To the Graduate Council:

I am submitting herewith a thesis written by Michele Odette Ricard entitled "Comparative Effects of Fossil Fuel Hydrocarbons on Microbial Activity in Freshwater Sediments." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ecology.

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COMPARATIVE EFFECTS OF FOSSIL FUEL HYDROCARBONS ON MICROBIAL ACTIVITY IN FRESHWATER SEDIMENTS

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ABSTRACT

Studies of freshwater sediment microbial activity were conducted using adenosine triphosphate (ATP) and dehydrogenase enzyme activities as measures of community viability and metabolic activity. A field study of two sites on Melton Hill Lake and a small pond in east Tennessee was conducted on a seasonal basis to provide background information on possible seasonal fluctuations in microbial activity in three different aquatic ecosystems. A microcosm experiment simulating conditions at one of the field sites was conducted to determine possible effects of hydrocarbons from chronic low-to moderate-level inputs of a diesel fuel marine (DFM) oil or a synthetic coal derived crude (ASO) oil on the microbial community. Adenosine triphosphate was extracted with 0.6 N cold sulfuric acid and ATP quantified via bioluminescence. A rapid dehydrogenase analysis procedure was developed by modifying several standard dehydrogenase analysis procedures. The correlation coefficients of the results of the field dehydrogenase analyses and microcosm dehydrogenase analyses using 10 and 60 minute incubations were correlated (α=0.0001) at r=0.907 for the field, and r=0.686 for the microcosm equilibration period and r=0.892 for the microcosm treatment period. The results of the field study showed that ATP content was significantly different (α=0.05) at the three sites, but not on a seasonal basis. Field dehydrogenase data showed site and seasonal effects on microbial activity.

Both oil treatments may have had significant effects on ATP content after the first oil treatment. The microcosm sediments were

not significantly different in ATP content at the end of the experiment. When the treatment period as a whole is considered, comparing treatment means, the hydrocarbon treatments appear to lower ATP content significantly. Dehydrogenase activity, when considered for individual microcosms on separate sampling days, was the same for all the microcosms immediately after the first treatment. After the second treatment, the ASO-treated microcosms were significantly lower in activity than the controls and DFM-treated microcosms. After the fourth hydrocarbon treatment, the DFM-treated microcosm sediments tended to have greater dehydrogenase activity than the controls, which tended to have greater activity than the ASO-treated microcosms. At the end of the experiment, after the fifth oil treatment, the controls had significantly greater dehydrogenase activity than the ASO-treated microcosms, while the DFM-treated microcosms tended to have activity midway between the controls and ASO-treated microcosms. When mean overall treatment statistics are compared for the treatment period, dehydrogenase activity in the ASO-treated microcosms was significantly lower than the controls and DFM-treated systems.

The results of this general examination of the effects of allochthonous hydrocarbons on microbial activity reveals a need for more detailed study.

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I. INTRODUCTION

A. Impact of Petroleum Hydrocarbons in the Aquatic Environment

Sediments as Recipients of Allochthonous Hydrocarbons

Sediments in aquatic ecosystems are the eventual recipients of all materials released in the environment, which are not immediately decomposed. Materials applied to the terrestrial environment are incorporated into sediments via surface runoff and erosion. Particulates in the air enter the sediment system from rainfall and fallout. Wastes from industrial, municipal or other sources reach water systems in treatment plant effluents. Transportation on waterways also yields hydrocarbon inputs from fuels and spills. Conservative estimates indicate that 0.2 million tons of petroleum hydrocarbons may be transported to the world's marine environment annually from municipal treatment plants (see Section IIA). These chronic inputs consist of home heating oils, industrial heating and lubrication oils, and used crankcase oil. Crude and refined oils released in spills along with biogenic oils account for the remaining 60% to 95% of the oils currently deposited in the marine environment.

Decomposition of Hydrocarbons in Sediments

Organic matter decomposition by detritus feeders in aquatic environments is vital to element cycling processes in the biosphere and is ultimately accomplished by sediment microorganisms. Sediment microorganisms can metabolize allochthonous oils by a variety of

biochemical pathways (Section IIA). Oils deposited in sediments can have a stimulatory or inhibitory effect on organic matter decomposition, oil decomposition and biogeochemical cycling. A stimulatory effect would occur if the added oil was readily metabolized by the decomposers. This would be especially marked in systems with naturally low levels of organic matter. An inhibitory effect could occur if the oils or oil components were toxic to metabolic processes or externally coated the organism.

B. Monitoring Microbial Activity in Sediments

Many methods are available for monitoring certain functions of an indigenous sediment microbial community (Section IIIB). Adenosine triphosphate (ATP) levels and the activity of dehydrogenase enzymes reflect the metabolic status of the microbial community in general. ATP and dehydrogenase enzymes are present only in viable cells due to their direct involvement in electron transport systems, and other anabolic and catabolic processes of aerobic and anaerobic microorganisms.

Utilization of ATP and dehydrogenase analyses, coupled with the use of microecosystems or microcosms, allows the effects of oils on the microbial community to be monitored in a controlled manner. Microcosms can be used for toxicity testing or screening oils, oil components, or other chemicals known or suspected to be entering a specific ecosystem, which the microcosm simulates.

C. Hydrocarbon Effects on Microbial Metabolism and Their Impact on ATP and Dehydrogenase Activity

Hydrocarbons can affect microorganisms externally and internally. External coatings of oils can interfere with ion and nutrient transport through the microorganism, solubilize cell membranes, and effect chemotaxis (Higgins and Gilbert 1977, Mitchell et al. 1972, Colwell and Walker 1977). Internal effects can be benign, positive or negative for the health of the microorganism or ecosystem. Aliphatic hydrocarbons, including higher alkanes with C_{10} to C_{18} can support growth on a wide range of bacteria, yeast, and fungi (Higgins and Gilbert 1977) thus contributing to ATP production and fueling respiratory activity. Initial mono-oxygenase oxidation of alkanes are often followed by oxidations conducted by specific dehydrogenases such as alcohol- and aldehyde-dehydrogenases (Higgins and Gilbert 1977). Negative internal effects might be caused by metabolites of the absorbed hydrocarbons (Colwell and Walker 1977). Some aromatics are metabolized by microorganisms (Colwell and Sayler 1978). Benzene, toluene, naphthalene, phenanthrene, anthracene and biphenyls are susceptible to degradation by some microorganisms (Higgins and Gilbert 1977). Ring cleavage is a multistep process beginning with initial enzymatic oxidations yielding diols, then dihydroxy derivatives and then ring cleavage to yield acids. Some oxidized aliphatic end products may pass into the tricarboxylic acid cycle returning some of the energy spent in the oxidation processes.

Petroleum hydrocarbons can cause a variety of effects on the microbial population. ATP and dehydrogenase analyses will give

general information about the viability of the microbial population but will not identify the organisms being affected or the hydrocarbon causing the effect. Activities of specific enzymes known to be functioning in hydrocarbon modification such as the mixed-function oxidases (MFOs) could indicate increased hydrocarbon utilization when studied with analyses of MFO end products from oil-derived hydrocarbon precursors.

D. Levels of Complexity in Toxicity Tests

Cairns and Dickson (1978) pointed out that one of the major difficulties in evaluating the toxicity of a chemical released into the environment is determining how much testing is required to delimit its effects satisfactorily. Toxicity testing can be costly in materials costs, time and personnel. Since the passage of the Toxic Substance Control Act (TOSCA) in 1976, toxicity testing of new chemicals is mandatory before they can be manufactured and put into general distribution and use. Toxicity testing is also required for the new synthetic fuels being produced from coal.

Cairns (1978) suggests using different levels of complexity for conducting toxicity tests: Tier I testing consists of comparatively short-term, generalized tests. Tier II testing is more complex and expensive using continuous flow rather than batch designs for test systems. Tier III testing is the most demanding in terms of the duration of the experiment and sophistication of the model ecosystem. Detail is emphasized by monitoring specific biological characteristics for specific organisms and utilizing chronic low-level doses of the

test material as opposed to acute tests that are more suitable at Tier I or II testing.

Cairns and Dickerson (1978) outlined a general toxicity testing procedure for materials entering aquatic ecosystems:

1. Compound Characterization--including chemical, physical, and biological characteristics of the test substance.
2. Receiving System Characterization--including chemical, physical and biological characteristics of the target aquatic system.
3. Testing Priority--where several substances are to be evaluated, the order in which the substances are to be examined needs to be determined.
4. Toxicity Evaluation--use of field and laboratory studies; laboratory screening, definitive laboratory tests, and chronic tests.
5. Time Required--coordinating laboratory and field tests to maximize equipment, personnel and previously attained information.

The steps taken for planning and implementation of this protocol are shown in Appendix Figure A-1 for laboratory studies, and Appendix Figure A-2 for field studies. These specific protocols can incorporate Tier I, II, and III testing into the evaluation process. The testing procedures and experiment designs of Cairns are some of many relevant to the toxicity testing of substances likely to be released into the environment. The procedures of Cairns and Dickerson are specifically designed for aquatic ecosystems.

E. Objectives of This Investigation

The need for investigating the effects of oil-derived hydrocarbons on sediment microbial communities was developed in the previous discussions and is described in more detail in Section II. An experiment combining the use of sediment microcosms, ATP and dehydrogenase analyses, and sediment texture and organic matter estimates with other chemical and physical ecosystem characteristic estimates, was designed to provide information on the ability of a freshwater sediment microbial community to accommodate inputs of oil-derived hydrocarbons. The objectives of the study were:

1. To measure microbial activity seasonally at several field sites using ATP and dehydrogenase analyses.
2. To assess the utility of microcosms to simulate the sediment environment found at one of the field sites.
3. To examine the effects of a marine diesel fuel oil (DFM) and a synthetic crude oil derived from coal (ASO) on microbial activity using microcosms as test systems.

The experimental design, the frequency and types of field measurements made, the treatments of the microcosms and the types of microbial activity indicators used show this experiment to have aspects of all three tiers. It was the intent of the investigation to obtain general information on the effects of the oil treatments on the microbial community which is typical of Tier I toxicity tests. The treatments simulate real, low-to moderate-level chronic oil inputs that is a feature of more complex test levels. The microcosm experiment lasted 203 days, which is a long duration for a microbial community and more typical of Tier III experiments.

II. LITERATURE REVIEW

A. Background

The objective of this literature review is to summarize current information on the effects of oils on sediment microbial communities. Investigations of the effects of oils on sediment microbial communities requires merging information from a variety of studies. The scope of oil pollution in aquatic environments is briefly discussed to establish the extent of the pollution problem. The emphasis is upon the use of biochemical indicators of microbial viability and metabolic activity. Sediment compositional features including organic matter, and texture are included in the review. Microbial ecological studies in aquatic ecosystems in general are examined to establish the types of information currently available. Specific metabolic pathways, oil components, and microorganisms illustrate microbial breakdown and biogeochemical cycling of oils and oil constituents. Physical, biochemical, and design features of microcosms that are important to testing the effects of oils on the sediment microbial community are examined. Finally, several conclusions are drawn from the review that point to a particular approach that would be useful in assessing the effects of environmentally realistic concentrations of oil in real aquatic sediment ecosystems.

The Extent of Oil Contamination of the Aquatic Environment

Sources of petroleum oil contamination of the environment can be classified into four major categories (Van Vleet and Quinn 1978):

Oil production operations, landbased discharges other than those directly related to oil production, natural oil seeps, and atmospheric deposition. Watercraft accidents are another potentially significant source of petroleum hydrocarbon release in the aquatic environment. A summary of estimated petroleum inputs into the marine environment is presented in Table 1 which shows current annual estimates range from 2 to 11 million tons. Of this annual input, 5% to 40% may be contributed by sewage treatment plant effluents. These effluents contain home heating oils, industrial heating and lubrication oils, and used crankcase oils. Van Vleet and Quinn (1977) reported total hydrocarbon concentrations from 5.41 mg/g dry weight sediment (5410 ppm) in surface sediments near an outfall of a sewage treatment plant on the Providence River, Rhode Island. Sediments at the mouth of the river contained 0.57 mg/g (570 ppm). These hydrocarbons were characterized as petroleum-derived based on gas chromatographic analyses. Shelton and Hunter (1974) showed sediment samples from New Jersey rivers to contain 0.026% to 38.3% oil (260 to 383,000 ppm) on a dry sediment basis (Table 2).

Population density and types of local industry affect the amount of oil deposited in the sediment. Some hydrocarbons found in sediments are from natural sources such as plant waxes. Mayo et al. (1978) found biogenic hydrocarbon content to range from 22 to 232 ppm in sediments near the site of a pipeline jet fuel and No. 2 heating oil spill in Maine. The hydrocarbon ranges in polluted areas were 9 to 7125 ppm about 5 years after the spill occurred. The studies by Van Vleet and Quinn (1977) and Mayo et al. (1978) also illustrate that

Table 1. Summary of Estimated Petroleum Inputs to the Marine Environment

Sources	Estimates Million Tons/Year
Marine Operation Losses	
Tankers	0.53 to 1.01
Ship operations; bilges, bunkering, etc.	0.05 to 0.61
Offshore Accidental Discharges	
Tankers	0.12 to 0.22
Non-tankers	0.02 to 0.14
Pipelines	<0.01
Offshore Oil Production	0.08 to <.38
Natural Marine Oil Seeps	0.2 to 7.0
Atmospheric Deposition	0.4 to 1.6
Land Based Discharges	
Refineries	0.2 to 0.3
Terminal transfer operations	0.01 to 0.25
Pipeline accidents	<0.01 to 0.03
Runoff, urban and river	1.9
Industrial wastes	0.08 to 1.98
Automotive wastes	1.03
Aviation wastes	0.05
Municipal wastes	0.2 to 0.3
Total Estimated Range	1.9 to 11.4

Modified from: Van Vleet, E. S. and J. G. Quinn, 1978, J. Fish. Res. Board Can. 35:536-543.

Table 2. Percent Oil in Bottom Sediments of Some Selected New Jersey Rivers

Site	% Oil on a Dry Weight Basis
Elizabeth River at South St. Bridge	0.57
Elizabeth River at Mattano Park	0.54
Lawrence Brook at Riva Ave. Bridge, Milltown	0.39
Lawrence Brook at Weston Mill Pond, Ryders Lane	0.26
Manasquan River at Rt. 70 Bridge	0.20
Passic River at Interstate 80 Overpass	1.45
Passic River at Horseneck Bridge	1.82
Rahway River at Rt. 27 Bridge	0.28
Rahway River at Milltown Rd. Bridge, Union, NJ	0.25
Raritan River at Landing Lane Bridge	0.37
Raritan River east of Victory Bridge	0.38
Toms River at Rt. 571 Bridge	0.15
Toms River at Rt. 166 Overpass	0.12
Woodbridge Creek at State St. Bridge N.	37.4
Woodbridge Creek at State St. Bridge S.	38.3

Modified from: Shelton, T. B. and J. V. Hunter, 1974, J. W. Pollut. Contr. Fed. 46:2172-2182.

the marine environment is the eventual sink for most terrestrial and freshwater oil discharges.

Microbial Degradation of Oil in Contaminated Aquatic Environments and in Vitro

Witkamp (1973) stated that the two most important aspects of microbial life in the environment are: (1) their role in breaking down organic matter preventing its accumulation to levels harmful to primary production, and (2) the mineralization of essential elements from organic detritus maintaining fertility and sustaining ecosystem productivity. Witkamp summarized the area of primary concern to microbial ecologists as the measurement and evaluation of the role of microbes in driving cycles of minerals critical to primary production.

There is an enormous volume of literature on the degradation of oil and oil constituents by microorganisms in vivo and in vitro. For this review some of the more recent reviews and on-site ecosystem studies are considered. This section includes information concerning microbial degradation of oils and oil constituents in water, soil, and sediment. Water and soil are considered because they contribute organisms, nutrients, and other materials to the sediment, and may encounter the oil before the sediments, influencing the character of the oil before it reaches the sediment microbial community.

Many studies have been conducted at sites of wrecked ocean-going vessels (Clark et al. 1978, Colwell et al. 1978, Butler and Levy 1978, Teal et al. 1978, Blumner and Sass 1972). Climate and the composition of the oil have a major influence on oil dispersal and degradation at the discharge site (Colwell and Sayler 1978). Oil

fractions that are not volatilized will be available for microbial degradation in the water column or in sediments (Atlas et al. 1978, Walker et al. 1974, Atlas and Bartha 1973, Atlas et al. 1976). Microbial degradation of petroleum in seawater was studied by Atlas and Bartha (1972) and Reisfeld et al. (1972). Since microbial degradation of oils is the main route of oil decomposition in the environment, the use of bacterial treatments of oil spills for increasing the rate of decomposition has been considered (LaRock & Severance 1973).

Due to their hydrophobic character, oils form micelles and coatings on suspended organic matter, clays, silt, or organisms in the water column and settle to the bottom by natural sedimentation processes. Sediment microbial communities with the ability to decompose some oil fractions are ubiquitous in the environment. The limiting factors to oil decomposition by sediment microbial communities are the concentration and composition of the oil, the frequency of the input, and the presence of available nutrients, O_2, pH, and temperature. Oil-exposed communities have a higher oil decomposition rate than similar non-exposed communities (Colwell and Sayler 1978). Marine sediments have been studied to determine how oils affect the growth potential and distribution of the sediment microbial communities (Walker et al. 1976, Stewart and Marks 1978).

Perry (1977) reviewed information on microbial metabolic pathways of cyclic hydrocarbons and related compounds. Most of the modifications involve oxidations. The pathways and metabolites have usually been investigated using single species and a specific substrate. Colwell and Walker (1977) reviewed the ecological features

of microbial degradation of petroleum in marine environments. Much of the information discussed was derived from oil spill studies. Comparisons of crude and refined oil composition and biodegradation revealed that biodegradation of n-alkanes was rapid. The amount of oil left unmodified depended on the relative initial amounts of n-saturates, resins, asphaltenes and other components, which are unique for each oil. Factors such as climate, nutrient availability and seasons were also considered.

Jurtshuk (1971) reviewed the mechanism of n-alkane oxidation by Corynebacterium sp. This organism uses mono-oxidases known collectively as mixed-function oxidases (MFOs) in the hydrocarbon oxidation process. Gibson (1971) discussed bacterial oxidation of hydrocarbons, particularly that of the pseudomonads. Oxygenases, conjugases, and epoxide hydrases were discussed. Benzene, toluene, ethylbenzene and naphthalene were used as model hydrocarbons for pseudomonad metabolite production. Higgins and Gilbert (1977) reviewed oil and hydrocarbon degradation by microorganisms by considering the various fractions separately. Aliphatics with saturated, straight chain configurations were most readily decomposed. Aromatics were oxidized by a more limited number of organisms. Uptake of hydrocarbons may be through adhesion of oil droplets to the microbial surface, or uptake of soluble components in the aqueous phase. The authors list microorganisms with known hydrocarbon degradation abilities, which included bacteria and yeasts. The MFO and rubredoxin oxidative pathways are two modes of xenobiotic oxidation identified in some microbes.

Microbial degradation of hydrocarbons in the environment often involves metabiotic processes or commensalism with one type of organism initiating the attack on the hydrocarbon and other organisms further decomposing the metabolite. Co-metabolism of hydrocarbons can also occur in some organisms. The hydrocarbon is modified along with organic compounds normally metabolized in the organism. Beam and Perry (1974) studied co-metabolism and commensalism among soil microorganisms in the degradation of cycloparaffinic hydrocarbons. They found that addition of hexadecane as a co-substrate increased $^{14}CO_2$ released from U-^{14}C cyclohexane in a mixed culture of microorganisms. The degradation of cyclohexane in a marine mud was also demonstrated.

Walker and Colwell (1974) found that indigenous sediment bacteria were able to degrade petroleum in a creek, which was rich in oil and mercury, located at Baltimore Harbor, Chesapeake Bay, Maryland. These bacteria were also found to be resistant to mercury which concentrated in the petroleum. Specific oil components such as phenanthrene, anthracene and naphthalene were used as substrates by soil pseudomonads for oxidative modification via ring-fisson (Evans et al. 1965, Davis and Evans 1964). Oxidation of carcinogens by Beijerinckia sp. was the subject of a study by Gibson et al. (1975). This microorganism was isolated from a polluted stream and has the ability to grow on biphenyls as a sole source of carbon and energy. The Beijerinckia sp. converted benzo(a)pyrene and benzo(a)anthracene to dihydrodiols.

Influences of Sediment Texture and Organic Matter Content on Microbial and Oil Interaction

To understand how microorganisms in sediments come into contact with oils, an understanding of the microhabitat of these organisms is needed. Microorganism habitats in sediments are similar to those in soils in that all the organisms are "aquatic" relying on pore water for growth. Sediments are usually water saturated and often have higher contents of organic matter than do soil habitats. Reviews of soil-sediment ecosystems as microbial habitats with an emphasis on surface interactions are provided by Marshall (1980), Bamforth (1977), and Hattori and Hattori (1976). Paerl (1980) described how microorganisms are often found attached to living and detrital surfaces in freshwater systems. This often serves as an ecological advantage since organic nutrients concentrate on these surfaces. The advantage may be dubious if oil pollutants are adsorbed since concentrating oils may lead to increased microbial uptake and modification. This increases the potential for reaching lethal doses in the microorganisms, especially when bioconcentrated materials settle into the sediments where they are subject to attack by the sediment decomposer community.

B. Toxicity Testing

Aquatic Microcosms as Tools in Toxicity Testing

Oil discharges occur with such frequency in the marine environment that several studies have been undertaken to establish what the ecosystem impacts of a discharge in a particular environment might be. An important tool in such experiments is the microcosm or microecosystem, which simulates a specific ecosystem on a reduced scale

allowing manipulation of various features such as toxicant dosage or temperature. Some microcosms are laboratory based housed in flasks, bottles, columns, and tanks to contain the test system whereas others are outdoor enclosures, which may link the bench top studies to a perturbed field site or a potential site of a synthetic fuels plant, petroleum refinery, tanker bay, or oil pipeline route.

A multidisciplinary program was organized to study the effects of sub-lethal inputs of pollutants, including oils, on the stability of marine ecosystems at Saanich Inlet, Canada. The experiments were termed CEPEX for Controlled Ecosystem Pollution Experiment. The studies utilized polyethylene "Controlled Ecosystem Enclosures" (CEEs) as test systems. Hodson et al. (1977) conducted a CEPEX using CEEs with an in vivo exposure of seawater bacteria to No. 2 fuel oil, and in vitro studies of the effects of four water soluble extracts from two refined and two crude oils on seawater bacteria from the control and oil exposed enclosures. The studies employed glucose utilization and hydrocarbon mineralization to assess the effects of the oil extracts on seawater bacteria from both CEEs. Estimates of bacterial population size were found to be similar throughout the 30 day experimental period for both the control CEE and the oil exposed enclosure. Uptake and mineralization of ^{14}C-glucose were proportionally inhibited by a particular concentration of No. 2 fuel oil over a range of oil concentrations. The assimilation of ^{14}C-glucose by the control and oil exposed bacteria was inhibited by the four aqueous oil extracts. The results were: (1) at concentrations below 300 µg/l the extracts inhibited glucose uptake by the oil exposed bacteria 12% less than the extract treated control CEE bacteria. At 80 µg/l Bunker C

oil extract simulated glucose uptake by the oil exposed bacteria 13% over that of extract treated bacteria obtained from the control enclosure; (2) the two refined oils (Bunker C and No. 2 fuel oil) were more inhibitory than the crudes (Louisiana and Kuwait) at concentrations above 300 µg/l. The refined oils at 1200 µg/l inhibited by 75% glucose uptake by the oil treated CEE bacteria as compared to the extract-treated control CEE seawater bacteria, while the crudes inhibited glucose uptake by 25%; and (3) mineralization of ^{14}C-labeled hydrocarbons by the control CEE seawater bacteria and oil treated CEE seawater bacteria were 0.5% of the added hexadecane after 72 hours. For fluorene, the amount mineralized was 0.9% by the control CEE bacteria and 0.6% by the oil-exposed CEE bacteria.

Lee et al. (1978) used enclosures similar to the CEEs for conducting controlled dose exposures of an aromatic hydrocarbon spiked Prudhoe crude oil. Microbial degradation accounted for a significant loss of naphthalene and to a lesser extent anthracene, in the spiked crude.

Large in situ microcosms were used in the CEPEX studies. Johnston (1970) used smaller microcosms--beach sand columns in containers (29 cm in diameter by 40 cm in height) for treatment with sand sorbed Kuwait crude oil. Gibbs and Davis (1976) using similar substrates in a 0.5-l container, also treated one of their microcosms with Kuwait crude oil sorbed onto dried sand. Oxygen utilization by the indigenous beach sand microorganisms was used as an indicator of metabolic activity in both experiments. The microcosms were studied for several months. The system of Gibbs and Davis recycled the seawater that passed through the columns, while Johnston did not

recycle the column effluent. Johnston found oil oxidation at 10°C in the heavily oiled microcosm (1.1 kg/m^2) to be 0.09 g/m^2/day. Lightly treated sand yielded a 0.04 g/m^2/day oil oxidation rate. Gibbs and Davis used an oil concentration of 700 g/m^2 in their treatments. About 12% of the added oil was oxidized after approximately 4 months.

To discern between the extent of oil decomposition in aerobic and anaerobic environments, Shelton and Hunter (1974, 1975) conducted sediment microcosm experiments in the presence and absence of O_2. The first experiment included O_2 in the overlying water in the microcosm, in a continuous flow system. Sediments were combined from the Elizabeth River and Woodbridge Creek in New Jersey to produce an estimated indigenous oil concentration of 8% on a dry weight basis. The sediment/water system was housed in 0.5-l bottles containing 225 g sediment. Distilled water enriched with N and P was used to supply nutrients and 1-alkyl-2 thiourea was added to prevent nitrification of NH_3. The experiment lasted approximately 8 months with biweekly measurements of dissolved oxygen, total organic carbon (TOC), biochemical oxygen demand, and pH in the overlying water. The sediment was analyzed for dry solids, volatile solids, total phosphorus, Kjeldahl nitrogen, and hexane and benzene extractable organics. Sediment samples were also analyzed from several New Jersey river sites to determine oil pollution ranges; their findings are presented in Table 2, page 10. Crude oil and a standard hydrocarbon mixture were chromatographed for comparison with the oiled sediment gas chromatograms. The oils in the sediments were of different composition than the crude, and changed during the experiment. A 4.45% loss of oils

from the sediments occurred. Shelton and Hunter attributed this to microbial degradation and/or leaching.

A similar experiment was performed using a closed system with O_2 free-water in sediment microcosms (Shelton and Hunter 1975). Water was changed weekly. Other features were identical to the previous aerobic microcosm experiment. Sediment oil component analyses included silica gel chromatography separation of hexane extracts into aliphatic, aromatic, and oxy-compound fractions with infra-red analysis conducted on each fraction, as well as the other analyses done in the aerobic experiment. Hydrogen sulfide was measured in the overlying water along with chemical oxygen demand, TOC and pH. The results of this experiment showed the water TOC loss to be 13.3 mg/wk/microcosm in the anaerobic system as compared with 9.1 mg/wk/microcosm in the aerobic system. Hexane extractable oils showed an 11.3% loss from the sediment in the second experiment compared with 4.45% under aerobic conditions. There was no observable change in the benzene extractable (aromatic) compounds from the oiled sediment under anaerobic conditions. Functional group analysis of the silica gel separated fractions showed a loss of oxy-compounds under anaerobic conditions. Shelton and Hunter concluded that the oils are lost more readily than other organic matter under anaerobic conditions, but that this may be due to increased leaching as a result of biochemical alteration rather than actual biochemical utilization of the oils.

Several aquatic microcosm experiments have been conducted by Dr. Jeffrey Giddings and associates at Oak Ridge National Laboratory (Giddings and Eddlemon 1977, 1978, 1979; Giddings et al. 1978, Giddings and Sayler personal communication). These studies used glass

aquaria and pond enclosures. Arsenic toxicity and compartmentalization, anthracene degradation and compartmentalization, and oil toxicity were studied in the microcosms. Various sizes and types of microcosms were examined. The effects of substrate type and tank size on primary production and arsenic compartmentalization were analyzed using east Tennessee reservoir sediments and Ottawa sand (Giddings and Eddlemon 1977). Results of this experiment showed substrate type to be important to primary production and degree of As partitioning. Larger microcosms with sediment were found to stabilize more rapidly than smaller microcosms.

An experiment using two 80-liter pond microcosms was conducted to test anthracene compartmentalization and breakdown in a freshwater ecosystem (Giddings et al. 1978). Anthracene was chosen to model polycyclic aromatic hydrocarbon (PAH) behavior in aquatic systems. The results indicated that the breakdown of anthracene in the microcosm may have been due to chemical rather than biochemical activity.

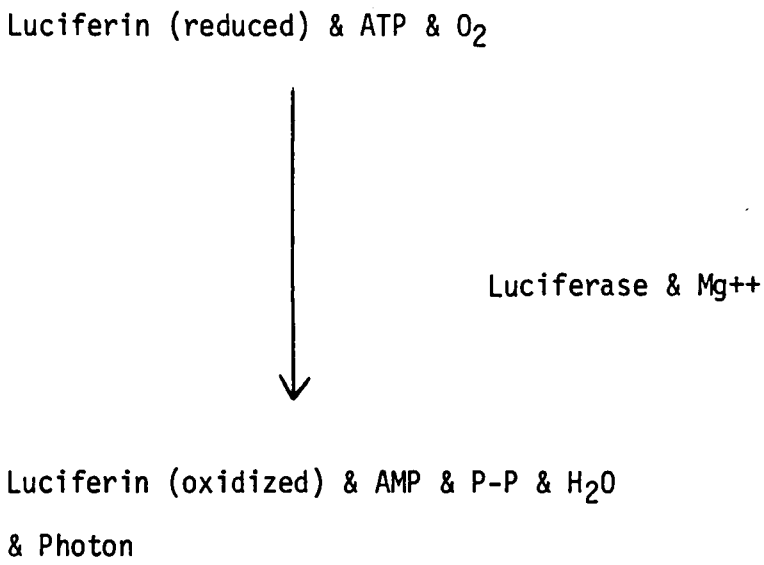
Giddings and Sayler (personal communication) conducted a pond microcosm experiment that determined the effects of a coal derived synthetic fuel oil on various components of the microcosm. The fuel oil affected the macrophyte productivity causing a drop in water dissolved oxygen. The microcosms showed some signs of recovery 4 to 6 weeks post treatment.

Measurement of Sediment Microbial Community Viability and Applications in Toxicity Tests

The microbial community in the environment can be assessed using a variety of different biological and biochemical parameters.

Plate counts on various types of media, direct microscopic counts, O_2 uptake, CO_2 evolution, enzyme activities, ^{14}C-substrate mineralization, and adenosine triphosphate (ATP) quantitation are analyses that include most components of the microbial community. There are limitations in using these characteristics. For example, anaerobic activity is not determined in measurements of O_2 uptake, in vivo populations may not be reflected in plate counts; and direct microscopic counts might include nonviable organisms. Holm-Hansen (1973a) listed some features of a good microbial community indicator; it should be a specific cell constituent which is present in all living cells and absent from dead cells and does not persist in the environment other than in viable cells.

ATP quantitation satisfies the criteria of Holm-Hansen. ATP is an energy rich compound, which is produced in photosynthesis, respiration, and substrate level phosphorylation, and thus is active in the electron transport system of all cells. Its analysis involves extraction from the cell and quantitation in the extract media, most commonly done using a bioluminescence reaction (Figure 1). ATP has been used for nearly 20 years to estimate microbial viability in soil, and in sediments and water in marine and freshwater ecosystems (Holm-Hansen 1973a,b, Witkamp 1973, Ausmus 1973, Paul and Johnson 1977, Lenhard et al. 1962, Karl and LaRock 1975, Lee et al. 1971a,b, Eiland and Nielsen 1979). The main differentiating feature between these various methods is in the extraction of the ATP from the cell and its associated media. Ausmus (1973) tested seven of the most common extraction methods for extraction efficiency using soil, litter, and a synthetic soil. The butanol, and cold sulfuric acid



One molecule ATP yields one photon

Figure 1. ATP quantitation using the luciferin/luciferase bioluminescence system.

Modified from: Holm-Hansen, 1973, Bullt. Ecol. Res. Comm. 17:215-222.

procedures were most efficient at >80% recovery of ATP. Lee et al. (1971a,b) and Karl and LaRock (1975) have used the cold sulfuric acid procedure to extract ATP from sediments. Karl and LaRock used low concentrations of a chelating agent (EDTA) to overcome the interference of indigenous cations that are sometimes present in high enough concentrations in marine sediments and calcareous sediments to lower ATP estimates.

Microbial community viability is also reflected by activities of enzymes specifically involved in energy generation, anabolism, catabolism, and respiration. The dehydrogenase enzymes are such a group of enzymes. They function in electron transport in cellular respiration by transferring electrons from substrates (organic or inorganic compounds) to acceptors such as O_2, NO_3^- and $SO_4^=$. Dehydrogenases in soils have been measured for approximately 30 years using added electron acceptors such as triphenyl tetrazolium chloride (TTC). TTC becomes a red colored formazan when reduced and can be extracted in organic solvents and quantified using spectrophotometry. Many researchers have measured dehydrogenase activity in a variety of soils using various incubation and extraction procedures (Casida 1977, Skujins 1973, Lewis et al. 1978, Lenhard 1965, Ruhling and Tyler 1973, Stevenson 1959, Klein et al. 1971, Ross 1971). Von Gudrun Malicky-Schatte 1973 used dehydrogenase activity to estimate microbial activity in lake sediments.

Both ATP and dehydrogenase levels have been measured in soils or sediments receiving toxic or polluting substances (Lennard 1965, Sayler et al. 1979, Sayler 1980, Lewis et al. 1978). The usefulness of both these analyses in toxicity testing of oil treated sediments is

especially apparent since these analyses can be carried out immediately on freshly collected sediment avoiding metabolic and population changes in the microbial community.

C. Summary

1. Aquatic environments receive a great quantity of allochthonous oils annually. These oils tend to accumulate in sediments where input exceeds the rate of decomposition.

2. The sediment microbial community is responsible for degrading incoming materials, including oils. Estimates of the viability of the sediment microbial community are needed at particular sites receiving or potentially receiving oil contaminants in order to determine if the oils will accumulate in that ecosystem.

3. Sediment texture and organic matter content is important in providing nutrients and niches for sediment microorganisms. Clays, organic matter, and microorganisms provide surfaces for the adsorbtion and concentration of oils in sediments.

4. Microcosms have been successfully used as test systems for estimating the effects of oils on indigenous sediment microorganisms. They permit administration of controlled dosages, replication, the environment is not polluted in the process and microcosms can be designed to reproduce field conditions.

5. Biochemical characteristics are available that can estimate sediment microbial community viability under normal and perturbed conditions.

6. Experiments combining the use of sediment microcosms, ATP and dehydrogenase analyses, and sediment texture and organic matter

estimates can provide information on the ability of the sediment microbial community to accommodate oil pollution. Such oil toxicity tests could be site specific for a particular sediment and oil.

III. METHODS

A. Field Site Seasonal Analysis of Microbial Activity

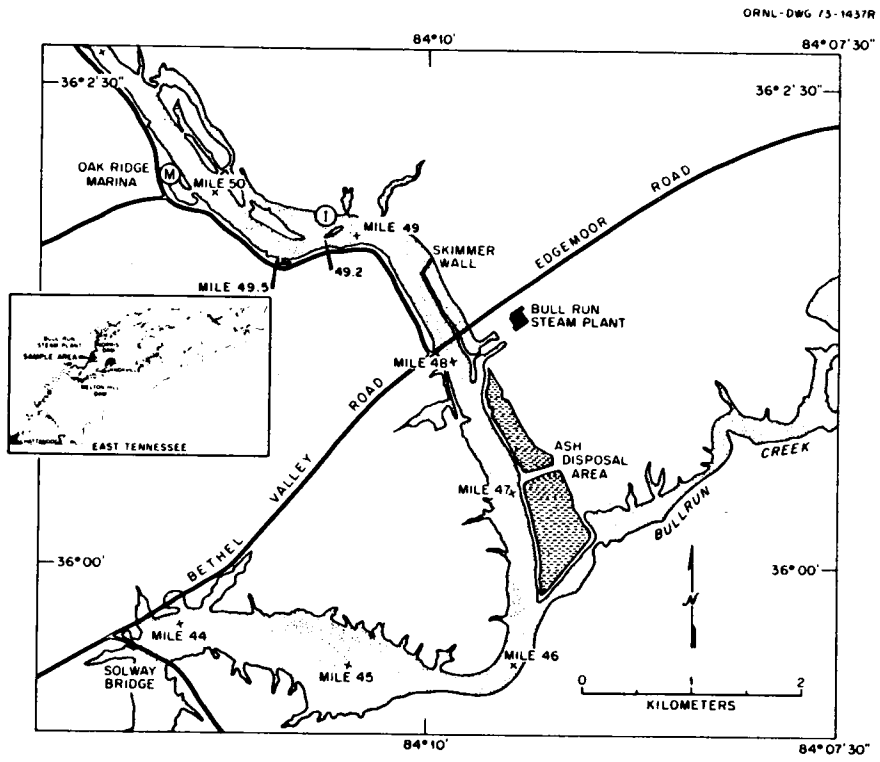
Field Site Locations

Three field sites were used for the seasonal analysis of sediment microbial activity. Two sites were located in Melton Hill Lake in east Tennessee (Figure 2). The third site was located in a small pond on the Oak Ridge National Laboratory Reservation, Oak Ridge, Tennessee.

Melton Hill Lake is part of the Tennessee Valley Authority (TVA) hydrological system used for flood control, power generation, transportation, municipal water supplies, and recreation. The lake is formed from a slow flowing section of the Clinch River. It is shallow and silty with the water level changing rapidly as affected by local precipitation and TVA management. The marina site is located at the Oak Ridge Marina, approximately 30 kilometers northwest of Knoxville, Tennessee. The island site is located 1.5 kilometers downstream from the marina site next to a small island about 100 meters long, located midlake at mile mark 49.2 (Figure 2).

The pond site is situated near the west portal entrance to Oak Ridge National Laboratory, next to the junction of Bethel Valley Road and the Target Range access road. This is a small pond 10 to 15 meters in diameter, spring fed with an outlet opposite the inflowing spring channel.

All three sites were chosen for their representiveness of local freshwater systems, accessibility, and previous investigation by



Ⓜ = Marina site.

Ⓘ = Island site; indicated by the arrow at mile 49.2.

Figure 2. Melton Hill Lake sampling site locations.

researchers at The University of Tennessee (UT) and/or the Environmental Sciences Division (ESD) at Oak Ridge National Laboratory (ORNL). The marina site was of particular interest because the sediments are chronically exposed to watercraft fuel. The marina site is located within an enclosure or levi that reduces water flow around the moored watercraft. The island site has been sampled extensively by graduate students working with Dr. Gary Sayler at The University of Tennessee, Knoxville. Some of their findings have been reported elsewhere (Pederson 1979, Pederson and Sayler 1981).

The island site has sediments that are easily collected, free of coarse fragments, and have substantial microbial activity. A coal barge dock was proposed for this particular area of Melton Hill Lake, which also added to the interest in monitoring this area for potential future effluent stress. The pond site was chosen because it represented a "clean," undisturbed area, receiving no foreign effluents other than possible road runoff from nearby Bethel Valley road.

The sediment removed at all sites was generally free of living plants. The marina sediments were totally free of visible detritus and macrophytes. The island sediment occasionally contained a few stalks of filamentous algae at the summer sampling, these were removed before sediment analyses. Melton Hill Lake does contain considerable amounts of M. spicatum L., which is controlled by TVA using 2,4-D. The pond contains filamentous algae, and macrophytes though the area sampled yielded sediments relatively free of live plant material, with any live plant material being removed before sediment mixing when the sediments were processed.

Field Sample Collection and Processing

Sediments were collected from each of the three field sites on four occasions: September 20, 1979 (late summer), November 26, 1979 (fall), January 23, 1980 (winter) and March 26, 1980 (early spring). Measurements of air and water temperature, sediment and water pH, and water dissolved oxygen (DO) were made at the time of sampling. The procedures used for these standard measurements are described in Section IIIE. Five to six cores were withdrawn at each site in 6 by 50 cm plastic core tubes, capping the ends of the tube to hold the sediment core in place with its accompanying overlying layer of water. Sediment cores at the island and pond sites were obtained by manually inserting the clean, distilled water rinsed core tubes into the soft sediments through a 0.5 meter water column. The marina samples were taken through a 3 meter water column using a gravity core sampler (Wildlife Supply Co., Saginaw, Michigan) attached to a winch.

After collection in the field, the cores were immediately taken to the laboratory at the Environmental Sciences Division, ORNL and held for approximately 18 hours at 4° to 5°C. Microbial activity of sediments is not affected by short-term refrigeration (Collins 1977). The cores were processed by decanting the surface water, and using the upper 3 to 5 cm of sediment for analysis. Three cores were retained for microbial activity assessment and sediment dry weight determinations, and the remaining cores were frozen for later estimates of sediment texture and organic matter content (Section IIIE). Sediment remaining from the winter and spring core microbial analyses was pooled at each season and frozen for organic matter and texture estimation.

The sediments used in the microbial activity analyses were placed in clean, sterile glass beakers. The standard glassware cleaning procedure used for all glassware in this study consisted of washing with detergent, rinsing with tap water, then 1 to 2 N HCl, followed by rinsing with distilled or deionized distilled water (Barnstead Ultrapure mixed-bed deionization column D0809, VWR Scientific, San Francisco, CA). The beakers of sediment were covered with sterile aluminum foil and placed in an ice bath. The sediments were thoroughly mixed using sterile glass stirrods immediately before subsampling. The sediments from each core were subsampled in duplicate for the analyses. One of the island cores at the fall field sampling was homogenized for a total of 40 seconds (s) at 10s intervals over a 60s period in a Waring Blender (Waring Products, New Hartford, CT) at setting 5 to break-up detritus and reduce replicate variability. Aliquots of sediment were dispensed using a clean, sterile, tipless, 3cc, disposable, plastic BD tuberculin syringe (VWR Scientific, San Francisco, CA), which was widened at the inlet to allow uniform filling of the syringe barrel. Sediment aliquots of 1 ml were dispensed when needed into the appropriate vessels for the analyses.

B. Microcosm Experiment

Preparation of the Microcosms

Six microcosms were prepared with sediments collected at the island field site. Field temperature, pH and DO measurements were made as previously described. The sediments were scooped with shovels into clean, plastic tubs. Melton Hill Lake water was obtained about 1.5 kilometers upstream from the island site and collected in clean 100-l

Nalgene polyethylene bottles (Nalge Co., Rochester, NY). The sediment and water was immediately taken to the laboratory. Sediment was placed in clean 76-l glass walled aquaria (Ramfab, Oak Ridge, TN) 31 cm wide, 60 cm long and 41 cm high, to a depth of 13 to 15 cm. Mats of partially decomposed leaves were obtained along with the sediments. The large mats of intact leaves were removed, while smaller sections of partially deteriorated leaf material were left as an integral part of the sediment. Several 300 ml portions of this sediment were frozen in glass jars for later addition to the microcosms to simulate natural sedimentation processes in the lake and to compensate for the sediment loss from sampling, and as the carrier for the oils during the oil treatment portion of the experiment. Some of these frozen sediments were used for sediment texture and organic matter content estimates. The lake water was carefully added to the tank to avoid disturbing the sediment. The water column in the microcosm was 22 to 24 cm deep.

The microcosms (Figure 3) were housed in a walk-in environmental chamber (Sherer Controlled Environmental Lab, model CEL 512-37, Sherer Envir. Div., Rheem Mfg. Corp., Marshall, MI). The temperature was maintained at 15±2°C for the duration of the experiment. No light was present except during sampling and sediment addition. This was done to avoid algal proliferation in the microcosms, and to simulate the light deficient conditions of the sediments beneath the silt-rich water of Melton Hill Lake. The microcosms were covered with plexiglass sheets to prevent excessive water evaporation caused by the forced air circulation system of the environmental chamber. The microcosms were allowed to settle for 1 week, then an aeration system was activated to maintain water DO at levels similar to the field site. The aeration

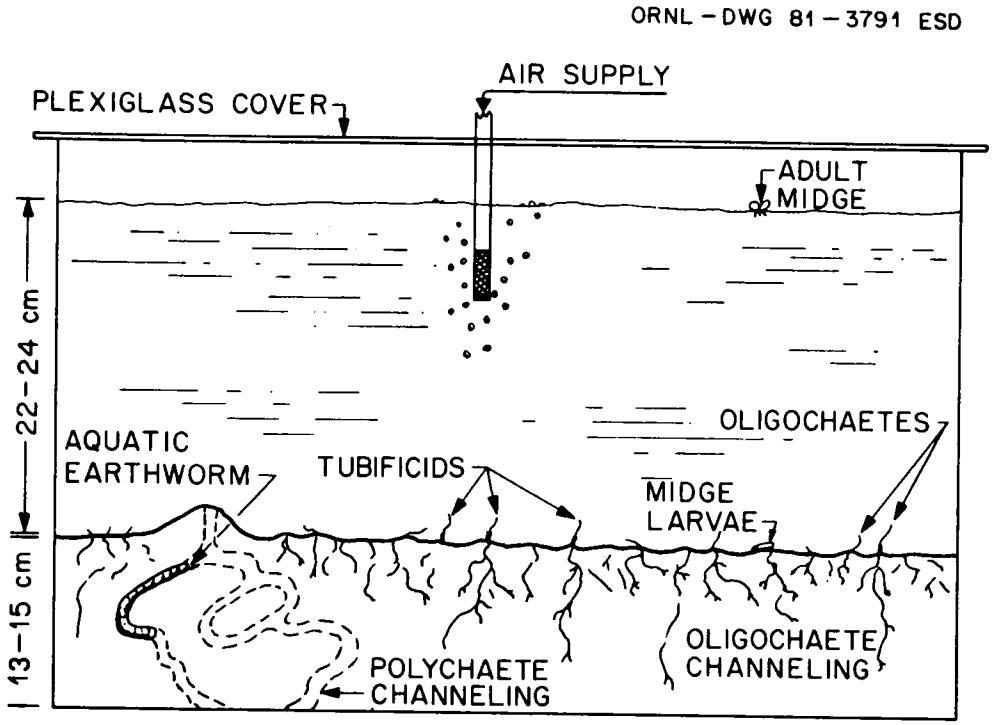


Figure 3. Microcosm illustration.

system consisted of glass gas dispersion tubes size 12-C connected with latex tubing to charcoal air filters (coconut charcoal in glass drying tubes, with glass wool plugs at the inlet and outlet) in turn connected to aquarium pumps (Whisper 500, Willinger Bros., Inc., Japan). Three microcosms were serviced by one pump. Screw clamps on the latex tubing controlled air flow along with the air flow controls on the pumps.

Microcosm Sampling and Treatments

A schedule of the microcosm experiment sampling and treatments is presented in Table 3. A 106-day equilibration/internal control period was used for obtaining background information on each microcosm before oil treatment. This allowed the microcosm sediment microbial community to stabilize after its establishment. During the equilibration period the microcosm sediment was sampled for microbial activity five times, three weeks apart, on days 14, 35, 56, 77 and 98. This also allowed each microcosm to be its own internal control for later comparison once oil treatments were initiated.

Sediment additions were made to each microcosm one week after sampling and two weeks before the next sampling. The sediments were added as sediment slurries. One jar of the frozen sediment reserved at the time of microcosm preparation was thawed overnight at room temperature. Excess water was drained and the sediment transferred to a clean beaker. One hundred milliliters of distilled water was added to the sediment and mixed. Fifty milliliters of this slurry was mixed with approximately 500 ml of microcosm water in another beaker and added to the microcosm that provided the water. The slurry was added

Table 3. Schedule for Microcosm Experiment

Date	Day	Event
8/1/79	0	Microcosms prepared
8/15/79	14	1st sampling of the equilibration/ internal control period
9/15/79	35	2nd sampling of the equilibration/ internal control period
9/26/79	56	3rd sampling of the equilibration/ internal control period
10/17/79	77	4th sampling of the equilibration/ internal control period
11/7/79	98	5th sampling of the equilibration/ internal control period
11/15/79	106	1st treatment with oiled slurries: 2 controls (A + E) 2 Diesel Fuel Marine (B + D) 2 Atmospheric Separator Overhead (C + F)
11/17/79	108	1st sampling of treated microcosms
11/22/79	113	2nd sampling of treated microcosms
12/1/79	122	3rd sampling of treated microcosms
12/6/79	127	4th sampling of treated microcosms
12/7/79	128	2nd treatment with oiled slurries
12/19/79	140	5th sampling of oil treated microcosms
12/28/79	149	3rd treatment
1/9/80	161	6th sampling
1/18/80	170	4th treatment
1/30/80	182	7th sampling
2/6/80	189	5th treatment
2/20/80	203	8th sampling End of Experiment

slowly to spread the sediment equally over the entire sediment surface. The microcosm water became murky and required several days to clear. A fine, silty new layer of sediment 2 to 4 mm thick was visible overlying the original surface sediment.

Measurements of environmental chamber air temperature, microcosm water and sediment temperature, water and sediment pH, and water DO were made at each sampling before sediment removal. The microcosm was subdivided into nine subunits by placing tape markers at the top of the tank rim, three on each side. Each subunit was randomly sampled 2 to 3 times by withdrawing approximately 2 cc of sediment at depths above 5 cm. The sediment was withdrawn using a sterile Nalgene polyethylene, 10-ml graduated pipette that was cut at the 9.5 ml graduation to form a uniformly wide opening, attached with latex tubing to a syringe barrel that provided suction for withdrawing a uniform, soft, sediment core. The sediments from the various subunits were pooled in sterile glass beakers to obtain material from throughout the microcosm sediment surface. The beakers were covered with sterile aluminum foil and the sediment allowed to settle about 0.5 to 1 hour before the surface water was decanted. During the last sampling on day 203 the sediment from microcosm F, one of the ASO treated units was allowed to settle less than 30 minutes while sediments from the control microcosms settled more than 1 hour. A sediment volume of approximately 150 ml was taken to provide sediment for additional microbial analyses (see Section IIIE).

The beakers of the collected sediments were brought to the laboratory and placed in an ice bath. The sediments were thoroughly mixed immediately before subsampling for the microbial activity and

dry weight determinations as described previously for the field sediments. Sediments from selected sampling days; 108, 113, and some sediment from the control microcosms on day 122 were homogenized in a Waring Blender for a total of 40s at 10s intervals before subsampling. A blending speed of 3 or 5 was used. Approximately 50 ml of sediment was homogenized without decanting the excess water from the sediment. Homogenization was used to break-up pieces of detritus thought to be a major factor in subsampling variation. Homogenization was discontinued as discussed in Section IV.

Characteristics of the Oils and Oil Treatments

The oils selected for the microcosm treatments were chosen from preliminary screenings of petroleum oils and H-coal, synthetic oils by Dr. Jeffrey Giddings using standardized algal toxicity tests (Giddings 1980). Water soluble extracts of the marine diesel fuel (DFM) EPA sample number 4616.7 and an Atmospheric Separator Overhead crude oil (ASO) EPA number 1312.6 were found to be the most toxic to the algae of the petroleum and synthetic oils tested. Characteristics of these oils are summarized in Table 4. The DFM oil is a fuel oil used by the United States Navy and is produced by standard petroleum refining methods. The ASO oil was produced at a pilot plant operated by Hydrocarbon Research Incorporated, Lawrenceville, New Jersey. This synthetic crude oil was produced during period 130-86-5A PDU 7 with the H-coal pilot plant operating in a fuel mode. Illinois coal number 6 was used in the liquifaction process. Atmospheric separator overhead oil is one of several oil fractions produced during the coal liquifaction process. A review of synthetic fuel production methods is provided by Worthy (1979).

Table 4. Hydrocarbon Components of the Oils Tested in the Microcosm Experiment

Hydrocarbon Fraction	% Weight of Whole Fraction: Diesel Fuel Marine (DFM) EPA # 4616	% Weight of Whole Fraction: Atmospheric Separator Overheads (ASO) EPA # 1312
Volatiles	1.02	34.72
Ether soluble bases	0.02	1.06
Insoluble bases	Not Collected (NC)	0.45
Water soluble bases	NC	NC
Ether soluble acids	0.11	0.94
Insoluble acids	NC	NC
Water soluble acids	NC	NC
Neutrals, total	97.51	54.88
Total	98.66	92.05
Neutrals Subfractions		
Saturates or Aliphatics	65.02*	11.88*
Mono and di aromatics	17.86	24.62
Polyaromatics	1.29	0.52
Residue	1.34	0.04
Total	85.51	37.06

*The low total yield of the neutral subfraction was attributed to the co-distillation of some aliphatics when the iso-propanol solvent was removed. The aliphatic portion for the DFM was estimated to be approximately 78%, and 30% for the ASO fraction.

Compiled from results supplied by: I. B. Rubin, Analytical Chemistry Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

The oil slurries used for treating the microcosms were prepared in the manner previously described in Section IIIB for preparing and dispensing the added sediments. Thawed sediment was drained and mixed with 100 ml of distilled water. This slurry was divided into three portions; one retained for the controls, one portion received 2 ml of DFM oil, and the third received 2 ml of ASO oil. The beakers were covered with Parafilm, secured with a rubber band and placed on a shaker for 5 to 48 hours at 21° to 27°C. The chamber housing the mixing slurries was usually dark during the mixing process. The beakers of sediment were covered with cardboard or plastic containers to shield them from light. After the sediments were slurried with the oils, they were distributed in the microcosms as described previously.

Glassware that was exposed to the oils or oiled sediments was separated from glassware used for the control samples. All glassware used in the microbial activity analyses that was exposed to oiled sediment was rinsed several times with acetone, cleaned with detergent, rinsed several times with each of the following solvents; acetone, distilled water, 2 N HCl, and deionized or distilled water. When further cleaning of oil-exposed glassware was required, the acetone-rinsed vessel was placed in a chromic acid bath for a minimum of 18 hours, followed by the usual cleaning procedure.

C. Adenosine Triphosphate Analysis

The cold, 0.6 N H_2SO_4 ATP extraction method of Karl and LaRock (1975) was used in these experiments. Modifications of the DuPont glassware preparation method was used (Instruction Manual for the Dupont 760 Luminescence Biometer). All reagents were analytical grade

unless otherwise specified. Sediment aliquots of 1 ml were dispensed into sterile, conical, 50-ml glass centrifuge tubes. The tubes were covered with sterile aluminum foil and the sediments rapidly frozen at -80°C by submerging the tube into an acetone/dry ice bath. The sediments were held 18 to 42 hours in a frozen state until the ATP could be extracted.

Several sets of ATP standards were used in the analysis:

1. Extraction Standards--a series of ATP standards were extracted and analyzed in the absence of sediment in order to estimate ATP loss from extraction and handling.
2. Recovery Standards--known amounts of ATP were added to 1 ml aliquots of sediment and extracted and analyzed to estimate the amount of ATP lost from the sample from ATP adsorbtion on sediment derived materials.
3. Internal Standards--known amounts of ATP were added to the extracts at the time of ATP quantitation to determine the extent of inhibition of the bioluminescence reaction by extract/sediment components (SAI photometer only).
4. Calibration Standards--known amounts of ATP were used to calibrate the photometers.

Five ml of 0.6 $\underline{N}$ H_2SO_4 at 4° to 5°C was added to the sediment, mixed on a vortex mixer (Sybron Thermolyne Maxi vortex mixer, Dubuque, IA) and set in ice. One ml of 0.025 $\underline{M}$ Tris buffer (Sigma, St. Louis, MO) pH 7.75, sterile, was added to the sediment samples and 1 ml ATP standard (Sigma, Equine ATP disodium salt) prepared in Tris buffer in a range of 10^2 to 10^4 ng ATP/ml was added to the recovery standards. The samples and recovery standards were mixed on a vortex

mixer 10s and centrifuged at 1500 rpm to separate the extract from the solids. Four ml of the extracts were transferred to sterile glass vials containing 1 ml of 0.048 M ethylenedinitrilo tetraacetic acid (EDTA, Mallinckrodt, St. Louis, MO) prepared in Tris buffer, sterile, and set in an ice bath. The pH of each extract was adjusted to 7.75 with 1.2 N NaOH and dilute HCl. The extracts were brought to volume with Tris buffer in 10-ml graduated cylinders on days before day 77 and subsequent days using 10-ml volumetric flasks, and the final pH rechecked. The extracts were transferred to glass vials, capped and immediately frozen in acetone/dry ice and stored frozen until ATP content analysis, usually 1 to 3 months.

A set of extraction standards was also prepared with each set of sediment samples. These standards were 1 ml aliquots of ATP standard, 5 ml of cold 0.6 N H_2SO_4 mixed on a vortex mixer for 60s, then 4 ml transferred to 1 ml of 0.048 M EDTA and the pH and volume adjusted as with the sediment samples. This procedure was abbreviated after day 108 onward and for the fall, winter and spring field samplings to add 3 ml of the cold acid to the 1 ml standard, mixed on a vortex mixer 10s and 4 ml transferred to the cold EDTA solution, pH and volume adjusted.

Less base was required to neutralize the samples prepared on day 182 and the winter field sampling. This was attributed to an error in the preparation of the extraction acid producing a 0.2 N acid (see Section IV).

ATP was quantified in the extracts using a DuPont 160 Luminescence Biometer (DuPont, Wilmington, DE) according to its accompanying instruction manual for samples from days 14, 77 and some

samples from day 108. A Science Applications Incorporated (SAI Technology Co., San Diego, CA) photometer model 3000, operated according to its accompanying manual was used for all other samples.

Lumit HS luciferin/luciferase was used with Lumit N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer pH 7.75 (Lumac Inc., Westlake Village, CA). The reaction mixture was prepared by combining one vial luciferin/luciferase per 5 ml buffer. This reaction mixture was used for the samples from days 14 and some samples from day 108. DuPont luciferin/luciferase at ≥100 units enzyme per vial was used for day 77. All other samples used DuPont luciferin/luciferase at the same activity level from lot number 10 26 79. The DuPont reaction mixture was prepared with 0.01 M morpholinopropane sulfonic acid (MOPS, Sigma, St. Louis, MO) and 0.01 M $MgSO_4$ (J. T. Baker Phillipsburg, NJ) pH 7.4 to 7.7, sterilized. One vial of the DuPont luciferin/luciferase was used per 6 ml buffer when the DuPont photometer was operated in a 6s peak mode. One vial of the DuPont luciferin/luciferase per 9 ml buffer was used when the SAI photometer was used in a 60s integration mode.

Extracts were thawed and placed in an ice bath along with the freshly thawed and/or diluted ATP calibration standards. Ten µl of extract or standard was used per 0.1 ml of reaction mixture in the DuPont instrument. One hundred µl extract or standard was used per 0.2 ml reaction mixture in the SAI photometer.

Internal standards were provided by adding known amounts of ATP standard to the extracts after an initial determination of the ATP content of the extract. The ATP fortified extract was then recounted in the photometer. Tris buffer was added to some extracts and the

extracts recounted to serve as a blank for the internal standards. Internal standards were not used for the samples analyzed on the DuPont photometer since a higher reaction mixture to sample ratio was used. This allows for greater dilution of inhibiting substances in the sample extract. The sample extracts from day 77 were centrifuged immediately before ATP analysis. This was done to remove the orange-gold precipate that forms in the extracts when they are neutralized.

D. Dehydrogenase Analysis

Several dehydrogenase analysis methods were modified and combined to obtain a procedure suitable for the rapid analysis of dehydrogenase activity in sediments (Ross 1971, Klein et al. 1971, Lewis et al. 1978, Casida 1977, Skujins 1973, Lenhard 1965, Stevensen 1959). One ml of sediment was added to 1 ml of sterile 0.01 M $CaCl_2$ in a sterile, air-tight sealing, disposable, 15-ml plastic centrifuge tube (Falcon or Corning, VWR Scientific, San Francisco, CA). The calcium chloride solution was used to simulate the ionic conditions present in water saturated soils (Grewling and Peech 1965). The samples were kept over an ice bath until 20 minutes before the start of the incubation period at which time they were placed in a 25°C water bath to equilibrate to 25°C. One ml of 3% triphenyl tetrazolium chloride (TTC, ICN Pharmaceuticals, Cleveland, OH) in sterile distilled or deionized water was added, the tube flushed with nitrogen gas, capped tightly and the contents mixed on a cortex mixer 10s and returned to the 25°C water bath. TTC is a hydrogen acceptor, which is reduced to triphenyl formazan (TF) by the indigenous microbial dehydrogenases. Air was flushed from the incubation vessels to prevent competition with O_2 for hydrogenation.

Short incubation times of 10 and 60 minutes were used to obtain estimates of dehydrogenase activities of the original microbial population. The linearity of the dehydrogenase activity over a 60 minute period was determined using marina sediment. Dehydrogenase activity was terminated by the addition of two 5 ml volumes of methanol. This killed the microorganisms and solubilized the TF. Blanks were prepared by adding methanol to the sediment and calcium chloride incubation medium, mixing, then adding the TTC and flushing with N_2.

The initial 10 ml of methanol was used to extract the TF for days 14 to 98. It was found that to recover all the TF produced, further extraction with methanol was required. The optimal extraction procedure used an initial 5 ml volume of methanol mixing 10s, a second addition of 5 ml methanol, the tube capped and shaken for 10s as in the original procedure. The samples were refrigerated, and further processed during the following 24 to 48 hours. The samples were brought to room temperature, resuspended, and centrifuged 10 to 15 minutes at 1700 rpm (Damom/IEC Division, Needham Hts., MA, CRU-5000 centrifuge) to separate the extract from the solids. This extract (fraction 1) was decanted into separate tubes. Five milliliters of methanol was added to the sediments, capped and refrigerated for later processing. Meanwhile fraction 1 was analyzed for formazan content using a dual beam spectraphotometer (Beckman ED, Fullerton, CA, or Perkin-Elmer/Hitachi 200, Norwalk, CT) set at 485 nm, calibrated with a series of TF standards (Sigma, St. Louis, MO) prepared in methanol ranging from 2 to 15 μg TF per milliliter solvent.

The second fraction was prepared by resuspending the sediments in the 5 ml methanol, mixed on a vortex mixer and transferring the

contents to a 50-ml centrifuge tube. The sediment was extracted with seven more 5 ml volumes of methanol delivered by a Repipet (Lab Industries model 3020-A-L, Berkeley, CA) for a final volume of 40 ml. This fraction was centrifuged and the TF content of the supernatant fluid quantified spectrophotometrically as described for the first fraction. Total TF produced was determined by adding the amount recovered in the two fractions adjusting for the extract volumes, and subtracting the blank.

E. Other Ecosystem Analyses

Organic Matter

Organic matter content of representative sediments was estimated via the Walkely-Black method, using sediments collected at each field sampling, sediment reserved from the microcosm preparation, and sediment from the control microcosms at the end of the experiment. The Walkely-Black method is a standard procedure for estimating organic carbon or organic matter in soils depending on the conversion factors used in computing the results from the raw data (Allison 1965, Grewling and Peech 1965, Broadbent 1965). Organic matter content is considered to be 1.7 to 2.0 times the organic carbon content (Broadbent 1965). A conversion factor of approximately 1.9 was used in the computations of this analysis.

Sediment Texture

Sediment texture was estimated for each field site seasonally and for the sediment used to prepare the microcosms. A standard hydrometer method was used (Day 1965, Cornell 1975). A predigestion

of the indigenous organic matter was made using hydrogen peroxide to allow free dispersion of the primary sediment particles.

Dry Weight and Water Content

Sediment dry weight and water content determinations were made using a standard soils drying method (Gardner 1965). One ml aliquots of sediment were weighed on an analytical balance (Mettler, Hightstown, NJ or Ainsworth, Denver, CO) then dried 18 to 24 hours at 105°C in a forced air drying oven (Stabil-therm, Blue M Electric Co., Blue Island, IL) in tared aluminum pans. The samples were cooled in a desiccator and reweighed.

pH

Hydrogen ion content was measured using combination electrodes and a portable pH meter (Corning model 610A, Corning, NY). In situ pH was measured by inserting a calibrated 45 cm combination electrode (Orion, Cambridge, MA, GX series 91-25) directly into the undisturbed water or sediment. A Corning plastic bodied combination electrode was used for some of the initial field and microcosm work. The pH was determined by collecting water or sediment in a beaker and measuring the pH.

Dissolved Oxygen

Dissolved oxygen (DO) was measured using a Yellow Springs Instrument (YSI, Yellow Springs, OH) model 51B or 54A-RC DO meter operated according to the instruction manuals. The DO probes were YSI 5739 with an immersible stirrer YSI 5795, and a YSI 5720 probe with an attached stirrer. Manual stirring of the water was used for some of

the early field and microcosm work. Submersible stirrers were used for the fall, winter and spring DO readings and automatic stirring was used throughout the treatment period in the microcosm study.

Temperature

Air, water, and sediment temperatures were measured in the field and laboratory with mercury thermometrs (Fisher Sci., Co., Pittsburg, PA) or temperature indicators incorporated into instruments such as the YSI DO probe, and the environmental chamber.

Other Microbial Analyses

At the end of the microcosm experiment, day 203, additional sediment was collected using the usual sampling method to yield approximately 150 ml sediment. About 100 ml of the sediment was taken to The University of Tennessee, Knoxville, where other microbial analyses were conducted. These analyses included: Total viable counts (TVC), protein, lipid and starch hydrolyzers, phenanthrene resistance, glucose, starch, protein and lipid mineralization, and naphthalene mineralization as described in a report by Sayler 1980a.

F. Data Analysis

Data processing was accomplished with a hand calculator (Texas Instruments SR-51A, Houston, TX) and/or the IBM 360/370 (White Plains, NY) computer at the Oak Ridge National Laboratory computer facilities. Statistical analyses were performed using the Statistical Analysis System (SAS) and its associated programs (Bark et al. 1979). Significance levels were set at 5% unless indicated otherwise. Sokal

and Rohlf's (1969) text on biometry was consulted for planning and conducting the statistical analyses.

IV. RESULTS

A. Observations and Comments on Sediment Biota

Examination of the Oak Ridge marina sediment during processing revealed no macroscopic organisms. The microcosm sediments were observed closely throughout the experiment. Midge larve or bloodworms were found in the island sediments collected on August 1, 1979 which were used for preparation of the microcosms. Adult midges were found in the microcosms during the first weeks of the experiment. Microcosm sediments were screened on day 35 for nematodes using a standard funnel collection technique described by Altman (1965). Few nematodes were recovered. Several types of oligochaetes were observed in the microcosms. Aquatic earthworms 8 cm long and 0.4 cm wide channeled throughout the sediment in control microcosm E, and microcosm B treated with DFM oil. These worms are usually found in sediments of shallow water less than 1 meter deep (Barnes 1963). Large piles of fresh casts signalled the openings of new burrows. Smaller oligochaetes were also active as shown by the number of smaller fresh cast piles. Many of these were tubificids identified by the tubules at the burrow openings (Barnes 1963). Tubificids are common in freshwater sediments. They are also known to inhabit organic polluted aquatic environments (Pennak 1953). The degree of protrusion and oscillation of these decomposers can indicate the extent of O_2 limitation in the ecosystem. Tubificids can be found in sediments in shallow and deep waters at densities of 8000/m^2 (Pennak 1953). The pond sediment contained snails with conical shells about 1 to 2 cm long. A sculpin fish 5 cm long was

collected in one of the winter field sampling cores at the pond site. Amphipods were observed in some pond cores and in the microcosms. These detritus feeders are found in unpolluted, freshwater systems where dissolved oxygen is abundant, moving in surface sediments and water (Pennak 1953, Barnes 1963). Amphipods were not observed later in the microcosm experiment when the interior of the microcosm was hidden by the collection of material on the aquaria walls.

The tubificids and aquatic earthworms were present in the microcosm sediment throughout the experiment as evidenced by new cast piles on the sediment surface. Small oligochaete cast pile density ranged around 0 to 10 cast piles per 3 cm^2 at the end of the equilibration period. At the end of the experiment, the small oligochaete cast pile densities were 1 to 8 per 3 cm^2 in the controls, and 8 to 13 per 3 cm^2 in the oil-treated microcosms. The particulates on the aquaria walls made cast pile counts more difficult to obtain toward the end of the experiment. The microcosms tended to have variable oligochaete activity throughout the experiment. One organism could produce several burrow openings, so that it may not be possible to equate cast pile counts with the number of oligochaetes.

B. Field Physical and Chemical Characteristics

The field physical and chemical data are presented in Appendix Table A-1. Seasonal differences in water (sediment) temperature were found. The dissolved oxygen content of the water, when considered as a function of water temperature was relatively stable throughout the year. The water at the island site was about 80% saturated with dissolved oxygen. The marina water was about 70% saturated and the

pond site approximately 90% saturated. These levels of dissolved oxygen in the water above the sediments indicated that O_2 is available in the surface sediments as introduced by diffusion, underwater currents, and water/sediment mixing by oligochaetes and benthic fish.

The water and sediment pH were also relatively stable throughout the seasons. The spring sampling yielded pH values more acidic than those obtained during the other seasons. This is perhaps due to the sluggish response of the pH electrode noted at this sampling. Pederson (1979) found water and sediment pH to be seasonally stable at sites adjacent to the island site, and nearly identical to those found in this study during the summer, fall, and winter.

C. Microcosm Physical and Chemical Characteristics

The microcosm physical and chemical data are presented in Appendix Table A-2. Water and sediment pH and temperature were quite constant throughout the experiment. Water dissolved oxygen levels were comparable with that of the island field site. Particulates collected on the walls of all the microcosms during the experiment increased to the extent that the microcosm interior could not be seen through the tank sides. Microscopic examination of this debris revealed no recognizable morphology of either a biological or geological origin. The particulates were probably aggregates of silt or clay attracted to the glass surface. Continuous additions of sediment to the microcosms could have supplied the material for a continuous build-up on the walls. The fine silty layer on the sediment surface also increased with time, again due to the additions of sediments throughout the experiment. Some dark lines were formed at

the sediment/water interphase along the sides of the aquaria. The thickness of these dark lines varied between the microcosms but were present in all units.

Hydrocarbon Treatment Concentrations

The amount of oil added to each microcosm per treatment was estimated to be 200 to 300 ppm based upon a 3 cm depth of surface sediment mixing by the oligochaetes. The final concentration of oil was estimated to be 1300 ppm at the end of the experiment, after five oil treatments. The actual concentration of hydrocarbons in the sediment is probably less due to loss of volatiles, and removal during sampling. Not all the oils were adsorbed on the sediments as evidenced by oil "slicks" that formed on the water surface during oiled sediment additions. These slicks were not visible 2 to 4 days post treatment. Comparison of these hydrocarbon concentrations with those found in the aquatic environment (Sections I and II) indicate that the microcosms received low to moderate inputs.

D. Sediment Texture and Organic Matter Content

Field and microcosm organic matter estimates are listed in Appendix Table A-3. Sediment texture was consistent during the months sampled in the field (Appendix Table A-4). Changes in the percentage of sand, silt, and clay seasonally at a particular site did not alter the textural classification to a significant degree. Organic matter was highest at the pond site, averaging 14%. The island site averaged 5% to 6% organic matter, and the marina site averaged approximately 3% organic matter. The microcosm sediments from the

control units showed a slight decrease in organic matter content from 7% at the start of the experiment to 5% at the end of the experiment. This may be more a feature of the sampling depth for the sampling of the microcosms at the end of the experiment, which comprised the finer textured surface material as opposed to sampling material characteristic of the total sediment thickness as was done when the microcosms were prepared.

E. Field Sediment ATP and Dehydrogenase Results

Field seasonal ATP content and dehydrogenase activity are presented in Figures 4-6. The 10 and 60 minute dehydrogenase activities had a correlation coefficient (r) of 0.907 at α=0.0001. The standard deviations were slightly greater for the shorter incubation time though both incubations yield similar overall trends in dehydrogenase activity with the 10 minute incubations somewhat more variable. Analysis of variance coupled with Duncan's multiple range tests show the 10 minute incubated summer dehydrogenase activity to be significantly higher when all sites are combined, followed by the fall activity, and the winter and spring activities significantly lower. The winter and fall dehydrogenase activities were not significantly different when comparing 60 minute dehydrogenase means. When comparing sites and combining the data for all seasons, the marina had significantly greater dehydrogenase activity, with the island and pond sites having similar activities. The most detailed comparison of means was conducted on season*site basis. The summer marina activity was especially high and the spring pond activity the lowest. The results of the 10 and 60 minute dehydrogenase activity Duncan's multiple range tests for season*site are presented in

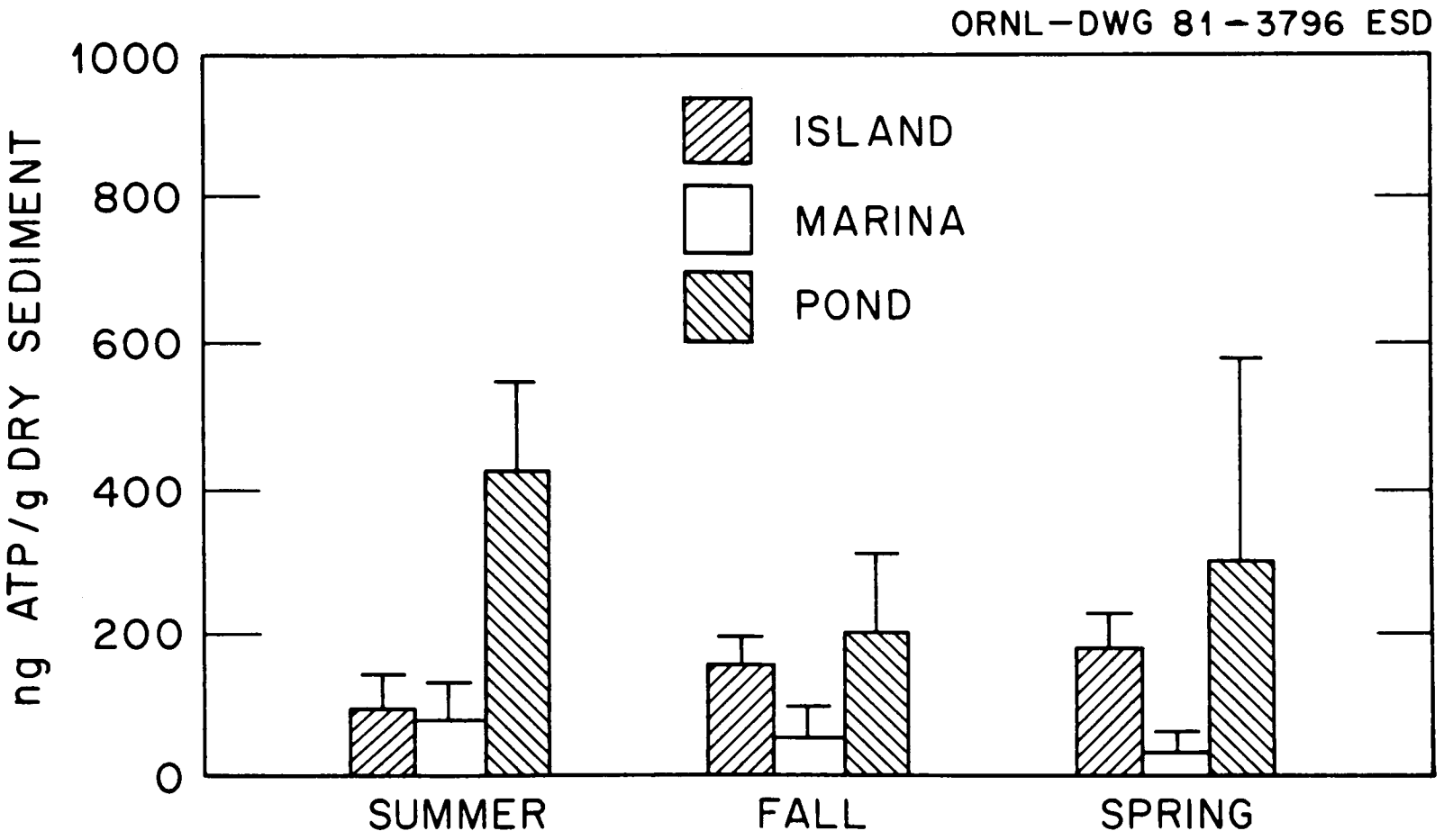


Figure 4. Field site mean ATP content of the sediment microbial community.

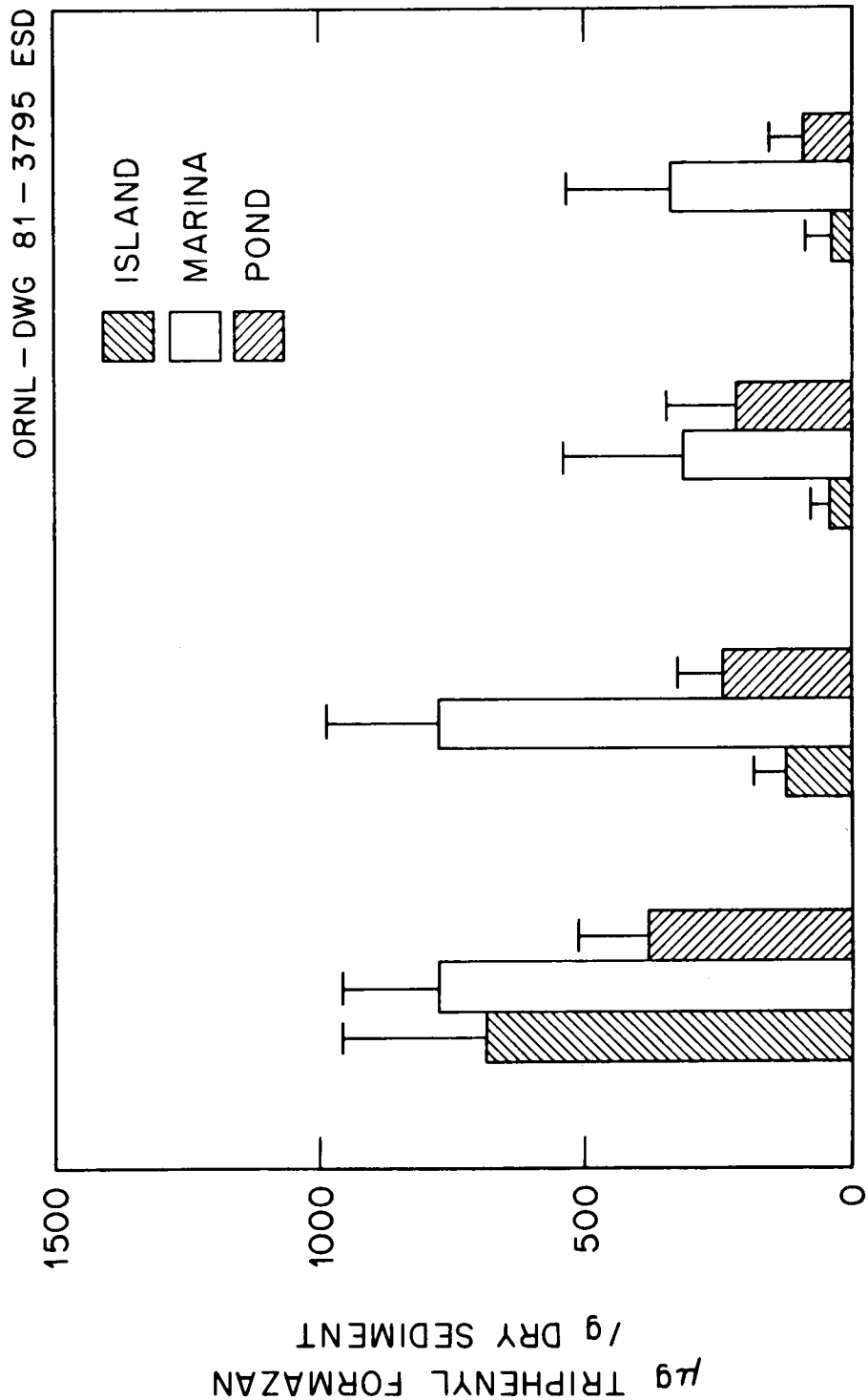


Figure 5. Field site mean 10 minute incubation dehydrogenase activities of the sediment microbial community.

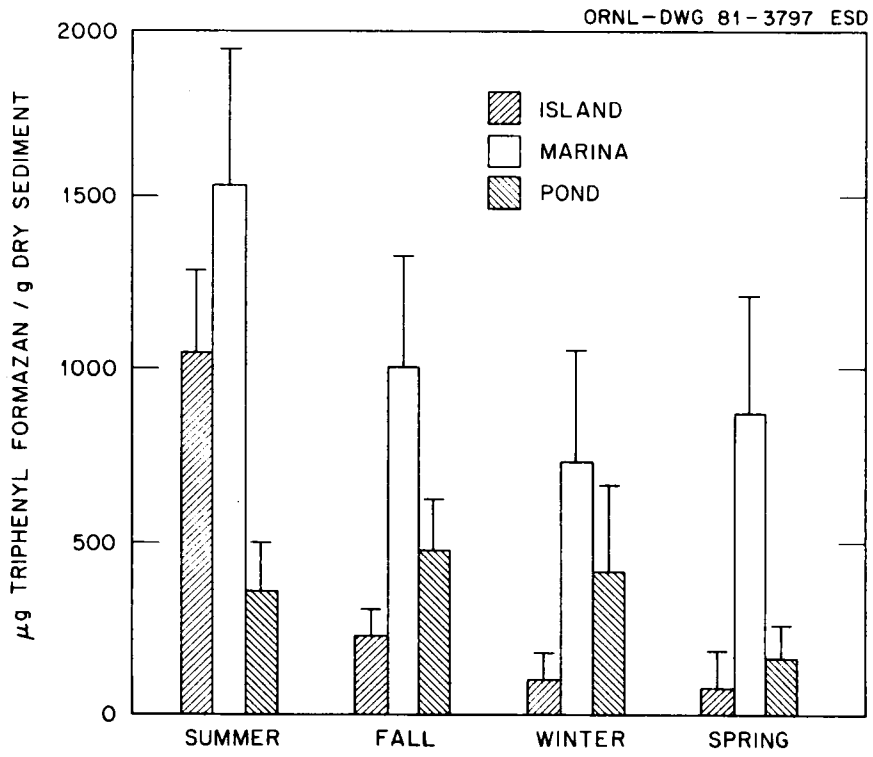


Figure 6. Field site mean 60 minute incubation dehydrogenase activities of the sediment microbial community.

Appendix Figures A-3 and A-4. Analyses of variance (ANOVA) F-statistic results are presented in Appendix Table A-5. Season, site and season-site interaction were significant in all cases.

Sediment ATP contents showed no seasonal variation and all sites had significantly different ATP contents as illustrated in the ANOVA results in Appendix Table A-5. The Duncan's multiple range tests groups for the field ATP contents are presented in Appendix Figure A-5. The marina site had the lowest ATP content, and the pond site the highest ATP content.

Correlation coefficients for the field dehydrogenase and ATP results are shown in Appendix Table A-6. Significant negative correlations were found using the 10 and 60 minute incubation dehydrogenase results and the ATP contents.

Field Site Core Replicability

Cores were taken randomly at each field site. The degree of similarity of these cores for a particular site during a specific sampling event or season was determined using analysis of variance and Duncan's multiple range tests. The cores taken at the island site showed ATP concentrations to be statistically similar during each season sampled. This was also true at the marina site. The pond cores showed some significant differences in ATP concentration at each season, with one core differing from the other two.

Island dehydrogenase activities measured over 60 minutes showed no significant differences among cores for the fall, winter and spring, with one core differing in the summer. The marina site yielded cores that were significantly different during each season. The pond core

60 minute dehydrogenase activities were not different during the summer and spring, while the fall and winter cores showed one core to have significant variation.

Ten minute dehydrogenase activities showed similar patterns at the various sites. At the island site, one core during the summer sampling was significantly different from the other cores, all other cores were similar at a particular season. The marina cores showed significant differences among cores at each season. The pond cores were not significantly different during the fall and spring samplings.

F. Microcosm Sediment ATP and Dehydrogenase Results

The ATP content and dehydrogenase activities as treatment means are presented in Figures 7-9. Dehydrogenase activities in microcosm sediments were moderate on day 14, then increased significantly on day 35 and dropped successively on days 56, 77, and 108. From day 108 until the end of the experiment, dehydrogenase activity was stable in the control microcosms. The microcosm 10 and 60 minute incubated dehydrogenase activities yielded a correlation coefficient of 0.686 for the equilibration period and 0.892 during the treatment period (α=0.0001). ATP content was relatively stable throughout the experiment with an increase in ATP content during the later part of the equilibration period, and a significant increase on day 127 for the controls, with a slight drop in ATP on day 122 in the controls.

Analysis of variance (ANOVA) demonstrated (Appendix Table A-7) that significant differences among microcosm ATP levels were attributable to the sampling day. The oil treatments were begun on day 106, with the first sampling on day 108. ANOVA for the treatment

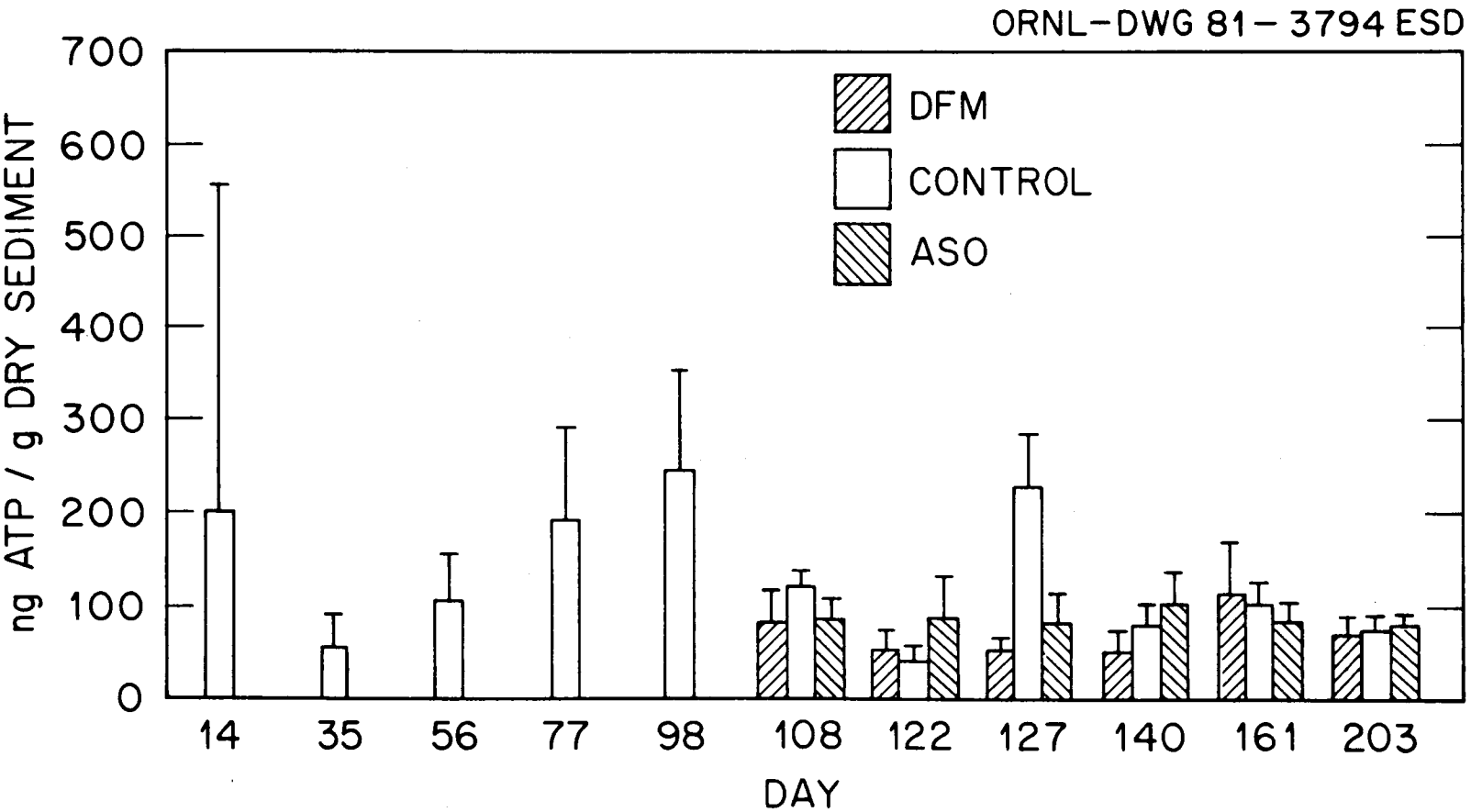


Figure 7. Microcosm experiment treatment means for sediment microbial community ATP content.

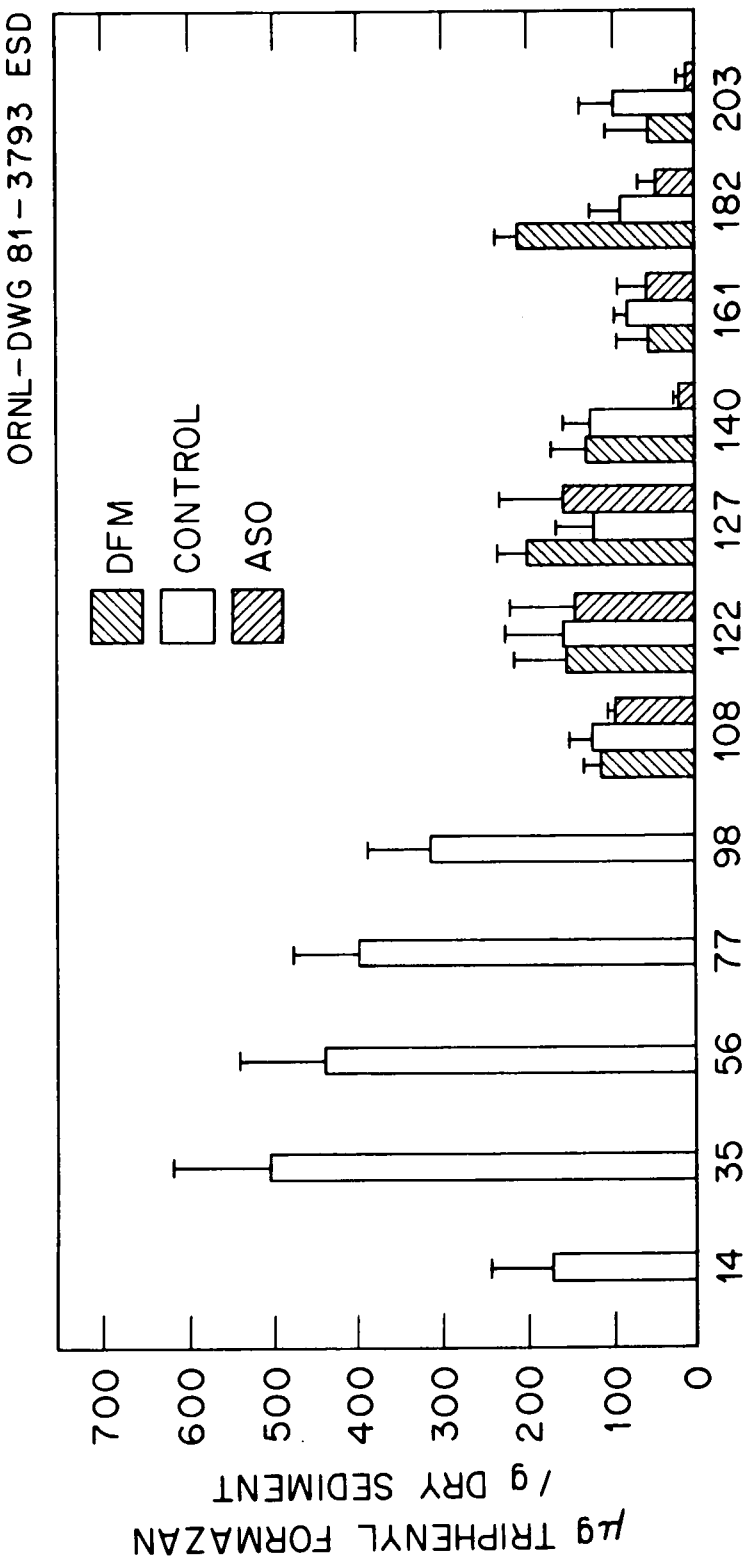


Figure 8. Microcosm experiment treatment means for 10 minute incubation dehydrogenase activities.

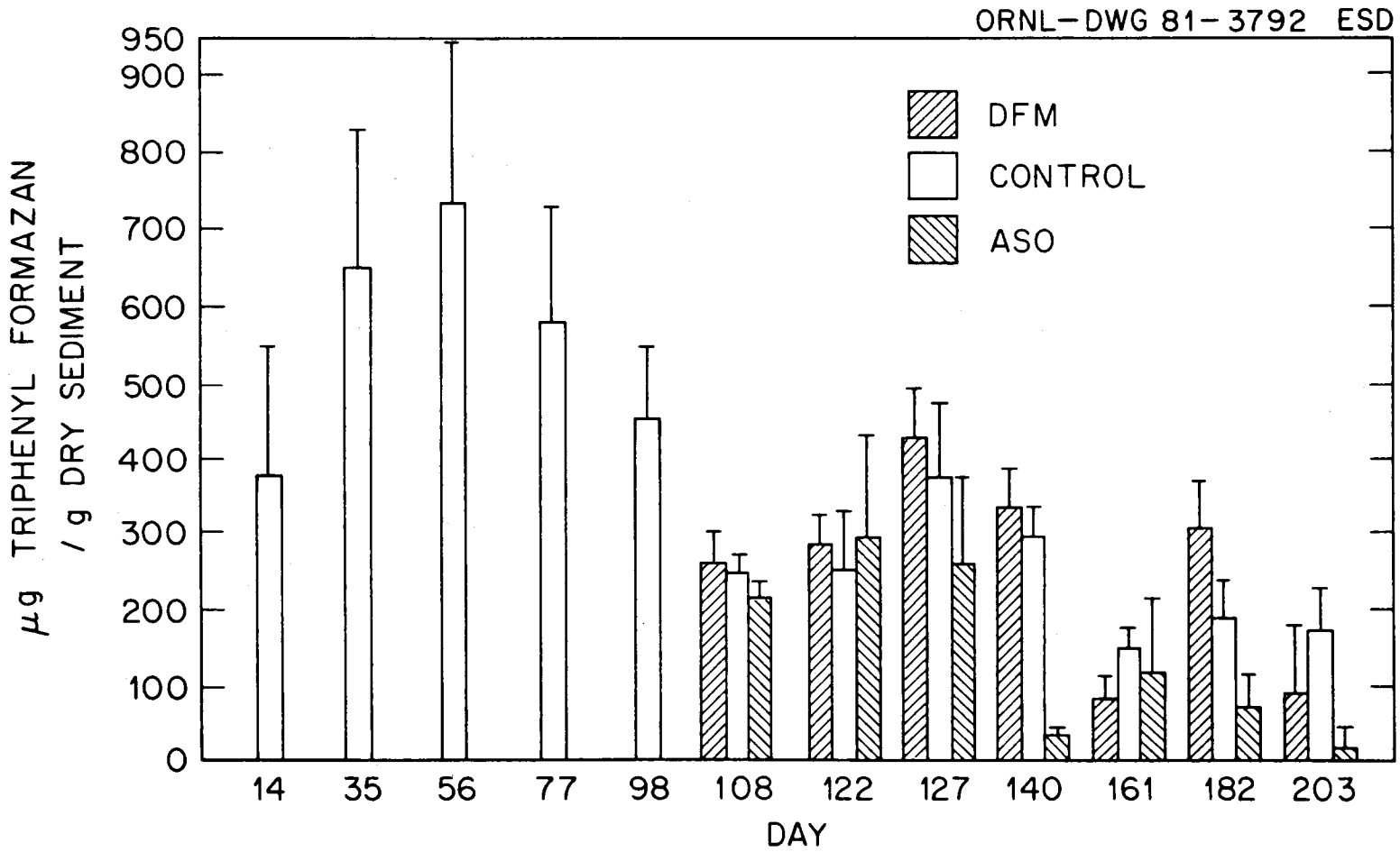


Figure 9. Microcosm experiment treatment means for 60 minute incubation dehydrogenase activities.

period showed significant effects attributable to the sampling day, treatment, and sampling day*treatment interactions for both microbial measurements. Duncan's multiple range tests for the overall mean during the treatment period showed the ASO treatments to have significantly lower mean dehydrogenase activities compared with the control and the DFM-treated microcosms, which were not significantly different from each other. The exception was the 10 minute dehydrogenase mixed model nested ANOVA where DFM treatments yielded significantly greater activity than the controls which were significantly greater than the ASO-treated sediment activities. When a day-by-day assessment of the data is made for the individual microcosms, a trend for lower dehydrogenase activity in the ASO-treated microcosms seems to be established by day 140, with a slight recovery on day 161, then a continued decline through the end of the experiment. The dehydrogenase activity of the DFM-treated microcosms tended to be greater than most other microcosms on day 182, but this increase was followed by a decrease on the last sampling day.

Sediment ATP levels, analyzed as overall treatment means, demonstrated that the controls had significantly higher overall mean ATP levels than either treatment, and that the treated sediments were not significantly different from each other. Inspection of the ATP results on a day-by-day basis reveals that the overall higher ATP content of the control microbial community is attributable to day 127 where a substantial increase in the control ATP content was found. The ATP content of the microcosms was not significantly different on days 108 and 203. During the interim, the individual microcosms showed some changing relationships in terms of ATP. The treatment replicates were not significantly different except for the ASO replicates on day 122.

Correlation analysis among mean ATP and dehydrogenase levels over time indicated a slight negative correlation during the equilibration period (Appendix Table A-8).

Microcosm Replicability

Microcosm replicates in the equilibrium period and treatment period of the experiment were compared using ANOVA and Duncan's multiple range tests. The microcosms were not significantly different in ATP concentration during the equilibration period when individual microcosm means were determined for all sampling days combined. On a day-by-day basis, microcosms were not significantly different on a particular sampling day with the exception of one microcosm on day 14. During the treatment period, the ATP concentrations of the controls were not significantly different on each sampling day. The DFM-treated microcosms were also not significantly different on each sampling day, while the ASO replicates were significantly different on 17% of the sampling days.

The overall mean microcosm 60 minute dehydrogenase activities were not significantly different during the equilibration period with the exception of one microcosm. During the treatment period these activities were significantly different on 14% of the sampling days for the controls, 29% for the DFM, and 43% for the ASO-treated microcosms. The microcosm that was significantly different in 60 minute dehydrogenase activity during the equilibration period later became one of the ASO-treated microcosms and may have contributed to replicate variation due to its generally lower level of dehydrogenase activity. Frequent sampling and changing microbial communities may have increased replicate differences around days 108 through 127 which was soon after the addition of new sediment and/or treated sediment.

The overall mean 10 minute incubated dehydrogenase activities in the microcosms were significantly different during the equilibration period. During the treatment period the control replicates were significantly different on 57% of the sampling days, the DFM replicates were significantly different on 29% of the sampling days, and the ASO-treated microcosms were significantly different on 43% of the sampling days.

The close degree of replicability shown by the microcosms in ATP concentration was further illustrated by a mixed model nested ANOVA conducted for the treatment period. Microcosm within treatment variation was not significant. This same type of ANOVA was conducted on the dehydrogenase results. Replicates were significantly different within treatments.

Other Microbial Community Analyses Results

The results of the analyses conducted at The University of Tennessee, Knoxville, using day 203 microcosm sediments are presented in Appendix Tables A-9 through A-12. Inspection of this data in comparison with the microcosm means for dehydrogenase activity and ATP content on day 203 showed no outstanding correlations.

The results of the additional microbial analyses are discussed in a report by Sayler (1980b). The microbial populations in the treated and control sediments were not significantly different, nor were the alkaline phosphatose activities. The ASO-treated microcosms yielded significantly greater glucose, starch, lipid, protein, and naphthalene mineralization rates. The DFM-treated microcosms had significantly greater starch, protein and lipid mineralization rates.

V. DISCUSSION

A. Hydrocarbon Effects on Sediment Biota

The activity of the small oligochaetes in the oil-treated microcosms may have increased as successive oil treatments were made. The oil-treated microcosms may have had more oligochaete activity over the whole sediment surface at the end of the experiment. The "newness" of the cast piles is a subjective estimate. The presumably greater number of cast piles on the oil-treated sediment surface may be an accumulation over time, and may not be a change in oligochaete population size or activity. The large aquatic earthworms appeared to survive the oil treatments since fresh casts were apparent on the last sampling day. The presence of cast piles does not reveal the health or size of the polychaete community.

If increased oligochaete activity is a real response to hydrocarbon inputs, it could affect the microbial community by increasing surface sediment mixing and aeration. This might increase the aerobic microbial population and the oxidation of hydrocarbons. The increased sediment mixing may increase nutrient availability by incorporating freshly sedimented surface material deeper into lower sediments. The increased activity of the oligochaetes may be due to the organisms seeking oil-free sediment, water, and detritus. Gordon et al. (1978) found that surface oil concentrations exceeding 600 ppm caused polychaetes to surface and in some cases die. Polychaete sediment-working rates were found to decrease in oil contaminated sediments.

A study of oligochaete and polychaete diversity, community size, and oil accumulation in tissues would add valuable information to future microcosm and aquatic ecosystem studies. Some studies have been conducted with marine fauna in oil-polluted areas (Leppäkoski and Lindstrom 1978). Specie diversity decreased in polluted areas, yet biomass remained high. Tubificids, midges, polychaetes and amphipods were found to recolonize sediments which had previously received oil refinery effluents. Polychaetes in marine environments with oil polluted sediments did not have markedly higher tissue levels of oil than polychaetes in nonpolluted areas (Gordon et al. 1978).

B. Statistical Analyses Selection

Statistical analyses were conducted using results from the 60 and 10 minute dehydrogenase incubations. Statistical analyses were conducted using averaged recovery standard computed ATP values for all of the data, and part of the data where ATP recovery was unusually low. Statistical analyses were originally conducted with and without the homogenized sediments, which consisted of one of the island site cores at the fall sampling, and the homogenized microcosm sediment from the controls on day 122. Inclusion of the homogenized sediments increased the range about the mean for the site or microcosm. This in some cases broadened the groupings in the multiple range test, but did not significantly alter the F-test results of the ANOVA. All the sediments from days 108 and 113 were homogenized and the data from day 108 were included in all statistical analyses presented here. The day 113 data showed significant effects from homogenization at a higher blending

speed and would bias the overall results, therefore that sample is not included in the results reported here.

The discussion and assessment of the data will focus on the statistical analyses computed without the homogenized sediments from the fall field sampling island core, and day 122. ATP values computed using overall average recovery standard correction were used only for the dates where ATP recovery was extremely low. This was done in the case of the homogenized samples because this yielded more balanced data sets without the increase in range brought about by the homogenized sediments. Homogenization appeared to decrease dehydrogenase activity and slightly increase ATP values in some cases. A decrease in dehydrogenase activity may be due to cell mortality caused during texture modification processed at faster blade speeds. An increase in ATP estimates might be attributed to mechanical release of ATP from the cells, accompanied by an increase in ATP complexation sites of a non-cellular origin as reflected in a lower ATP recovery standard value. Use of the accompanying ATP recovery standard value in the computation of the final ATP value compensates for the increase in sorbtion surface area brought about by disruption of sediment particles. This relationship was also noted by Jenkinson and Oades (1979) when they used an acid extraction medium and sonication of soils.

The use of the accompanying ATP recovery standards for a particular date and treatment is still considered by the author to reflect better actual sediment ATP recovery for a particular extraction. Thus, the average ATP recovery standard values are used only on the dates where an unexplained low recovery of ATP from the recovery standards (less than 10%) was obtained. This includes the winter and

spring field samplings and days 14, 161, and 203 in the microcosm experiment. Consideration of these selected groups of data will also simplify the discussion and assessment of the results and statistical analyses.

ATP values obtained for the winter field sampling and day 182 of the microcosm experiment were approximately ten fold lower than that of any other date and thus are not included in the results presented here. The accompanying dehydrogenase values were not exceptionally different though the field values were one of the lowest of the seasons examined. This suggests that the recovery of ATP was not complete in these samples presumably due to the probable use of a weaker acid extractant. The pH of the sediment/acid extractant would be higher using a lower normality acid. The stronger acid provides better recovery of ATP by more complete acid hydrolysis of the microbial cells and increased protonation of the triphosphate group (Lee et al. 1971a, Lehninger 1970). Three of the ionizable protons have pK' values around 2 to 3, the fourth is at 6.5. The degree of protonation of the triphosphate moiety is important in shielding the molecule's active sites from other charged sediment particles where complexation could occur. Addition of the acid extract to the EDTA solution allows some ions in the extract to be chelated and eliminated from further contact with the ATP (Karl and LaRock 1975). Neutralization of the extract is required for the bioluminescence reaction. This step precipitates iron and aluminum hydroxides out of solution which tend to co-complex the ionized ATP anion at neutral pH (Lee et al. 1971a, Lehninger 1970).

C. Dehydrogenase Analyses Incubation

The shorter incubation time of 10 minutes may be preferable in limiting microbial metabolic changes that would not be indicative of their initial or *in vivo* metabolic state. The shorter incubation time has the disadvantage of possibly increasing error between replicate subsamples or within samples by mechanical handling and timing of the beginning and ending of the incubation period. A few seconds difference is a larger percentage of a 10 minute incubation than a 60 minute incubation though still minor in both cases. More samples can be handled with greater ease using a 60 minute incubation.

The linearity of dehydrogenase activity during 60 minutes was checked using sediment from the spring sampling at the marina site. The linear regression correlation coefficient was 0.959 when comparing the means of triplicate samples at seven incubation times. This indicates a fair fit in a linear model. The position of the y-intercept, the rapid increase in TF during the first 5 minutes and the 60 minute-TF concentrations being two to three fold greater than the 10 minute concentrations rather than the predicted six fold indicates that an expotential model may provide a better fit of the data; $y=a+b \log x$. Expression of dehydrogenases activity is best reported in terms of TF accumulation over a set time period.

D. Field Microbial Communities

Field data suggests that there are different microbial communities at the three sites, in terms of numbers and/or species as reflected by significant differences in ATP contents. Dehydrogenase

activity indicates a seasonal, temperature effect on microbial activity. High dehydrogenase activity and low ATP may suggest a rapid turnover of ATP. The lower organic matter content at the marina site might suggest fewer nutrients are available at that site. This may not be the case since organic matter estimates reveal no information about the availability of nutrients and organic carbon to the microorganisms. An area of heavy human and waterfowl use such as the marina may add more dissolved nutrients and organics to the aquatic system as opposed to the pond site where organics and nutrients may be in intact plant material. Studies of inorganic nutrient levels, and an increased number of sampling events during each season are needed to better define changes in the microbial community. Usage of specific nutrients and estimates of community diversity could be obtained by conducting analyses using substrate mineralization rates, and media containing various substrates. A test oil or oil components could be incorporated into some of these media. The marina site may contain a higher population of fuel hydrocarbon degrading organisms since that site is exposed to watercraft fuels.

E. Microcosm Simulation of Field Microbial Activity

The sediment used for the microcosms was collected at the island site in the summer. The sediment and water temperature and dissolved oxygen concentration in the water of the microcosms was adjusted to simulate the island site in the summer. The summer sampling at the island site showed the mean dehydrogenase activity to range around 1042±237 µg TF/g oven-dry sediment, and 690±261 µg TF/g oven-dry sediment for the 10 minute incubation. The mean microcosm

dehydrogenase activities were 376±194 µg TF/g oven-dry sediment for the 60 minute incubation, and 221±151 µg TF/g oven-dry sediment for the 10 minute incubation. These means are not similar for the field site and microcosms with both field activities three fold higher than the microcosm activities.

The field ATP concentration was 94.8±49.3 ng ATP/g oven-dry sediment at the summer sampling and 133±72.4 ng ATP/g oven-dry sediment for the microcosms. The field and microcosm sediment ATP contents are similar. ATP content in the microcosms appears to reflect that in the field during the summer, while the microcosm dehydrogenase activities are lower than the field. The differences between the field and microcosm dehydrogenase activities are not necessarily unexpected since dehydrogenase activity appears to be more variable than ATP concentrations as reflected by the field cores at all sites and the microcosms during the equilibration period, and the treatment replicates during the treatment period. ATP content in cores and microcosm replicates is reproducible whereas the dehydrogenase activities are less consistent among replicates. Averaging microbial community characteristics over time in the microcosms cannot give a totally realistic comparison of the microcosms and the field site. Other factors, such as inorganic nutrient depletion, could be occurring in the microcosms. One sampling event during a season may not show all the community changes that may occur in that season. Realistic comparisons of the microcosms and field site should include many more characteristics and sampling events than were measured here.

Pederson (1979) found rates of methanogenesis in Melton Hill Lake to be similar to those found in microcosms of a similar natural

sediment system. She also found the microcosms, which were similar in size to the ones used in this experiment, to have low biological, chemical and physical variability among replicates.

F. Microcosm and Core Replicability

As mentioned in previous sections, the microcosm microbial biochemical status was consistent in terms of ATP analyses throughout the experiment among replicates. ATP content was also consistent among cores at a particular site indicating that the cores were representative of the site though the sites receive irregular inputs of soil and other material from surrounding watersheds. Currents in Melton Hill Lake will also cause changes in sediment substrate layers and composition. Leaf fall in the nearby deciduous forests contributes considerable organic matter to the island and pond sites and no discernible leaf material to the marina site. Inclusion of part of a leaf in a core could contribute substantial microbial activity to a core since microorganisms tend to accumulate on detrital surfaces.

Dehydrogenase activity showed more significant differences between field site and microcosm replicates. The equilibration period of the microcosm experiment showed the 60 minute dehydrogenase activity to be consistent for all but one of the microcosms. The random selection of microcosms to receive treatments included this microcosm as one of the ASO-treated replicates. The low dehydrogenase activity of this microcosm continued throughout most of the experiment. This contributed to some of the differences between the ASO-treated replicates. Both ASO-treated replicates were significantly lower in dehydrogenase activity at the end of the experiment than the controls

though the replicate with nondeviate dehydrogenase activity during the equilibration period showed more fluctuation in activity in the treatment period. A significant microcosm within treatment F-statistic was revealed in the mixed model nested ANOVA.

The 10 minute dehydrogenase activity of the control microcosms showed variability between replicates throughout the equilibration and treatment periods. This suggests that this measure of metabolic activity may be more sensitive to or easily disturbed by environmental or microbial inter or intra populational changes. This variability may also be due to natural biological rhythm in the organism itself such as feeding, movement, reproduction or dormancy. The shorter dehydrogenase incubation time may accentuate these short-term metabolic changes and may reflect more acutely possible methodological bias in sample processing.

Larger coefficients of variation were found for the microcosm and core ATP means, and overall models in the associated ANOVAs. This may account, in part, for the seemingly better replicability of ATP content among microcosm and cores. The 60 minute dehydrogenase activities had lower coefficients of variation than the 10 minute activities.

In order to obtain better replication in both the field, and among microcosms, more replicates may be required. The microbial activity for a site on a particular sampling day was determined by taking the mean of the three core values. The treatment effects in the microcosm experiment were compared using both the treatment replicate means, and the individual microcosm activities. Averaging of both the core and microcosm microbial community characteristics combines replicate variation which may be acceptable in the case of ATP

content, and may be masking significant replicate differences in the case of dehydrogenase activity in the microcosms and at the marina site. The overall results of the microcosm experiment, viewed from averaging microcosm replicates or assessing the individual microcosms separately, appear to show the same trends.

Sediment microbial communities may be of markedly small physical size, situated on a section of a detritus, or a clay particle. The cores and pooled microcosm sediment would thus contain numerous microbial microhabitats with perhaps different associated microbial communities. Obtaining statistically and biologically similar replicates may be a nearly impossible task since sediments and sediment microbial communities are naturally heterogeneous. Replicability of methodologically practical samples will most likely not be consistent with currently accepted statistical limits since the samples are probably biologically diverse.

G. General Toxicity of the Hydrocarbon Mixtures

The composition of the oils (Table 4, page 37) studied reveals some differences in the gross composition of the refined petroleum fuel oil and the coal-derived synthetic crude oil (Rubin 1980). Thirty-five percent of the ASO oil consists of volatile components. Its neutral fraction is composed of 25% mono- and di-aromatics and 12% to 30% aliphatic or saturates. The DFM has volatile components amounting to 1%, 98% neutral components of which 65% to 78% are aliphatic or saturates and 18% are mono- or di-aromatics. The amount of hydrocarbons reaching the microcosm sediments in the ASO-treated microcosms will be less from loss of volatile compounds than that in the DFM-treated

microcosms. Of the hydrocarbons reaching the microbial community in the microcosm sediment, a greater portion may be potentially utilizable in the DFM-treated microcosms. The DFM oil contained a higher percentage of aliphatics or saturates. N-alkanes in these fractions have the greatest potential for microbial biodegradation and assimilation (Higgins and Gilbert 1977). Aromatic compounds are less likely to be assimilated by microorganisms than aliphatics, and if metabolized in the microorganism, they are usually via co-metabolic pathways which consume ATP in the cell but do not necessarily regenerate ATP since the end products may not be carbon or energy sources (Higgins and Gilbert 1977). Polyaromatic hydrocarbons (PAHs) are most noted as producing potential mutagens in eucaryotic organisms, and possibly some procaryotes through the activity of mixed-function oxidases (Walburg 1979, Higgins and Gilbert 1977, Gunsalus and Marshall 1971). Intracellular modification of the ring system through oxidation yields electrophilic regions of the PAH which have an affinity for the nucleotide bases of DNA and other genetic materials (Walburg 1979, Gibson et al. 1975). The DFM oil contains 1.3% PAHs compared with 0.5% in the ASO oil. The identity of the PAHs in these fractions has not been reported. A high content of phenanthrene or anthracene would yield a less mutagenically toxic PAH fraction than one containing 1,2-benzanthracene, 3,4-benaphenanthrene or benzo(a)pyrene (Walburg 1979).

The results of analyses conducted at ORNL using mammalian toxicity tests (Smith 1980 personal communication) and the Ames assay (Rao et al. 1980, Epler, Rao and Larimer 1980) indicates that neither of these oils are particularly toxic. The mammalian tests showed the

DFM oil to be nontoxic using standard acute oral and intraperitoneal tests, and dermal and eye irritation tests. The ASO oil was slightly toxic in acute oral tests. The microbial *in vitro* tests showed ASO and DFM type oils to yield 0% mutagenic activity.

Giddings (1980) found freshwater algal cultures to have a 1% to 29% reduction of photosynthetic activity when treated with water soluble fractions of the DFM oil. The basic fraction contributed to the greatest decrease in photosynthesis. Water soluble fractions of the ASO oil caused 40% to 92% decrease in photosynthetic activity in the algal cultures. The basic and acidic fractions were both particularly toxic to one or the other of the two algal species tested.

Pederson (1979) measured rates of methanogenesis from sediments treated with hydrocarbons derived from oil. The sediments were from microcosms prepared with natural freshwater sediment and water, including the associated plants and animals. Methanogenesis rates were not significantly different between the control and hydrocarbon-treated sediments at the end of the experiment.

H. Assessing Hydrocarbon Effects on ATP, Dehydrogenase Activity and Other Microbial Community Characteristics

When considering the treatment period as a whole, comparing means from combined replicate data, the DFM oil treated microcosms showed some increases in dehydrogenase activity, but generally were similar to the control microbial populations. The substantial input of aliphatic hydrocarbons provided by the DFM may have occasionally stimulated growth and respiration in the microbial community. The ASO

oil with its significant contribution of aromatics may initially stimulate some metabolic pathways. This activity may have been mainly from co-metabolic processes that may not produce suitable end products for ATP regeneration, thus lowering dehydrogenase activity at the end of the experiment and perhaps causing an eventual decrease in ATP levels if the experiment continued past day 203.

The negative correlations, which occasionally occurred between ATP concentrations and dehydrogenase activity, may indicate that control mechanisms may shunt metabolic energy supplied from ATP away from growth processes when ATP pools begin to become depleted decreasing growth and rebuilding the ATP pool. These negative correlations were noted in the field sediments and microcosm equilibration period.

Chapman and Atkinson (1977) describe that a very narrow range exists in the cellular or microbial adenylate energy charge ratio during growth and normal metabolism; $(ATP+1/2ADP)/(ATP+ADP+AMP)=0.8$ to 0.95. This ratio must be quite critical to the organism and must be tightly regulated since growing bacteria can have an ATP turnover rate of one second or less. Adenine nucleotides are intermediates in the biosynthesis of nucleic acids, histidine and the nucleotide cofactors NAD, and NADP, which are important in electron transfer mediated by the dehydrogenases. The adenine nucleotides form an energy transfer system which couples all metabolic processes, and they regulate the activities of many metabolic enzymes. If the overall ATP concentration is changing for the whole microbial community, then there must be a significant change in the community population, metabolic or viability status.

The significant decreases seen in the microcosm experiment between the control sediment microbial community ATP concentrations and the oil treated microbial communities seem to indicate changes in the microbial community at some time during the treatment period. All the microcosms had similar ATP concentrations initially; after treatment the treated replicates responded in a coordinated manner. The overall effect of the treatments was to produce reduced ATP concentrations in treated microcosms below that of the controls. The treatment period mean higher ATP content of the controls can be attributed to day 127. The higher ATP content of the controls on this day may be due to the increased disturbance of the community by the repeated samplings before that day. The oil treatments may have suppressed microbial activity. The addition of new sediment along with increased sediment mixing caused by withdrawing sediment at the time of sampling may have increased ATP production if more nutrients and oxygen were made available.

Another possibility is that the control ATP concentration on day 127 may be returning to the level of the equilibration period. Previous samplings on days 108 and 122 contained the newly added sediment in which the microbial community may not have been fully established. The oil treatments may have been retarding the establishment of the microbial community in the treated microcosms. Both the establishment of the microbial community in the new sediment and an increase in nutrients and aeration may be affecting the ATP concentrations of the microcosms during this time.

The activity of the dehydrogenase enzymes in microorganisms is responding to a variety of controls; needs for energy or a particular

substrate, availability of cofactors and electron acceptors and the possible presence of toxic substances. It may be expected that unless severe metabolic stress is placed on the microorganism or community that ATP concentrations might be stable even in a slightly perturbed system. Dehydrogenase activities are responding to ATP controls and may show more variability in response to shunting metabolic activity away from growth to maintenance of ATP pools. The temporary stimulation of dehydrogenase activity in the DFM oil treated systems on day 182 did not appear to alter ATP levels on day 203.

The results of the additional microbial analyses conducted at the end of the microcosm experiment seem to indicate an increase in mineralization rates of most test substrates in the oil-treated sediments. The ASO-treated sediment microbial community also showed an increased mineralization rate for the di-aromatic hydrocarbon naphthalene. The microbial communities, in terms of overall size, were not significantly different. The ATP contents of the communities on the last sampling day were not significantly different, while the drhydrogenase activity of the ASO-treated microbial community was substantially lower than in the controls or DFM-treated microcosms. These results may suggest that microbial community size may be comparable in all microcosms, but different catabolic pathways may be operative. The microbial community in these sediments is most likely to be predominantly heterotrophic, aerobic, and facultative anaerobic organisms. These organisms can metabolize some aliphatics and aromatics through a variety of pathways. Some aromatics such as naphthalene, phenols, benzene, and toluene might be degraded to

catechol which can pass into the β-ketoadipate pathway in some organisms (Stanier et al. 1976). The end products of this pathway are acetyl CoA and succinate both of which are used for ATP generation via the tricarboxylic acid (TCA) cycle and respiration. Saturates, and mainly n-alkanes, are oxidized in some microorganisms to fatty acids which may produce end products for the TCA cycle and respiration. ATP regeneration in the latter case may be more efficient since fewer steps are required to yield the TCA cycle components, and less energy expended during decomposition of suitable aliphatics than aromatics.

Microbial community growth does not yet appear to have been stimulated to a statistically significant degree by the increased substrate mineralization rates in the oil-treated microcosms. The regeneration of ATP in the oil-treated sediments may be adequate to replenish that consumed in the extra degradative steps required to decompose the more complex hydrocarbons, but not sufficient to increase overall community size. Community composition may be different in the various treated sediments and controls. This could also account for the differences in mineralization rates found at the end of the experiment.

VI. CONCLUSIONS

The field sites showed significant differences in ATP content of the microbial community in the sediments. The pond sediments had the highest ATP content, followed by the island, then the marina site. These differences were site specific and not seasonal. The dehydrogenase activities were both site specific and affected seasonally. The summer sampling yielded the highest dehydrogenase activities. The fall dehydrogenase activity was similar to the winter activity in the 60 minute analyses, but different from the winter when comparing 10 minute analyses. The winter and spring dehydrogenase activities were not significantly different at both incubations. The marina site had significantly higher dehydrogenase activity than the other two sites. These results indicate a difference in community size, composition and/or growth state at various sites, and metabolic activity during various seasons. The cores or site subsamples taken at each site seemed to be representative of a particular site with the possible exception of the marina dehydrogenase activity which was variable among cores at all samplings.

The microcosms appeared to simulate the island site in terms of ATP content and physical/chemical features and showed lower overall dehydrogenase activity. Simulation of the field site may be improved by incorporation of a flow-through system in the microcosm design. This would better imitate the field dynamics, but would increase the complexity of the experimental design physically, mechanically, and in the control of treatments and evaluation of results. Measurement

of nitrogen, phosphorus and other inorganic nutrient levels would add valuable information about community growth, and future growth potential.

Both treatments may have had significant effects on ATP content after the first oil treatment. The microcosms were not significantly different in ATP content at the end of the experiment. When the treatment period as a whole is considered, comparing treatment means, the hydrocarbon treatments appear to lower ATP content significantly.

Dehydrogenase activity, when considered for individual microcosms on separate sampling days, was the same for all the microcosms immediately after the first treatment. After the second oil treatment, the ASO-treated microcosms were significantly lower in activity than the controls and DFM-treated microcosms. After the fourth oil treatment the DFM-treated microcosms tended to have greater dehydrogenase activity than the controls, while the controls tended to have greater activity than the ASO-treated microcosms. At the end of the experiment, after the fifth oil treatment, the controls had significantly greater dehydrogenase activity than the ASO-treated microcosms, the DFM-treated microcosms tended to have activity midway between the controls and ASO-treated microcosms. When mean overall treatment values are compared for the treatment period, dehydrogenase activity in the ASO-treated microcosms is significantly lower than the controls and DFM-treated microcosms. This was in part contributed to by an initial low dehydrogenase activity in one of the ASO-treated replicate microcosms.

The objective of this study was to examine field freshwater sediment microbial communities to determine if significant metabolic

activity changes occurred during the year in different aquatic ecosystems. Also to determine if chronic low-to moderate-level hydrocarbon inputs from a refined petroleum oil and a synthetic coal-derived crude oil had any discernible effects on microbial community viability and metabolic activity. The complexity of natural systems demands that a wide variety of chemical, physical, biochemical, and physiological characteristics be evaluated in order to understand the natural changes occurring in a sediment microbial community. Human induced changes in the microbial community, caused by released effluents and pollutants into the aquatic system, need to be differentiated from the natural community variation found in different sediment substrates and climates. Much information can be derived from a microcosm experiment if all its components, such as the soil and water chemistry, macroorganisms, microorganisms, test substance dissimilation, and ecosystem modeling, are thoroughly assessed. The results of the general studies reported here indicate a need for more detailed studies since some significant effects were found.

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APPENDIX

I. Chemical and Physical Characterization of Effluent.

II. Chemical and Physical Characterization of the Target Ecosystem.

III. Determination of Testing Priority.

IV. Laboratory Screening Test
- A. Determine relative sensitivity of specific life stages of fish, invertebrates, and microbial community to the effluent.
- B. Determine the effects of critical environmental parameters on toxicity to fish, invertebrates, and microbial community.
- C. Determine the stability of the toxicological characteristics of the effluent.
- D. Screen effluent for fish tainting.
- E. Determine the potential for bioconcentration.

V. Evaluating the Results of I-IV
- A. Should future studies be conducted with all three elements of the Rood chain? NO ↘ VI, YES ↘ VIII
- B. Should future studies be conducted to define the relationship between toxicity and environmental parameters? NO ↘ VII, YES ↘ VIII
- C. If the effluent undergoes modification in the ecosystem, are the secondary products toxic? NO ↘ VII, YES ↘ VIII
- D. Are additional fish flesh tainting tests needed? NO ↘ VI, YES ↘ VIII
- E. Is there bioconcentration of effluent components? NO ↘ VII, YES ↘ VIII

VI. Redefine Research Needs and Proceed to Step VIII.

VII. No more testing required unless indicated by field tests.

VIII. Definitive Laboratory Tests
- A. Determine the relative sensitivity of the life cycle and developmental stages.
- B. Determine the extent of environmental factors affecting toxicity.
- C. Define the cause of the effluent's instability and factors affecting it.
- D. Define the range of concentrations producing fish flesh tainting.
- E. Define the degree of bioconcentration.

Figure A-1. Laboratory study toxicity evaluation protocol.

IX. Evaluating the Results of VIII
   A. Which element of the food chain should be used to evaluate the effects of sublethal exposure of the effluent on growth, reproduction and physiology?
   B. Is there a potential for biomagnification?

X. Chronic and Biomagnification Tests
   A. Determine the toxicant effects on growth, reproduction and physiology.
   B. Determine how the ecosystem and the toxicant interact for expression of toxicity in reproduction, growth and physiology.
   C. Determine the potential for biomagnification.

XI. Data Base Developing for Evaluating Effects on Target Ecosystem.

Modified from: J. Cairns, Jr. and K. L. Dickson, 1978, Field and laboratory protocols for evaluating the effects of substances on aquatic life. Journal of Testing and Evaluation 6:81-90.

Figure A-1 (Continued)

I. Characterization of Waste.

II. Characterization of Target Ecosystem.

III. Determination of Priority.

IV. Field Screening Test
- A. Determine the effects of the effluent on the invertebrate, periphyton and microbial communities.
- B. Determine if the effluent components can undergo biomagnification.

V. Evaluating the Results of I-IV
- A. Is there a significant effect on the invertebrate, periphyton and microbial communities? NO ↘ VI, YES ↘ VII
- B. Is there biomagnification? NO ↘ VI, YES ↘ VII

VI. No more tests required unless indicated by laboratory tests.

VII. Food Chain and Public Health Studies: Extended Study.

VIII. Extended Field Studies
- A. Determine the effects of the effluent on major elements of the aquatic food web.
- B. Determine the effects of the effluent on primary productivity and decomposer activity; microbial activity.

IX. Evaluating the Results of VII-VIII
- A. Is species diversity and functional capacity of the aquatic community significantly affected by the effluent? NO ↘ VI, YES ↘ X
- B. Is there an effect on primary productivity and decomposer activity? NO ↘ VI, YES ↘ X

X. Definitive Field Study
- A. Define the extent of response of the affected group of aquatic organisms.
- B. Define the extent and conditions under which an affect occurs on primary productivity and decomposer activity.

Figure A-2. Field study toxicity evaluation protocol.

XI. Evaluating the Results of X
   A. Is the effluent having a significant effect on aquatic life? NO ↘ VI, YES ↘ XII
   B. Are the effluent effects going to have an impact on human health? NO ↘ VI, YES ↘ XII

XII. Controls on effluent production and discharge.

XIII. Data base developing for evaluating effects on target ecosystem.

Modified from: J. Cairns, Jr. and K. L. Dickson, 1978, Field and laboratory protocols for evaluating the effects of substances on aquatic life. Journal of Testing and Evaluation 6:81-90.

Figure A-2 (Continued)

Table A-1. Field Physical and Chemical Data

Date	Site	Air Temp °C	Surface Water Temp °C	Bottom Water Temp °C	Water pH	Surface Water DO ppm	Bottom Water DO ppm	Sediment Temp °C	Sediment pH
9/20/79	Island	--	20.7	20.1	6.8	6.3*	4.8*	20.1	7.2
	Marina	--	18.5	18.2	6.8	5.8*	3.7*	18.2	7.2
	Pond	19.8	--	16.4	6.8	--	6.2*	17.0	6.2
11/26/79	Island	14.0	--	13.8	7.2	--	8.6	--	6.8
	Marina	14.0	13.8	13.6	7.4	7.8	7.7	--	7.3
	Pond	13.5	--	13.9	6.6	--	8.9	13.8	6.2
1/23/80	Island	1.2	--	7.0	7.4	--	>15.0	--	7.2
	Marina	1.2	7.5	7.5	7.6	9.6	9.2	--	7.4
	Pond	3.5	--	12.0	4.6	--	10.0	--	5.1
3/26/80	Island	12.5	--	11.1	5.6	--	8.8	--	5.7
	Marina	12.5	10.8	10.2	5.7	8.0	8.4	--	6.0
	Pond	15.0	--	13.2	4.4	--	10.5	--	4.5

*Measured with manual mixing of the water around the probe membrane.

Table A-2. Microcosm Physical and Chemical Data

Day	Treatment	Microcosm	Water °C	Water pH	Water DO ppm	Sediment °C	Sediment pH
0	Micro. Prep.		14.5	7.6	--	15.0	7.3
14	Equilibration	A	16.1	7.7	--	16.2	7.4
		B	16.1	7.6	--	16.0	7.4
		C	16.5	7.6	--	16.1	7.4
		D	16.0	7.8	--	16.6	7.4
		E	16.0	7.5	--	15.9	7.4
		F	15.4	7.7	--	15.6	7.4
35		A	16.6	7.0	8.0*	16.4	7.1
		B	16.3	7.2	8.2*	16.4	7.0
		C	16.6	7.4	8.5*	16.7	7.0
		D	16.8	7.6	8.6*	16.6	7.0
		E	16.3	6.8	8.7*	16.4	6.8
		F	16.1	6.9	8.9*	16.0	7.0
56		A	16.0	8.3	7.3*	16.1	7.2
		B	16.2	8.2	7.2*	16.4	7.2
		C	16.2	8.2	7.2*	16.5	7.2
		D	16.7	8.4	6.6*	16.8	7.4
		E	16.3	8.4	7.5*	16.6	7.2
		F	16.2	8.2	7.6*	16.4	7.0
77		A	15.4	8.0	6.0*	15.2	7.8
		B	15.2	8.2	4.8*	15.5	8.0
		C	15.4	8.2	4.3*	15.5	7.8
		D	15.7	8.2	4.4*	15.6	7.8
		E	15.2	8.2	5.0*	15.3	7.8
		F	15.3	8.2	4.8*	15.3	7.8
98		A	14.8	7.8	7.7	14.8	7.9
		B	14.8	7.8	7.7	14.8	7.8
		C	14.9	7.8	7.6	15.0	7.8
		D	15.0	8.0	8.2	15.0	7.7
		E	14.7	8.0	8.1	14.8	7.8
		F	14.6	8.0	8.3	14.7	7.7
108	ASO	C	16.1	7.9	8.1	16.3	7.6
		F	15.8	8.0	8.3	16.1	7.7
	Control	A	15.8	7.8	7.9	16.0	7.8
		E	16.2	8.0	7.6	16.2	7.6
	DFM	B	16.1	7.9	8.4	16.1	7.9
		D	16.2	8.0	8.0	16.6	7.7
113	ASO	C	15.7	7.7	8.6	15.8	7.6
		F	15.2	8.1	8.2	15.3	7.6
	Control	A	15.4	8.1	8.9	15.6	7.8
		E	15.8	7.7	8.2	15.9	7.8

Table A-2 (Continued)

Day	Treatment	Microcosm	Water °C	Water pH	Water DO ppm	Sediment °C	Sediment pH
	DFM	B	15.8	8.2	8.4	16.0	7.8
		D	15.6	8.1	8.4	15.9	7.8
122	ASO	C	15.0	7.8	8.4	15.0	7.6
		F	15.0	8.0	8.5	15.0	7.7
	Control	A	15.0	7.8	8.2	15.0	7.6
		E	15.1	7.8	7.9	15.2	7.3
	DFM	B	15.0	8.0	8.2	15.0	7.6
		D	15.0	8.0	8.4	15.3	7.6
127	ASO	C	15.4	8.0	6.9	15.8	7.8
		F	15.2	8.4	8.8	15.4	8.0
	Control	A	15.0	8.2	6.8	15.2	7.8
		E	15.3	8.0	7.6	15.8	7.6
	DFM	B	15.4	8.2	6.2	15.6	7.9
		D	15.3	8.4	8.6	15.8	7.8
140	ASO	C	15.6	8.0	7.8	15.8	7.6
		F	15.2	8.2	8.2	15.5	7.6
	Control	A	15.1	8.2	8.5	15.2	7.5
		E	15.3	7.8	8.0	15.5	7.2
	DFM	B	15.4	8.2	8.3	15.6	7.6
		D	15.6	8.1	8.2	15.7	7.4
161	ASO	C	15.6	8.0	8.0	15.8	7.5
		F	15.2	8.0	7.8	15.6	7.5
	Control	A	15.2	7.9	8.6	15.7	7.4
		E	15.5	7.9	7.9	15.7	7.4
	DFM	B	15.6	8.2	8.5	15.9	7.8
		D	15.7	8.0	8.4	16.0	7.6
182	ASO	C	15.7	7.7	7.9	16.0	7.2
		F	15.6	7.8	8.1	15.8	7.4
	Control	A	15.5	8.2	8.0	15.8	7.6
		E	15.4	7.9	8.1	15.7	7.2
	DFM	B	16.0	8.1	8.2	16.0	7.8
		D	15.6	7.8	7.6	16.0	7.2
203	ASO	C	15.6	7.7	8.4	15.6	7.2
		F	15.6	7.6	8.0	15.8	7.2
	Control	A	15.1	7.8	8.0	15.6	7.3
		E	15.7	7.8	8.3	15.6	7.4
	DFM	B	15.6	8.2	8.8	15.5	7.8
		D	15.5	7.8	8.2	15.6	7.3

*Measured with manual mixing of the water around the DO probe membrane.

Table A-3. Organic Matter Content of Field and Microcosm Sediments

Sediment		Core Mean ± SD*	%CV	Site Mean ± SD**	%CV
Microcosm(s)	8/1/79	7.2 ± 0.64	8.9		
A	2/20/80	5.1 ± 0.24	4.8	5.1 ± 0.14	2.8
E		5.1 ± 0.01	0.11		
Island core 1	9/20/79	4.6 ± 0.07	1.4	4.5 ± 0.17	3.7
core 2		4.4 ± 0.23	5.1		
core 1	11/26/79	5.2 ± 0.33	6.3	5.5 ± 0.45	8.1
core 2		5.8 ± 0.28	4.8		
core 1	1/23/80	4.7 ± 0.40	8.5	5.0 ± 0.37	7.5
core 2		5.4 ± 0.02	0.35		
CS[Δ]		4.8 ± 0.23	4.7		
core 1	3/26/80	7.9 ± 0.26	3.3	7.2 ± 0.64	8.9
core 2		6.8			
CS		6.8 ± 0.19	2.8		
Island overall mean		5.6 ± 1.1	19.8		
Marina core 1	9/20/79	3.2 ± 0.01	0.34	3.3 ± 0.07	2.2
core 2		3.4 ± 0.02	0.50		
core 1	11/26/79	3.3 ± 0.01	0.42	3.0 ± 0.28	9.3
core 2		2.8 ± 0.01	0.40		
core 1	1/23/80	3.2 ± 0.01	0.35	3.2 ± 0.07	2.1
core 2		3.2 ± 0.003	0.09		
CS		3.2 ± 0.14	4.5		
core 1	3/26/80	3.4 ± 0.02	0.49	3.4 ± 0.09	2.7
core 2		3.6			
CS		3.3			
Marina overall mean		3.2 ± 0.22	6.8		
Pond core 1	9/20/79	12.4 ± 0.37	3.0	12.2 ± 0.39	3.2
core 2		11.9 ± 0.27	2.2		
core 1	11/26/79	20.1 ± 0.63	3.1	18.0 ± 2.6	14.6
core 2		15.9 ± 1.7	10.5		
core 1	1/23/80	12.2 ± 0.03	0.23	14.4 ± 2.6	18.1
CS		16.7 ± 0.33	2.0		
core 1	3/26/80	13.0 ± 0.72	5.6	12.5 ± 0.55	4.4
core 2		12.2 ± 0.02	0.14		
CS		12.3 ± 0.59	4.8		
Pond overall mean		14.1 ± 2.8	20.0		

*Mean of duplicate samples.
**Mean of all replicates.
[Δ]Combined slurries.

Table A-4. Percent Sand, Silt, Clay and Textural Class in Field and Microcosm Sediments

Sediment		% Sand	% Silt	% Clay	Textual Class*
Microcosm sediment	8/1/79	21	54	25	Silt loam
Island core 1	9/20/79	34 > 32 ± 2.1	42 > 44 ± 2.8	24 > 24 ± 0.71	
core 2		31 > CV = 6.5%	46 > CV = 6.4%	23 > CV = 3 %	Loam
core 1	11/26/79	24 > 25 ± 1.4	60 > 58 ± 2.8	16 > 17 ± 1.4	
core 2		26 > CV = 5.7%	56 > CV = 4.9%	18 > CV = 8.3%	Silt loam
core 1	1/23/80	34 > 35 ± 1.4	47 > 46 ± 1.4	19 > 19	
CS**		36 > CV = 4.0%	45 > CV = 3.1%	19 >	Loam
core 2	3/26/80	36 > 34 ± 2.8	40 > 45 ± 7.1	24 > 21 ± 4.2	
CS		32 > CV = 8.3%	50 > CV = 15.7%	18 > CV = 20%	Loam
Island overall mean		30 ± 5.5	49 ± 6.7	21 ± 3.3	
		CV = 18%	CV = 14%	CV = 16%	Loam
Marina core 1	9/20/79	0	45	65	Silty clay/clay
core 1	11/26/79	0	48	66	Silty clay/clay
CS	1/23/80	0	38	68	Clay
CS	3/26/80	0	34	71	Clay
Marina overall mean		0	41 ± 6.4	68 ± 2.6	
			CV = 16%	CV = 3.9%	Clay
Pond core 1	9/20/79	0	72	32	Silt loam/silty clay loam

Table A-4 (Continued)

Sediment			% Sand	% Silt	% Clay	Textual Class*
Pond	core 1	11/26/79	0	75	32	Silt loam/silty clay loam
	CS	1/23/80	3	67	30	Silty clay loam
	CS	3/26/80	4	69	27	Silt loam
Pond overall mean			2 ± 2.1 CV = 118%	71 ± 3.5 CV = 4.9%	30 ± 2.4 CV = 7.8%	Silty clay loam

*Based on USDA system--soil texture triangle (Donahue et al., 1971).

**Combined slurries.

Table A-5. Field Data Statistical Analyses (ANOVA)

Variable	Classification	N	DF	F-stat.	α	Sig.Δ
10 DEH	Season	72	3	34.83	.0001	S
	Site		2	36.52	.0001	S
	Season* site		6	5.93	.0001	S
	Model = Season site Season* site		11	19.37	.0001	S
60 DEH	Season	72	3	24.52	.0001	S
	Site		2	64.88	.0001	S
	Season* site		6	6.48	.0001	S
	Model = Season site Season* site		11	22.02	.0001	S
ATP	Season	54	2	1.34	.2716	NS
	Site		2	24.86	.0001	S
	Season* site		4	2.80	.0369	S
	Model = Season site Season* site		8	7.95	.0001	S

Δ = Significant at α = 0.05; NS = Not Significant at α = 0.05.

Table A-6. Field Correlation Coefficients

Variables	Classification	N	r	α
ATP + 60 DEH	Overall	26	-0.589	.0016
	Summer	9	-0.866	.0025
	Fall	8	-0.727	.0410
	Summer Pond	3	-0.999	.0073
ATP + 10 DEH	Overall	26	-0.440	.0246
	Summer	9	-0.742	.0221
	Fall	8	-0.708	.0495
10 DEH + 60 DEH	Overall	35	0.907	.0001

Table A-7. Microcosm Statistical Analyses

Variable	Classification	N	DF	F-statistic	α	Significance α = 0.05
ATP Eq$^{\Delta}$	Day	89	4	3.65	.0086	S
10 DEH Eq	Day	90	4	36.13	.0001	S
60 DEH Eq	Day	89	4	14.26	.0001	S
ATP Eq	Day	89	4	3.90	.0071	S
	Microcosm		5	0.79	.5461	NS
	Day* Microcosm		20	1.34	.1922	NS
	Model		29	1.60	.0641	NS
10 DEH Eq	Day	90	4	80.56	.0001	S
	Microcosm		5	12.96	.0001	S
	Day* Microcosm		20	3.23	.0002	S
	Model		29	15.58	.0001	S
60 DEH Eq	Day	89	4	50.71	.0001	S
	Microcosm		5	11.23	.0001	S
	Day* Microcosm		20	9.18	.0001	S
	Model		29	15.26	.0001	S
ATP Trt$^{\Delta}$	Day	107	5	9.24	.0001	S
	Microcosm		5	5.21	.0004	S
	Day* Microcosm		25	5.78	.0001	S
	Model		35	6.19	.0001	S
10 DEH Trt	Day	126	6	34.64	.0001	S
	Microcosm		5	28.46	.0001	S
	Day* Microcosm		30	6.71	.0001	S
	Model		41	13.45	.0001	S
60 DEH Trt	Day	125	6	68.51	.0001	S
	Microcosm		5	39.51	.0001	S
	Day* Microcosm		30	8.57	.0001	S
	Model		41	21.11	.0001	S
ATP Trt	Day	107	5	7.65	.0001	S
	Treatment		2	10.39	.0001	S
	Day* Treatment		10	9.02	.0001	S
	Model		17	8.78	.0001	S
10 DEH Trt	Day	126	6	17.10	.0001	S
	Treatment		2	20.66	.0001	S
	Day* Treatment		12	5.40	.0001	S
	Model		20	10.43	.0001	S
60 DEH Trt	Day	125	6	33.35	.0001	S
	Treatment		2	35.83	.0001	S
	Day* Treatment		12	7.17	.0001	S
	Model		20	17.89	.0001	S
ATP Trt	Day	107	5	9.24	.0001	S
	Treatment		2	12.55	.0001	S
	MC (Treatment)		3	0.31	.8190	NS
	Day* Treatment		10	10.88	.0001	S
	Day* MC (Treatment)		15	2.37	.0079	S
	Model		35	6.19	.0001	S

Table A-7 (Continued)

Variable	Classification	N	DF	F-statistic	α	Significance α = 0.05
10 DEH Trt	Day	126	6	34.64	.0001	S
	Treatment		2	41.87	.0001	S
	MC (Treatment)		3	19.52	.0001	S
	Day* Treatment		12	10.94	.0001	S
	Day* MC (Treatment)		18	3.90	.0001	S
	Model		41	13.45	.0001	S
60 DEH Trt	Day	125	6	68.51	.0001	S
	Treatment		2	73.61	.0001	S
	MC (Treatment		3	16.78	.0001	S
	Day* Treatment		12	14.70	.0001	S
	Day* MC (Treatment)		18	4.48	.0001	S
	Model		41	21.11	.0001	S

ΔEq = Equilibration period, Trt = Treatment period.

Table A-8. Microcosm Correlation Coefficients

Variables	Classification	N	r	α
ATP 10 DEH Eq$^{\Delta}$	Overall	30	-0.338	.0674
ATP 60 DEH Eq	Overall	30	-0.365	.0472
ATP 10 DEH Trt$^{\Delta}$	Overall	36	-0.264	.1197
ATP 60 DEH Trt	Overall	36	-0.0267	.877
ATP 10 DEH Trt	Day 77	6	-0.824	.0439
ATP 10 DEH Trt	Day 122	6	-0.845	.0342
10 DEH + 60 DEH Eq	Overall	30	.686	.0001
10 DEH + 60 DEH Trt	Overall	42	.892	.0001

$^{\Delta}$Eq = Equilibration period, Trt = Treatment period.

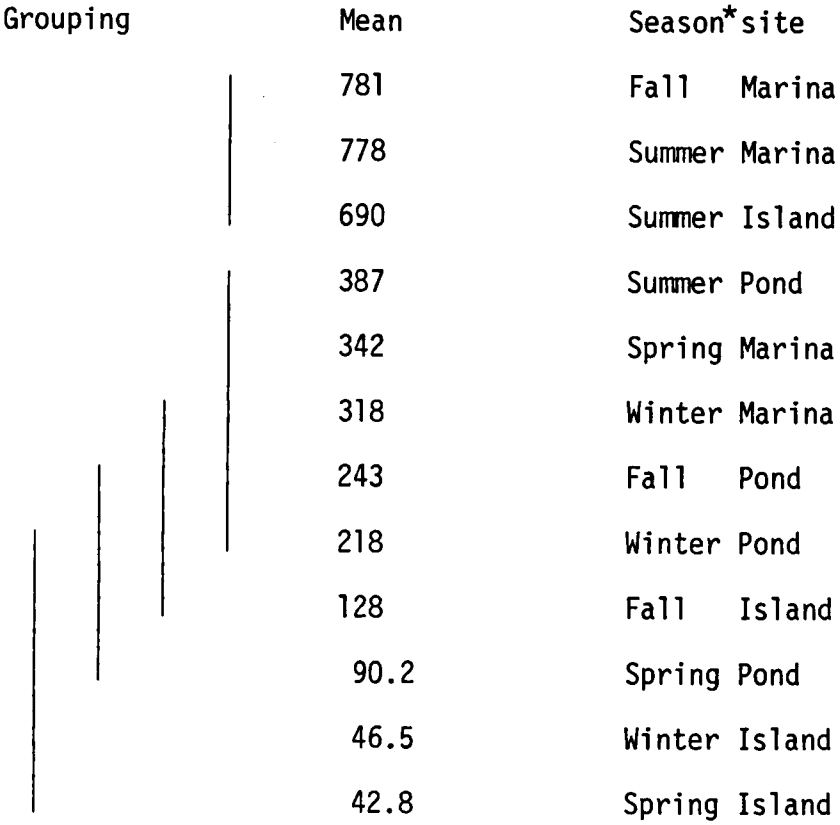

Figure A-3. Duncan's Multiple Range Test for 10 Minute Dehydrogenase Activity.

Means connected by a line are not significantly different. Alpha level = 0.05, DF = 60, MS = 23022.5, N = 6.

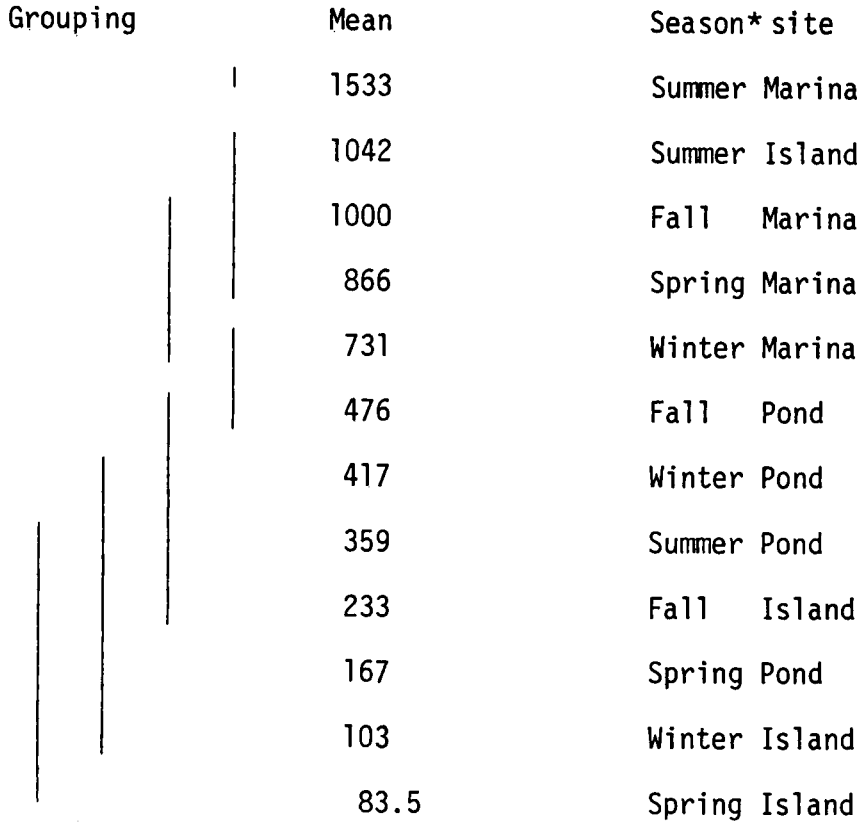

Figure A-4. Duncan's Multiple Range Test for 60 Minute Dehydrogenase Activity.

Means connected by a line are not significantly different. Alpha level = 0.05, DF = 60, MS = 55831.4, N = 6.

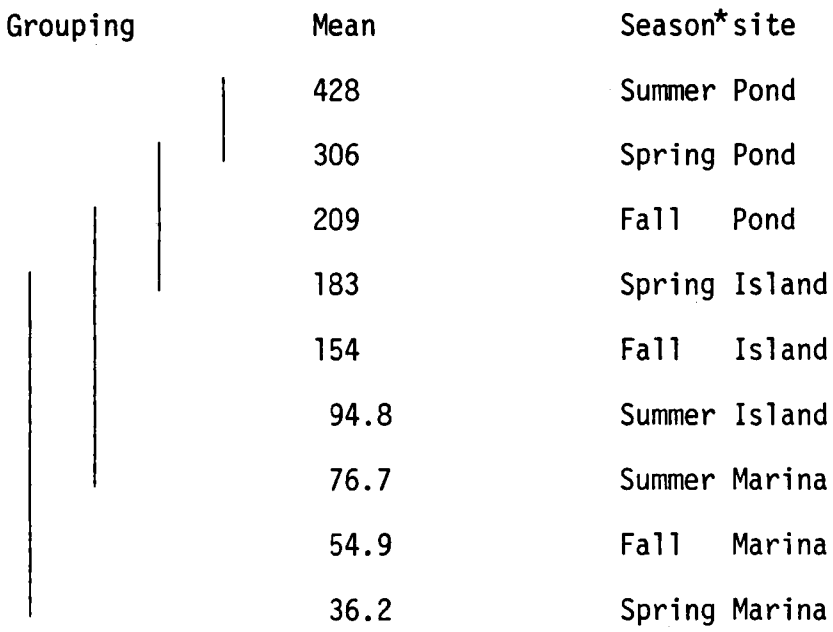

Figure A-5. Duncan's Multiple Range Test for ATP in Field Sediment.

Means connected by a line are not significantly different. Alpha level = 0.05, DF = 45, MS = 12491.7, N = 6.

Table A-9. Naphthalene Mineralization

Microcosm	pg CO_2 Respired/g Dry Wt. Sediment	
	Sterile Control	Experimental
ASO C	739.9 ± 27.9	1272.9 ± 163.7
ASO F	819.1 ± 99.4	1446.6 ± 109.9
DFM B	909.8 ± 103.7	1057.3 ± 205.5
DMF D	840.8 ± 46.2	1203.8 ± 95.0
CONT A	613.2 ± 29.3	1008.4 ± 48.7
CONT E	663.5 ± 61.2	974.9 ± 15.7

Results provided by: Dr. Gary Sayler, Microbial Ecology, The University of Tennessee, Knoxville.

Table A-10. Microbial Population Densities

Microcosm	Viable Counts/g Dry Wt. Sediment				
	Total Viable Cells (TVC)	Phenanthrene Resistant	Protein Hydrolyzer	Starch Hydrolyzer	Lipid Hydrolyzer
ASO C	$1.0 \times 10^7 \pm 3.6 \times 10^6$	$1.6 \times 10^6 \pm 6.7 \times 10^5$	4.7×10^6	2.0×10^6	8.6×10^6
ASO F	$9.5 \times 10^6 \pm 4.3 \times 10^6$	$3.3 \times 10^6 \pm 5.5 \times 10^5$	6.8×10^6	6.8×10^5	5.9×10^6
DFM B	$1.2 \times 10^7 \pm 7.7 \times 10^6$	$9.7 \times 10^5 \pm 6.8 \times 10^5$	1.0×10^7	4.8×10^6	9.4×10^6
DFM D	$1.6 \times 10^7 \pm 5.0 \times 10^6$	$6.1 \times 10^6 \pm 2.1 \times 10^6$	1.5×10^7	4.4×10^5	7.6×10^6
CONT A	$1.7 \times 10^7 \pm 1.3 \times 10^6$	$1.4 \times 10^6 \pm 4.6 \times 10^5$	5.4×10^6	2.6×10^6	6.6×10^6
CONT E	$8.8 \times 10^6 \pm 4.5 \times 10^6$	$1.2 \times 10^6 \pm 3.0 \times 10^5$	3.8×10^6	1.9×10^6	5.0×10^6

Results provided by: Dr. Gary Sayler, Microbial Ecology, The University of Tennessee, Knoxville.

Table A-11. Organic Matter Mineralization

Microcosm	μg CO_2 Respired/g Dry Wt. Sediment			
	Protein	Starch	Glucose	Lipid
ASO C	$1.1 \times 10^{-3} \pm .2 \times 10^{-3}$	$3.6 \times 10^{-2} \pm .5 \times 10^{-2}$	$4.9 \times 10^{-3} \pm 1.3 \times 10^{-3}$	$1.2 \times 10^{-3} \pm .1 \times 10^{-3}$
ASO F	$1.2 \times 10^{-3} \pm .1 \times 10^{-3}$	$5.2 \times 10^{-2} \pm .2 \times 10^{-2}$	$6.7 \times 10^{-3} \pm 1.7 \times 10^{-3}$	$1.4 \times 10^{-3} \pm .1 \times 10^{-3}$
DFM B	$1.0 \times 10^{-3} \pm .4 \times 10^{-3}$	$3.9 \times 10^{-2} \pm .4 \times 10^{-2}$	$4.6 \times 10^{-3} \pm .5 \times 10^{-3}$	$1.3 \times 10^{-3} \pm .7 \times 10^{-3}$
DFM D	$8.5 \times 10^{-4} \pm .9 \times 10^{-4}$	$3.7 \times 10^{-2} \pm .2 \times 10^{-2}$	$4.3 \times 10^{-3} \pm .2 \times 10^{-3}$	$1.1 \times 10^{-3} \pm .1 \times 10^{-3}$
CONT A	$6.1 \times 10^{-4} \pm .2 \times 10^{-4}$	$2.5 \times 10^{-4} \pm .6 \times 10^{-2}$	$3.6 \times 10^{-3} \pm .7 \times 10^{-3}$	$3.6 \times 10^{-4} \pm 1.2 \times 10^{-4}$
CONT E	$6.7 \times 10^{-4} \pm .3 \times 10^{-4}$	$3.0 \times 10^{-2} \pm .2 \times 10^{-2}$	$4.1 \times 10^{-3} \pm .1 \times 10^{-3}$	$3.3 \times 10^{-4} \pm .5 \times 10^{-4}$

Results provided by: Dr. Gary Sayler, Microbial Ecology, The University of Tennessee, Knoxville.

Table A-12. Phosphatase Activity

Microcosm	nM PNP/g Dry Wt./Hr.
ASO C	1240.4 ± 140.7
ASO F	749.6 ± 81.7
DFM B	1075.2 ± 340.4
DFM D	618.2 ± 141.6
CONT A	858.5 ± 145.5
CONT E	709.4 ± 280.7

Results provided by: Dr. Gary Sayler, Microbial Ecology, The University of Tennessee, Knoxville.

VITA

Michele Odette Ricard was born in Sacramento, California. She later moved with her family to Corvallis, Oregon where she graduated from high school in 1970. A strong interest in the sciences led to the completion of a Bachelor of Science from the University of Oregon in June 1974. Studies at Oregon State University followed by two years as a technical assistant at the Environmental Protection Agency in Corvallis contributed to a growing concern for the influences of xenobiotics in organisms and the environment. Ongoing research at The University of Tennessee, Knoxville and Oak Ridge National Laboratory concerning toxicity testing involving aquatic ecosystems provided the opportunity for attainment of a Master of Science degree in Ecology from UT in March 1981.

The author is a member of the American Association for the Advancement of Science. Ms. Ricard plans to continue in research developing model systems for toxicological testing.