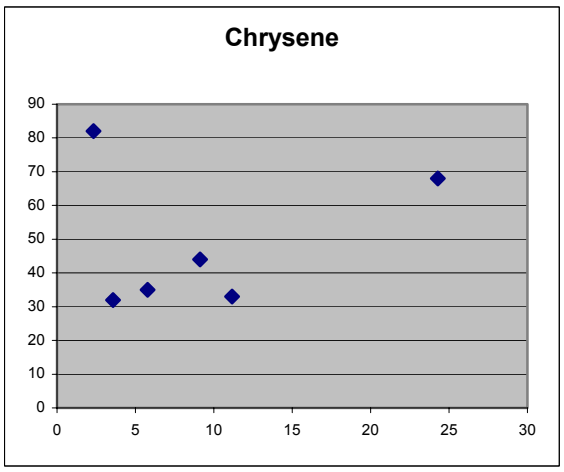












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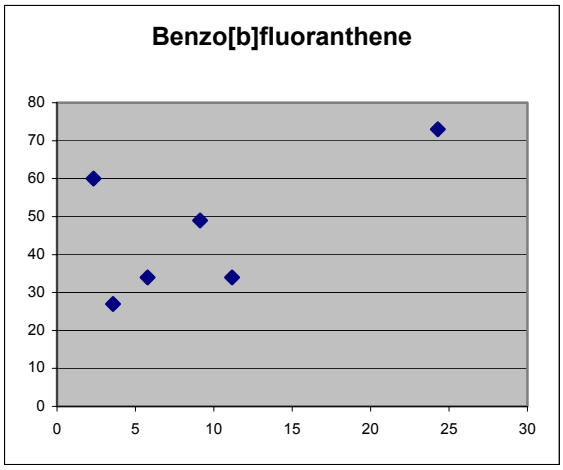
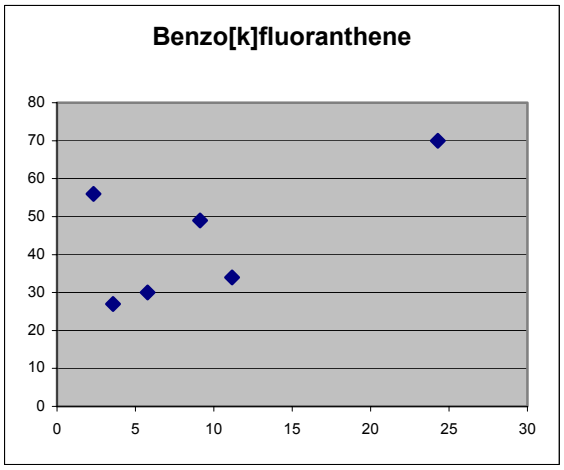
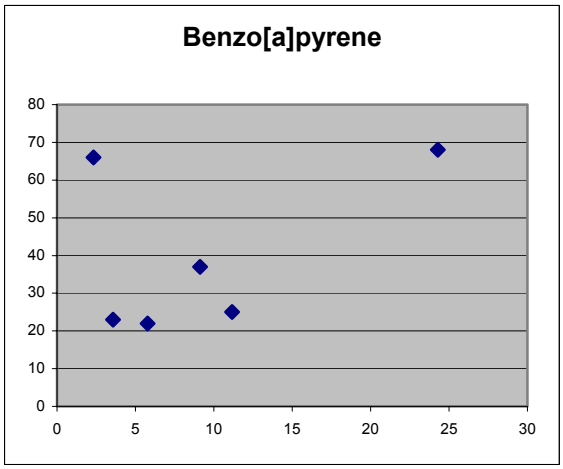
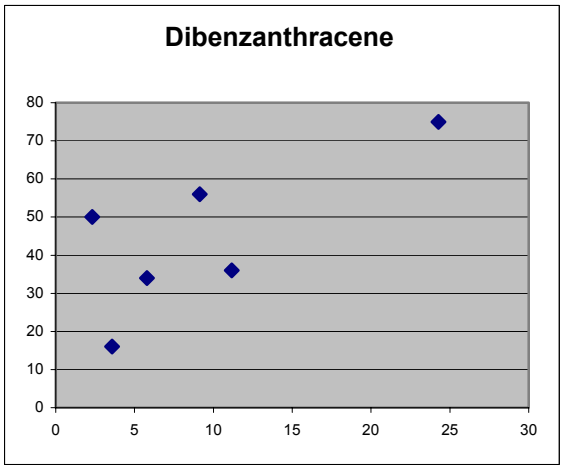
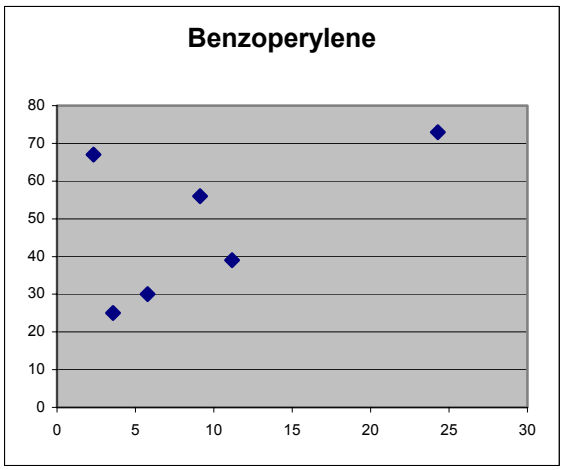
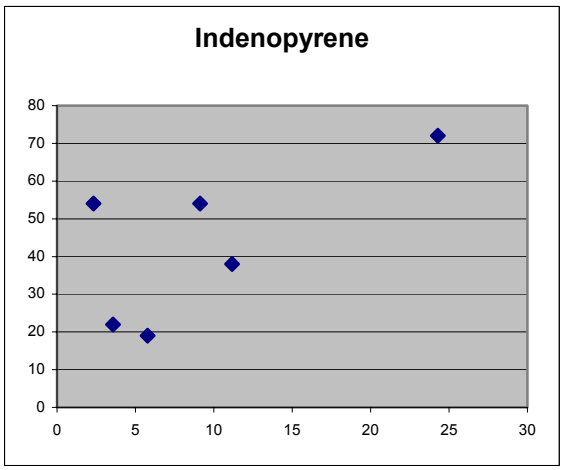




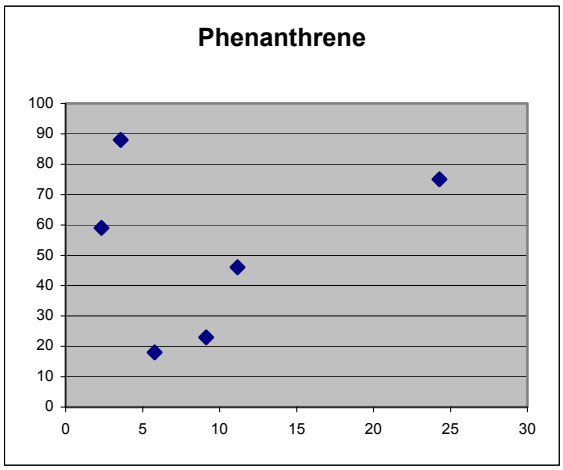


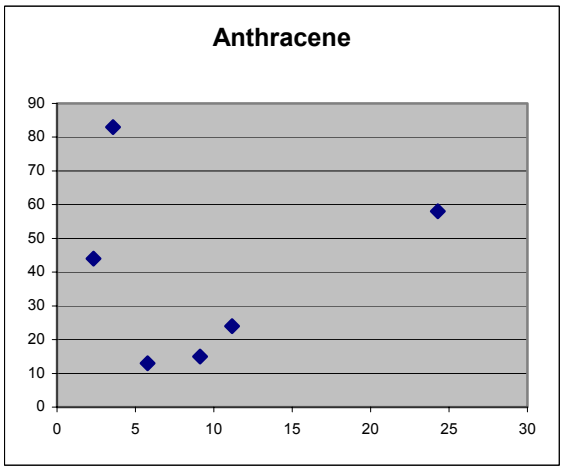


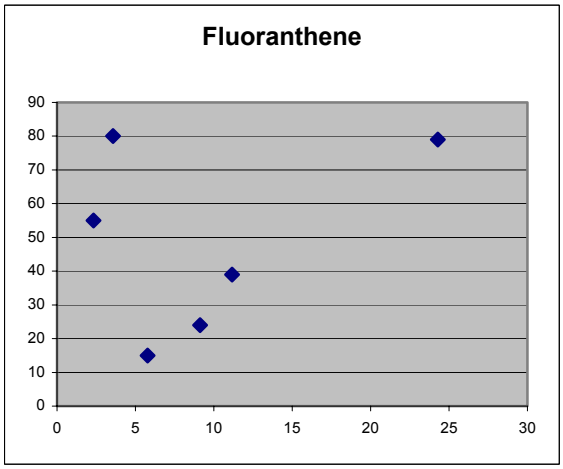


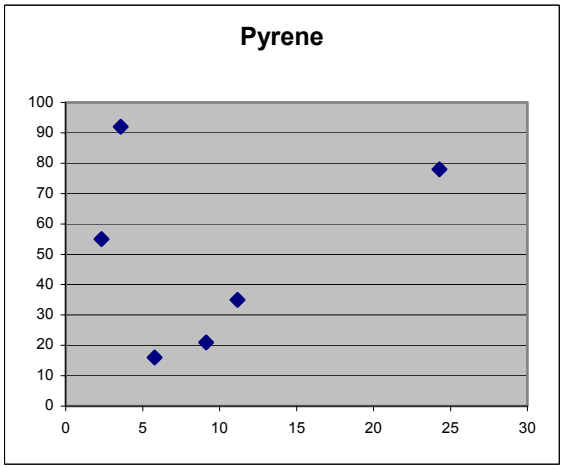


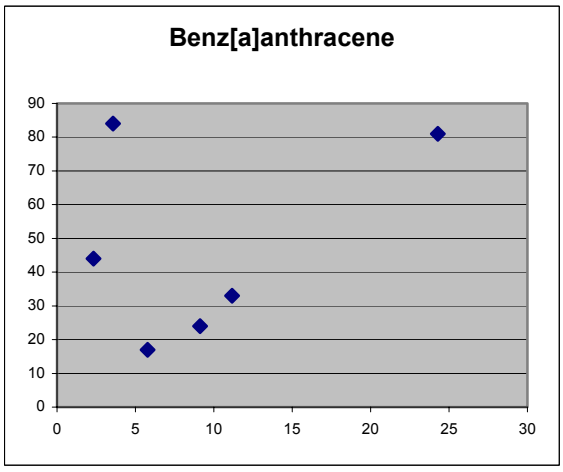
PAH recovered (% of no-oxidant control – "y" axis) vs. TOC.
TIMEPOINT #3 – 80 DAYS SEQUESTRATION

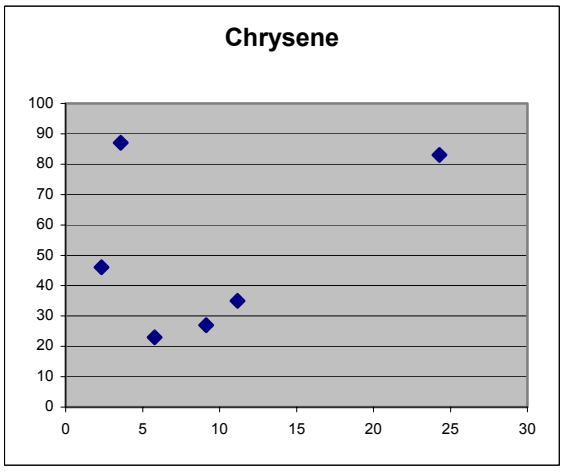




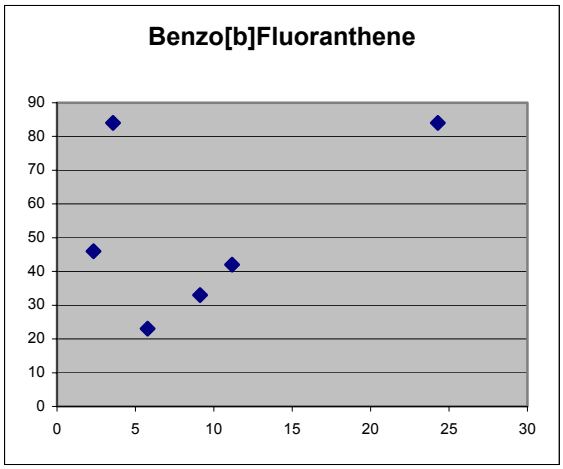


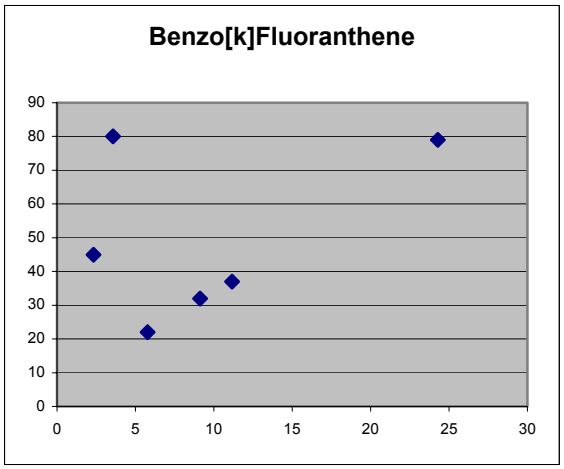


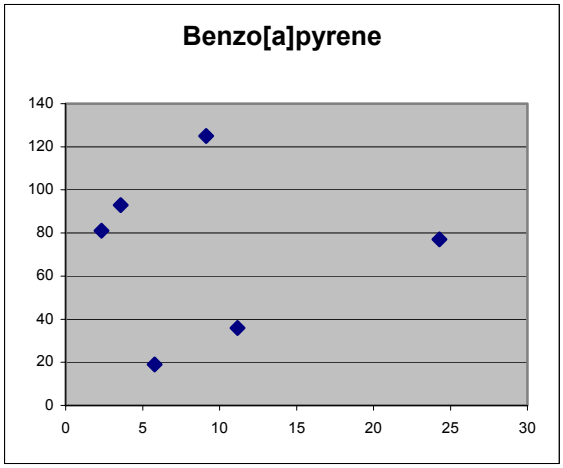


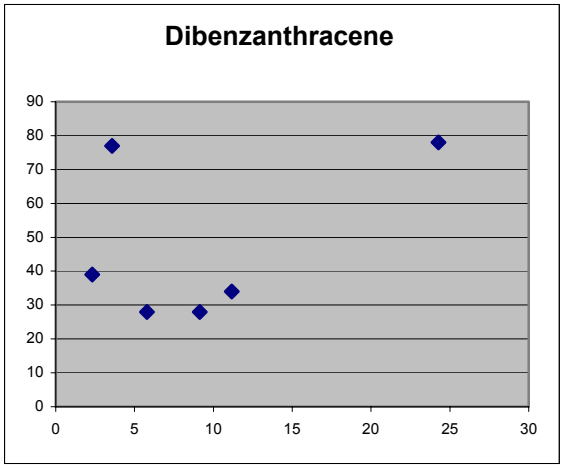


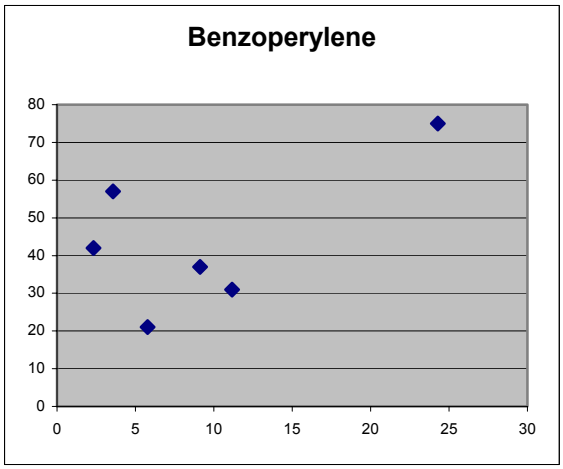
Timepoint #3 (cont.)

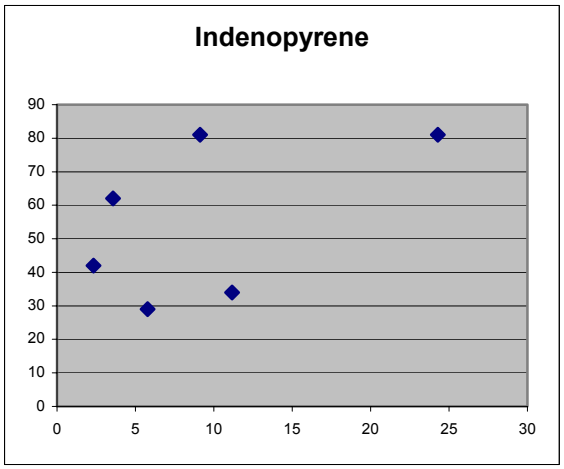












Fenton's Reagent Generation of Intermediates and Their Identification

Instrumentation

The GC/MS system that we have available in the Environmental Science and Technology Center is a ThermoFinnigan TraceGC PolarisQ ion trap system equipped with an AS2000 liquid sample autosampler. This GC/MS system can be operated in the electron ionization (EI) mode as well as positive and negative chemical ionization (+/- CI) modes. In addition, this GC/MS is capable of an MS^n analysis up five times. All of these features are highly valuable for the identification and confirmation of unknown compounds. The samples are introduced into the GC through a programmed temperature vaporizing (PTV) injector. This type of injector can be used as a regular split/splitless injector or as a PTV injector, which is ideal for large volume injection as well as for the analysis of polycyclic aromatic hydrocarbon (PAH).

Quantitative Analysis

The protocol that we use for the quantification of PAH compounds is the modified EPA method for the analysis of semivolatile organic compounds (Method 8270C) by GC/MS selected ion mass (SIM) mode. This method is used to determine the concentrations of PAH as well as other semivolatile organic compounds in extracts from soil, sediment and water samples. The column that is used for this analysis is a 30 m × 0.25 mm ID 0.25 μm film thickness silicone-coated fused silica capillary column (Restek DB-5 MS or equivalent). Five internal standards are used to quantify the compounds of interest (naphthalene-d_8, acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12}, and perylene-d_{12}).

Qualitative Analysis

The same GC/MS conditions are used for the qualitative analysis of semivolatile organic compounds. The peaks in the total ion chromatography (TIC) for each sample are identified using interactive chemical information system (ICIS™) algorithm. The spectrum of each identified peak is enhanced using the combined algorithm and searched

with the spectra in the Nation Institute of Standards and Technology (NIST) library. The spectra indices and probability for the matches are used as a guideline for the identification of the unknown compound. The GC elution time as well as the spectra generated by +/- CI and MS^n modes of a known standard can all be used to confirm the identity of the unknown compound.

100 mg of five individual PAHs (phenanthrene, anthracene, fluoranthene, pyrene, and benzo(a)pyrene) were added to Fenton's reactions (25 mL water, 0.85 mL 30% H_2O_2, 250 μL 1 M $FeSO_4.7H_2O$) as granules in duplicating serum bottles. The first set was taken down from the shake table after 2 weeks, while the second set was taken down after 4 weeks. Two different extractions were made with the separatory funnel liquid-liquid extraction (EPA Method 3510C), once when at the pH of 7 (adjusted with 0.5 M NaOH) and once at the pH of 2 (adjusted with 1M H_2SO_4). Water was removed from these extracts with hexane rinsed anhydrous $NaSO_4$. Methylene chloride in each extract was evaporated down to a final volume of approximately 2 mL.

Each extract was analyzed with modified EPA Method 8270. The chromatogram generated by each extract was qualitatively analyzed by Xcalibur 1.2 using ICIS peak identification algorithm (parameters: peak smoothing = 7, area noise factor = 2, and peak noise factor = 30). The identified peaks were limited to peaks that were at least 1% of the highest peak height. The spectra of the identified peaks were enhanced using the combined algorithm (parameters: peak top region = 4 points, background subtraction = 5 points on the left and right of the peak start and peak end regions, respectively). The enhanced spectra were searched with spectra in the NIST library; five top hits are displayed with the spectra indices and probability for the matches.

<u>Neutral Extracts</u>

- Of the 5 PAHs (phenanthrene, anthracene, fluoranthene, pyrene, and benzo(a)pyrene), fluoranthene appears to be most easily degraded by Fenton's reaction (~60% left), possibly because not all made up of benzene rings; however, pyrene was the second most easily degraded by Fenton's reaction (~70% left).

- For some reason, there appears to be more degradation after 2 weeks than after 4 weeks.
- For phenanthrene, all intermediates are composed of 2 benzene rings and some "break-down" structure (e.g., 5-C ring ot 6-C ring that is not aromatic).
- For anthracene, there was some rearrangement → get phenanthrene, some intermediates found in phenanthrene reaction, and some methylation (C1-of 3-ring benzene).
- For fluoranthene, the most abundant intermediate was more complex (4-ring benzene); other intermediate were products of hydration, rearrangement, & methylatio.n
- For pyrene, all intermediates were composed of 2-3 benzene ring, some oxygenated.
- For benzo(a)pyrene, there was an elimination of 1 benzene ring, and most intermediates were seen in pyrene reaction.

Phenanthrene Intermediates

- In the 2-week extract, 5 chemical fragments were identified, while only 3 fragments were identified in the 4-week extract.
- Phenanthrene was 94% of the total peak area in the 2-week extract and was 97% of the total peak area in the 4-week extract.
- The most abundant intermediate of phenanthrene was dibenzothiophene (2-3% of total peak area).
- The second most abundant intermediate of phenanthrene was Anthracene-maleic anhydride Diels-Alder adduct or Pentacyclo[6.6.5.0(2,7).0(9,14).0(15,19)]nonadeca-2,4,6,9,11,13,16-heptaen-16-ol-18-one (~1% of total peak area)
- The other 2 fragments that were identified in the 2-week extract but not in the 4-week extract were relatively small; they are Fluorene or 1H-Phenalene and Anthracene, 9,10-dihydro-

Anthracene Intermediates

- In the 2-week extract, 7 chemical fragments were identified, while 6 fragments were identified in the 4-week extract.
- Anthracene was 87% of the total peak area in the 2-week extract and was 94% of the total peak area in the 4-week extract.
- The most abundant intermediate of anthracene was N-Hydroxymethylcarbazole or Carbazole (3-6% of total peak area).
- The second most abundant intermediate of anthracene was phenanthrene (1-3% of total peak area).
- The third most abundant intermediate of anthracene was C1-anthracene or C1-phenanthrene (~ 1% of total peak area).
- The forth most abundant intermediate of anthracene was Dibenzothiophene (~ 1% of total peak area).
- The fifth most abundant intermediate of anthracene was Fluorene or 1H-Phenalene (~ 1 % of total peak area).
- The peak that was only identified in the 2-week extract but not in the 4-week extract was quite small; it was identified as Dibenzofuran-2-sulphonic acid.

Fluoranthene Intermediates

- In the 2-week extract, 9 chemical fragments were identified, while 7 fragments were identified in the 4-week extract.
- Fluoranthene was ~ 60% of the total peak area.
- The most abundant intermediate of fluoranthene was pyrene (~ 26% of total peak area).
- The second most abundant intermediate of fluoranthene was Acephenanthrylene, 4,5-dihydro- or 1H-Indene, 1-(phenylmethylene)- or Anthracene, 9-ethenyl- or Naphthalene, 1-phenyl- (6-8% of the total peak area).
- The rest of the intermediates were less than 0.5% of the total peak area; they were identified as: anthracene, phenanthrene, methyl-phenanthrene/methyl-anthracene, and methyl-pyrene/methyl-fluoranthene.

Pyrene

- In the 2-weekextract, 12 chemical fragments were identified, while 9 chemical fragments were identified in the 4-week extract.
- Pyrene was ~ 70% of the total peak area.
- The most abundant intermediate of pyrene was most likely Benzene, 1,1'-(1,3-butadiyne-1,4-diyl)bis- (~3% of the total peak area)
- The second most abundant intermediate of pyrene was not identifiable with great certainty; but may possibly be Phenanthrene, 9-methoxy (~1% of total peak area)
- The third most abundant intermediate of pyrene was Benzo[b]naphtho[2,1-d]furan or Benzo[kl]xanthen (~1% of total peak area)
- The rest of the intermediates were less than 0.5% of the total peak area; they were identified as: Phenanthrene, Anthracene, 4H-Cyclopenta[def]phenanthrene, 1H-Indene, 1-(phenylmethylene)- or Acephenanthrylene, 4,5-dihydro- or Anthracene, 9-ethenyl- or 1,9-Dihydropyrene or 3,10B-Dihydrofluoranthene ($C_{16}H_{12}$; MW = 204 compound)

Benzo(a)pyrene

- In the 2-weekextract, 9 chemical fragments were identified, while 7 chemical fragments were identified in the 4-week extract.
- Benzo(a)pyrene was 90-97% of the total peak area.
- The most abundant intermediate of benzo(a)pyrene was pyrene (~7% in the 2-week extract; but <0.5% in the 4-week extract).
- The second most abundant intermediate of benzo(a)pyrene was identified as Perylene, 3-methyl- or 11H-Indeno[2,1-a]phenanthrene or 13H-Dibenzo[a,h]fluorene ($C_{21}H_{14}$; MW=266 compound) (~1% of the total peak area in both extracts).
- The rest of the intermediates were less than 0.5% of the total peak area; they were identified as: Fluoranthene, Triphenylene or Benz[a]anthracene or Chrysene ($C_{18}H_{12}$; MW=228 compounds), and possibly Dibenz[a,h]anthracene, 5,6-dihydro- or Phenanthrene, 9-ethyl-3,6-dimethoxy-10-methyl-.

Fenton's Reagent Generated Intermediate Identification for Anthracene

Five milligrams of anthracene was completely dissolved in 50 mL ethanol in duplicating serum bottles. Five milliliters of 1M $FeSO_4.7H_2O$ was then added to each bottle. The pH of each solution was subsequently adjusted to 3.5 - 4.5 using 1M H_2SO_4. Finally, 5-mL of 30% H_2O_2 was added to initiate Fenton's reaction. Fenton oxidation of the first bottle was terminated after 30 minutes, while the reaction in the second bottle was allowed to react for 75 minutes.

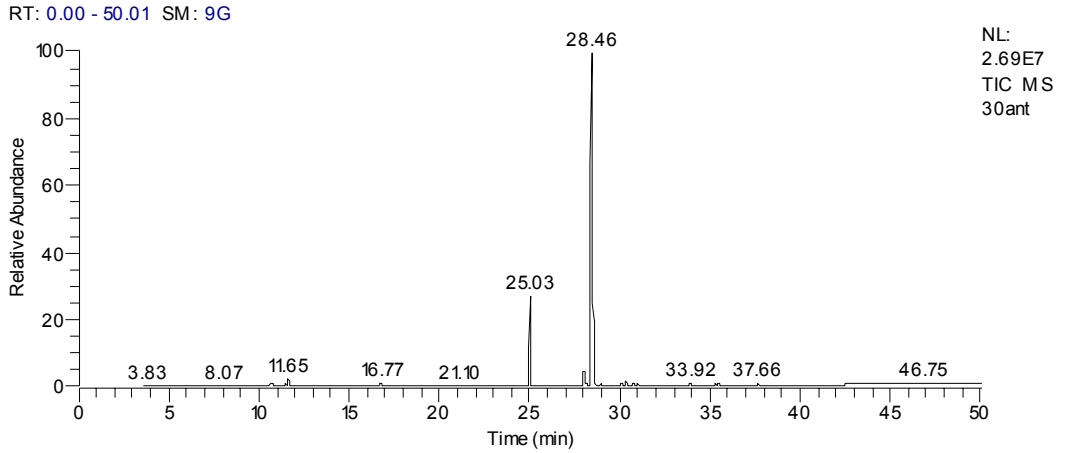
Each of these two samples was then extracted with 50-mL methylene chloride two times using the separatory funnel liquid-liquid extraction (EPA Method 3510C). Water was removed from these extracts with hexane rinsed anhydrous $NaSO_4$. Methylene chloride/ethanol in each extract was completely evaporated down with high purity nitrogen TurboVap system. The extracts were re-dissolved in 2-mL acetone.

Each extract was analyzed with modified EPA Method 8270. The chromatogram generated by each extract was qualitatively analyzed by Xcalibur 1.2 using ICIS peak identification algorithm (parameters: peak smoothing = 7, area noise factor = 2, and peak noise factor = 30). The identified peaks were limited to peaks that were at least 1% of the highest peak height. The spectra of the identified peaks were enhanced using the combined algorithm (parameters: peak top region = 4 points, background subtraction = 5 points on the left and right of the peak start and peak end regions, respectively). The enhanced spectra were searched with spectra in the NIST library; five top hits are displayed with the spectra indices and probability for the matches.

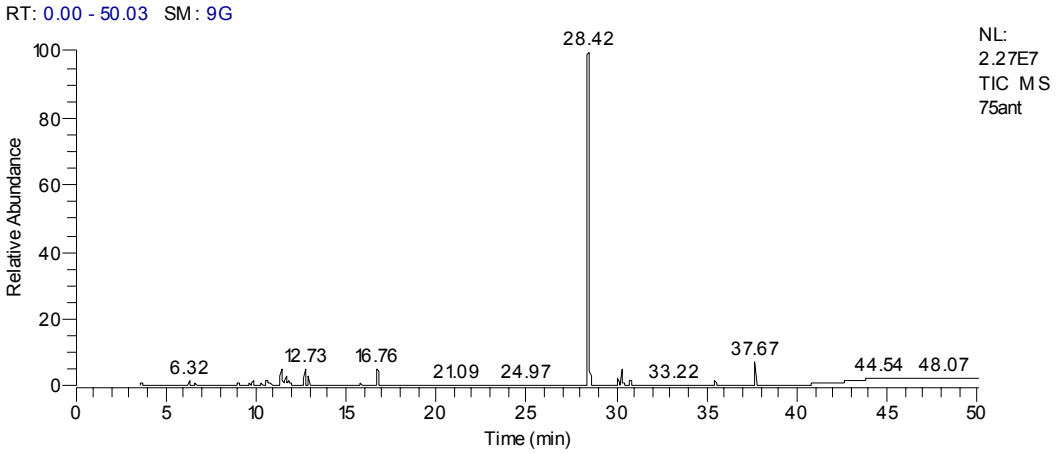
First following figure shows the results of total ion chromatography (TIC) of GC/MS after 30-minute Fenton oxidation of anthracene. Anthracene was not completely oxidized at this time. The main oxidation product of anthracene was 9,10-anthracenedione (28.46 minute). Traces of anthrone (28.00 minute) and 2-hydroxy-9,10-anthracenedione (35.45 minute) were also observed. The chemical properties of anthracene and its Fenton oxidation products are listed in the following table.

Second figure shows the results of total ion chromatography (TIC) of GC/MS after 75-minute Fenton oxidation of anthracene. After 75 minutes of reaction, anthracene was completely oxidized. The main oxidation product at the 75-minute mark was still 9,10-anthracenedione (28.42 minute). The only other oxidation product that was measurable was 2-hydroxy-9,10-anthracenedione (35.45 minute).

After the addition of 30% H_2O_2, the pH of the solution was drastically reduced to as low as 1.7. This low pH is much lower than the optimal pH for the Fenton's reaction. The optimal pH may be maintained if 30% H_2O_2 is slowly introduced to the reaction, for instance, with the use of a syringe pump. We will also investigate and identify the Fenton oxidation products of 9,10-anthracenedione (i.e., anthracene main Fenton oxidation product) by: (1) using 9,10-anthracenedione as the starting material and (2) add more Fe^{2+} and/or H_2O_2 to the 75-minute reaction. These similar conditions and strategies will also be used to explore the Fenton oxidation products of other PAHs such as phenanthrene, fluoranthene, pyrene, and benzo(a)pyrene.

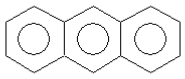
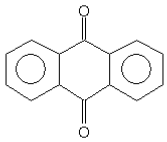
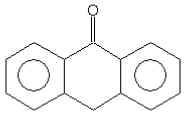
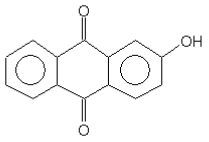


Total Ion Chromatography of GC/MS After 30-Minute Fenton Oxidation of Anthracene.



Total Ion Chromatography of GC/MS After 75-Minute Fenton Oxidation Of Anthracene.

Chemical Properties of Anthracene and Its Fenton Oxidation Products.

Name	Structure	Molecular Formula	Formula Weight
anthracene		$C_{14}H_{10}$	178.23
9,10-anthracenedione		$C_{14}H_8O_2$	208.21
anthrone		$C_{14}H_{10}O$	194.23
2-hydroxy-9,10-anthracenedione		$C_{14}H_8O_3$	224.21

Biological and Chemical/Biological Treatment of Heavily Crude Oil-Contaminated Soils: Contaminant Removal and Effects on Risk

- Using an oilfield soil which is ~15% crude oil-derived hydrocarbons by weight
- Fenton's reagent (single and multiple doses) tested, combined with biological treatment or independently, and compared with biodegradation only
- Microtox® analysis in progress on soils from various treatments; will be compared with TPH reductions
- Best conditions will also be scaled-up for earthworm toxicity tests.

Relative Abilities of Alkane and PAH-Degrading Strains to Survive Fenton's Reaction in Solid-State Systems and Effects on Chemical-Biological Treatment

- Previous experiments have indicated that several PAH-degrading species (*i.e. Mycobacterium*) were better able to withstand Fenton's reaction-generated ·OH than other bacteria.
- Two contaminated field soils (one from oilfield, one from combined MGP/oilfield)
- Populations of total heterotrophic bacteria, alkane (hexadecane)-degrading strains, and PAH (phenanthrene) degraders are being assessed, both before and after Fenton's treatment.
- Same experiment is being conducted in a commercial topsoil, spiked with oil and coal tar and inoculated with two distinct (distinguishable) oil degraders (*Arthrobacter* sp. and MVAB Hex1) and two PAH-degraders (*Burkholderia* sp. and *Mycobacterium austroafricanum*).
- At conclusion of experiment, contaminant removal will also be determined, and toxicity tests (Microtox®) run.
- Best conditions will also be scaled-up for earthworm toxicity tests.

Isolation And Characterization Of A New Genus Of Hydrocarbon-Degrading Bacterium (MVAB Hex1) from Oilfield Soils (See accompanying manuscript)

- Bacterium does not fit in any known genus, based on 16s rDNA gene sequence
- Strain is able to degrade high-molecular-weight, heavily-branched aliphatics (pristane and squalane), and to use these as sole sources of carbon and energy for growth - this is the first non-*Mycobacterium* ever identified with this ability
- Isolate is unusual in that it requires long-chain alkanes (C_{16} and above) for good growth to occur - only a handful of strains have ever been described with this requirement
- Strain accumulates high levels of poly-β-hydroxyalkanoate (wax ester) in inclusion bodies during growth on some alkanes (especially hexadecane and squalane)

Further Experiments

- Solid-state and slurry culture for remediation of heavily-contaminated oilfield soils
- Examination of the lipid content of the membrane, hydrophobicity, etc, as these relate to and confer the ability to take up HMW and branched alkanes.
- Chemical characterization of wax ester inclusion bodies, to help determine the pathways for metabolism of squalene and related compounds, and to determine if wax ester synthesis could be exploited for commodity chemical production.
- Determinations of the relative abilities of low- and high-molecular-weight alkanes to enhance retention of PAH on soils. If HMW alkanes are shown, as expected, to enhance PAH retention, inoculation with this isolate will be examined as a means to biodegrade this fraction, freeing "solvated" PAH for degradation by other bacteria (including Mycobacteria)

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APPENDIX – PUBLICATIONS AND PRESENTATIONS

Paper Published in Proceedings of GTI's Site Remediation Technologies & Environmental Management in the Utility Industry. December 2-6, 2001

Environmental Risk Reduction After Application of Chemically Accelerated Biotreatment to Hydrocarbon-Contaminated Soils Under Water-Saturated and Unsaturated Conditions

J. Robert Paterek, William Bogan, Lisa Lahner, and Vesna Trbovic, GTI and Nancy Comstock, National Energy Technology Laboratory, US EPA

Abstract

Our laboratory is studying various forms and factors of chemically accelerated biotreatment (CAB) for hydrocarbons contaminated soils, i.e. polynuclear aromatic hydrocarbons; benzene, ethylbenzene, toluene, and xylenes (BTEX); and aliphatic moieties. As the biodegradation capacity of the contaminated soils is the decisive parameter in the CAB technology, we are investigating the effects of delivering nutrients (nitrogen and phosphorus moieties) to soils under simulated *in-situ* conditions to maximize biodegradation. Nitrogen and phosphorus containing compounds that are gases under expected field conditions are a major research area. In order to determine effectiveness of these additions it was necessary to first identify candidate soil that is suitably nutrient-limited. Slurry-phase bioreactors were the method to assess nutrient effects on contaminant degradation under "ideal" conditions, *i.e.* in systems where issues such as nutrient and contaminant bioavailability are minimized. In order to determine whether the Microtox® solid-phase test would be suitable for assessing the potential toxicity of the E&P soils to be used in remediation experiments and in determinations of environmentally acceptable endpoints, this assay was investigated. All procedures were followed according to manufacturer's instructions, and clean coarse sand (the same sand used to dilute the soil for column experiments) was used as a control. Solid-phase Microtox® analyses proved to be applicable to the experimental systems under study. In addition, soil moisture content (a_w) was evaluated in both the nutrient study and the Microtox® evaluation.

Keywords

Bioremediation, environmentally acceptable endpoints, risk-based technology, chemically accelerated biotreatment.

Introduction

Gas and oil exploration and production (E&P) activities are conducted in 33 U.S. states under a wide variety of environmental and operational conditions. The pipeline transmission and distribution of natural gas and petroleum involves all 50 states. On the exploration and production sites, there are an excess of 800,000 wells producing gas and oil with 30,000 new wells completed annually (based on 1991 surveys). Add this number to the 500,000 abandoned wells and a major potential environmental liability is indicated.

The research reported is part of a DOE/FERC project that targets waste pits that can be remediated on-site or *in-situ*, as well as soil contaminated by accidental release of petroleum and natural gas-associated organic wastes from pipelines or during transport. Based on GTI's experience with a form of Chemically-Accelerated Biotreatment (CAB) for the remediation of Manufactured Gas Plants (MGP-REM), use of these technologies at E&P sites could save the industry an estimated $150 Million to $200 Million over the next ten years.

The most common contaminants associated with E&P sites are:

- hydrocarbons associated with the natural gas condensates, such as benzene, toluene, ethyl benzene, and the xylenes which are commonly designated as BTEX;
- polynuclear aromatic hydrocarbons (PAHs) from the formation's natural gas deposits (liquid or solids under the conditions of pressure and temperature in many reservoirs);
- PAHs associated with the drilling materials;
- fuel oil and diesel fuel that is often a component of the drilling materials;
- heavy metals, such as arsenic, barium, cadmium, chromium, lead, mercury, selenium, silver, and zinc;
- biocides and scale/corrosion inhibitors; and
- organic and inorganic sulfur compounds (mercaptans and hydrogen sulfide, respectively).

The technology under development, i.e. CAB to mitigate possible contaminated sites, combines two powerful and complimentary remedial techniques: 1) chemical oxidative treatment using Fenton's Reagent (hydrogen peroxide and iron salts); and 2) biological treatment, primarily using native aerobic microorganisms (Paterek *et al.*, 1994, Srivastava *et al.* 1994). This integrated process generates environmentally benign products including carbon dioxide (CO_2), inorganic salts, biomass, and water. A schematic illustration of the CAB Process is presented in the Figure 1.

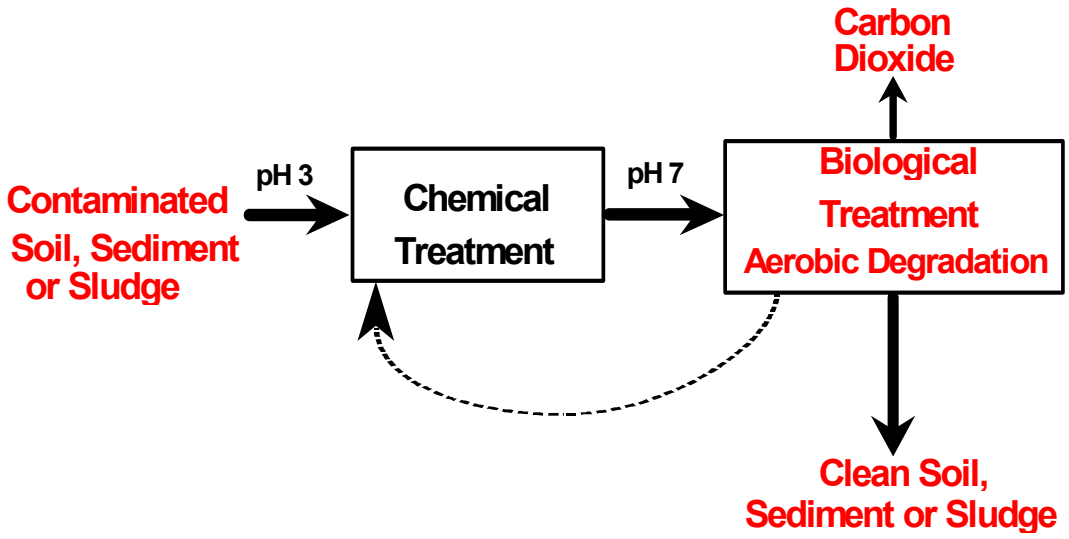

Figure 1: Schematic Diagram of the CAB Process

Application of CAB to E&P sites and other gas industry sites (Paterek *et al*. 1993) with treatment endpoints based on risk, bioavailability and EAEs (Harkey *et al*. 1997) utilizes engineered systems to supply microbe-stimulating materials and pretreatment chemicals to:

- encourage the growth and activity of targeted microorganisms,
- minimize mass-transfer problems, and
- optimize environmental conditions for degradation and detoxification reactions.

These systems must be effective to decrease the time required to destroy or detoxify the contaminants (Grey *et al*. 2000), thus decreasing the overall liability of the gas/petroleum industry partner that is responsible for the site, as well as his cleanup costs. The technology must also be efficient enough to meet or surpass the regulatory requirements. This technology will also be more reliable that the existing technologies of landfilling, landfarming, and composting. Cost reduction will be realized by shortening the treatment time, due to the option of applying the *in-situ* mode of the CAB process. The use of low-cost and industrially available chemicals will eliminate excavation, transport, and land use costs.

Materials and Methods

Determination of Nutrient Limitations in E&P Soils

As a first step towards determining the ability of gaseous nutrient sources to support degradation of oil-derived aliphatic hydrocarbons in petroleum-contaminated E&P soils,

it was necessary to first identify a candidate soil that is suitably nutrient-limited. This was accomplished through the use of slurry-phase cultures, as we have used throughout this reseach to assess nutrient effects on contaminant degradation under "ideal" conditions, *i.e.* in systems where issues such as nutrient and contaminant bioavailability were minimized.

Homogenized soil samples (500 mg) were mixed with 50 ml sterile media (0.1ml Wolfe's Vitamins (2 $mg \cdot l^{-1}$ biotin, 2 $mg \cdot l^{-1}$ folic acid, 10 $mg \cdot l^{-1}$ pyridoxine HCl, 5 $mg \cdot l^{-1}$thiamine HCl, 5 $mg \cdot l^{-1}$ riboflavin, 5 $mg \cdot l^{-1}$ nicotinic acid, 5 $mg \cdot l^{-1}$ pantothenic acid, 0.1 $mg \cdot l^{-1}$ cyanocobalamine, 5 $mg \cdot l^{-1}$ *p*-aminobenzoic acid, 5 $mg \cdot l^{-1}$ thioctic acid), 0.1ml Trace Minerals (100 $mg \cdot l^{-1}$ $ZnSO_4$, 300 $mg \cdot l^{-1}$ H_3BO_3, 300 $mg \cdot l^{-1}$ CoCl, 10 $mg \cdot l^{-1}$ CuCl) and 0.8 ml N- & P-free (pH 7.2) Winogradsky medium (62.5 $g \cdot l^{-1}$ $MgSO_4 \cdot 7H_2O$, 31.25 $g \cdot l^{-1}$ NaCl, 1.25 $g \cdot l^{-1}$ $FeSO_4$, 1.25 $g \cdot l^{-1}$ $MnSO_4$) per 100 ml of sterile deionized water) in 125-ml serum bottles. Cultures were incubated at room temperature (approx. 25 ºC), with constant shaking at 170 rpm.

In order to assess the degree of N- and P-limitation on PAH degradation inherent in each soil, ^{14}C-hexadecane mineralization was measured in cultures of each of two crude oil-contaminated wellhead soils which received no supplemental N or P, N only (as NH_4Cl), P only (as KH_2PO_4), or both N and P. CO_2 traps were made by wrapping stainless steel wire around the necks of 12 x 32 mm borosilicate glass autosampler vials and pushing the wire through 20-mm Teflon silicone-lined septa. These assemblies were placed in the serum bottles, which were then crimped with aluminum seals. Syringes were used to inject 1 ml of 0.5M NaOH into each CO_2 trap. Periodically, the CO_2-containing NaOH solution was withdrawn from the traps, mixed with 5 ml of Ultima Gold® high-flashpoint LSC cocktail solution (Packard, Meriden, CT), and counted in a liquid scintillation counter (Packard Model 2200CA Tri-Carb). Fresh NaOH was then added to the CO_2 traps. Cultures containing ^{14}C- hexadecane typically received *ca*. 60,000-80,000 dpm of labeled hexadecane in 20 µl of methanol.

Six combinations were then investigated for nitrogen (N) and phosphorus (P) supplementation: NH_4Cl/KH_2PO_4; N_2O/KH_2PO_4; NH_4Cl/TEP; NH_4Cl/TBP; N_2O/TEP and N_2O/TBP. Within each condition, duplicate cultures were employed. In all cases, addition of N and P sources was normalized on a molar basis to provide 9.2 mM N and 3.7 mM P. When N_2O was used, it was added by injection to sealed bottles.

Toxicity Determinations with Microtox® Solid-Phase Test

In order to determine whether the Microtox® solid-phase test would be suitable for assessing the potential toxicity of the E&P soils to be used in solid-state remediation experiments and in determinations of environmentally acceptable endpoints, this assay was run on one of the oil-contaminated soils (AB). Microtox® Solid-Phase assays, marketed as test kits by Azur Environmental, Carlsbad, California, will be used to obtain soil toxicity data. In these tests, toxicity is determined by a decrease in metabolism, and thus a decrease in light output, which can be sensed by a modified spectrophotometer. Microtox® software then calculates the EC_{50} and 95% confidence intervals from replicates of each sample analyzed. Microtox® Solid-Phase tests was used to determine

baseline toxicity in the soil samples selected for study. The manufacturer's protocols were used for all Microtox® tests. Coarse sand was used as a control.

Results And Discussion

Determination of Nutrient Limitations in E&P Soils

The extent of hexadecane mineralization in soil-slurry bioreactors containing indigenous microbes from the two oil-contaminated E&P soils are shown in Figure 2 below. Both of the two soils tested evidenced very significant limitations for nitrogen; this was most notable in soil AB, in which the amount of $^{14}CO_2$ released through hexadecane mineralization was approximately 17-fold higher in N-supplemented conditions versus those with no nutrient addition. Similar patterns were seen in soil C, although the degree of stimulation by supplemental N was not as high as in soil AB. The other notable difference between the two soils was in the significance of the phosphorus limitation. While soil AB supported significantly higher mineralization when both N and P were added (vs. N only), the two conditions behaved virtually identically in soil C.

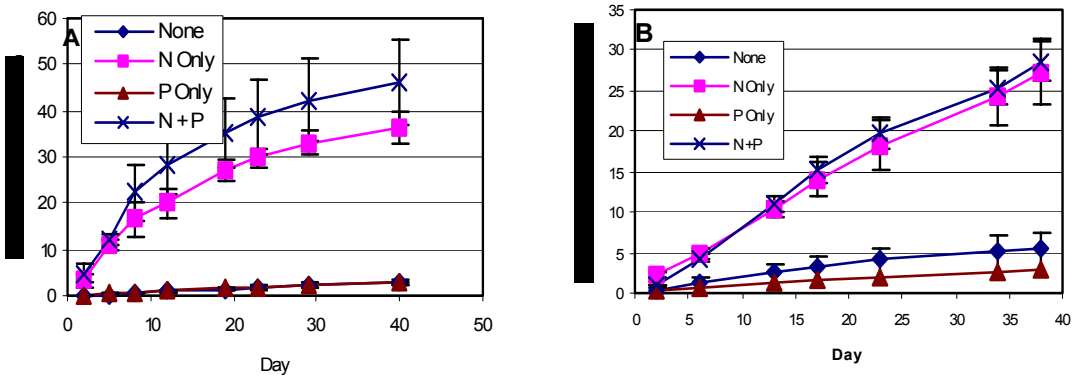

Figure 2 – Effects Of N And P Addition On Mineralization Of Hexadecane By Microbes Indigenous To Oil-Contaminated E&P Soils. Graph A = Soil AB; Graph B = Soil C

Thus, it can be concluded that, in terms of degradation of oil-related hydrocarbons, the microbes in soil AB are strongly limited by their available N supply, and, when N is added in sufficient levels, a significant P limitation also becomes apparent. In contrast, soil C contained sufficient P supplies that N alone was the limiting factor for hydrocarbon degradation. Given that the goal of the experiments undertaken here was to evaluate potential gaseous N and P sources for their effects on hydrocarbon remediation, soil AB was selected for further work, due to the fact that it was found to be limited for both nutrients.

The ability of the indigenous microbes in soil AB to mineralize hexadecane was then investigated under different conditions of nitrogen and phosphorus supplementation. The results of this experiment are shown in Figure 3 below.

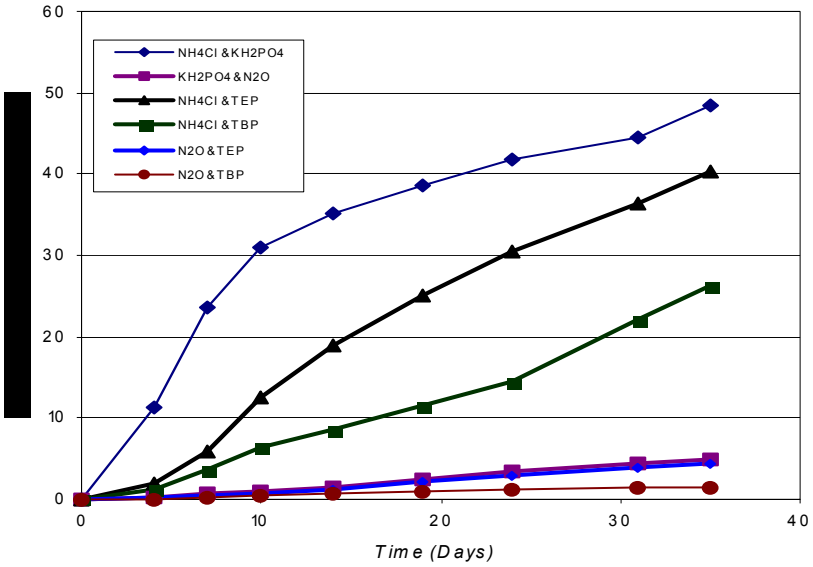

Figure 3 – Ability of Gaseous N and P Compounds to Overcome Nutrient Limitations and Support Hexadecane Mineralization by Indigenous Microbes in Soil AB.

These results clearly show that use of TEP as an alternative to inorganic phosphate to support remediation of aliphatic hydrocarbons is quite feasible in this soil. Mineralization of hexadecane with TEP as a phosphorus source was nearly equal to that with KH_2PO_4. TBP supported considerably less hexadecane mineralization, and use of N_2O as a source of N was found to be extremely deleterious to the performance of the microorganisms in this soil. Other tests with soils with PAHs as the target for biodegradation indicated the opposite effect. Environmental conditions of the treatment regime, such as aw, pH, application of chemical oxidants, i.e. Fenton's reagent impact the effectiveness. These factors can be determined and incorporated into the treatment technology.

Conclusions

These results support our previous observation that each soil and its associated microorganisms is unique. Due to the unique features of these soils and there response to nutrient stimulation, each soil with its comittent contaminants must be evaluated to determine the most effective treatment parameters to met or exceed the treatment endpoints. These endpoints can be determined using traditional analytical methods or risk or toxicity associated techniques, such as solid-phase Microtox®.

Acknowedgements

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A NOVEL GENUS AND SPECIES OF OBLIGATELY HYDROCARBONOCLASTIC, SQUALANE-DEGRADING BACTERIUM ISOLATED FROM OILFIELD SOILS

Running Title: Novel Squalane-degrading Bacterium

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Enrichment culture techniques, using hexadecane as a sole carbon source, were used to isolate an alkane-degrading bacterium, MVAB Hex1, from chronically crude oil-contaminated soil from a Southern Illinois oilfield. On the basis of 16s rRNA gene sequence, this isolate appears to belong to a previously undescribed genus, most closely related (*ca*. 94% sequence similarity) to *Acinetobacter*. The isolate grew weakly in defined media without hydrocarbons; however, growth was greatly enhanced by alkanes. This was observed with the straight-chain aliphatics hexadecane and heptadecane, although not with shorter-chain ($\leq C_{15}$) hydrocarbons; growth was also greatly enhanced by the branched aliphatic hydrocarbons pristane and squalane. The latter of these was most intriguing, as catabolism of squalane has hitherto been reported only for *Mycobacterium* species. Although unable to utilize mono- or polycyclic aromatics as sole carbon and/or energy sources, the isolate did show slight fluorene-mineralizing capability in LB medium, which was partially repressed by hexadecane. In contrast, hexadecane supplementation greatly increased mineralization of ^{14}C-dodecane, which was not a growth substrate. BIOLOG testing further emphasized the isolate's extremely narrow substrate range, as only Tween 40 and Tween 80 supported significant growth. Microscopic examination (SEM and TEM) revealed a slightly polymorphic coccoidal to bacillar morphology, with hydrocarbon-grown cells tending to be more elongated. When grown with hexadecane, MVAB Hex1 accumulated large numbers of electron-transparent intracytoplasmic inclusion bodies. These were also prevalent during growth in the presence of squalane. Smaller inclusion bodies were occasionally observed with pristane supplementation; they were, however, absent during growth on crude oil.

Introduction

Bacterial biodegradation of crude oil, together with the inherent possibility for microbial bioremediation of hydrocarbon pollution in the environment, first gained widespread attention in the early 1970's (5, 38). In the intervening years, considerable knowledge has accumulated towards elucidating the biochemical mechanisms underlying bacterial degradation of the various chemical fractions present in crude oils. Four main classes of hydrocarbon are present in oils - namely, alkanes/aliphatics, aromatics, heteroatom (N, S, O)-containing compounds, and asphaltenes (42), the ratios of which vary from one oil to another. Many bacteria are known to degrade the aliphatic constituents of crude oils; for example, straight-chain aliphatics up to at least C_{40} to C_{44} are known to be biodegradable (31, 32). Branched-chain alkanes, particularly those with anteiso-terminal branching (*i.e.* substitution on carbons immediately adjacent to the terminal carbon(s)), are considerably less amenable to biodegradation than their unbranched counterparts, due to their poor susceptibility to β-oxidation (33). In fact, one such hydrocarbon, pristane (2,6,10,14-tetramethylpentadecane), has often been used as an internal standard in estimating the amount of biodegradation and weathering which has occurred to other fractions of oils (4 & refs. therein). More recently, however, species of *Acinetobacter*, *Alcaligenes*, *Nocardia*, *Brevibacterium*, *Corynebacterium*, and *Rhodococcus*, have been shown to be able to biodegrade pristane (4, 24, 26, 29, 30). A longer and more heavily substituted alkane, squalane (2,6,10,15,19, 23-hexamethyltetracosane), has to date only been shown to be catabolized by two species of *Mycobacterium* - *M. fortuitum* and *M. ratisbonense* (6). Aromatic hydrocarbons, particularly those with four or fewer fused benzene rings, are also readily degradable (For review, see (39)). Recently, several bacterial species, most of which have been classified as *Mycobacterium* species (or a closely-related

undescribed genus) have come to light which are capable of simultaneously degrading aliphatic and aromatic crude oil constituents (25, 37, 41), although this was once viewed as exceedingly rare (17). Strains harboring both of these abilities are clearly particularly promising for site-remediation applications.

The work presented herein describes the isolation and initial characterization of a bacterium from a chronically heavily-contaminated oilfield site in southern Illinois. The bacterium, whose 16S rRNA gene sequence is sufficiently distinct from the closest known genus (*Acinetobacter*) so as to warrant inclusion in a new genus, has been found to have a very narrow range of growth substrates. Several standard microbiological media only supported significant growth when long-chain ($\geq C_{16}$) alkane hydrocarbons were added; furthermore, BIOLOG testing of the isolate showed good growth only on Tween surfactants. In addition to growth on straight-chain aliphatics, the isolate was found to be able to grow very well on two model branched alkanes, pristane and squalane.

Methods and Materials

Chemicals

Fluorene (98%), phenanthrene (98%), and pyrene were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Fluoranthene (Practical grade) was obtained from Eastman Organic Chemicals (Rochester, N.Y.). Quant grade linear alkane hydrocarbons (*n*-decane through n-heptadecane) were from PolyScience Corporation (Niles, IL). The following ^{14}C-radiochemicals were obtained from Sigma Chemical Co. (St. Louis, MO): 9-^{14}C-fluorene (14.2 mCi/mmol), 4,5,9,10-^{14}C-pyrene (58.7 mCi/mmol), 1-^{14}C-dodecane (4.1 mCi/mmol), and 1-^{14}C-hexadecane (2.2 mCi/mmol). 3-^{14}C-fluoranthene (45 mCi/mmol) was from Moravek Biochemicals (Brea, CA).

Isolation and Identification of Alkane-degrading Bacteria from Site Soil

The site soil was reportedly contaminated with oil pumped from the Salem limestone formation (Mississippian Age), at a depth of approximately 3400 feet; contamination was very heavy, with TPH (total petroleum hydrocarbon) levels averaging 150,000 ppm (data not shown). Samples (*ca*. 4 grams) of soil were added to 50 ml of mineral salts medium (MSM) (10), which was then further supplemented with 800 µl of either hexadecane or dodecane. After 3 days of shaking, the resultant enrichment cultures were then subcultured into fresh MSM (at the rate of 300 µl of primary culture to 50 ml medium), again supplemented with 800 µl of alkane. After one additional day of incubation, cultures were plated onto gelrite gellan gum plates (11), which were then overlaid with a layer of alkane (approximately 100 µl per plate) as a sole carbon source. Colonies were subcultured onto R2A agar (Difco), overlain with hexadecane, until pure; the isolate, which was given the designation MVAB Hex1, was then identified by sequencing a 1533-base pair stretch of its 16S rRNA gene, using primers spanning positions 5 to 1540 in the corresponding *E. coli* sequence (MIDI Labs, Newark, DE). The resultant sequence was matched against the GenBank database using BLASTN 2.2.2 (2).

Further analyses compared the sequence data obtained for the MVAB Hex 1 isolate with those for 21 published genospecies of *Acinetobacter* (19). Sequences were aligned using Clustal W (40). The phylogenetic tree was generated using the neighbor-joining method with the Kimura (22) two-parameter model for nucleotide change.

Delineation of Substrate Range

Growth data for the MVAB1 bacteria on various hydrocarbons were obtained using the following method. Wells in a 96-well plate were filled with 150 μL of LB media, 50 μL of MVAB1 bacteria suspended in LB (A_{600} ~ 0.2), and 5 μL of hydrocarbon. The hydrocarbons used were decane, dodecane, tetradecane, pentadecane, hexadecane, heptadecane, pristane and squalane. For each hydrocarbon, three wells were inoculated with the MVAB1 suspension, and 2 wells served as non-inoculated controls. Five additional wells with only media were used as a blank control. The experiment was also conducted using MSM media, using the same procedure as above (in place of LB media). The plates were incubated at room temperature on a shaker (~180 rpm). Readings on the plates were done at A_{600} (MRX-II Plate Reader, Dynex Technologies), approximately twice daily. The ability of the isolate to utilize individual PAHs as sole carbon sources was checked on PAH-sublimated gel plates as previously described (1, 11). Gel plates supplied with benzene, toluene, or naphthalene vapors were used to assess possible growth on these substrates. Growth on non-petrochemical compounds was assessed using the BIOLOG procedure; BIOLOG testing was conducted, using standard methods, by Geneva Laboratories, Inc. (Elkhorn, WI).

Mineralization Experiments Effects of Hexadecane Addition on Mineralization of Dodecane and PAHs

Two sets of mineralization experiments were conducted; the first of these examined conversion of ^{14}C-hexadecane to $^{14}CO_2$ in different media, and the second assessed the ability of the MVAB Hex 1 isolate to mineralize various other hydrocarbons (aliphatic and aromatic) in the presence or absence of hexadecane. All mineralization experiments

were conducted in crimp-top 125-ml serum bottles as previously described (10). Cultures containing ^{14}C-hydrocarbons typically received *ca*. 50,000-100,000 dpm of ^{14}C; radiolabeled hydrocarbons were added in 10 µl of methanol. Non-labeled hydrocarbons (ca. 10 mg per culture bottle) were added to bottles aseptically: Solvent-dissolved PAH was added to bottles prior to autoclaving (28), whereas liquid alkanes were added to media just prior to inoculation. Typically, addition of non-labeled hydrocarbons was done at the rate of 10 mg per 50-ml culture.

Microscopy

Preparation of samples for Scanning Electron Microscopy (SEM) was based on previously-published methods (12, 18). Briefly, samples were prepared on 0.2-micron black polycarbonate Millipore filters, which were sputter coated with gold before the bacteria were introduced. The bacteria were harvested from agar plates in 50 mM phosphate buffer (pH 7). Media and extracellular debris were removed with five exchanges of phosphate buffer through vacuum filtration. The sample was then fixed with 3% glutaraldehyde in phosphate buffer for one hour, followed by a wash with phosphate buffer. The secondary fixative was 1% osmium tetroxide in phosphate buffer for 20 minutes, again followed by a buffer rinse. After the secondary fix, dehydration was begun. The concentrations of ethanol used were: 30%, 70%, 90%, 100%, and 100%; each change of dehydrant was held for 20 minutes. After the second 100% ethanol step, the samples were ready for critical point drying (CPD), which was done using a Model K-850 Critical Point Drier (Emitech Ltd., Ashford, Kent, UK). A transitional fluid of liquid CO_2 was used for drying. The samples were then gold sputter

coated and viewed using a Hitachi S3500-N scanning electron microscope, using a 10 KV electron beam at a working distance between 8 and 10 mm.

For transmission electron microscopy, cells were fixed on the surface of the agar culture plate by flooding with 2.5 % glutaraldehyde buffered with 0.1M sodium cacodylate buffer and fixing at room temperature for 45 minutes. After buffer washing, cells were fixed with 1% osmium tetroxide in the same buffer on ice for 45 minutes, washed with distilled water, and then stained with 1% aqueous uranyl acetate for 30 minutes. Samples were dehydrated in an ethanol series and embedded in Spurr resin. Sections were cut with diamond knives on an LKB Nova ultramicrotome, post-stained with uranyl acetate and lead citrate, and observed and photographed on a Zeiss EM-10CA transmission electron microscope.

Results

Isolation and Identification

An isolate, designated MVAB Hex1, was obtained from oil-contaminated site soils through the use of very short-term enrichment cultures in which hexadecane was added as a sole supplemental carbon source. This isolate grew poorly on R2A agar in the absence of hydrocarbon, producing small (~0.5 mm), translucent off-white colonies; in contrast, growth was much more vigorous in the presence of an overlayer of liquid hexadecane. In the latter case, colonies tended to be much larger, and frequently showed convergent growth along the path of the streak used to inoculate the plate. When the isolate was subcultured from hexadecane-overlain plates to hydrocarbon-less R2A plates, growth remained vigorous for 1-2 successive transfers, then began to decrease, eventually

reaching the same poor level of growth seen when cultured directly to these plates. No growth was observed on MacConkey agar.

Based on the 16S rRNA gene sequence of the MVAB1 isolate, the highest degree of similarity observed was to *Acinetobacter junii* (19 & refs. therein), with a 94% sequence homology. This level of sequence identity is at the lower limit present among the 21 genospecies of *Acinetobacter* recognized by Ibrahim et al (19), and it is therefore ambiguous, based on this data alone, as to whether a confident genus-level match can be made. However, alignment of the 21 sequences used in the previous phylogenetic analysis of *Acinetobacter* (19) with the MVAB Hex1 sequence revealed no less than 36 individual residues in which a strict consensus was present among all of the *Acinetobacter* strains, and a different nucleotide was present in the same position of MVAB Hex1. A phylogenetic tree showing the relationship of the Hex1 isolate to the 21 genospecies of *Acinetobacter* is represented in Figure 1.

Biochemical examination of the isolate using BIOLOG plates showed strong growth on only two compounds; namely, Tween 40 and Tween 80. Weak growth was also seen on acetic acid, and equivocal results were obtained in the cases of α-hydroxybutyric acid, muconic acid, α-keto butyric acid, and sebacic acid. These data produced a "match" to *Acinetobacter johnsonii* genospecies 7 (Prob = 80, Sim = 0.68, Dist = 2.25); in fact, however, the substrate range of the MVAB Hex1 isolate is considerably narrower than the latter species: According to the BIOLOG database, *Acinetobacter johnsonii* generally ($\geq$ 70% frequency) gives positive reactions with the three compounds listed above, but also methyl pyruvate (100% of isolates), α- and β-hydroxybutyric acid, lactic acid, propionic acid, bromosuccinic acid, D- and L-alanine, L-proline, L-glutamic acid, and L-

pyroglutamic acid. Other sources (7) state that 100% of *Acinetobacter* genospecies 7 strains grew on lactic acid, bromosuccinic acid, alanine, asparagines, and pyroglutamic acid. None of these wells yielded an unequivocally positive reaction in the case of the MVAB Hex1 isolate, although slightly equivocal results were observed with α-hydroxybutyric acid, as described above.

Growth on Alkane Hydrocarbons

In the absence of additional hydrocarbon supplementation, the Hex1 isolate exhibited weak growth in LB broth, as it had on R2A agar plates in the absence of a liquid hydrocarbon overlayer. However, when hexadecane was added to LB, growth was greatly enhanced; the isolate was also able to grow in MSM with hexadecane as a sole carbon and energy source. Similar results were observed (Table 1) for heptadecane and octadecane, as well as for the branched-chain aliphatics pristane and squalane.

No growth or PAH-clearing ability was observed for the Hex1 isolate on any PAH (fluorene, anthracene, phenanthrene, or pyrene) -sublimated plates. Similarly, there was no growth on gel plates which were exposed to vapors of benzene, toluene, or naphthalene as sole carbon sources.

Degradation of Alkane Hydrocarbons and Fluorene

The MVAB Hex1 isolate was able to mineralize ^{14}C-hexadecane in both MSM and LB liquid media; mineralization was more rapid and extensive in the latter of these (Figure 2). The culture's ability to mineralize a shorter-chain alkane (dodecane) was also assessed, as were the $^{14}CO_2$ releases from several polycyclic aromatic hydrocarbons (fluorene, phenanthrene, pyrene, and benzo[*a*]pyrene). All of these tests were conducted

in LB medium. Mineralization of dodecane was low in LB cultures which were supplemented only with the C_{12} hydrocarbon, with slightly more than 2% (of an initial concentration of 200 mg/l) mineralized in 53 days (Figure 3b). This finding agrees well with the growth-substrate data, which indicates that dodecane, when added alone, is a poor substrate for growth of MVAB Hex1 (Table 1). In contrast, when 10µl of hexadecane was added to ^{14}C-dodecane-spiked cultures, the amount of $^{14}CO_2$ generated from the dodecane increased by approximately sixfold.

Although the Hex1 isolate was apparently (based on gel plate data) incapable of using any PAH as a sole C source, its possible ability to co-metabolize PAH during growth in LB was also assessed. Of the four PAHs tested, mineralizations of three (phenanthrene, pyrene, and benzo[*a*]pyrene) were very low (less than 1% of the input ^{14}C released as $^{14}CO_2$), below the stated radiochemical purity of the parent PAH. Only fluorene-spiked cultures evidenced sufficient $^{14}CO_2$ releases (approximately 2.5% in 53 days) to be confident that some mineralization of the input PAH was actually occurring. As opposed to the behavior seen with dodecane, addition of 10 µl of hexadecane to these cultures significantly reduced the amount of mineralization; the amount of ^{14}C generated from ^{14}C-fluorene by hexadecane-supplemented cultures was consistently approximately 40% lower than that observed with LB cultures which lacked hexadecane supplementation (Figure 3a).

Microscopy

SEM examination of the MVAB Hex1 isolate revealed a somewhat polymorphic morphology, with both cocci (diameter ≈ 0.6-0.8 µm), and short bacilli (0.6-0.8 x 1.0-1.2 µm) present, as shown in Figure 4a. The dimensions of cells grown on R2A plates in the

absence of hydrocarbon appeared similar to those of cells harvested from pristane-overlain plates (Figure 4b).

When cells were visualized by transmission electron microscopy, they were found to contain large numbers of electron-transparent inclusion bodies. These were found to be of two types, similar to those seen with *Rhodococcus* (3). Using the nomenclature from the latter work, we observed both spherical "ET1" inclusions, which were by far the predominant inclusion type (Figure 5a-c, e-f), and non-spherical "ET2" bodies; the latter were sometimes disc-shaped (Figure 5b), and sometimes much more irregular in structure (Figure 5c-d). The largest and most prevalent inclusion bodies were seen in hexadecane-grown cells; frequency and size were somewhat decreased in squalane-grown cells (Figure 5e), and further diminished in cells grown on pristane (Fig. 5f). Cells grown in the presence of crude oil contained no inclusions, as did those cultured in the absence of hydrocarbon (Figure 5g & h, respectively).

Discussion

Growth of the MVAB Hex1 isolate in the absence of hydrocarbons was found to be poor on three standard complex microbiological media (R2A agar, MacConkey agar and LB broth), but became very vigorous in both cases when the medium was supplemented with hexadecane or other alkanes. The isolate displayed strong growth on C_{16}-C_{18} linear alkanes (hexadecane, heptadecane, and octadecane), as well as on the branched-chain aliphatics pristane and squalane. The latter of these is of particular interest, as this isolate is the first non-*Mycobacterium* species known to degrade and/or grow on this particular compound; to date, the only identified species capable of growth on squalane have been *Mycobacterium fortuitum* and *Mycobacterium ratisbonense* (6). The weakness or absence of growth observed with shorter-chain (C_{10}-C_{15}) hydrocarbons is analogous to a

phenomenon seen with *Arthrobacter nicotianae* strain KCC B35, which grows better on long-chain (C_{20}-C_{40}) alkanes than on shorter (C_{10}-C_{18}) aliphatics (31). In the latter case, the authors pointed out that their culture was isolated from a post-Gulf War environment in which many of the short-chain aliphatics from oil spills had been biodegraded and/or removed via weathering, leaving a disproportionate amount of heavy alkanes. The MVAB Hex1 isolate can clearly take up and catabolize short-chain alkanes (at least dodecane - Figure 3b); failure to use these hydrocarbons as sole growth substrates may indicate that one or more of the strain's alkane-degrading enzymes are only induced by aliphatics with chain length of C_{16} or above.

BIOLOG testing results further substantiated the isolate's extremely narrow range of suitable growth substrates; strong growth occurred on only two of the 95 potential substrates – the surfactants Tween 40 and Tween 80. The significance of this finding is clear, inasmuch as these are the only substrates included in the BIOLOG system which contain long-chain alkyl moieties within their molecular structures. No growth was observed on any of the other substrates included in the BIOLOG system, including those which are generally diagnostic (7) for any of the 14 recognized DNA-DNA hybridization groups of *Acinetobacter*, the closest known genus (see below) to the Hex1 strain based on 16s sequence data. For example, of 14 *Acinetobacter* hybridization groups (genomospecies) examined, 11 have at least one substrate for which every known strain of the genomospecies gives a positive reaction (7); for some cluster groups (*e.g.* genomospecies 2), the number of such substrates is as high as 24. The only two groups for which this is not true, genomospecies 8 and 12, still have several substrates which give positive reactions for at least 60% of known strains. None of these substrates

supported growth of the MVAB Hex1 isolate; thus, the BIOLOG results for this isolate also strongly support placing it in a genus distinct from *Acinetobacter*.

Such a limited growth-substrate range for a bacterium is fairly rare. Thermophilic bacteria are known to occasionally possess this trait (27, 43). Among non-thermophiles, however, we are only aware of two instances in which a bacterial strain has been documented to have a range of growth substrates as narrow as MVAB Hex1. One such strain, apparently related to *Alcaligenes*, reportedly grew on C_9-C_{18} alkanes, but was unable to utilize any of the sugars or amino acids against which it was tested (9); this strain was, however, able to grow on propionic acid (8), which the MVAB Hex1 isolate is unable to utilize. Furthermore, the range of potential substrates against which this strain was screened was far less broad than the 95 substrates employed in the BIOLOG procedure. Cabezali and Cubitto isolated "many" strains of hydrocarbon-degraders from the Bahía Blanca estuary; of these, one unclassified bacterium was only capable of growth on linear alkanes, fatty acids, and ethanol (13, 14). Like MVAB Hex1, both of the above-described strains were isolated from chronically oil-polluted sites. Taken together, these findings suggest that chronically petrochemical-contaminated sites may have a high propensity for giving rise to bacteria which can only degrade petrogenic hydrocarbons.

The 16S rRNA gene sequence obtained for the MVAB Hex1 strain is also sufficiently divergent from known species as to fall outside existing genera, and apparently constitutes a new genus. The sequence differs the closest *Acinetobacter* species (*A. junii*) by approximately 6%, which is roughly equal to the maximum amount of divergence seen among the 21 recognized genospecies of *Acinetobacter* (19). However, the MVAB

Hex1 isolate exhibited sufficient differences from all of the published *Acinetobacter* genospecies sequences (divergence at at least 36 positions which are strictly conserved among all *Acinetobacter* sequences) that we are confident in placing it outside the genus entirely. Only one 16S rRNA sequence in the GenBank database exhibits sufficient identity to the MVAB Hex1 isolate so as to be viewed as congeneric; the partial (339 bp) sequence (Accession # Z69268) obtained from a groundwater bacterium (designated JN3c) present in an alkaline spring at Maqarin, Jordan, displayed 99.1% homology to Hex1. Furthermore, a BLAST search reveals that the JN3c sequence, like that of MVAB Hex1, exhibits no more than 94% homology to any other sequences in the GenBank database; included among the strains which matched JN3c at this level were numerous isolates of *Acinetobacter*. The JN3c bacterium, however, has so far not been cultured, and the sequence information was obtained exclusively by isolating environmental DNA (Dr. Karsten Pedersen, Göteborg University, personal communication); therefore, at present, the strain is unavailable for any morphological and/or biochemical comparisons. Morphologically, MVAB Hex1 is similar to *Acinetobacter* in that it occurs as both spheres and short rods, although the dimensions which we observed by SEM and TEM are slightly smaller than those typically given (23) for *Acinetobacter* (0.9-1.6 μm x 1.5-2.5 μm). In the case of *Acinetobacter*, the transition from bacillar to coccoidal morphology occurs in stationary phase (23), and has been observed to occur in response to nutrient limitation (21).

Accumulation of lipids and/or partially metabolized hydrocarbon in intracellular inclusion bodies as a means of sequestering carbon has been noted previously in several genera, including *Acinetobacter* (20, 34, 35) and *Rhodococcus* (3). This phenomenon

occurs as a consequence of nutrient-limited growth, most often an excess of available carbon relative to nitrogen. The ultrastructure of inclusion bodies in *Acinetobacter* appears to vary depending on strain and growth substrate: Strain HO1-N grown on hexadecane appeared to accumulate mostly small spherical ("ET1") inclusions (35), while large, disc-shaped "ET2" inclusions predominated in the same strain grown on hexadecanol (36) and in strain M-1 during growth on hexadecane (20). We observed the most and largest inclusion bodies (of both morphologies) in MVAB Hex1 cells during growth on R2A agar in the presence of hexadecane, followed by squalane; few inclusions were observed in pristane-grown cells, and essentially none in cells cultured on crude oil. It should be noted that, in rich liquid media (LB), cell growth was far more rapid on hexadecane or squalane than on pristane (See "Day 2" data in Table 1). Limitation of other nutrients may therefore have more rapidly become a factor in these cases than with squalane- or pristane-supported growth, resulting in more extensive wax ester sequestration. The fact that no inclusion bodies were formed during growth on crude oil may be due to the (relative) slowness of growth under these conditions; another possible scenario is that the isolate may possess the ability to obtain other, potentially limiting nutrients (*e.g*. N) from compounds in the oil. This possibility is currently being examined, as is the exploitation of the MVAB Hex1 strain for site-remediation purposes.

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MVAB Hex1 isolate has been deposited in the GenBank database under the accession number AF513979.

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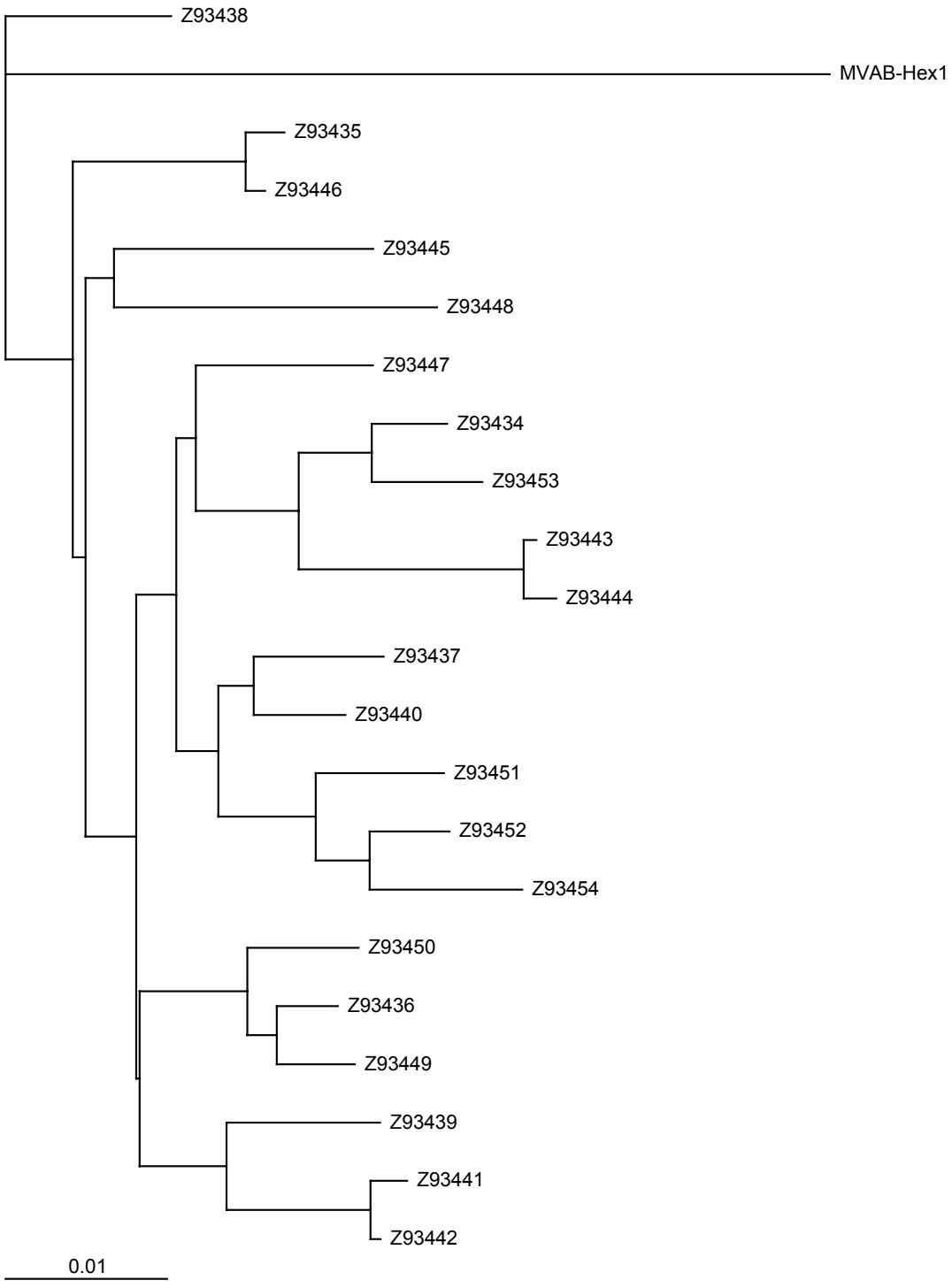
Table 1 – Effect of hydrocarbon supplementation on liquid-culture growth (measured as increase in A_{600}) of the MVAB Hex1 isolate. Experiments were conducted in LB broth and in MSM; hydrocarbon addition rate was ~ 5mg/well (total culture volume per well ≈ 150 µl). Non-inoculated (hydrocarbon + media) control wells were included for each individual condition. "-" indicates that no measurable growth occurred.

	LB Medium		MSM
	Day 2	Day 4	Day 4
n-Decane	-	-	-
n-Dodecane	-	-	-
n-Tetradecane	-	-	-
n-Pentadecane	-	-	-
n-Hexadecane	0.267	0.815	0.112
n-Heptadecane	0.221	0.983	0.140
Pristane	0.055	0.736	0.344
Squalane	0.284	0.529	0.232
(Inoculated) Media-only control	- (-0.004)	- (-0.004)	- (-0.002)


Z93438
MVAB-Hex1
Z93435
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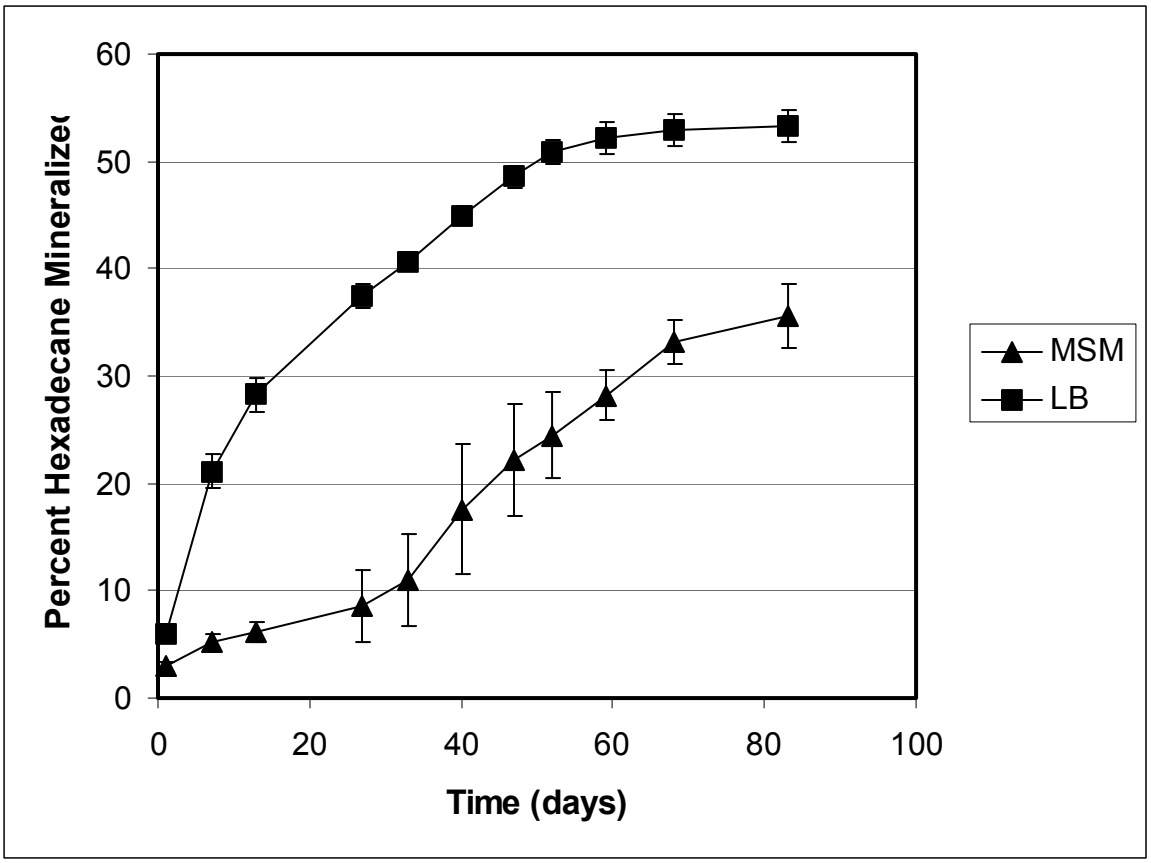
Percent Hexadecane Mineralized
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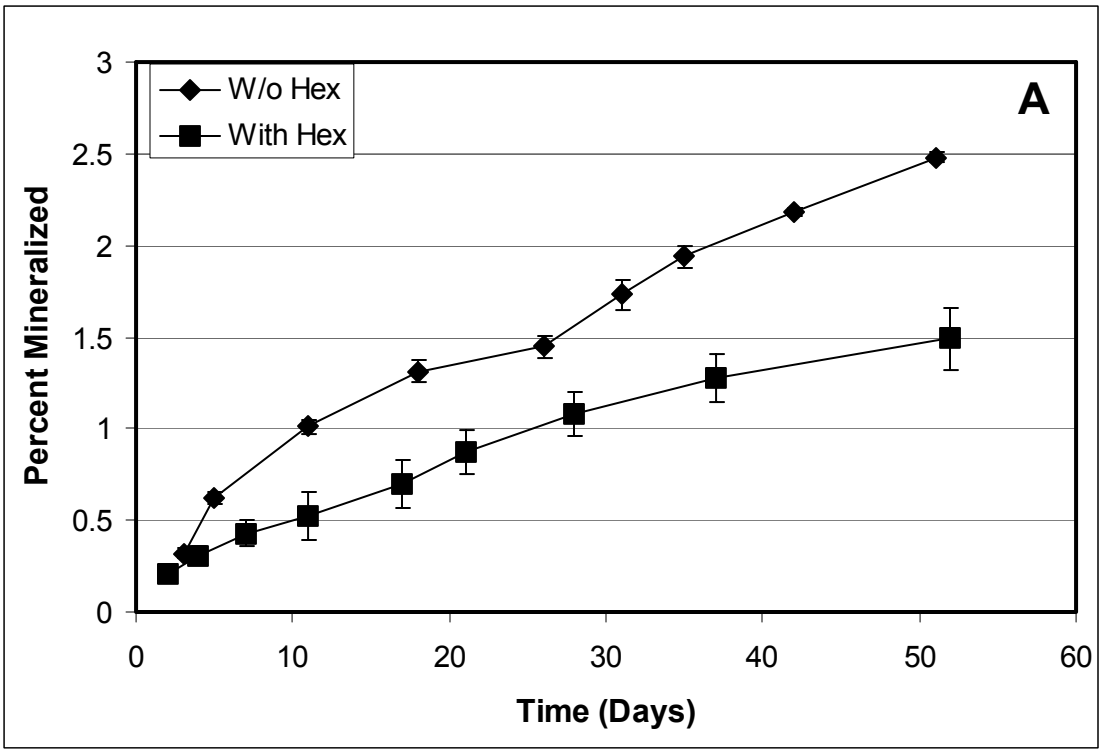
50
40
30
20
10
0
0
20
40
60
80
100
Time (days)
MSM
LB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Percent Mineralized
Time (Days)
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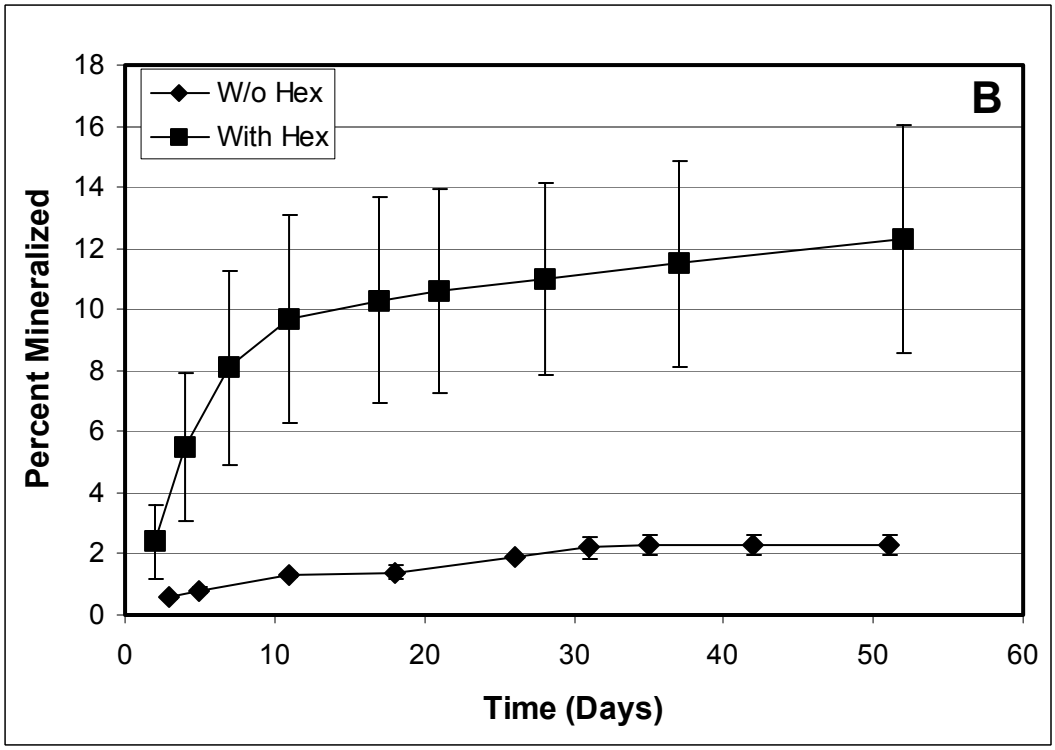
B
W/o Hex
With Hex
Percent Mineralized
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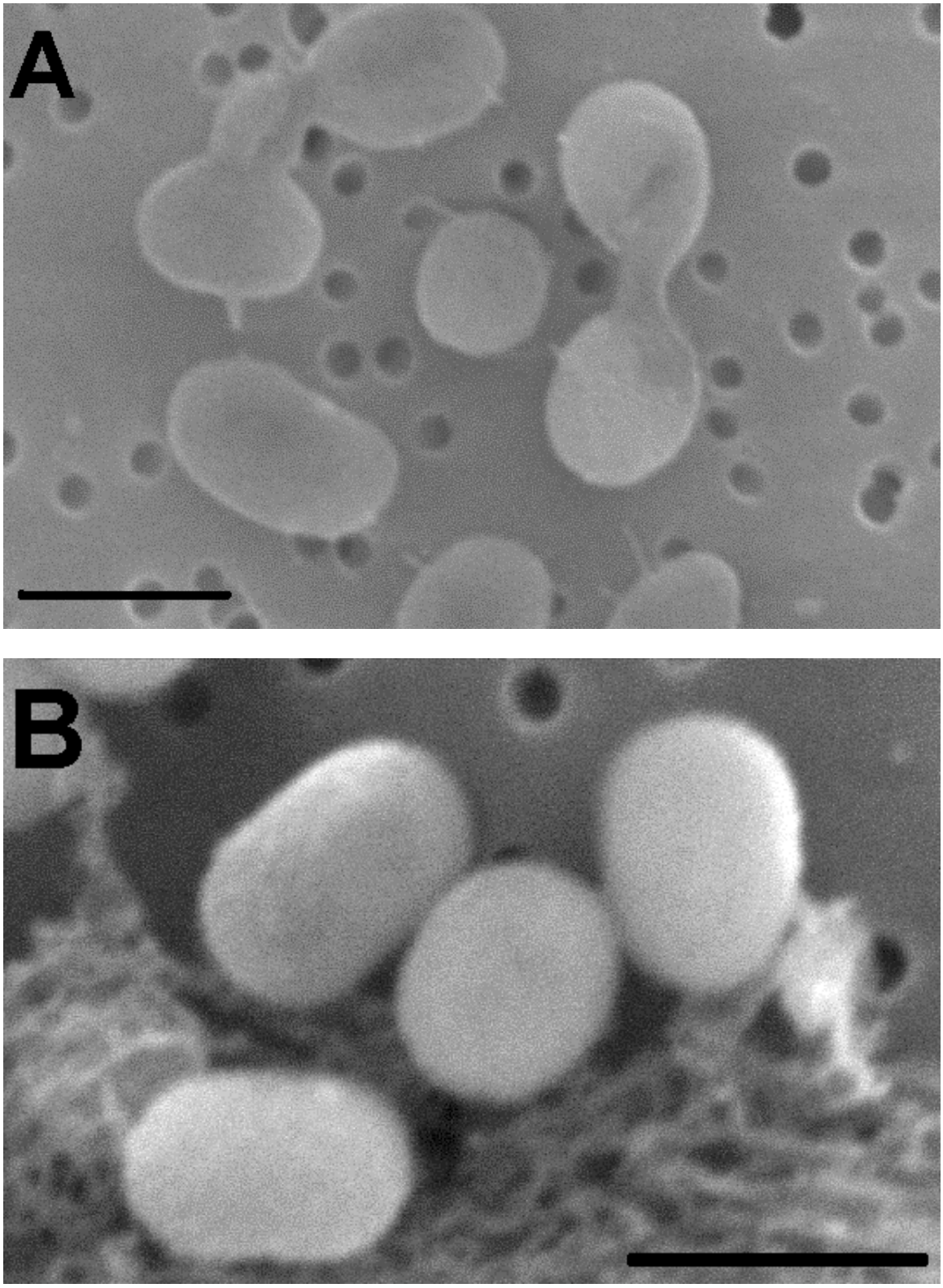
A
B

Figure 1 – Phylogenetic tree showing relationship of 16S rDNA of MVAB Hex1 and *Acinetobacter* genospecies. Tree was constructed using the dnadist and neighbor joining method programs included in the PHYLIP package (15, 16). Bootstrap values (100 replications) were generated by the neighbor joining method. Designations for *Acinetobacter* sp. are as used for a previous phylogenetic analysis (19), and are identified using the GenBank Accession numbers of their 16S rRNA gene sequences.

Figure 2 – Mineralization of ^{14}C-hexadecane (initial hexadecane concentration = 1.2 mM) by MVAB Hex1 grown in either mineral salts medium or LB broth.

Figure 3 – Mineralization of fluorene (**A**) and dodecane (**B**), each added at the rate of 10mg/40 ml, by MVAB Hex1 grown in LB broth in the presence or absence of 1.2 mM hexadecane.

Figure 4 – SEM images of MVAB Hex1. Cells were taken from R2A agar plates, with either no hydrocarbon supplementation (**A**) or an overlay of liquid pristane (**B**). Bars denote 1 micron.

Figure 5 – Transmission electron microscopy of MVAB Hex1. All cells were taken from R2A agar plates after 3-5 days growth in the presence or absence of liquid hydrocarbon overlay. (**A**) Hexadecane-grown cells showing large number of small spherical "ET1"-type inclusion bodies. Hexadecane-grown cells were observed to also accumulate disc-shaped to amorphous "ET2" wax ester inclusions, either alone (**B**) or together (**C**) with ET1 bodies. (**D**) ET2-containing cell in which inclusion bodies show evidence of intracellular membranes. (**E**) Squalane-supplemented cells showing ET1 inclusions, which also occurred, albeit smaller and more rarely, in cells cultured on pristane (**F**). Crude oil-grown cells (**G**) and those grown in the absence of hydrocarbon (**H**) lacked detectable inclusions. In all frames, bar denotes one micron.