BENZ(a)ANTHRACENE IN BENTHIC MARINE ENVIRONMENTS: BIOAVAILABILITY, METABOLISM, AND PHYSIOLOGICAL EFFECTS ON THE POLYCHAETE *Nereis virens*

by

Anne Elizabeth McElroy

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Signature of Author.....
Department of Biology, Massachusetts Institute of Technology and the Joint Program in Oceanography, Massachusetts Institute of Technology/Woods Hole Oceanographic Institution, February, 1985.

Certified by.....
John M. Teal
Thesis Supervisor

Accepted by.....
John J. Stegeman
Chairman, Joint Committee for Biological Oceanography, Massachusetts Institute of Technology/Woods Hole Oceanographic Institution.

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Doctor of Philosophy

ABSTRACT

The fate of [^{14}C-12] benz(a)anthracene (BA) was followed in benthic microcosm experiments in the presence and absence of the polychaete *Nereis virens*. In concert with chemical analysis of BA and its metabolites in all components of the system, physiological and biochemical effects of exposure on *Nereis* were investigated. BA was introduced in three ways: already sorbed to the entire sediment reservoir; directly into the water column; or incorporated into a gelatin-based diet. Experiments ran from 4 to 25 days. Activity of BA and BA metabolic products was followed in sediments, worm tissue, and in the water column. $^{14}CO_2$ activity in the water column was also measured. Growth, oxygen consumption, ammonia excretion, adenylate nucleotide pools, and mixed function oxygenase activity of the worms were also monitored.

The presence of worms and the mode of introduction had significant effects on the fate of BA in this system. In experiments with sediments uniformly labeled with BA, worms increased flux of BA from the sediment, and after nine days, their presence lead to increased rates of microbial mineralization of BA to CO_2. In experiments where BA was added directly to the water column, worms mixed BA into the sediment, but had no net effect on removal of BA in the sediment to the water column. BA added to the water column and deposited at the sediment-water interface was more available for uptake by worms, microbial mineralization to CO_2, and removal to the water column than BA sorbed to bulk sediments. Regardless of mode of introduction, worms were able to accumulate BA. However, bioavailability of BA previously sorbed to sediments was less than BA added to the water column and allowed to settle at the sediment-water interface. Of the three modes of introduction studied, BA ingested in a geletin-based diet was most available for accumulation by *Nereis*.

Length of exposure and mode of introduction had significant effects on accumulation and metabolism of BA by *Nereis*. Of total activity accumulated, the proportion remaining as parent compound decreased with time, and was inversely correlated with relative efficiency for accumulation. The relative amounts of different metabolic products were also affected by time and mode of exposure. In all experiments, most activity recovered from worms was present as metabolic products with only

2 to 23% remaining as parent compound. A significant portion (from 33 to 51%) of total activity was not extractable, indicating incorportation into macromolecular components.

Physiological and biochemical effects of BA exposure on Nereis were minimal. Subtle alterations in adenylate nucleotide pools were observed after 6 days in experiments with either sediment-sorbed BA or BA added directly to the water column. After 25 days of exposure to sediment-sorbed BA, worms showed increased rates of oxygen consumption and ammonia excretion. No significant changes in growth or activity of the mixed function oxygenase system were observed.

These experiments demonstrated: (1) that the presence of a large burrowing polychaete can have significant effects on the fate of PAH in the benthos; (2) that source can have significant effects on both fate and metabolism of PAH in the benthos; (3) that Nereis virens is capable of accumulating and metabolizing BA from the sediment, water column, or ingested food; and (4) that incorporation into cellular macromolecules is a major fate of accumulated BA.

Thesis supervisor: John M. Teal
Chairman, Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA

Thesis Committee

John M. Teal: Supervisor
Biology Department, W.H.O.I.

Judith M. Capuzzo
Biology Department, W.H.O.I.

John W. Farrington
Chemistry Department, W.H.O.I.

Michael Marletta
Department of Nutrition and Food Sciences, M.I.T.

Jerry M. Neff
Battelle New England

John J. Stegeman
Biology Department, W.H.O.I.

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To my family

John, Marian, and Mary McElroy,
and Josie

ABBREVIATIONS

ACN: acetonitrile
ADP: adenosine triphosphate
AHH: aryl hydrocarbon hydroxylase
AMP: adenosine monophosphate
ANOVA: analysis of variance
ATP: adenosine triphosphate
BA: benz(a)anthracene
BP: benzo(a)pyrene
BSA: bovine serum albumin
C_{18}: octadecylsilane
$CHCl_3$: chloroform
CPM: counts per minute
DEE: diethyl ether
DPM: disintegrations per minute
EC: energy charge
EDTA: ethylene diamine tetracetic acid
EtOH: ethanol
GDW: gram dry weight
GWW: gram wet weight
HPLC: high pressure liquid chromatography (chromatograph)
Hx: hexane
LDH: lactate dehydrogenase
LSC: liquid scintillation counting (counter)
MeOH: methanol
MFO: mixed function oxygenase
MK: myokinase
NADH: nicotine adenine dinucleotide reduced form
NADPH: Nicotine adenine dinucleotide phosphate reduced form
O.D.: optical density
PAH: polycyclic aromatic hydrocarbons
PEP: phosphoenol pyrophosphate
PK: pyruvate kinase
SNK: Student-Neuman-Keuls test
Tris: tris(hydroxymethyl)aminomethane
UV: ultraviolet

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CHAPTER 1:
INTRODUCTION

PAH in the environment:

The annual discharge of petroleum hydrocarbons into the world's oceans has been estimated at 1.9 to 11.1 million tons (Connel and Miller, 1980,1981). The single largest component of petroleum hydrocarbon input Is chronic land-based discharges, such as precipitation run-off and sewage, associated with the routine use of fossil fuels in industrialized areas (NAS, 1975; Hoffman et al., 1984). Once introduced into the aquatic system, non-volatile hydrocarbons, due to their characteristically low water solubility, do not remain dissolved for long. They are removed from solution either by direct mixing to the sediment surface, by adsorption onto particles, or by uptake into aquatic organisms. The importance of sediments as reservoirs for hydrocarbons has been well documented in natural (Hites et al., 1980; Royal Society,, 1980; Wakeham and Farrington, 1980; Farrington and Tripp, 1977; NAS, 1975), as well as experimental (Gearing et al., 1979, 1980).

Although less abundant on a per weight basis than aliphatic hydrocarbons, polycyclic aromatic hydrocarbons (PAH) constitute a significant, long-lived, and biologically active portion of the total hydrocarbons in the benthic environment. The primary source of PAH to sediments globally is thought to be the combustion of fossil fuels (LaFlamme and Hites, 1979; Wakeham and Farrington, 1980). In the coastal zone land-based runoff (Hoffman et al., 1984; Prahl et al. 1984) and input from unburned coal (Tripp et al., 1981) can also be major sources of PAH to the benthos. Blumer and Sass (1972) demonstrated that biochemical degradation selectively removes alkanes prior to removing the complex mixture of

cyclic and aromatic hydrocarbons. Long-term studies of an oil spill in a New England salt marsh showed that aromatic hydrocarbons were retained in sediments at least 6.5 years after the spill. The aromatic hydrocarbons remaining were relatively enriched in the heavier molecular weight fractions (Teal et al., 1978). Prudhoe Bay crude oil experimentally weathered under a variety of simulated intertidal weather conditions also became enriched in the heavier tri- and tetra-aromatic hydrocarbon fractions (Riley et al., 1980-81).

Complex PAH mixtures in marine sediments are ubiquitous, and have been detected in remote pristine areas including the Nares abyssal plain, Walvis Bay, the Cariaco Trench, and the Gulf of Maine (Giger and Blumer, 1974; Farrington, et al. 1977; La Flamme and Hites, 1978). Coastal sediments have elevated PAH levels due to their proximity to industrial regions. Analysis of sediment profiles in various parts of the world indicates that PAH input has increased dramatically since 1900 (Hites, et al., 1977: Grimmer and Bohnke, 1975). The deposition and retention of substantial concentrations of PAH in surface sediments emphasizes the need for understanding the fate and effects of these compounds in benthic ecosystems. Organisms living in the sediment will be continually exposed to PAH present in pore waters, on particles, in detritus (both within and upon the sediment), and in other organisms.

Factors affecting the distribution of PAH in the benthos:

A multitude of factors can affect the distribution of PAH in the benthos. In nearshore areas physical processes such as tidal pumping, wind-driven and tidal currents, and storm events can influence deposition, resuspension, and advection of PAH. The distribution of PAH can

also be affected by biological activity. PAH can be transformed either chemically or biologically to more reactive products. Photooxidation of PAH leads to a wide range of products of including quinones (Zafiriou, 1977). Lee and Takahashi (1977) reported photolysis to be a major factor in removal of benzo(a)pyrene in the watercolumn of CEPEX enclosures. Sorption of PAH onto sediment particles, even under full illumination, reduces their susceptibility to photolysis (Zepp and Schlotzhauer, 1979), and in most benthic environments, ambient light levels are low. Although photooxidation and large scale physical processes can affect PAH in the benthos, particularly in intertidal and nearshore environments, the following discussion will focus on interactions between biological processes and PAH in the benthos.

Infaunal organisms can alter the chemical composition of sediment, pore water, and overlying water (Aller, 1978; Aller & Yingst, 1978; Fisher et al., 1980). Burrowing organisms also have an impact on microcirculation near the sediment surface (Eckman, 1978). These effects result from: (1) irrigation of subsurface burrows for feeding and respiration; (2) deposition of mucous both within burrows and at the sediment surface; and (3) movement of sediment for burrow and tube construction, and feeding. Few reports exist on how these activites affect the distribution of hydrophobic compounds residing in sediments. Gordon et al. (1978) estimated that observed densities of the polychaete Arenicola marina could remove all sediment-bound oil resulting from an oil spill in Chedabucto Bay, Nova Scotia in a period of 2-4 years. Lee et al., (1978) found the presence of the polychaete Capitella sp. increased the removal rate of several PAH from sediments. More recently Karickhoff and Morris

(1985) found bioturbation by oligochaetes increased flux of sediment-sorbed chlorinated hydrocarbons to the water column by a factor of 4 to 6.

The presence of infaunal organisms can also affect both removal and microbial mineralization of PAH. Gardner et al. (1979) compared rates of removal and mineralization of sediment spiked with anthracene, flouranthene, benz(a)anthracene (BA), and benzo(a)pyrene (BP) in the presence and absence of Capitella sp. Capitella increased the removal rate of all PAH from the sediment. The effect of the worms was most pronounced with the larger (>3 ring) PAH. Furthermore, mineralization rates of BA added to sediments taken from depth in chambers with worms were higher than in sediments taken from depth in chambers without worms.

The tendency for organic molecules to sorb onto particles is largely determined by the organic content (Means et al., 1979) and size (Steen et al., 1978) of the particulate matter available. Karickhoff (1979) calculated the relationship between empirically determined partition coefficients, normalized to organic content of the sorbant (K_{oc}), to octanol-water partition coefficients (K_{ow}) of the organic compounds. Bioconcentration of organic pollutants in marine organisms has also been found to correlate with K_{ow} (Neely et al., 1974; Vieth et al., 1979). In addition to particulate matter, organic colloids have been shown to be important sorbants for organic compounds (Means and Witjayaratne, 1982). Boehm and Quinn (1973) found that dissolved organic matter (DOM) increased the solubility of hydrocarbons in seawater, and that removal of DOM increased accumulation of hydrocarbons by filter feeding bivalves (Boehm and Quinn, 1976). Leversee et al. (1982) reported that accumulation of several PAH

by Daphnia were affected by the presence of synthetic (Aldrich humic acids) or natural DOM, or natural particulate matter. Particulate size and organic content are also known to influence feeding behavior of benthic organisms. Particulate protein content has been shown to affect both selection (Taghon, 1982) and feeding rate (Taghon and Jumars, 1984) of deposit feeding polychaetes. Several species of deposit feeders have demonstrated the ability to selectively feed on small sediment particles (Taghon, 1982). These studies clearly illustrate that the physical/chemical properties of organic compounds and available sorbant material can affect the chemical- and bio-availability of organic pollutants.

PAH Metabolism

Metabolic transformation of PAH greatly alters their chemical and biological reactivity. Therefore this process can have profound effects on both the fate of PAH in the environment, and their effects on organisms.

Bacteria are the only organisms able to completely mineralize PAH to CO_2 (See review by Atlas, 1981). Although both bacteria and fungi are capable of metabolizing PAH, mineralization rates of the larger PAH (greater than three rings) in natural systems have been found to be slow, and no known microbes are capable of using these larger PAH as a sole carbon source (Atlas, 1981). Lee and Takahashi (1977) attempted to measure BP and fluorene mineralization in water taken from CEPEX enclosures that had been spiked with PAH and petroleum hydrocarbons. They detected no mineralization of fluorene and could detect only very low (1 ± 0.7 ug/l-day) rates for BP, which gave a turnover time of 1,400 days for BP in the enclosure. Lu et al. (1977) were also unable to detect

mineralization of BP in an aquatic microcosm where BP was added to the water column. Saltzman (1982) measured microbial mineralization of PAH spiked to sediments taken from North Sea oil fields and only occasionally found significant rates of BP degradation, whereas naphthalene was always mineralized.

Lee and Ryan (1983) compared microbial mineralization of several PAH spiked into either seawater or sediment-seawater slurries taken from both clean and oil-contaminated environments. PAH containing fewer than four rings (naphthalene, methylnaphthalene, and phenanthrene) were mineralized in water samples while other larger PAH (BA, chrysene, fluorene, and anthracene) were not, unless illuminated with sunlight. However, in sediment-seawater slurries all PAH studied were degraded. Since microbial activity on sediment particles exceeds that in the water column the increased degradation of PAH associated with sediment particles is not surprising. Hinga et al. (1980) found 29% of a water column spike of BA, in the MERL mesocosms at the University of Rhode Island, was mineralized to CO_2 in 230 days. In this experiment it was not possible to distinguish between either BA mineralized in the water column and that mineralized at the sediment-water interface or between microbial degradation and photooxidation.

Herbes and Schwall (1978) looked at mineralization of PAH spiked into undiluted sediment taken from clean and oil-polluted environments. They found turnover times for added PAH to be faster in sediments from contaminated areas and faster for the lower molecular weight PAH (naphthalene and anthracene) than for the higher molecular weight PAH (BA and BP). In sediment from oil contaminated areas, naphthalene was degraded

within hours, whereas the turnover time for BA was approximately 400 days, and that for BP was greater than 3.3 years. Gardner et al. (1979) using sediment from benthic microcosms containing PAH and petroleum hydrocarbons, found mineralization rates of BA spiked to sediment-seawater slurries taken from surface sediments to be higher than in sediments taken from depth. Since microbial mineralization requires oxygen (Gibson, 1976), populations of PAH mineralizers should be localized at the sediment water interface where oxygen would not be limiting.

In concert, these studies indicate that although microbial mineralization of two and three ring PAH may be an important process, the significance of this pathway for larger PAH in the water column is negligible. It is more likely that photooxidation and sorbtion onto sinking particles are the primary processes responsible for removing larger PAH from the water column. Once on the bottom, mineralization of the larger PAH, particularly at the sediment-water interface, may take on greater importance.

In mammals, PAH metabolism is initiated by a NADPH-dependent enzyme system where cytochrome P-450 is the terminal oxidase. This enzyme system is commonly referred to as a mixed function oxygenase (MFO) system because it splits molecular oxygen, adding one atom of oxygen to the substrate, while the second atom forms a water molecule. This system is responsible for the metabolism of a wide variety of foreign compounds in addition to PAH, and is also responsible for metabolizing endogenous substrates such as steroid hormones. Fish and invertebrates possess MFO systems that are similar to those found in mammals (see reviews by Stegeman, 1981 and Lee, 1981), and some evidence for MFO activity has

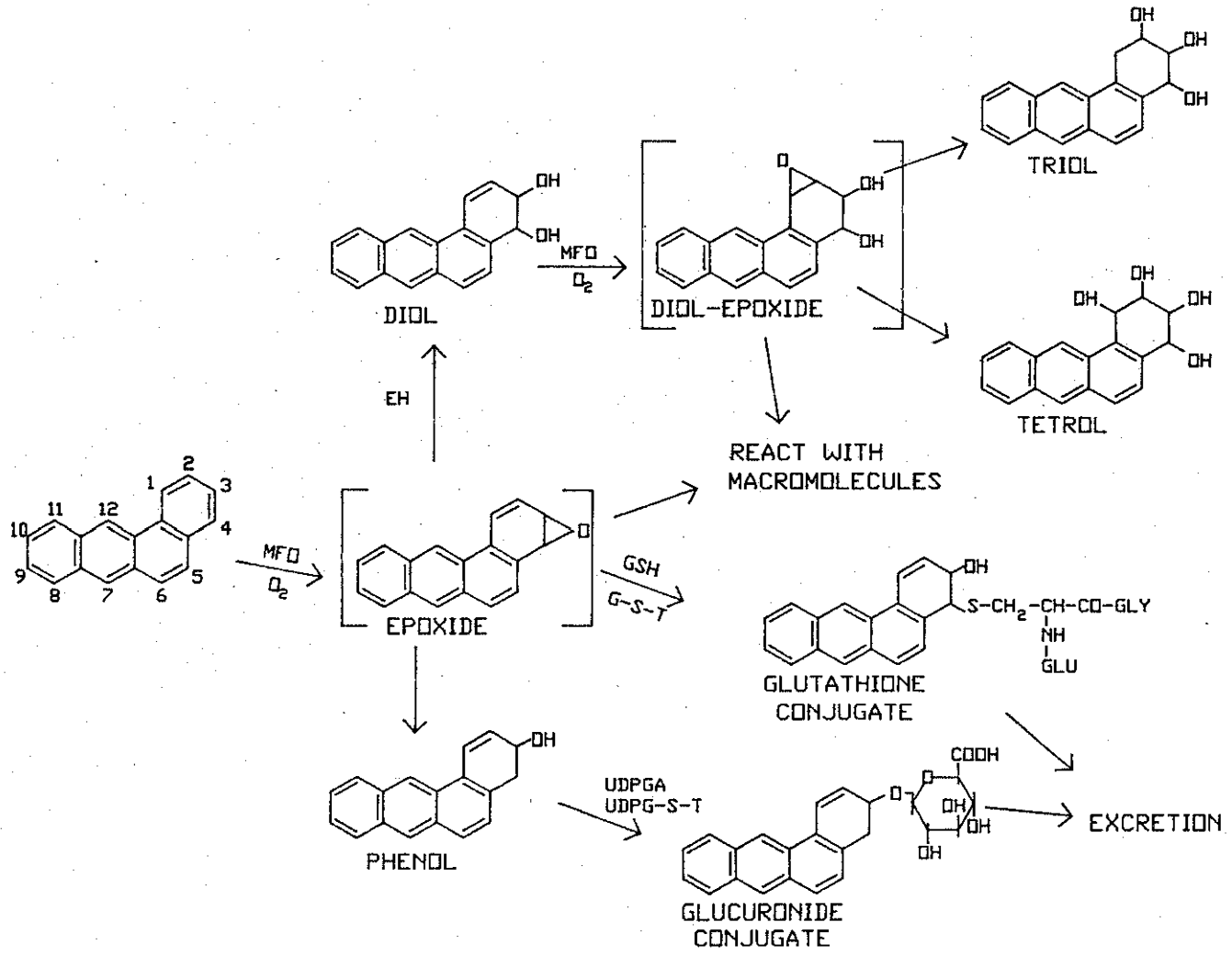
been observed in most aquatic eukaryotic organisms (Neff, 1979).

A schematic of MFO metabolism of BA is shown in Figure 1.1 The MFO system inserts oxygen onto the ring forming a very reactive epoxide intermediate. The epoxide can rearrange to a phenol, can be converted to a diol either by spontaneous rearrangement or via the enzyme epoxide hydrolase (EH), can be conjugated to form a water soluble metabolite through the action of enzymes such as glutathione-S-transferase or UDP glucuronosyl transferase, or, because it is a strong electrophile, can covalently bind to nucleophiles such as cellular macromolecules. Polar metabolites can be further metabolized by the MFO system at other ring positions, which can lead to the formation of a very reactive diol-epoxide intermediate. The diol-epoxide can also bind to cellular macromoleucles or can be hydrolyzed to triol or tetrol metabolites.

This figure illustrates the dichotomous nature of PAH metabolism in eukaryotic organisms. It leads to the production of water soluble conjugated metabolites that can be excreted, and to the production of highly reative electrophiles that can spontaneously bind to DNA, RNA, and proteins. PAH induced carcinogenesis requires that the PAH be activated (metabolized) before binding to DNA (Sims and Grover, 1974; Jerina and Daley, 1974). Wood et al., (1977) have demonstrated that the 3,4-diol metabolite of BA is 10 to 20 time more tumorogenic than the parent compound when applied topically to mouse skin.

One important aspect of the MFO system is that it can be induced by exposure to a wide variety of compounds including PAH and PCBs. Induction involves the <u>de novo</u> synthesis of new cytochromes P-450 with increased ability to metabolize specific substrates. Experimental evidence

Figure 1.1: Possible metabolic fate of benz(a)anthracene in eukaryotic organisms. MFO= mixed function oxygenase. G-S-T= glutathione S-transferase. GSH= reduced glutathione. UDPGA= uridine diphosphate glucuronic acid. UDPG-S-T= UDP glucuronosyl S-transferase. Glu= glutamate. Gly= glycine.


TRIOL
TETROL
DIOL
MFO
O_2
DIOL-EPOXIDE
EH
REACT WITH
MACROMOLECULES
MFO
O_2
EPOXIDE
GSH
G-S-T
S-CH$_2$-CH-CO-GLY
NH
GLU
GLUTATHIONE
CONJUGATE
PHENOL
UDPGA
UDPG-S-T
COOH
EXCRETION
GLUCURONIDE
CONJUGATE

indicative of induction include enhanced hydroxylation activity of MFO substrates, elevated concentrations of cyctochrome P-450, and by stylized reponses to selective inhibitors (Stegeman, 1981). However, compounds that stimulate the MFO system do not necessarily initiate commensurate increases in the activity of conjugating enzymes (Statham et al., 1978; Balk et al., 1980). Therefore previous exposure to compounds which can induce the MFO system might increase the production and residence time of reactive PAH metabolites binding to cellular macromolecules.

Most characterization of the MFO system has been done in vitro using purified proteins in reconstituted systems or microsomal membrane preparations. This type of investigation has produced much useful information on what factors can affect MFO activity and substrate specificity, and or the kinetics of metabolite formation. However, in the intact system in a functional organism there are many additional processes that may affect MFO activity such as availability of substrates and cofactors, the inhibition by reaction products or other compounds, the presence of enzymes and substrates for conjugation, and the scavenging of reactive intermediates by cellular nucleophiles. Investigation of in vivo metabolism is required to actually determine the fate of accumulated PAH in organisms.

In vivo metabolism of PAH has been observed in virtually all species of fish investigated, including teleosts (Lee et al., 1972; Ahokas et al., 1975; Lu et al., 1976; Roubal et al., 1977; Statham et al., 1978; Varanasi et al., 1979; Solbakken, 1980; Lech and Bend, 1980; Richert et al., 1982; Varanasi and Gmur, 1981) and elasmobranchs (Solbakken and Palmork, 1980). In vivo PAH metabolism has also been observed in many species of crustacea (Corner et al., 1973, 1976; Lee et al., 1976; Lu et

al., 1977: Sanborn and Malins, 1977; Herbes and Rissi, 1978; Palmork and Solbakken, 1980; and Leversee et al., 1981); in echinoderms (Malins and Roubal, 1982) and in a few polychaetes as described below. Attempts to measure *in vivo* metabolism have produced negative results in two coelenterates (Lee, 1975) and the bivalve mollusc *Mytilus edulis* (Lee et al, 1972a), although evidence of *in vivo* PAH metabolism has been observed in the gastropod mollusc *Physa* (Lu et al., 1976).

Lee and coworkers have demonstrated that *Nereis virens* has a functional MFO system (Singer et al., 1979). They have reported increases in AHH activity and cytochrome P-450 concentrations in *Nereis* collected from polluted environments in Maine and have also shown an occasional increase in these parameters after feeding *Nereis* food contaminated with PAH or PCBs in the laboratory (Lee et al., 1981b). They have also shown *in vitro* metabolism of BP to primarily phenolic and diol metabolites (Fries and Lee, 1984). *In vivo* metabolism of chlorinated hydrocarbons such as lindane (Goerke and Ernst, 1980) and a pentachlorobiphenyl (Goerke, 1984) has been reported in *Nereis virens*, but *in vivo* PAH metabolism has not been investigated. Lee et al. (1979) have reported increased AHH activity in another polychaete *Capitella* sp. after long-term exposure to petroleum hydrocarbons. Rossi and Anderson (1977) reported that another polychaete, *Neanthes arenaceodentata*, could metabolize naphthalene. However, Augenfeld et al. (1983) did not find direct evidence for *in vivo* metabolism of sediment-sorbed phenanthrene, chrysene, or BP by the lugworm *Abarenicola pacifica*.

Biological and chemical availability of PAH:

Tissue concentrations of individual PAH such as BP observed in

aquatic organisms varies from undetectable to several ppm (Neff, 1979). A consistent trend for the selective retention of the larger, more hydrophobic components of oil has been observed in both field and laboratory studies. Polychaetes collected from the New York Bight and from Buzzards Bay showed preferential retention of PAH with three or more rings (Farrington et al., 1985). Similar findings were reported for benthic invertebrates in an extensive study of chemical contamination in central and southern Puget Sound (Malins et al., 1980). Preferential accumulation of aromatic hydrocarbons over lighter molecular weight other components in fuel oil has been observed in the laboratory for oysters, clams, polychaetes, limpets, shrimp, and fish (Stegeman and Teal, 1973; Neff et al., 1976; Clement et al., 1980; Farrington et al., 1982). Laboratory studies of accumulation of one to 5 ring aromatic hydrocarbons have demonstrated a correlation between number of rings and accumulation and retention in Coho salmon (Roubal et al., 1977) and a deposit feeding clam Macoma inquinata (Roseijadi et al., 1978).

Analysis of benthic organisms and the sediment they inhabit supports the premise that PAH from different sources are not equally available for accumulation by benthic organisms. Shaw and Wiggs (1980) found petroleum derived hydrocarbons in the filter feeding bivalve Mytilus edulis whereas coal-derived hydrocarbons were found in a deposit feeding bivalve Macoma baltica from the same area. Work in the MERL mesocosms, looking at the addition of #2 fuel oil to the water column, showed different patterns of PAH accumulation in sediment, limpets living on the walls near the sediment-water interface, and polychaete worms living in the sediments (Farrington et al., 1982). Similar patterns were observed between PAH in

the polychaete Nephtys incisa and sediments collected in the New York Bight (Farrington et al., 1985).

Accumulation of dissolved PAH in organisms has been well documented, with bioaccumulation factors ranging up to many orders of magnitude (Neff, 1979). Uptake of PAH is a function of water solubility, with the smaller and more soluble PAH being taken up more rapidly than the larger and less soluble PAH. PAH retention is a function of the rate of excretion via metabolic processes, and the rate of passive processes such as desorption of accumulated PAH out of the organism. Therefore, smaller PAH will be both taken up and lost more rapidly than larger PAH. Similarly, accumulation of PAH in organisms such as fish that are capable of rapid PAH metabolism, is frequently much lower than that seen in organisms without active PAH degrading systems such as molluscs, due to the rapid turnover of these compounds (Stegeman, 1981).

Accumulation of PAH via trophic transfer has frequently been observed, although this mode of uptake seems to be more variable. Rossi (1977) was unable to detect accumulation of naphthalene from labeled detritus eaten by the deposit feeding worm Neanthes arenaceodentata. However, Elder et al. (1979) found Nereis virens was capable of dietary accumulation of PCB mixtures in labeled clam tissue. Dietary accumulation of a variety of PAH has been documented in larval bivalves (Drobosky and Epifanio, 1980), in crustacea (Corner et al., 1976; Lee et al., 1978); and in fish (Dixit & Anderson, 1977; Palmork & Solbakken, 1980). Lee et al. (1976) found uptake of BP in larval blue crab to be more efficient from food than from water, and Corner et al. (1976) found similar results for uptake of naphthalene in Calanus. Dobrosky and Epifanio (1980)

however, found the opposite trend for BP accumulation in larval bivalves. Recently Rubinstein et al. (1984) reported dietary transfer of PCBs from Nereis virens to the demersal fish Leiostomus xanthurus to be more efficient than direct uptake from contaminated sediments.

The bioavailability of PAH sorbed to sediments is not well understood. Since there will always be a dissolved phase whenever particulates or even colloidal material such as DOC are present, discriminating between uptake from particulate versus uptake from solution is extremely difficult. To adequately assess the importance of uptake from dissolved vs. sorbed PAH, exacting comparisons between concentration factors, total accumulation, and especially rates of uptake via the two sources would have to be made.

Factors which affect sorptive capacity of sediments also affect flux of PAH from sediments and, potentially, bioaccumulation. Gardner et al. (1979) found that 2 to 5 ring PAH were lost most rapidly from sediments with the lowest sorptive capacity. Flux was highest from medium sands with large particle sizes and low organic carbon content, intermediate from fine sands with smaller particle sizes, and lowest from marsh peat with the highest organic carbon content and smallest average particle sizes.

Roesijadi et al. (1978) exposed a filter feeding clam, Protothaca stamina, a deposit feeding clam Macoma inquinata, and a deposit feeding worm, Phascolosoma agassizii, to sediment containing Prudhoe Bay crude oil. The deposit feeders accumulated hydrocarbons to a greater extent than the suspension-feeder. However, Rossi (1977) was unable to detect accumulation of naphthalene from sediment or food in the deposit feeding

worm Neanthes arenaceodentata, whereas Lyes (1979) observed naphthalene accumulation from sediments by the worm Arenicola marina and Courtney and Langston (1978) observed PCB accumulation from sediments by two other polychaetes. Other investigators have detected accumulation of PAH from sediments by fish (Varanasi and Gmur, 1981). Even though it seems that most organisms can accumulate PAH from sediment reservoirs, concentrations in organisms greatly exceeding that in the sediment reservoir are rarely observed in marine organisms.

Several investigators have found similar total amounts of accumulated PAH in organisms exposed to either sediment or water sources, although concentration factors for water uptake were much higher, as would be expected based on partitioning. Roesijadi et al. (1978) looked at accumulation of phenanthrene, chrysene, and BP from labeled sediments by Macoma inquinata either suspended above, or in direct contact with the sediment. In a 7 day exposure, net uptake was of the same order of magnitude for all PAH regardless of whether or not the clams were in direct contact with the sediment. Courtney and Langston (1978) compared uptake of the PCB mixture Arochlor 1254 from sediment and seawater by two deposit feeding polychaetes. Again, similar absolute amounts of PCBs were accumulated from both sources. However, in this case the concentration in sediment was 1 ppm whereas that in the water column was 1 ppb.

The time course for biological uptake and loss of PAH from sediment seems to be much longer than for that from the water column for compounds with very low water solubility. After exposure of the deposit feeding clam Macoma baltica to sediment-sorbed PAH for 45 days, tissue concentrations of BP were still increasing at an exponential rate whereas

concentration of chrysene had already peaked and was beginning to decline, and concentration of phenanthrene was declining exponentially (Roesijadi et al. 1978). Fowler et al. (1978) compared accumulation of the PCB mixture Penoclor DP-5 from sediment and from water by the polychaete Nereis diversicolor. Accumulation from the water column attained equilibrium after only two weeks, while accumulation from sediment attained equilibrium after two months. Both uptake and depuration was dose dependent with worms in contact with higher concentrations of PCBs taking longer to reach equilibrium. Calculations based on concentration factors from the sediment (3 to 4), from the water column (800) and from ambient PCB levels in sediment and water indicated that the water column could only contribute one percent of the observed PCBs accumulated at equilibrium.

Since most PAH in the marine environment are deposited in sediment reservoirs, it is unfortunate that the chemical and biological availability of these pools are not better understood. Based on current literature, it seems that sediment PAH reserves, particularly those near the sediment water interface and within the bioturbated zone are more available for bioaccumulation and degradation. Although the efficiency of uptake from particulates appears to be much less than that from the dissolved form, the sheer mass of PAH located in the sediment dictates that sediments are a major source of PAH to benthic organisms. PAH already accumulated in marine organisms are available for uptake by predators with efficiencies similar to that seen for uptake from the dissolved form. The potential importance of dietary transport of PAH by mobile predators such as fish into pristine environments or into the

human food chain warrents further investigation. Recent field evidence by Malins et al. (1985) suggests that this vector may be important in Puget Sound.

Metabolic fate of PAH in the benthos:

Although numerous studies have investigated different factors affecting the fate of PAH in the benthos as described above, there have been only a few attempts to construct a complete mass balance of what actually happens to PAH in relatively undisturbed benthic systems. Using the 13,000 l MERL mesocosms at the University of Rhode Island, Hinga et al. (1980) followed BA added to the water column in all components of the system for 230 days. Degradation of BA proceeded rapidly in the water column. The activity of the parent compound accounted for 70% of the total activity in the water column on day one, but by day 9 this had been reduced to 20%. The actual extent of BA degradation was probably even higher since only the lipid portion of the extract was analyzed for metabolic products and 30 to 40% of total activity in the water column was not lipid extractable. By day 60, the only activity left in the water column was as CO_2 from mineralized BA. Concentration of $^{14}CO_2$ in the water column peaked on day 12 and slowly decreased throughout the rest of the experiment. A cumulative total of 29% of BA added to the microcosm was mineralized to CO_2 by the end of the experiment.

BA was rapidly deposited at the sediment-water interface, with maximum concentrations in the top centimeter found in the first sediment samples taken on day 10. Polar metabolites of BA were found in all cores analyzed, although this was not done until day 86. As time increased, BA was mixed into the sediment. Significant levels of polar metabolites (up

to 95%) were found to depths of 6 cm in some cores. Due to the rapid disappearance of BA from the water column and the presence of BA polar metabolites in the sediment, it is likely that a major portion of BA mineralization to CO_2 and metabolism to polar intermediates occurred near the sediment-water interface, and that net production of CO_2 resulted from removal of BA from the sediment.

A similar experiment was conducted by McElroy et al., (1982; Appendix 1) where ^{14}C-BA was added to the water column in two 230 l recirculating microcosms each containing a 0.25 m^2 sediment box. As was seen in the MERL experiment, BA disappeared from the water column in a matter of days. The production of $^{14}CO_2$ followed kinetics similar to those reported by Hinga et al. (1981). The concentration of BA in surface sediments increased rapidly and after 42 days over 97% of recovered activity was found in the top 2 cm of the sediment reservoir. In this experiment most of the BA present in sediment cores appeared as polar metabolites as early as nine days after introduction of isotope to the chambers. A population of the deposit feeding worm Nephtys incisa recovered from the chambers after 42 days of exposure also contained very little unmetabolized BA. Since this experiment was run in the dark, it is believed that all degradation occurred via metabolism.

Herbes and Schwall (1978), as part of a comparative study of microbial transformation of PAH in pristine and petroleum-contaminated sediments, constructed a mass balance of PAH added directly to undiluted sediments. The percent of parent PAH remaining in the sediment after 24 hours was 7% for naphthalene, 78% for anthracene, 82% for BA, and 78% for BP. Incubations for BA and BP were continued for 26 days, during which a

significant decrease in the amount of BA occurred whereas no significant change in the amount of BP remaining was observed. For naphthalene, a significant portion of degraded PAH was mineralized to CO_2 (approx. 50% in 48 hours), or bound to microbial biomass (approx. 28% in 48 hours) with only a few percent recovered as polar metabolites in the sediment. For anthracene, a much greater percentage (approx. 10% after 128 hours) was found in the polar fraction as compared to 4% as CO_2 and 23% bound. For both BA and BP after 14 days about 2% of activity was bound to microbes, 3 and 5% respectively found as polar compounds and 2.1 and 0.1% recovered as CO_2. This study supports work described earlier indicating that microbial mineralization of the larger sediment-sorbed PAH is not a rapid process.

Varanasi and Gmur (1981) and Augenfeld et al. (1982) followed the fate of PAH from labeled sediments into fish, and deposit feeding worms and clams. Varanasi and Gmur reported rapid uptake and metabolism of naphthalene and BP in English sole. Bulk concentration of BP in the sediment over ten days did not change although bulk concentrations of naphthalene decreased. Chromatographic analysis of sediment and pore water extracts showed that BP remained unmetabolized whereas approximately 7 and 14% of naphthalene activity in the sediment and pore water extracts was found as polar metabolic products. Augenfeld at al. (1982) reported that after 60 day exposures to sediment sorbed phenanthrene, chrysene, or BP both Macoma and Abarenicola accumulated PAH; but polar metabolic products were not detected in either organisms or sediments.

These experiments indicate that the bioavailability of PAH for uptake and metabolism is highly dependent on their individual chemical

properties and roughly decreases with decreasing solubility. However, in some cases even the larger PAH such as BA and BP are available for accumulation and metabolism by benthic organisms. A comparison between the two microcosm experiments where BA was added to the water column (Hinga et al., 1981; and McElroy et al., 1982, Appendix 1) and the experiments with labeled sediments (Herbes and Schwall, 1978) also indicate that PAH may be differentially available, depending on the degree to which they are associated with particles.

Effects of PAH exposure on marine organisms:

The effects of PAH on aquatic organisms have been the object of numerous investigations. The responses are highly species specific and are also dependent on factors such as life history stage, salinity, temperature, and reproductive status. The range of accute toxicities of specific PAH as indicated by LC_{50} vary from less than 10 parts per billion to greater than 1 parts per thousand (Neff, 1979). Although toxicity testing generates an easily defined endpoint, this type of investigation yields little information on mechanisms of toxicity, or environmentally disruptive levels.

Organisms maintain homeostasis through a variety of systems operating at all levels of biological organization from biochemical to community levels of structure. The ability of any one or combination of systems to mitigate disruption may ameliorate effects before they become evident at higher levels. Adverse effects of petroleum hydrocarbons have been observed at all levels of organization in many marine organisms (Capuzzo, 1985). Effects at one level of organization leading to demonstrable effects in a higher level of organization have been documented

(Capuzzo et al., 1984). Others have demonstrated saturation of an adaptive response in one system leading to disruption in other systems (Brown et al., 1982). The time required to observe measurable effects is highly dependent on the magnitude of disruption, the sensitivity of the component under investigation and the level of biological organization studied. In general the lag time for response increases with each level of organization, with effects at the biochemical and cellular level occurring on a time course of seconds to days, while those at the population or community level occuring on a time course of months to decades (NAS, 1971). From a predictive standpoint, investigation at the initial levels of response, such as physiological and biochemical levels, has the advantage of speed, simplicity, and the potential of indicating specific mechanisms of toxicity.

Physiological measurements of stress are confounded by the extreme variability of these indices to both intrinsic and extrinsic factors. Responses are often not dose dependent, particularly if the contaminant can be metabolized or sequestered. The effects of experimental manipulation and natural variables such as season, light, or nutritional status can often produce effects of greater magnitude than the treatment under investigation. A combination of measurements designed to evaluate the status of individuals, either integrated into a single index at one level of organization such as scope for growth (Bayne et al., 1975), or by following energetic parameters at adjacent levels of organization (Capuzzo, 1985) might provide a less variable and better appraisal of the general response of an organism. With any of these approaches it is essential to either have very detailed information on variability of

these indices in natural populations, or to compare responses between closedly matched experimental and control organisms under identical conditions.

Aromatic hydrocarbons have been found to produce a variety of physiological and biochemical effects on marine organisms. (See reviews by Neff, 1979 and Capuzzo, 1985). Effects observed at the cellular and biochemical level include chromosomal aberrations and increased rates of sister chromatid exchange (Hooftman and Vink, 1981); induction of mixed function oxygenase activities (Stegeman, 1981, Lee, 1981); alterations in free amino acid ratios (Augenfeld et al., 1980-81); increases in lysosomal fragility (Moore et al. 1978); disruption of glucose metabolism (Riley et al., 1981); changes in respiratory pigment concentrations (Crider et al., 1982); and reduced affinity of respiratory pigments for oxygen as well as alterations in cardiac output, heart rate, and hemolymph pH (Sabourin, 1982); alterations in neurotransmitter levels (Fingerman and Short, 1983), disruptions in RNA polymerase and RNAase activities (Viarengo and Moore, 1982);, changes in glycogen content (Augenfeld et al., 1983); alterations in adenylate nucleotide pools (Ivanovici, 1980); disruption of lipid metabolism (Stegeman & Sabo, 1976; Capuzzo et al., 1984); and liver hypertrophy (Fletcher et al., 1982). Effects of petroleum hydrocarbons observed on whole animals include alterations in oxygen consumption rates (Anderson et al, 1974; Gilfillin and Vandermeulen, 1978; Edwards, 1978; Laughlin and Neff, 1980), ammonia excretion rates (Vargo, 1981; Capuzzo and Lancaster, 1981), feeding rate (Gordon et al., 1978), time to metamorphosis in larval invertebrates (Capuzzo et al., 1984), growth (Hauschildt-Lillge, 1982), and reproduc-

tive success (Hose et al., 1982).

The sensitivity of marine worms, particularly polychaetes, to petroleum hydrocarbons appears to be quite variable. George (1971) found variations in population abundance in two species of cirratulid worms to be no different after an oil spill, and although oil was found to depress feeding rates of Arenicola marina, populations of this worm were found existing in sediments severely impacted by an oil spill (Gordon et al., 1978). Nevertheless, effects of petroleum hydrocarbons on marine worms have been documented at community and population levels affecting species composition and abundance (Elmgren et al., 1980; McLusky et al., 1980); at the whole animal levels causing increased mortality (Mohamed, 1974), reduced growth and feeding rates, and fertility (Lee et al., 1981, Hauschildt-Lillge, 1982; Augenfeld, 1980; Fries and Lee, 1984); and at the biochemical level inhibiting activities of glycolytic enzymes such as phosphofructokinase (Blackstock, 1980), increasing mixed function oxygenase activity (Lee et al., 1979, 1981), and decreasing tissue glycogen (Augenfeld et al., 1983) and alanine concentrations while increasing tissue lipid concentrations (Carr and Neff, 1984). Toxicities of water soluble fractions of several test oils to different species of worms is highly species specific ranging by over one order of magnitude (Carr and Reish, 1977), with refined oils being generally more toxic than crude oils (Rossi et al., 1976).

Problems of experimental design

Many of the studies mentioned above either documented effects on endemic populations in environments contaminated with hydrocarbons or have measured responses of individual organisms to water soluble oil

fractions. In either case the mixture of chemicals present is large and complex. Detailed chemical analysis of the mixtures to show how they may change over the duration of the experiment is often not conducted, so actual exposure concentrations and accumulated concentrations are not precisely known. In addition to adequate chemical characterization in laboratory studies, it is also important to maintain and test organisms in an environment that simulates as closely as possible natural physical and chemical conditions. For instance, when investigating the response of burrowing organisms to a sediment associated compound it is important to study the organism in a system containing sediment. DeWild (1973), Vernberg (1977), and Pamatmat (1982) have all reported differences in metabolic rates of burrowing organisms depending on whether or not sediment was available. Accumulation of and responses to chemical contaminants by burrowing organisms has also been shown to be affected by the presence or absence of sediment (Pesch & Morgan, 1978; Fowler et al., 1978). The maintenance of water quality is another important variable requiring consideration. Depletion of oxygen and build up of excretory products in static bioassay systems can affect physiological parameters. (Prosser, 1973). Higher basal metabolic rates have been reported for a mysid shrimp depending on whether a static or flow through experimental system was used (Smith and Hargrave, 1984).

Scope of thesis research:

Previous investigations have clearly demonstrated that PAH in the benthic environment are influenced by an interactive collage of physical, chemical, and biological process. Of the many processes involved, metabolic transformation of PAH is one that has only begun to be investigated

on an environmental scale. The numerous investigations on the effects of PAH on benthic organisms have documented a myriad of effects, although the results are so varied, it is difficult to make meaningful generalizations. In reading the literature, the lack of investigations of realistic exposures to a chemically well-characterized contaminant is apparent.

The goal of the thesis research described here was to investigate both the fates and effects of PAH in the benthos. An interdisciplinary approach was used, focusing on interactions between benthic organisms and PAH in the benthic environment. Emphasis was placed on the role of metabolic transformations, bioavailability from different sources, and sublethal effects on infaunal organisms. Due to the interactive nature of these processes, it was decided that from an environmental perspective they could not be studied in isolation. Attempting to investigate so many different processes in one system limited the resolution possible for analysis of any one process. Nevertheless, the results from this investigation of BA, a model hydrophobic pollutant compound, in an intact benthic system, support the validity of this approach, and contribute to the understanding of the fate of PAH in the marine environment, and animal/sediment/chemical interactions.

The presentation of this thesis was organized to minimize repetition, while logically separating the results into managible units for easy reading. The introduction provides a brief backround to the relevant literature. The second chapter describes the experimental approach and analytical methods. This investigation involved five major experiments. Portions of the results from all experiments are divided between the next three chapters: (3) the fate of BA in the system; (4) <u>in vivo</u>

metabolism of BA by the polychaete Nereis virens; and (5) the physiological effects of BA exposure on Nereis. Chapter 6 summarizes the entire investigation. The results of a previous investigation on the fate of BA in a large recirculating benthic microcosm conducted by our laboratory are presented in Appendix 1.

CHAPTER 2:
EXPERIMENTAL APPROACH

Experimental system:

The experimental design of this study was directed to simultaneously measure both the fates and effects of a higher molecular weight (>3 rings; Neff, 1979) PAH in a model benthic system. In order to carry out a multidisciplinary study of a system as complex as the benthos, investigation was centered on a single PAH, benz(a)anthracene (BA), and a single benthic organism, the polychaete Nereis virens. BA was chosen as a representative PAH because, due to its low water solubility it is thought to be primarily associated with particles. Although BA is classified as a known mutagen (Ames, 1972), not much is known of its in vivo metabolism or sublethal effects in marine organisms. Radioisotopes were used to allow construction of a complete budget for total BA and BA metabolites. Nereis virens was chosen as a representative infaunal organism because it is omnivorous, relatively large and hardy, its life history and physiology are relatively well known, and it has been shown to possess the MFO system necessary for PAH metabolism (Lee et al., 1979).

An attempt was made to design an experimental system that would provide a realistic benthic environment from the standpoint of both the biology of Nereis and the geochemistry of BA. A flow-through system was used to mimic the real flow regime in the near-shore benthos by providing a constant supply of oxygenated water to the worms and removing worm metabolic products. Natural sediments were used since attempting to duplicate the physical and chemical characteristics of sediment, such as grain size, organic carbon content, and surface properties that affect both biological and geochemical processes, would have been very diffi-

cult.

The benthos receives contaminant inputs from a variety of sources including the bulk sediment reservoir, the water column, and large objects such as detritus and animal carcasses that may be deposited. In an attempt to study how bioavailability is affected by source, BA was introduced in three ways: (1) already bound to sediment, simulateing input from dumping activity; (2) directly into the water column, simulating input from urban run-off or spilled oil; and (3) as labeled food, simulating input from organisms or detritus contaminated with PAH acquired elsewhere. All of these exposure senarios were directed toward understanding the fate of BA in the subtidal benthos. Factors which might be important to the fate of BA in intertidal or shallow benthic environments such as photoxidation, turbulence, or tidal pumping were not considered.

PAH are not known to be acutely toxic at concentrations present in most environments. Because this study was aimed at sublethal effects of PAH exposure and at PAH metabolism, a multilevel bioenergetic approach was used to monitor the physiological effects of BA exposure. Animal bioenergetics are known to be affected by exposure to oil (Capuzzo, 1985). Energy is a common currency used at all levels of organization in an organism, thus it is reasonable to consider that disruption in the bioenergetics at one level may precede disruption at the next level.

A schematic of the exposure chamber developed for these experiments is shown in Figure 2.1. This chamber was designed with two purposes in mind: (1) measurement of whole chamber metabolic rate and (2) measurement of the concentration of BA and BA metabolites in the sediment, worms, and water column. Each chamber enclosed a total volume of two liters. In

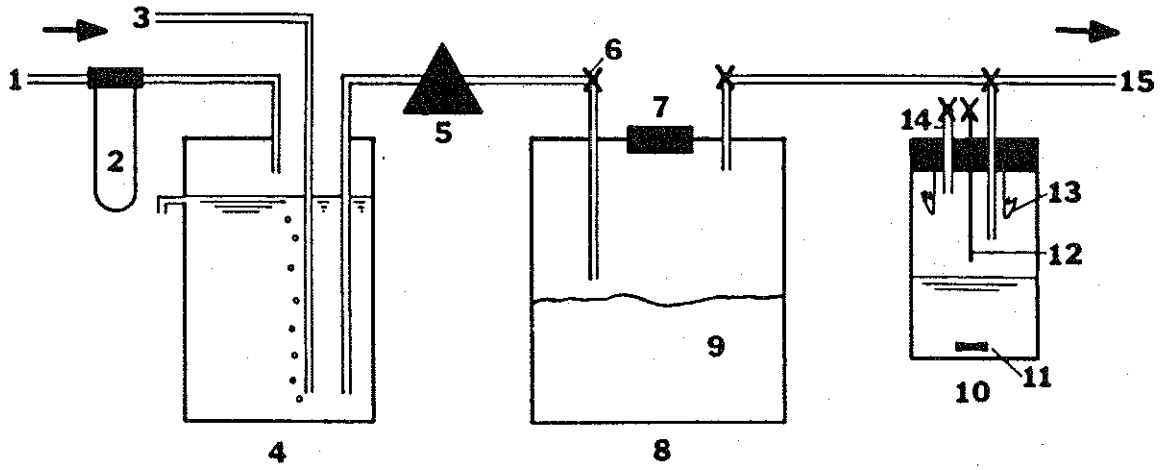


Figure 2.1: Schematic diagram of experimental system. 1. seawater inlet; 2. 1 um honeycomb filter; 3. air line; 4. seawater reservoir; 5. peristaltic pump; 6. 3-way stopcock; 7. silicon stopper; 8. exposure chamber; 9. sediment; 10. CO_2 trapping chamber; 11. magnetic stirring bar; 12. acid injection port; 13. wicks to collect CO_2; 13. vent; 15. waste outlet.I

most experiments the chambers contained 450 grams dry weight (gdw) of sediment with a water column of 1.4 liters (l) of seawater. The system was composed almost entirely of glass to minimize sorption of organic compounds and was designed to be both gas and water tight. Silicon rubber was used for tubing connections and the central stopper, and plastic 3-way stopcocks were used to take samples from the inlet and outlet ports. Chambers were held together by two Plexiglass rings connected by 4 threaded rods which sandwiched the chamber top to the bottom, a small silicon rubber gasket forming the seal.

All chambers received water from a common head tank of filtered seawater maintained at saturated oxygen tensions with vigorous air bubbling. A constant flow of water was driven by peristaltic pumps (Harvard, Model 1203 in experiment 1 and 2 and Cole Palmer Masterflex Model 7508 in experiments 3-5). Water entered the chambers near the sediment surface and exited near the top to provide good mixing. Chamber effluent could either be collected for sampling, diverted into a CO_2 trapping chamber, or discarded. The entire experimental system was enclosed in a temperature controlled room maintained at $14^{o}C$ and illuminated continuously by red light.

Standard experimental design included nine chambers run simultaneously, allowing for three different treatment regimes with three replicate chambers for each treatment. One set of chambers contained worms not exposed to BA, the second contained worms exposed to BA, and the third received BA but did not contain any worms. This design allowed comparisons to be made between the effects of BA on the worms and the effects of the presence of worms on the distribution of BA in the system.

The only exception to this general plan was in the experiment with BA labeled food (exp. 5). Since this was a feeding experiment, chambers without worms were not run in favor of having additional chambers for replication of control and exposed groups.

Natural sediments were collected with a 0.1 m Van Veen grab sampler from a depth of 10 meters in Buzzards Bay, MA (41°32'2"N, 70°45'05"W) and stored frozen until use. These sediments contained 2% organic carbon (Perkin-Elmer CHN Elemental Analyzer Model 240) and were composed of 8.5% sand and 91.5% silt and clay sized particles (determined by wet sieving). Before each experiment sediments were thawed and rinsed with seawater filtered to 1 um. The sediment-seawater slurry was homogeneous in appearance without any visible impurities. After thawing sediments were kept cold until introduction into experimental chambers.

Nereis virens were obtained from the Back River Estuary in Boothbay, ME. This is a relatively unpolluted area with 30 °/oo salinity water and sediment of a grain size similar to that of the sediment used in these experiments. Worms were maintained at the Environmental Systems Laboratory (ESL) in a flow-through seawater system containing natural sediment. In addition to sediment, the worms were fed either minced Mytilus edulis tissue (exp. 1-3) or a formulated diet (Leavitt, 1985) which was supplemented with 20% Mytilus edulis tissue. Prior to use in an experiment, worms were acclimated at the experimental temperature of 14°C for at least one week.

In experiments with sediment-sorbed BA, labeled or control sediments were added to the chambers and allowed to settle before initiation of flow. Approximately 8 hours later, initial cores were taken and the

worms added. During all experiments water samples were collected at regular intervals for determination of oxygen and ammonia concentrations, and for extraction of total BA, and $^{14}CO_2$. At the end of the experiment (and also near the middle in the month-long sediment exposure experiment) cores were taken, and then worms were gently teased out of the sediment.

The experimental plan was modified slightly for experiment 4 where BA was introduced directly into the water column, and in experiment 5 where BA was introduced as labeled food. In experiment 4, after the BA spike was introduced, output from the chambers was diverted back into the chambers to conserve isotope. After isotope addition, water was pumped over the sediment surface for 2 days during which radioactivity in the water column was monitored. At this point, the worms were added and 8 hours later the chambers were switched back to the normal flow-through mode to avoid hypoxia and/or high ammonia concentrations. In the feeding experiment (exp. 5) worms were added and oxygen consumption and ammonia production rates were measured for two days prior to food introduction.

The exposures used in this study were of relatively short duration covering periods of days to weeks. The experiments were set up in this way to focus on metabolism of BA and the initial effects of sublethal exposure on worm bioenergetics. Previous work by Malins and his co-workers on <u>in vivo</u> PAH metabolism in fish and invertebrates indicated that the process is rapid, occurring on a time course of hours to days (Varanasi and Malins, 1977). Previous work in our laboratory, looking at the fate of BA introduced into the water column of a large benthic recirculating chamber, showed substantial metabolism of BA in a period of days to weeks (McElroy et al., 1982; Appendix 1).

Analytical methods:

Chemicals:

BA was purchased from Aldrich Chemical Co., Milwaukee, WI, and ^{14}C-BA and ^{3}H-BP from Amersham-Searle, Skokie, IL. Authentic standards for BA and BP metabolites were provided by Dr. David Longfellow at the National Cancer Institute. Aquasol, Protosol, phenethylamine, and Count-off were obtained from New England Nuclear, Boston, MA and Scintiverse II from Fisher Scientific. All other chemicals and enzymes were obtained from Sigma Chemical Co. All solvents were obtained from Burdick and Jackson.

Glassware:

All glassware and equipment used in these experiments was washed using standard laboratory procedures. In addition, all glassware used for BA analysis was solvent rinsed with two rinses with methanol followed by two rinses with chloroform. Glassware used for biochemical analyses was soaked in 10% hydrochloric acid after washing and rinsed again in distilled water. After use with radioisotopes all glassware was soaked overnight in a 2% solution of Count-off, rinsed and cleaned as described above.

Light:

In order to focus only on the biochemical transformations of BA in these experiments it was necessary to avoid photooxidation. Consequently, the experiments, and all analyses involving samples used for metabolite determination were carried out under red light.

Quantification:

Total BA concentration was calculated using the specific activity of

isotope used in each experiment and the extraction efficiencies determined empirically for each type of analysis. Extraction efficiencies were based on recovery of knwon amounts of isotope added to unlabeled samples. [^{14}C] activity was quantified in all samples by liquid scintillation counting (LSC) using a Beckman LS100-C counter and either Aquasol or Scintiverse II as scintillants. Counting efficiencies were determined using either ^{3}H- or ^{14}C-toluene as internal standards. Unless indicated otherwise, all data are presented as the mean $\pm$ the standard error. Statistical analyses employed (Zar, 1984) are described in either in the text or in figure legends. When necessary, data were transformed to either reduce the variance or approximate a normal distribution prior to using parametric statistics. The significance level required to demonstrate a statistical difference between means was 0.05.

Dosing:

[12-^{14}C]Benz(a)anthracene of specific activity 49mCi/mMol was obtained in sealed 100 uCi ampules. A fresh ampule was used for each experiment. The isotope spiking solution was made up in a carrier of acetonitrile and unlabeled BA. Specific activities of the spiking solutions used in each experiment were 4.60×10^{-3}, 4.67×10^{-3}, 4.76×10^{-3}, 3.61×10^{-2}, and 2.15×10^{-1} uCi/ug BA in experiments 1-5 respectively. Prior to dosing, each spike mixture was chromatographed on HPLC (See section 3.10 for analysis conditions.) to assure purity of $\geq$ 98%. If the spike mixture did not meet these specifications it was purified using Sep-Pak (Waters Assoc.) cartridges following the procedure outlined in Figure 2.2. Once purity was assured the spiking mixture was used to dose the chambers as described below.

Figure 2.2

ISOTOPE PURIFICATION USING WATERS SEP-PAK CARTRIDGES

Dry isotope under N_2

Rinse C_{18} Sep-Pak with 10 ml Hx

Transfer isotope to Sep-Pak in $\leq$ 2 ml Hx

Elute with 3 ml 90:10 Hx:DEE

Dry under N_2

Check purity on HPLC if $\leq$98% continue

Rinse silica Sep-Pak with 10 ml Hx

Activate with 2.0 ml H_2O

Deactivate with 1:1 MeOH:H_2O

Transfer isotope to Sep-Pak in 53:27:20 MeOH:EtOH:H_2O

Elute with 3 ml Hx

Dry with Na_2SO_4

Check purity on HPLC

Sediment spiking:

The sediment labeling method was adapted from methods of Karickhoff (1980), and was designed to minimize microbial oxidation and maximize binding of BA to sediment particles. Three equal portions of washed sediments were placed into gallon glass jars and diluted with filtered seawater to form a slurry which was approximately 50% seawater and 50% sediment. Two jars each received a solution containing BA in acetonitrile. A third jar received an equal volume of uncontaminated acetonitrile. The jars were sealed, shaken vigorously by hand then strapped onto a rotary shaking table. Jars of sediment were continuously mixed on the shaking table for at least 24 hours at 4°C, interupted periodically by vigorous hand shaking to resuspend all sediment. After the equilibration period the labeling solution was removed from the sediment by centrifugation (500 g, 5 min, 4°C) and the sediment resuspended in clean filtered seawater. Direct counts of the labeling solution after sediment removal indicated that less than 1% of the isotope added remained in solution. The contents of each gallon jar were then split equally between three replicate chambers. After the sediment settled, flow was initiated and the experiment started 10 to 20 hours later. In three separate sediment dosing experiments (1,2, & 3) this sediment labeling procedure resulted in BA concentrations in the sediment at the beginning of each experiment of 7.93$\pm$.51, 8.61$\pm$1.46, and 6.23$\pm$.24 ug/gdw respectively.

Water column spiking:

Just prior to spike introduction, chambers containing washed sediment were switched from flow-through to a recycling mode, where chamber

effluent was pumped directly back into the chamber. Each chamber received either 2.0 ml of a spiking solution containing BA in acetonitrile or 2.0 ml of acetonitrile alone. Water samples were taken at regular intervals to follow the course of BA removal from the water column for the next two days. After 47 hours small sediment samples were taken with a Pasteur pipette and counted to ensure that isotope from the water column had settled out on the sediment surface. Worms were then added to each chamber, and flow switched from recycle mode to flow-through mode eight hours later, purging BA remaining in the water column.

Preparation of BA labeled food:

[^{14}C]Benz(a)anthracene was incorporated into a formulated diet containing lyophilized Mytilus edulis soft tissue. Two batches of the diet, each sufficient to yield 20 individual portions were prepared using the following ingredients:

1.69 ml water
0.66 g gelatin
0.19 g freeze dried Mytilus powder
0.094 g cod liver oil
90 ul hexane (with or without ^{14}C-BA).

Cod liver oil was placed in a small aluminum weighing pan and either hexane (for the control diet) or the BA spike in hexane (for the labeled diet) was mixed in. Next the dried Mytilus and the gelatin were mixed together and mixed into the cod liver oil. The water (heated to steaming) was then quickly stirred into the mixture and the pan heated to 100°C for a few seconds until the gelatin dissolved. The pan was immediately placed on ice as soon as the gelatin melted.

This procedure produced a circular slab of food approximately 6 cm in diameter and 1 mm thick with an elastic consistency. The slab was cut

into pieces which weighed 102.3±4.2 mg each. Three extra pieces of labeled food not used in the experiment were extracted according to the methods outlined in Figure 2.3. The concentration of BA in the extracted food was 17.8±0.9 ug/gdw, representing 84.0% of the label added; 98.5% of activity was recovered in the organic extract, 0.2% in the aqueous extract, and 1.3% in the aqueous precipitate (See section 3.12 for explanation of extraction protocol). HPLC analysis of the organic extract of the labeled food showed that >98% of label was present as unaltered BA (Figure 2.4).

Worm weights:

Wet weight of individual worms was taken just prior to placing them in exposure chambers at the beginning of each experiment and just after removal at the end of each experiment. Worms were removed from sediment by gently stirring the sediment until they swam to the surface where they could be retrieved without damage and placed in a beaker of seawater. After being blotted quickly, worms were weighed to the nearest mg on a Cahn Model TA4100 balance.

In calculations involving worm weights, the starting weight of worms was used in the week-long experiments where worm weight did not change, (exp. 1,2,4,5). For the month-long experiment (exp. 2), where worms lost weight, daily weights were calculated from the total weight loss assuming a linear decline. When necessary, dry weights were calculated from wet weights using a linear regression of wet and dry weights taken for a group of control worms shown in Figure 2.5.

Oxygen consumption and ammonia production:

Oxygen consumption and ammonia production were calculated by differ-

Figure 2.3

EXTRACTION OF BENZ(a)ANTHRACENE LABELED FOOD

Add 0.8 ml of steaming H_2O to sample maintained at 50°C

Stir to dissolve

Rinse spatula with 2 x 1 ml hot MeOH

Vortex

Add 1 ml $CHCl_3$

Shake well, sonicate 10 min

Add 1 ml H_2O and 1 ml $CHCl_3$

Shake well, sonicate 10 min

Centrifuge 5 min low speed

Aqueous phase — **Organic phase**

Add 2 ml $CHCL_3$

Stir 5 min at 50°C

Sonicate 5 min

Centrifuge

Aqueous phase — **Organic phase**

LSC

Combine organic phases

Dry with $NaSO_4$

HPLC and LSC

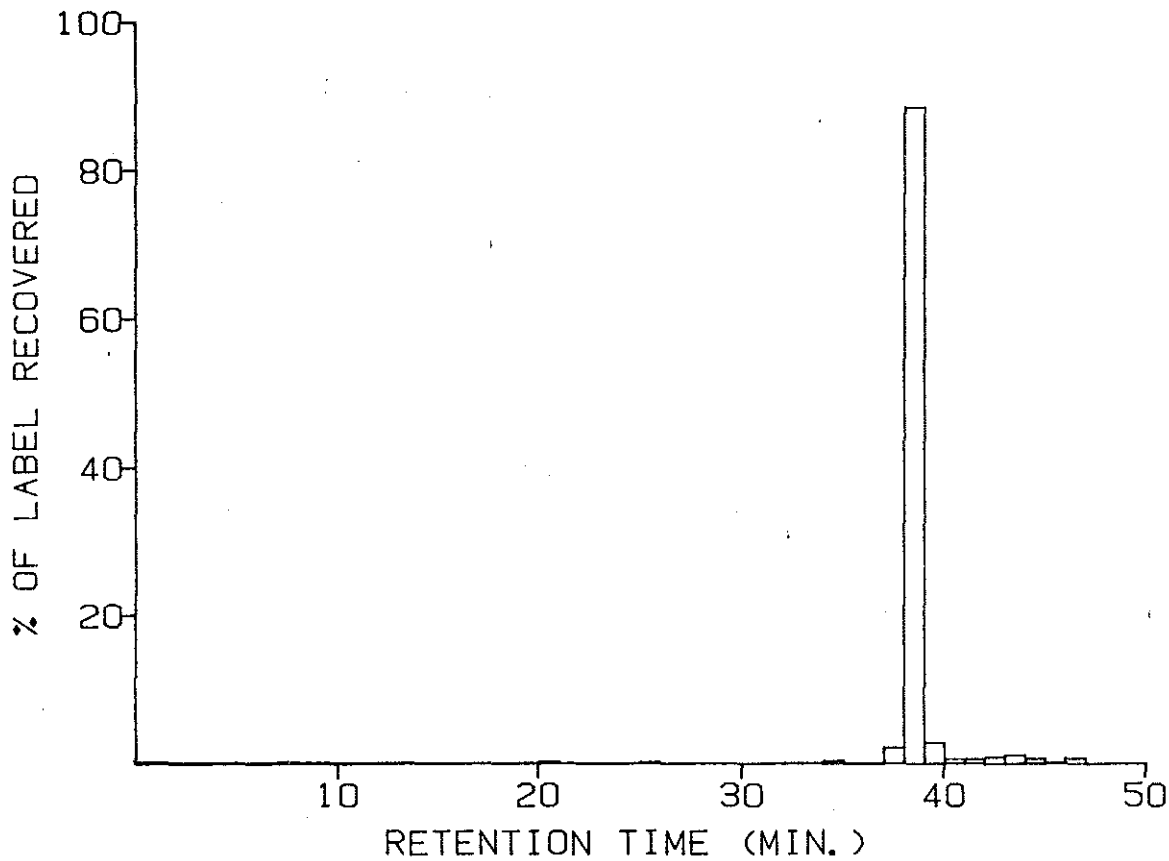


Figure 2.4: ^{14}C HPLC chromatogram of BA labeled food.

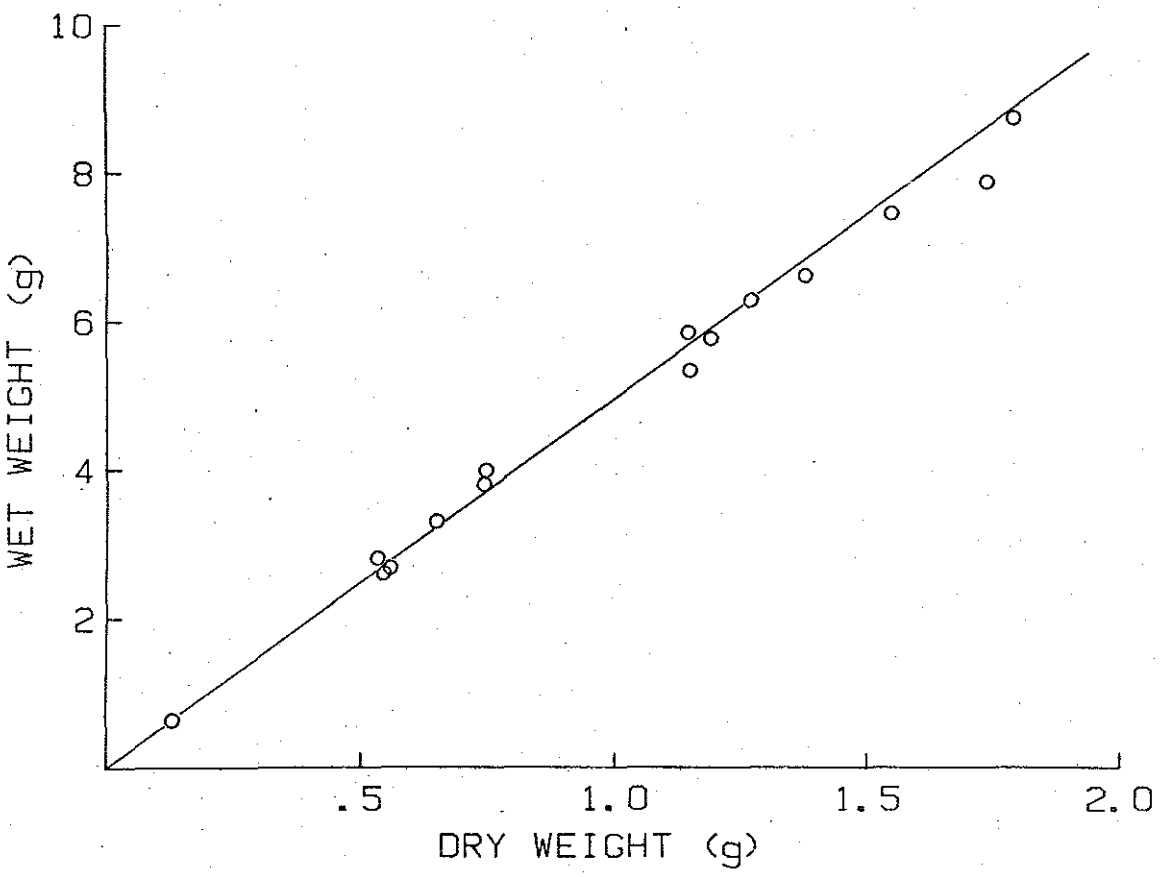
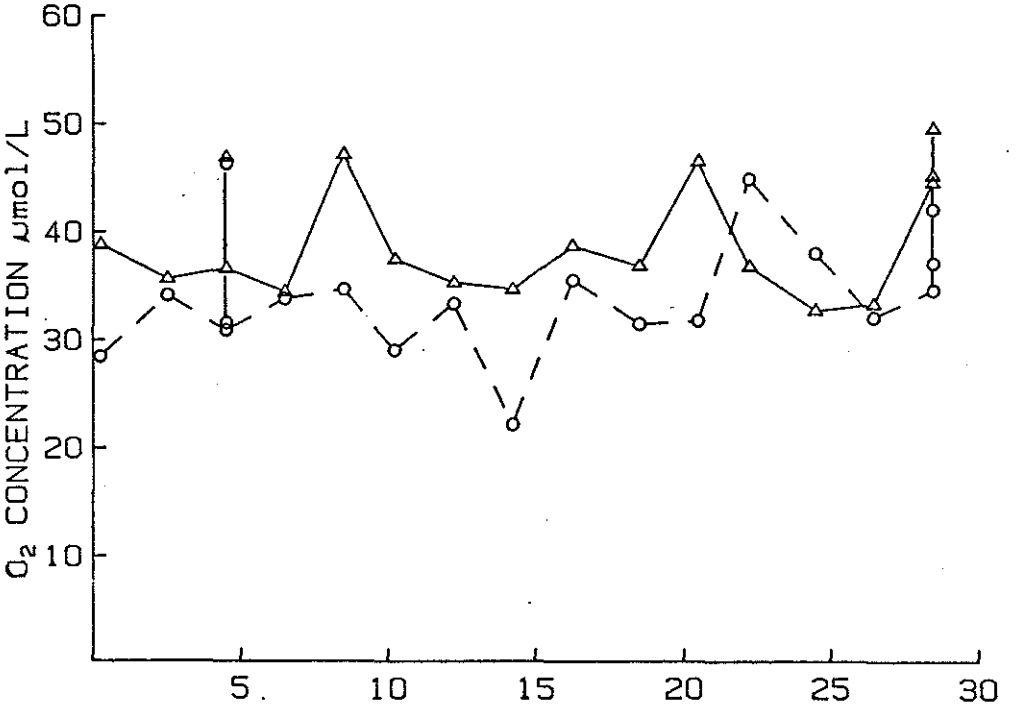


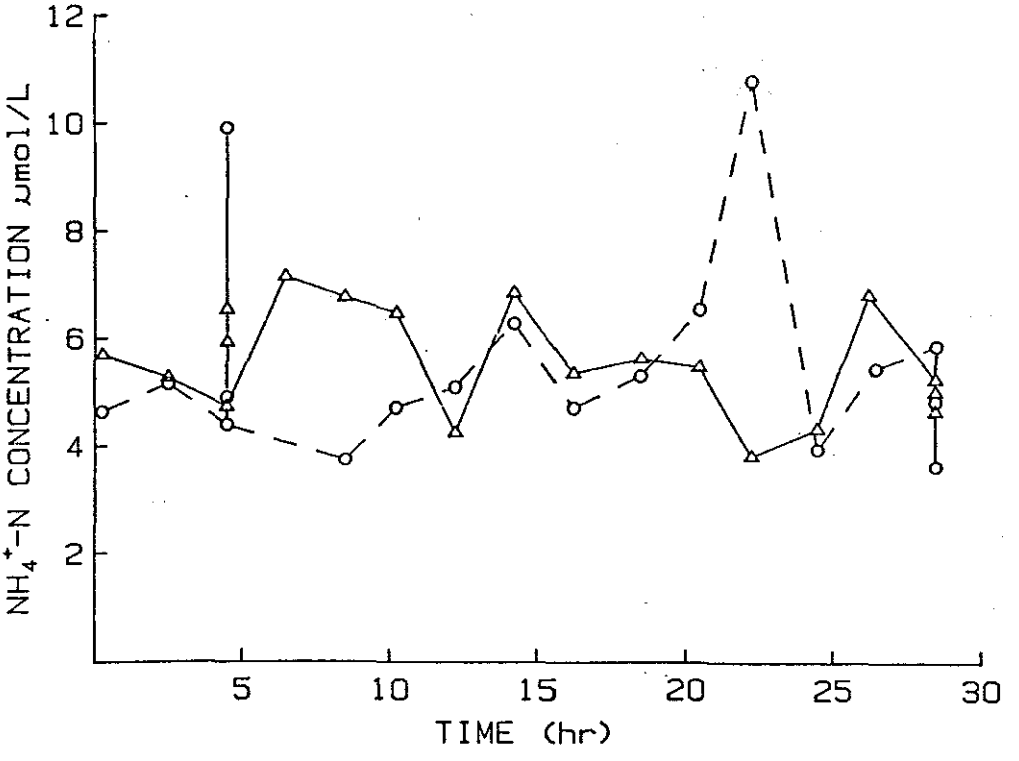
Figure 2.5: Wet weight/dry weight linear regression for <u>Nereis</u> <u>virens</u>

ence from concentrations in water entering and leaving the chambers. Residence time of water in the chambers was maintained at 2 to 2.3 hours. Each chamber contained 4 worms. No attempt was made to correct for non-worm metabolic activity in the chambers. Although metabolic measurements were made on chambers without worms, due to the absence of irrigated burrows these were not considered to be valid controls for non-worm metabolism. Comparisons between rates in control chambers and chambers receiving BA are made assuming that BA had no measurable effect on microbial metabolism. Although it would have been best to test this assumption directly, several factors suggest that the assumption is reasonable. Bacteria cannot use larger PAH such as BA as a sole carbon source, (Gibson, 1976), therefore extensive metabolism of the molecule is needed before easily metabolizable fragments are produced. Especially considering the relatively abundant source of carbon already in these sediments, it is unlikely that cometabolism of BA would support increased microbial activity (Perry, 1979). In addition, low and consistent rates of microbial mineralization of BA observed in the 25 day experiment with sediment-sorbed BA (See Chpater 3) suggest that the presence of BA alone was not responsible for stimulation of microbial activity sufficent to make a measureable impact on whole chamber respiration.

Samples were taken at the same time each day to minimize any diel periodicity in activity. This was probably unnecessary, because in one experiment, measurements of oxygen consumption and ammonia production taken every two hours for 28 hours showed no pronounced diel patterns (Figure 2.6). This observation is supported by previous work, by Scott (1976) who found no periodic diel fluctuations in Nereis virens respira-

Figure 2.6: Diurnal cycle of whole chamber oxygen consumption and ammonia production. Values represent single measurements on one chamber over time. Circles represent control chambers. Triangles represent chambers with BA. Multiple values at one time point show range for all replicate chambers at that time.


60
50
40
30
20
10
O_2 CONCENTRATION µmol/L
5
10
15
20
25
30


12
10
8
6
4
2
NH_4^+-N CONCENTRATION µmol/L
5
10
15
20
25
30
TIME (hr)

tion when worms were kept under constant illumination or darkness.

O:N ratios (the atomic ratio of oxygen consumed to nitrogen produced) were calculated from measured oxygen consumption and ammonia excretion rates. Ammonia has been shown to be the primary excretory product of marine polychaetes (Hult, 1969; O'Malley and Terwilliger, 1975). In addition to ammonia analysis, total nitrogen was analyzed in one set of samples using persulfate oxidation at 110°C (Grassholff, 1976) followed by standard analysis on a Technicon autoanalyzer. The variation between duplicate samples was large; however, the results indicated that 73±14% of total nitrogen in the chamber effluent was in the form of ammonia.

Water samples were drawn from the inlet and outlet ports of the chambers into glass syringes that were immediately capped and placed on ice until analysed. Dissolved oxygen was measured using a polarographic oxygen electrode (Radiometer Copenhangen E5046 pO_2 electrode with either a Radiometer PHA930 O_2 analyzer or a Strathkelvin 781b O_2 meter) housed in a temperature controlled water jacket maintained at the same temperature as the exposure system. The electrode was calibrated between zero and 100% saturation using a zeroing solution (Radiometer) and 14°C seawater made saturated by sparging with air for 30 minutes. Measurements were made on each sample until readings stabilized at ± .05 ppm. Although measurements for each chamber were usually made on single samples, duplicate samples taken periodically had an average coefficient of variation of 1.26%. Since all chambers received water from a common source, oxygen concentration was measured only in three randomly picked inlet ports and the average of these measurements was used to calculate the

oxygen consumption measured in samples taken at the outlet ports of each chamber.

Ammonia was measured as NH_4^+-N using the methods of Solorzano (1969). Water samples were collected at the same time as those for dissolved oxygen. Because of its low ammonia content, water in the head tank feeding the chamber was used for analytical blanks and to dilute ammonium chloride standards for calibration. Therefore, ammonia concentration in the water entering the chambers was taken to be zero. Ammonia concentration was routinely determined from a single analysis of a single sample from each chamber. However, duplicate analyses of single samples, and duplicate samples taken from individual chambers periodically indicated that the average coefficient of variation between duplicate analyses and duplicate samples was 2.65% and 3.30%, respectively.

Oxygen consumption and ammonia production rates were calculated from the difference in concentration between water entering and leaving the chambers multiplied by the volume of the water column in the chambers divided by the residence time of water in the chambers. Calculated rates were then normalized to the wet weight of worms in each chamber.

BA mineralization to carbon dioxide:

Dissolved CO_2 was stripped from 250 ml water samples collected directly into traps as pictured in Figure 2.1. The traps contained 0.5 ml of saturated potassium hydroxide which kept the pH of the sample above 10 to prevent CO_2 loss during collection; two trapping wicks each containing 0.3 ml of a quaternary amine as a trapping agent; and a stirring bar. Protosol was used as a trapping agent in experiments 1-2, and phenethylamine was used in experiments 3-5. After water collection the

traps were sealed, acidified with sulfuric acid to a pH less than 2, and stirred for 14 to 24 hours. The only exception to this was in experiment 1 where the traps were stirred for only 12 hours. Carbon 14 activity in carbonate collected on the wicks was then quantified by LSC after neutralization with Tris buffer and the addition of a small amount of water.

Extraction efficiency of the CO_2 stripping procedure determined using ^{14}C labeled bicarbonate was 54.0% using Protosol and 86.6% using phenethylamine as trapping agents. Chemical- or photo-oxidation of dissolved BA in water samples during the stripping procedure would lead to overestimation of BA mineralization. This possibility was assessed by running the extraction on seawater spiked with ^{14}C-BA. 8.33% of the total BA concentration in seawater could be liberated to CO_2 using the procedure outlined above. To avoid overestimation of BA mineralization due to this analytical problem, in cases where measurable amounts of BA were found in the water column, the concentration of $^{14}CO_2$ measured was reduced by a factor of 0.0833 times the concentration of BA observed in at that time in water samples.

Total ^{14}C activity in the water column:

Total concentration of BA (parent plus metabolic products) was determined by several different methods. The standard procedure involved concentrating a 50 to 600 ml water sample on a C_{18} Sep-Pak (Waters Assoc.) cartridge, eluting the sample with 10 ml of acetonitrile, reducing the volume by evaporation, and quantification by LSC. In the first experiment, water samples were extracted twice with 25 ml of ethyl acetate, the volume of the extract reduced by evaporation, and activity quantified by LSC. In the third and fourth experiments, in addition to

the Sep-Pak method, 750 ml samples were extracted two times each with 20 ml of chloroform and a 1:1 mixture of methanol/chloroform. BA metabolites present in some of these extracts were analyzed using HPLC as described below. In the fourth experiment, activity was initially high enough to allow direct quantification of unextracted samples by LSC. A comparison of the various methods used is shown in Table 2.1. Solvent extraction of large volume samples gave the best estimate of total activity and was comparable to results obtained with direct counting of unextracted samples. The Sep-Pak method underestimated total activity by a factor of 1.81$\pm$.22. Unfortunately, due to the low activity of most water samples and the limitations on doing many replicate large volume extractions, it was necessary to use the Sep-Pak method for routine analysis, and correct flux measurements for the low efficiency of extraction.

Adenylate nucleotide pools in individual worms:

Methods for adenylate nucleotide extraction were adapted from those of Karl and Holm-Hansen (1978) and Walsh and Somero (1981). After the worms were weighed at the end of the experiment, a cross-sectional piece of worm tissue containing approximately 3 segments and having an average weight of 0.5 g was quickly removed from just behind the pharynx and freeze-clamped clamped in liquid nitrogen cooled tongs. Time elapsed between tissue removal and clamping was less than 5 seconds. The frozen tissue was homogenized in liquid nitrogen cooled mortars with 3.0 ml of ice-cold 0.6 N sulfuric acid. After thawing at 0°C the precipitate was removed by centrifugation for 30 minutes at 3000 g at 4°C, and duplicate 1.0 ml aliquots of the extract neutralized with 0.5 ml of 1.2 N sodium hydroxide and buffered with 1.5 ml of a solution containing 12 mM EDTA

Table 2.1

COMPARISON OF DIFFERENT METHODS FOR QUANTIFYING TOTAL ^{14}C ACTIVITY IN WATER SAMPLES

	All values normalized to DPM/100 ml				
	Day 1	Day 2	Day 3	Day 4	Day 5
Direct Counts 1 or 2 ml samples averages for 3 chambers					
Worms	6700	1650	1450	1450	
No Worms	7550	1700	1600	1450	
Sep-Pak 100 ml samples per chamber times 3					
Worms	8240	999	938	683	793
No Worms	9240	994	1072	791	904
Organic Exctract 250 ml samples per chamber times 3					
Worms			1840		1750
No Worms			2020		1080

and 100 mM Tris pH 7.0. Buffered extracts were frozen and stored at -70°C for less than two weeks until analysis.

Concentrations of ATP, ADP, and AMP in these extracts were measured spectrophotometrically using NADH coupled enzymatic reactions employing methods (Figures 2.7 and 2.8) modified from those described in Bergmeyer (1974). Concentrations were calculated from standards for each nucleotide measured each day of analysis. Samples were run in duplicate. The average coefficients of variation were 10.8, 8.7, and 7.5 % for ATP, ADP, and AMP respectively.

Mixed function oxygenase activity in whole worm homogenates:

Mixed function oxygenase activity was measured radiometrically using microsomes prepared from whole worm homogenates and [^{3}H-U]benzo(a)pyrene as a substrate. After removal of the head and the first few sections for adenylate nucleotide analysis, the remainder of the worms from each chamber were pooled and homogenized in a solution containing 150 mM potassium chloride and 50 mM Tris pH 7.4. The homogenate was then split, one fraction frozen for BA analysis as described below, and the other used for microsome preparation. Microsomes were sedimented by centrifugation at 4°C using sequential spins of 800 g for 10 minutes, 12,000 g for 10 minutes, and 39,000g for 90 minutes following the methods of Lee et al. (1979). In the last three experiments two modifications were made. Sedimented microsomes were washed by resuspension in fresh homogenation buffer and resedimentation. Additionally, the protease inhibitor PMSO (phenylmethyl-sulfonylflouride) was added to the homogenate and the supernatant before each 90 minute spin. After isolation microsomes were resuspended in a buffer containing 1 mM EDTA, 1 mM dithiothreitol,

Figure 2.7

ADENOSINE TRIPHOSPHATE ASSAY

Reaction Protocol:

Mix .833 ml Buffer
.020 ml NADH
.127 ml Sample

Incubate 10 min room temperature

Read at 340 nm E_1

Add .020 ml Enzyme mixture

Incubate 30 min 30°C

Read at 340 nm E_2

ATP = $E_2 - E_1$

Solutions:

Buffer	Triethanolamine hydrochloride	18.6 mg/ml
	K_2CO_3	3.39 mg/ml
	$MgSO_4 \cdot 7H_2O$	1.0 mg/ml
	EDTA $\cdot$ $Na_2H_2 \cdot H_2O$	0.4 mg/ml
	Glycerate 3-phosphate	3.30 mg/ml
NADH:	6.81 mg/ml in 5% $NaHCO_3$	

Enzyme Mixture:

Glyeraldehyde-3-phosphodehydrogenase EC No. 1.2.1.12	209 U/ml
3-Phosphoglycerate phophokinase EC No. 2.3.2.3	333 U/ml

Enzymes in 3.2 M $(NH_4)_2SO_4$

Standards in 20 mM Tris pH 7.7

Figure 2.8

ADENOSINE DIPHOSPHATE AND MONOPHOSPHATE ASSAY

Reaction Protocol:

Mix .670 ml Sample
.050 ml PEP in $MgSO_4$-KCl
.020 ml NADH
.010 ml LDH

Incubate 10 min room temperature

Read at 340 nm E_1

Add .020 ml PK

Mincubate 15 min 30°C

Read at 340 nm E_2

Incubate 15 min 30°C

Read at 340 nm E_3

ADP = $E_2 - E_1$ **AMP** = $E_3 - E_2$

Solutions:

PEP: 6.0 mg/ml in $MgSO_4 \cdot 7H_2O$ 123 mg.ml
KCl 400 mg/ml

NADH: 8.52 mg/ml in 5% $NaHCO_3$

LDH: 1900 U/ml in 3.2 M $(NH_4)_2SO_4$ EC No. 1.1.1.27

PK: 710 U/ml in 3.2 M $(NH_4)_2SO_2$ EC No. 2.7.1.40

MK: 630 U/ml in 3.2 M $(NH_4)_2SO_4$ EC No. 2.7.4.3

Standards in 20 mM Tris pH 7.7

AMP standards also contained ATP

20% glycerol, and 50 mM Tris pH 7.4.

The radiometric BP hydroxylase activity assay (Figure 2.9) was adapted from methods originally described by Van Cantfort et al. (1977) and modified by Stegeman and Binder (1980). The assay was run at 30 °C for 30 minutes using assay mixtures killed at time zero for blanks. The assay and blanks were run in triplicate for each sample. Following these procedures, the rate of BP oxidation was linearly related to time (up to 30 minutes) and microsomal protein concentration (up to 1 mg). Microsomal protein concentration was determined using the methods of Lowry et al. (1951) using bovine serum albumin as a standard.

High pressure liquid chromatography (HPLC):

Reverse phase HPLC was used to separate metabolic products from parent BA in concentrated organic extracts of sediment, water, and worm homogenates. Separations were carried out using either a Dupont Model 840 or Perkin-Elmer Model Series 4 liquid chromatograph equipped with fixed wavelength detection at 254 nm and reverse phase 5 um 25 cm analytical columns (either slurry packed Dupont Zorbax, or Altech 605-RP, or Rainin Microsorb) equipped with an Upchurch guard column dry packed with Polyosil (Rainin Inst.) 25-40 um irregularly shaped C_{18} packing. HPLC analyses were run at 35 °C with gradient elution from solvent A (water) to solvent B (methanol/ethanol 2:1) using a three step linear program on: (1) the Dupont chromatograph, changing from 45 to 35% A in 15 min, 35% to 20 % A in 30 minutes, and jumping to and holding at 0% A for 15 minutes at a flow rate of 1.5 ml/min; and (2) on the Perkin-Elmer chromatograph changing from 50 to 35% A in 15 min, 35 to 0 %A in 25 min, and holding at 0% A for 10 minutes at a flow rate of 1.0 ml/min.

Figure 2.9

BENZO(a)PYRENE HYDROXYLASE ASSAY

Reaction Mixture:

Mix .070 ml buffered microsomes

.010 ml ^{3}H-BP

Equilibrate at 30°C

Start reaction with .020 ml NADPH

Shake 30 min at 30°C

Stop reaction with .300 ml 150 mM KOH 85% DMSO

Remove unmetabolized BP by 3 Hx extractions
1.0 ml Hx
Vortex 10 seconds
Centrifuge 5 min 2.5 K RPM 4°C
Remove Hx by aspiration

Neutralize portion of aqueous extract for LSC

Solutions:

Buffer: 1.4 mg/ml BSA in 50 mM Tris pH 7.4

NADPH: 2.5 mg/ml in buffer

BP: .078 mg/ml in Buffer

Aliquots from the entire run were collected for quantification of BA and BA metabolites. Identity of BA and BA metabolites was based on retention times of authentic standards provided by the National Cancer Institute. A chromatograph showing representative metabolite class standards using the Perkin-Elmer chromatograph is shown in Figure 2.10. Background corrected DPM in each fraction collected were summed for the entire run and activity assigned to a particular metabolite or class of metabolites presented as a percentage of total activity recovered.

Sediment sampling and BA analysis:

Sediment cores were taken in duplicate from each chamber at the beginning and end of each experiment. The only exceptions being the month-long sediment exposure experiment where cores were also taken in the middle of the experiment, and the feeding experiment where cores were taken only at the end. Twelve mm diameter cores, approximately 5 cm deep were taken with butyrate plastic core tubes and immediately frozen until analysis. In most experiments whole cores were analyzed. However, in the water column exposure experiment one set of duplicate cores from the end of the experiments were cut into three sections comprising the top centimeter, the second centimeter, and the remainder of the core, before extraction.

In the month-long sediment exposure experiment and the water column exposure experiment, an extra set of cores was taken at the end of the experiment from each chamber. Cores from chambers with worms and from chambers without worms were pooled and size fractionated by wet sieving prior to freezing. Fractions analyzed included a known volume of filtrate passing through a glass fiber filter (Whatman type GF/F), the size

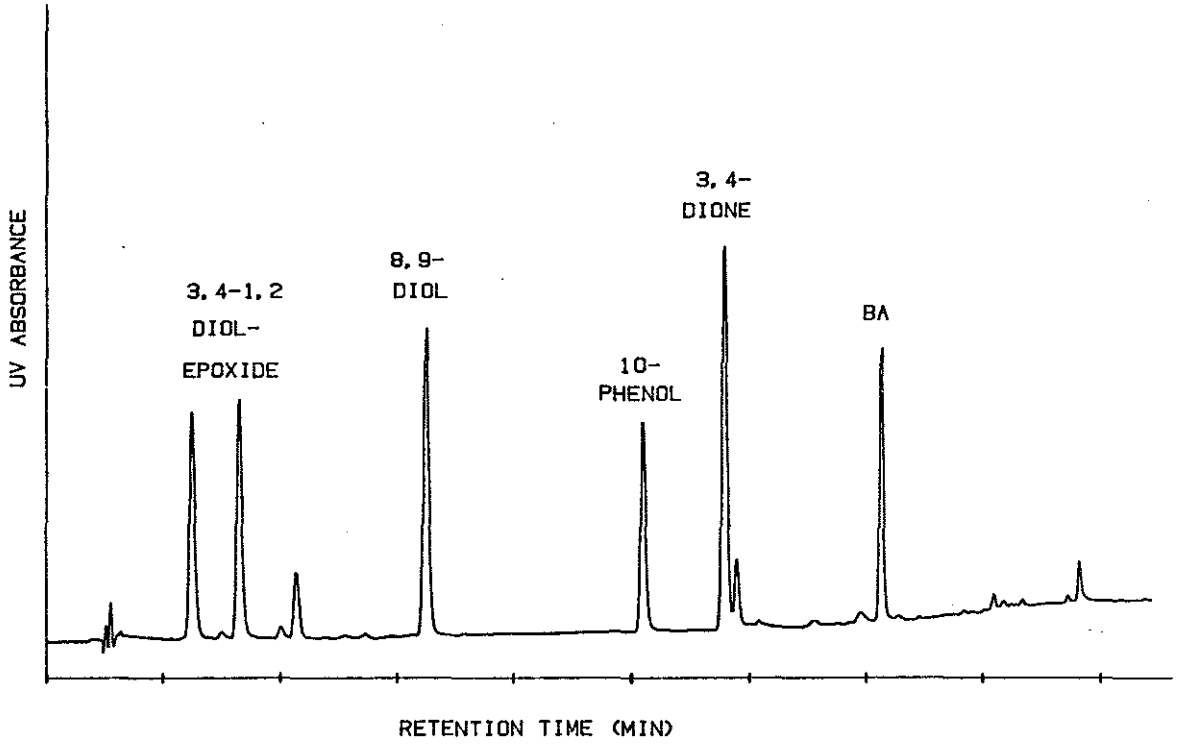


Figure 2.10: UV chromatogram of HPLC separation of representative BA standards

fraction passing a 35 um screen but collected on the glass fiber filter, the fraction passing a 125 um screen but collected on the 35 um screen, the fraction passing a 250 um screen but collected on the 125 um screen, and the fraction collected on the 250 um screen. Nothing was collected on a 500 um screen. Although sieving was done as gently as possible, fecal pellets were disaggregated during this process. With the exception of the filtrate sample all fractions were collected on preweighed glass fiber filters.

Sediment samples were extracted for BA and BA metabolites as described schematically in Figure 2.11, using a general lipid extraction scheme modified from that of Bligh and Dyer (1959). In this method BA and metabolites are recovered in the organic extract, and conjugated metabolites are recovered in the aqueous extract. Subsamples of the organic extract were analyzed for total lipid, determined gravimetrically, and total BA concentration determined by LSC. Total BA concentration was normalized to the dry weight of the sediment measured after extraction. The organic extract was further analyzed for the presence of metabolic products by HPLC as described above. Total BA concentration in the aqueous extract was determined after drying in an oven at 70°C and rehydration before LSC. Extraction efficiency was determined by spiking unlabeled sediment samples with ^{14}C-BA prior to extraction. Efficiency of BA extraction was found to be 90.7%.

Worm homogenates, BA extraction and analysis:

Frozen whole worm homogenates pooled from the worms in each chamber were freeze-dried before extraction. The extraction scheme for worm tissue is described schematically in Figure 2.12, and was modified from

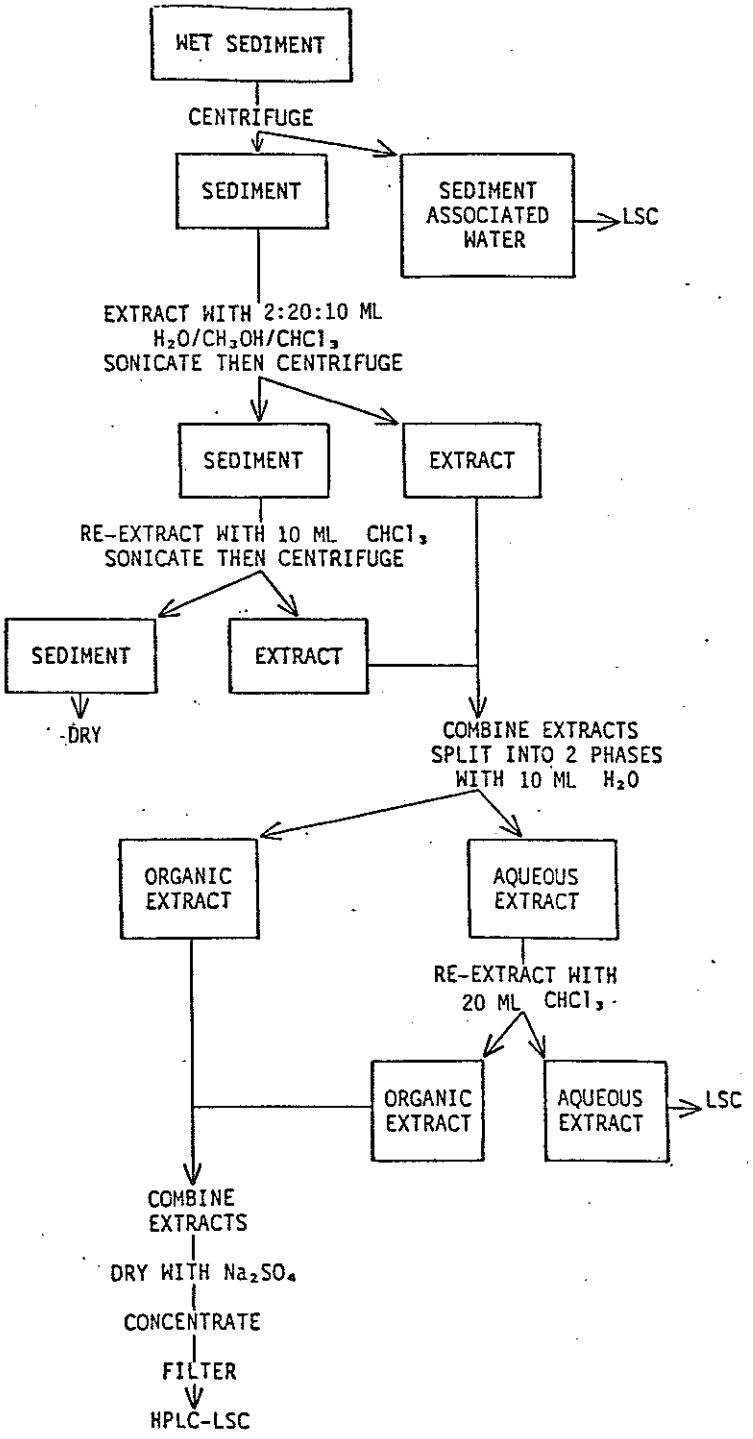


Figure 2.11: Sediment extraction protocol

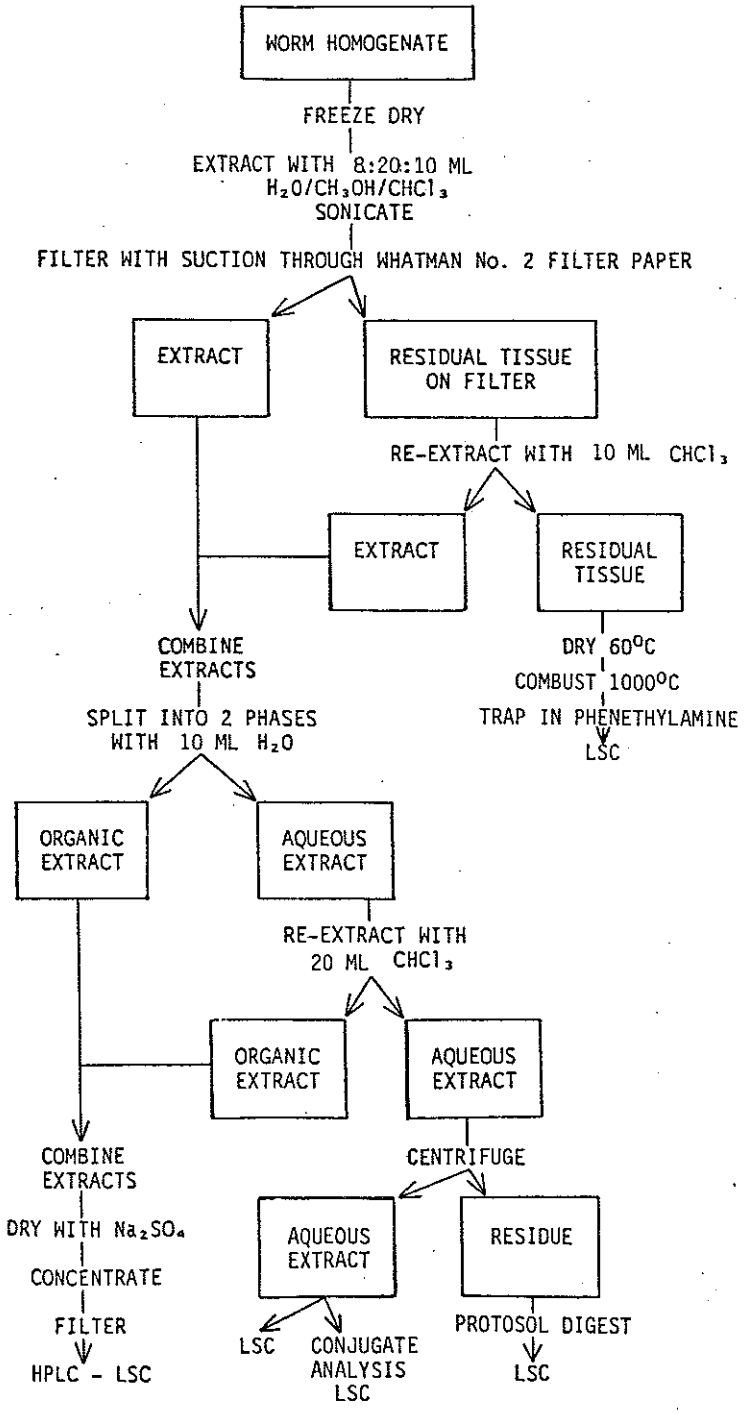


Figure 2.12: Worm extraction protocol

that of Bligh and Dyer (1959), Baird et al.(1977), and Varanasi and Gmur (1981). As in the sediment extraction scheme, BA and oxygenated metabolites were recovered in the organic extract, and conjugated metabolites in the aqueous extract. In the worm extraction sequence a fluffy precipitate was present in the aqueous extract. After separation by centrifugation the aqueous precipitate was digested with Protosol at 35°C overnight, bleached with hydrogen peroxide, neutralized, and the radioactivity remaining quantified by LSC. Unextractable activity remaining in worm tissue after extraction was analyzed by combusting the worm residue at 1000°C and bubbling the effluent through phenethylamine in a Vigreux column. Radioactivity trapped in the phenethylamine was quantified by LSC. Efficiency of the worm extraction procedure was estimated by spiking unexposed hydrated worm homogenates with ^{14}C-BA prior to extraction. Extraction efficiency for BA was found to be 95.6%. Subsamples of organic extracts of worm tissue were analyzed for total lipid, total BA activity, and the presence of BA metabolites on HPLC as described above.

The aqueous extract of worm homogenates was found to contain a significant fraction of total radiolabel incorporated, so an attempt was made to quantify what type of conjugated metabolites had been formed. Methods used for conjugate analysis were modified from those of Varanasi and Gmur (1981) and from methods supplied by Sigma Chemical Co. These are described schematically in Figure 2.13. After concentration and removal of residual solvent by rotary evaporation at 40°C, aqueous extracts were treated with the enzymes B-glucuronidase and arylsulfatase to convert sulfate and glucuronide conjugates back to organic extractable, polar metabolites. The aqueous extracts treated with the conjugate

Figure 2.13

CONJUGATE ANALYSIS

Reation Mixutre:

2.0 ml Concentrated Worm Aqueous Extract
2.0 ml Sample Buffer
0.1 ml Enzyme Mixture

Incubate at 35°C for 20 hours

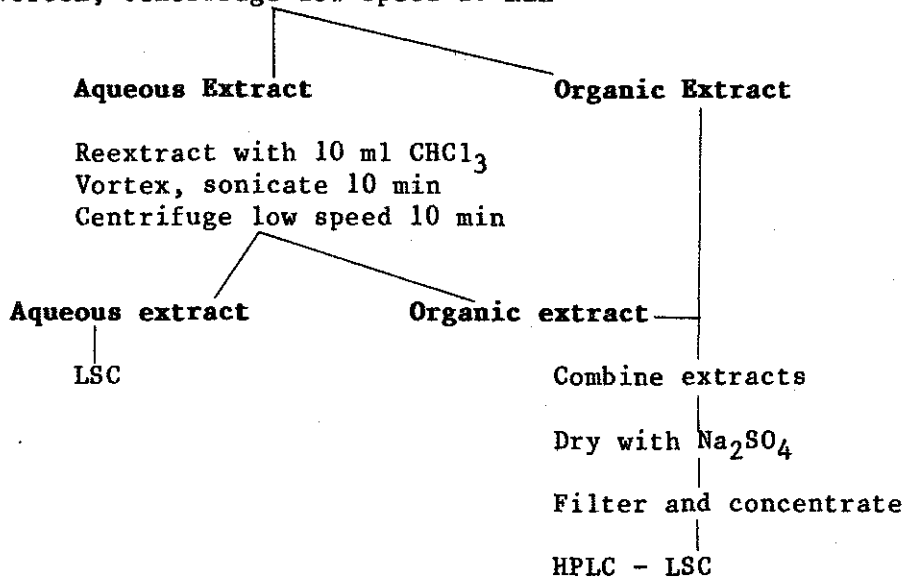
Extraction Protocol:

Add 5 ml $CHCl_3$ 10 ml MeOH

Vortex, sonicate 10 min

Add 5 ml $CHCL_3$ and 5 ml MEOH

Vortex, centrifuge low speed 10 min



Solutions:

Sample Buffer: 200 mM Acetate pH 5.0

Enzyme Mixtures: Aryl sulfatase 500 U/ml EC No. 3.1.6.1
B-glucuronidase 10,000 U/ml EC No. 3.2.1.31
in 75 mM potassium phosphate pH 6.8

cleaving enzymes were then re-extracted using the procedures outlined in Figure 2.12. The increase in activity found in the organic extract (in excess of that extracted from aqueous extracts incubated without enzymes) was then compared to the original activity contained in the aqueous extract before conjugate cleavage to determine the amounts of sulfate and glucuronide conjugates originally present. Possible inhibition of the enzymes by the worm aqueous extract was tested using extracts spiked with known concentrations of para-nitrophenyl sulfate and phenophthalein glucuronide under conditions where conjugate cleavage can be monitored spectrophotometrically. Under these assay conditions, unconjugated product formation showed a linear dependance on substrate concentration (Figure 2.14).

The assignment of radioactivity into specific metabolite classes was based on the operationally defined extraction and seperation techniques described above. A schematic describing these designations is shown in Figure 2.15. Values for total BA recovered from the worms were calculated from the sum of radioactivity in the organic and aqueous extracts and unextractable radioactivity in the aqueous precipitate and the worm residue.

The data are expressed as radioactivity in the form of BA or BA metabolic products recovered from the worms. However, no attempt was made to inhibit bacteria associated with the worms by antibiotics. Consequently there was no way to distinguish between metabolites formed by Nereis itself, and metabolites produced by attached or enteric bacteria.

Whole worm homogenates were used for analysis of worm BA and BA metabolic products. Worms were not allowed to depurate their gut con-

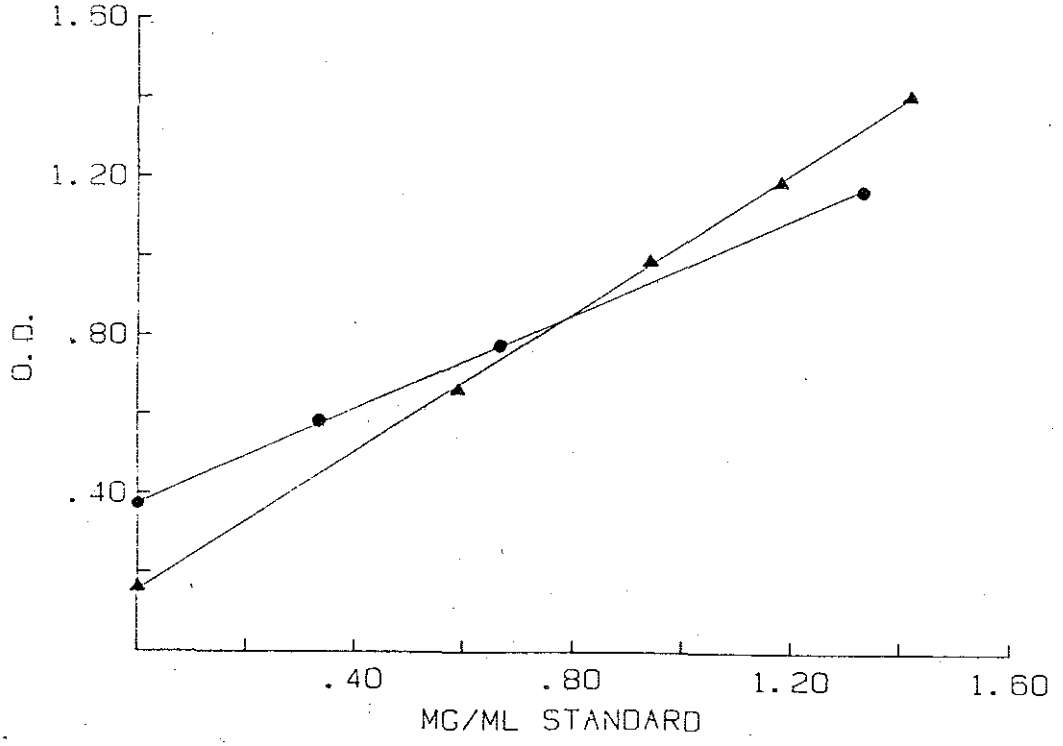
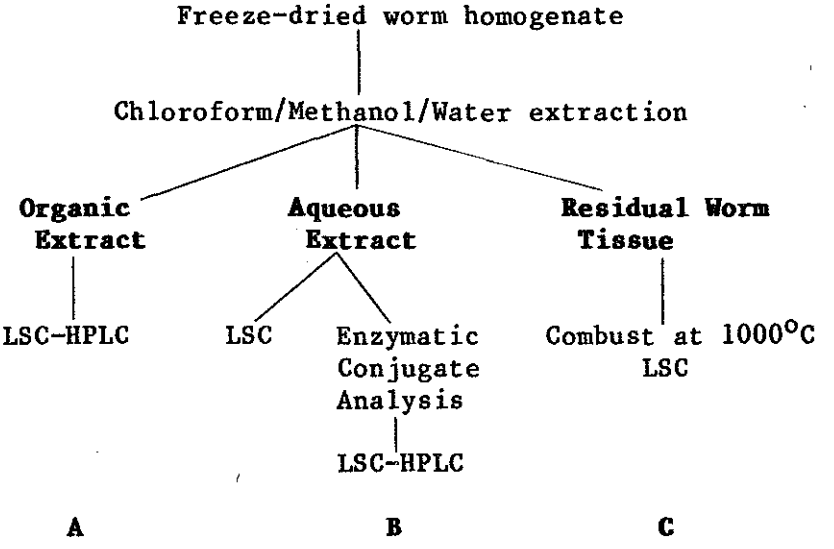


Figure 2.14: Concentration curve for B-glucuronidase and aryl sulfatase assay using indicator substrates added to worm aquesous extracts. Circles show arylsulfatase assay. Triangles show B-glucuronidase assay. Concentrations of products read at 410 nm for the arylsulfatase assy and 540 nm for the B-glucuronidase assay.

Figure 2.15

ANALYTICAL DETERMINATION OF METABOLITE CLASSES



A: Contains BA and Polar Metabolites

B: Contains Conjugates Metabolites

C: Contains Unextractable Radioactivity

tents, nor were their intestines removed prior to homogenation. Therefore, it is likely that most worms contained some sediment in their guts, which in experiments 1-4 would have contained measurable quantities of isotope. Depuration studies on control worms indicated that intestinal contents only accounted for 3% of the total wet weight of the worms.

CHAPTER 3:
THE FATE OF BENZ(a)ANTHRACENE IN BENTHIC MICROCOSMS: INFLUENCE OF *Nereis virens* AND MODE OF EXPOSURE

Results:

Results from experiments with BA labeled sediment, with BA added to the water column, and with BA introduced as labeled food will be presented separately. For each type of experiment detailed information on the fate of BA in sediment, worms, and water column will be presented, then these values will be used to calculate an overall mass balance of BA recovered from the system.

Experiments with sediment-sorbed BA:

Three experiments were conducted where sediment was labeled with BA prior to placement in the exposure chambers. The dose to the sediment was approximately the same in all experiments. In the first two experiments measurements were taken for 6 days, and in the third experiment measurements were taken for 25 days.

Concentration of BA in sediment cores for both the 6 day and the 25 day experiments with sediment-sorbed BA are presented in Table 3.1. Although the sediments used in these experiments were labeled in bulk with continuous mixing, the variability in BA concentration in the sediment was considerable. Analysis of replicate cores taken at the same time in the same chamber gave coefficients of variation ranging from 5.6 to 14.3 averaging 10.2$\pm$2.5%. In all three experiments concentration of BA in the bulk sediments did not change between initial and final cores, nor did the presence of worms have a measurable effect. Total BA concentration from all cores taken in the two 6 day experiments were indistinguishable (8.53$\pm$.33 and 8.86$\pm$.73 ug/gdw). However, mean sediment total

Table 3.1

BENZ(a)ANTHRACENE CONCENTRATION IN SEDIMENT SAMPLES FROM EXPERIMENTS WITH SEDIMENT-SORBED BENZ(a)ANTHRACENE

	ug/gdw		
	Initial	Mid	Final
Experiment 1			
6 days			
With worms	8.94±.25 (3)		7.65±.86 (4)
Without worms	8.42±.39 (2)		9.04±.40 (3)
Experiment 2			
6 days			
With worms	10.96±1.43 (3)		8.96±.45 (3)
Without worms	6.35±1.83 (2)		8.10±.65 (2)
Experiment 3			
25 days			
With worms	6.14±.42 (3)	6.46±.55 (2)	6.93±.25 (5)
Without worms	6.29±.31 (3)		6.89±.19 (3)

Values presented as mean ± SE (n).
Within each experiment BA concentrations in initial and final cores, and from cores taken from chambers with and without worms were compared using the Student's t-test. No significant differences between means were observed.

BA concentration from all cores taken from the 25 day experiment with sediment-sorbed BA was less, 6.60±0.15 ug/gdw.

The sediment extraction scheme used in these experiments yields two fractions, an organic fraction containing BA and polar metabolites, and an aqueous fraction containing very polar conjugated metabolites. In all sediment samples analyzed, an average of only 0.04% of total radioactivity recovered was in the aqueous extract (Table 3.2). Even though activity in the aqueous fraction represented a small fraction of total activity, in the 25 day experiment the presence of worms caused a significant increase in activity in the aqueous sediment extract. The same trend was seen in the two short-term experiments, although in this case differences between chambers with and without worms were not statistically significant.

HPLC analysis of the organic fraction indicated very little evidence of polar BA metabolites in any of the sediment exposure experiments. Representative chromatograms of organic extracts from sediment samples from each of the sediment exposure experiments are shown in Figures 3.1 and 3.2. Unmetabolized BA in all sediment samples amounted to an average of 98.0±.2% of total radioactivity recovered from the chromatogram. No peaks, other than BA, containing greater than 1% of total radioactivity were observed.

BA concentration in sediment cores taken at the end of the 25 day experiment and fractionated according to particle size is presented in Table 3.3. In order to have enough material, cores from the three replicate chambers were pooled before fractionation, thus there is no estimate of variability in these data. No large differences were apparent between

Table 3.2

% OF TOTAL RADIOACTIVITY RECOVERED IN AQUEOUS EXTRACTS OF SEDIMENT IN EXPERIMENTS WITH SEDIMENT-SORBED BENZ(a)ANTHRACENE (EXPERIMENTS 1,2, & 3)

	T-Zero		T-Mid	T-Final	
	Worm	No worm	Worm	Worm	No worm
Experiment #1	.0563 ±.0132 (3)	.0504 ±.0110 (2)		.0558 ±.0402 (3)	.0438 ±.0013 (3)
Experiment #2	.0196 ±.0080 (3)	.0108 ±.0042 (2)		.0620 ±.0402 (3)	.0155 ±.0052 (2)
Experiment #3	.0254 ±.0040 (3)	.0273 ±.0032 (3)	.0449 ±.0241 (2)	.0958 ±.0258 (3) *	.0217 ±.0050 (3)

Values expressed as mean ±SE (n).

Means within each experiment compared using arcsin transformed data by ANOVA and SNK.

T-F worm ≠ any other mean in exp.3 except T-Mid $p<.025$.

Figure 3.1: ^{14}C HPLC chromatogram of organic extracts of sediment cores taken at the beginning (a) and end (b) of experiment 2. Overlay on x axis refers to retention time windows corresponding to different classes of authenticBA metabolite standards. Abreviations: SF= solvent front; D-E= diol-epoxide. Non-polar refers to all activity eluting after BA.

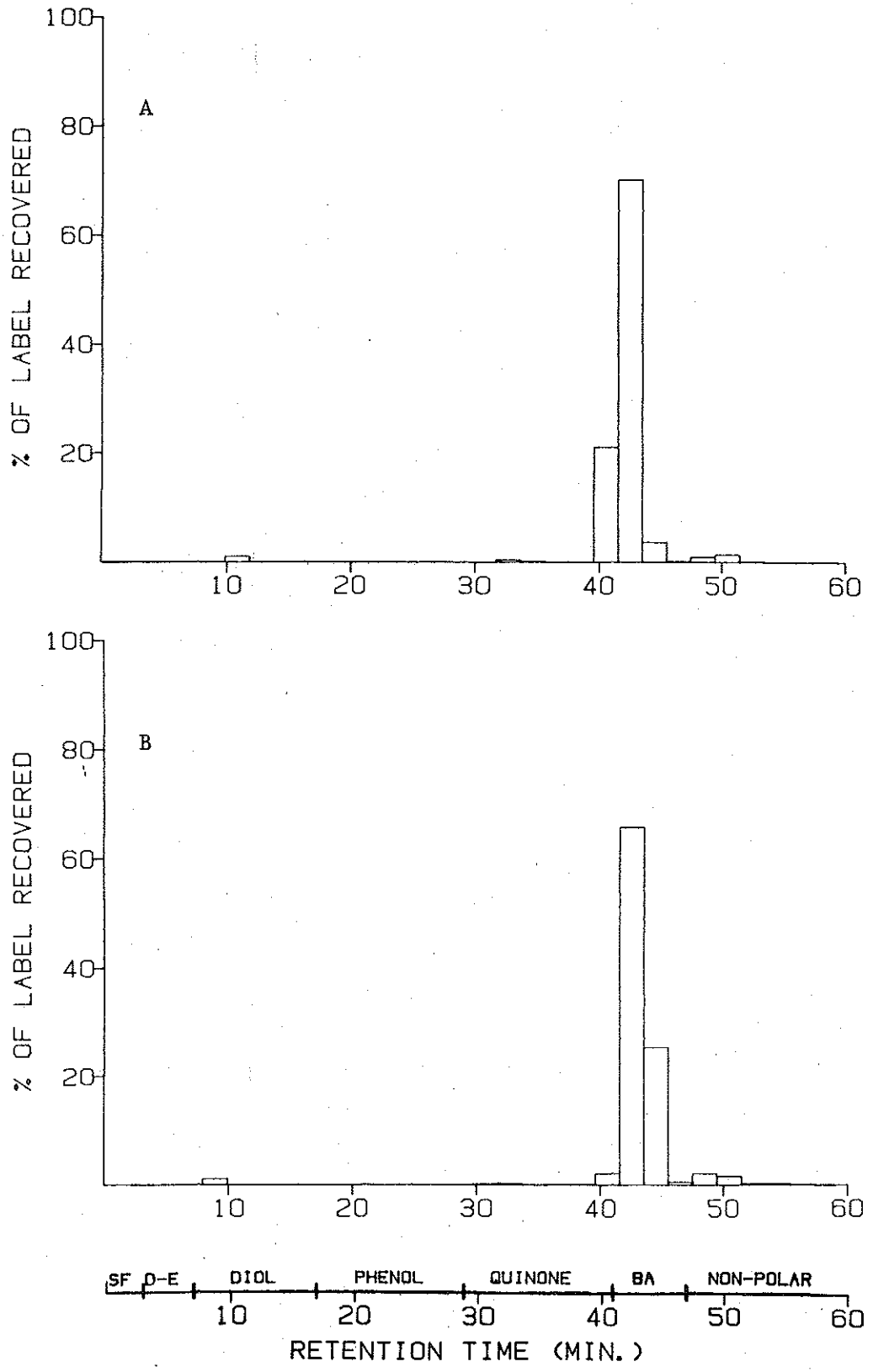

A
B
% OF LABEL RECOVERED
100
80
60
40
20
10
20
30
40
50
60
SF
D-E
DIOL
PHENOL
QUINONE
BA
NON-POLAR
RETENTION TIME (MIN.)

Figure 3.2: ^{14}C HPLC chromatogram of organic extracts of sediment cores taken at the beginning (a), middle (b), and end (c) of experiment 3. Overlay on x axis refers to retention time windows corresponding to different classes of authentic BA metabolite standards. Abreviations: SF= solvent front; D-E= diol-epoxide. Non-polar refers to all activity eluting after BA.

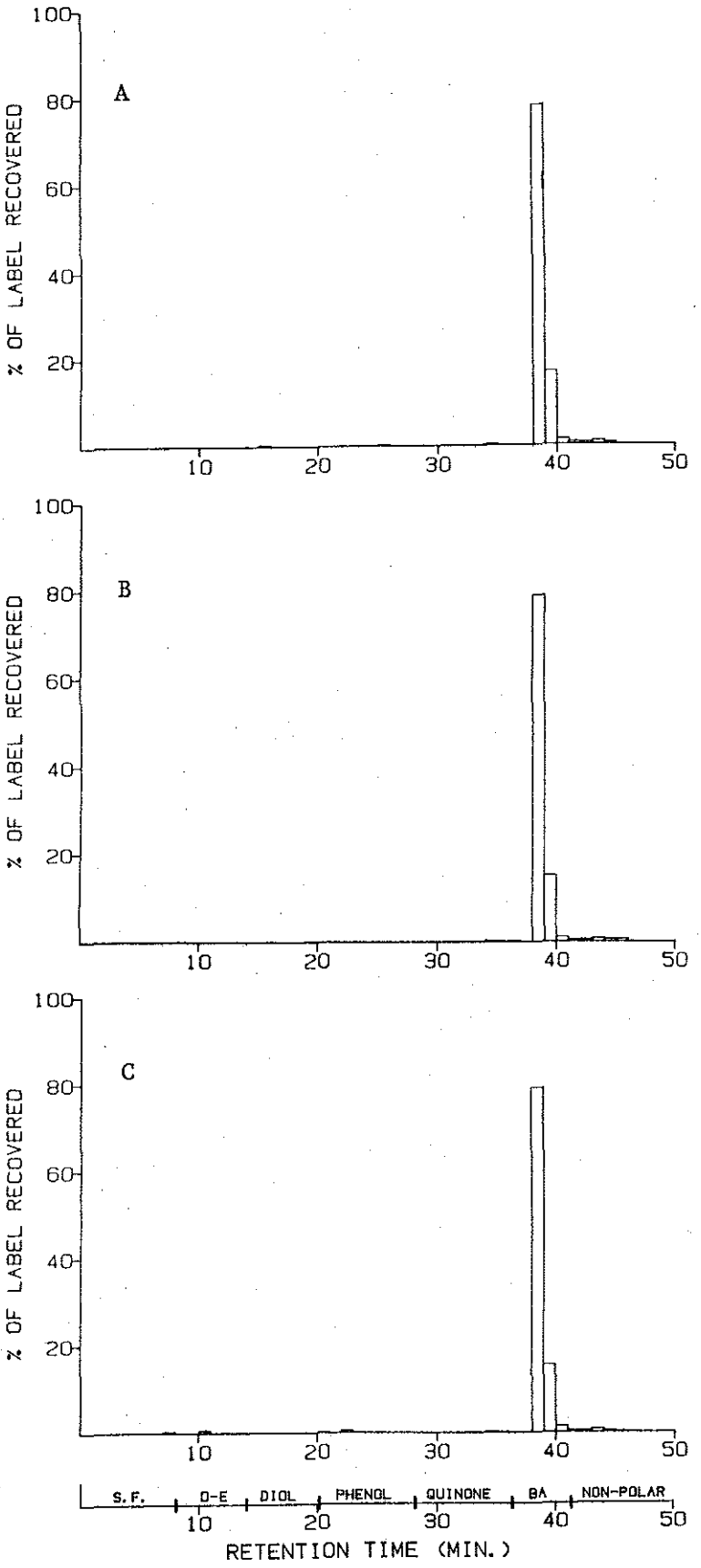

A
B
C
% OF LABEL RECOVERED
100
80
60
40
20
10
20
30
40
50
S.F.
D-E
DIOL
PHENOL
QUINONE
BA
NON-POLAR
RETENTION TIME (MIN.)

Table 3.3

SIZE FRACTIONATION OF RADIOACTIVITY IN SEDIMENT CORES TAKEN IN THE 25 DAY EXPERIMENT WITH SEDIMENT-SORBED BENZ(a)ANTHRACENE (EXPERIMENT 3)

% of Total Activity Recovered
Normalized to gdw

	With worms	Without Worms
Filtrate	1.4	1.0
Gff-37 um	76.7	70.8
37-125 um	19.8	24.8
125-250 um	1.8	2.4
> 250 um	.50	1.0

chambers with and without worms. Most of the BA is associated with 1-37 um sized particles, and approximately 97% is present on particles less than 125 um.

The sediments remained oxidized throughout each experiment. At no point was any darkening due to a redox discontinuity observable. To verify the apparent oxidized state of the sediment, E_h was measured at the end of the 25 day experiment using platinum electrodes. E_h averaged +221 mV (14°C, pH 6.9) at depths of 1 to 5 cm in all three experimental treatments.

Concentrations of total BA (parent and metabolites) recovered from worms harvested at the end of 6 and 25 day exposures to sediment-sorbed BA are given in Table 3.4. Significantly more BA accumulated in worms exposed for 25 days than in worms exposed for only 6 days, with worm concentrations exceeding those in the sediment after 25 days. Total BA accumulated was $5.02 \pm .27$ and $5.28 \pm .44$ for the two 6 day experiments and $13.08 \pm .44$ ug/gdw for the 25 day experiment. Compared to total BA concentration in the sediments, this corresponds to bioconcentration factors (ppm worm/ppm sediment) of 0.656, 0.589, and 1.89.

Activity in individual water samples was not high enough to allow HPLC analysis for the presence of metabolic products. Therefore, activity in seawater samples must be considered a sum total of parent BA and whatever metabolic products may have been present. In all experiments with sediment-sorbed BA, flux to seawater was greater in chambers with worms than in chambers without worms although variability between replicate chambers was high (See Figures 3.3-3.5). The somewhat lower rates seen in the first experiment may be due to the seawater extraction tech-

Table 3.4

BENZ(a)ANTHRACENE ACCUMULATION INTO WORM TISSUE IN EXPERIMENTS WITH SEDIMENT-SORBED BENZ(a)ANTHRACENE (EXPERIMENTS 1,2, &3)

	ug/gdw	CF
Experiment 1 6 day exposure	5.02 ±.27 (3)	.656 ±.092
Experiment 2 6 day exposure	5.28 ±.44 (3)	.589 ±.050
Experiment 3 25 day exposure	13.08 ±.44 (2) *	1.89 ±.09

Values expresed as mean ±SE (n).

CF = ug/gdw in worms / ug/gdw sediment.

Means for concentrations in worms compared between experiments by ANOVA and SNK. Exp.3 ≠ Exp.1 or Exp.2 $p<.005$.

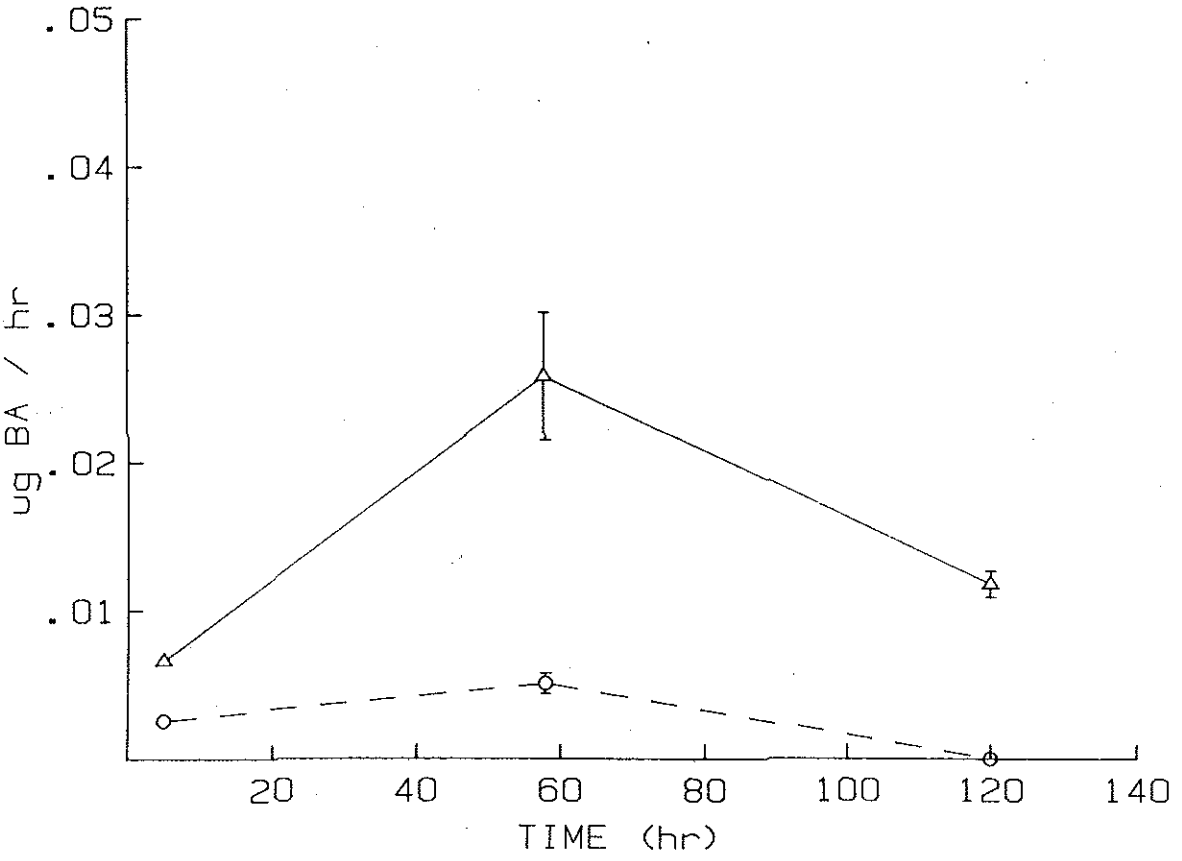


Figure 3.3: BA flux to water column in experiment 1. Circles represent chambers without worms. Triangles represent chambers with worms. Values expressed as mean ± SE n=3.

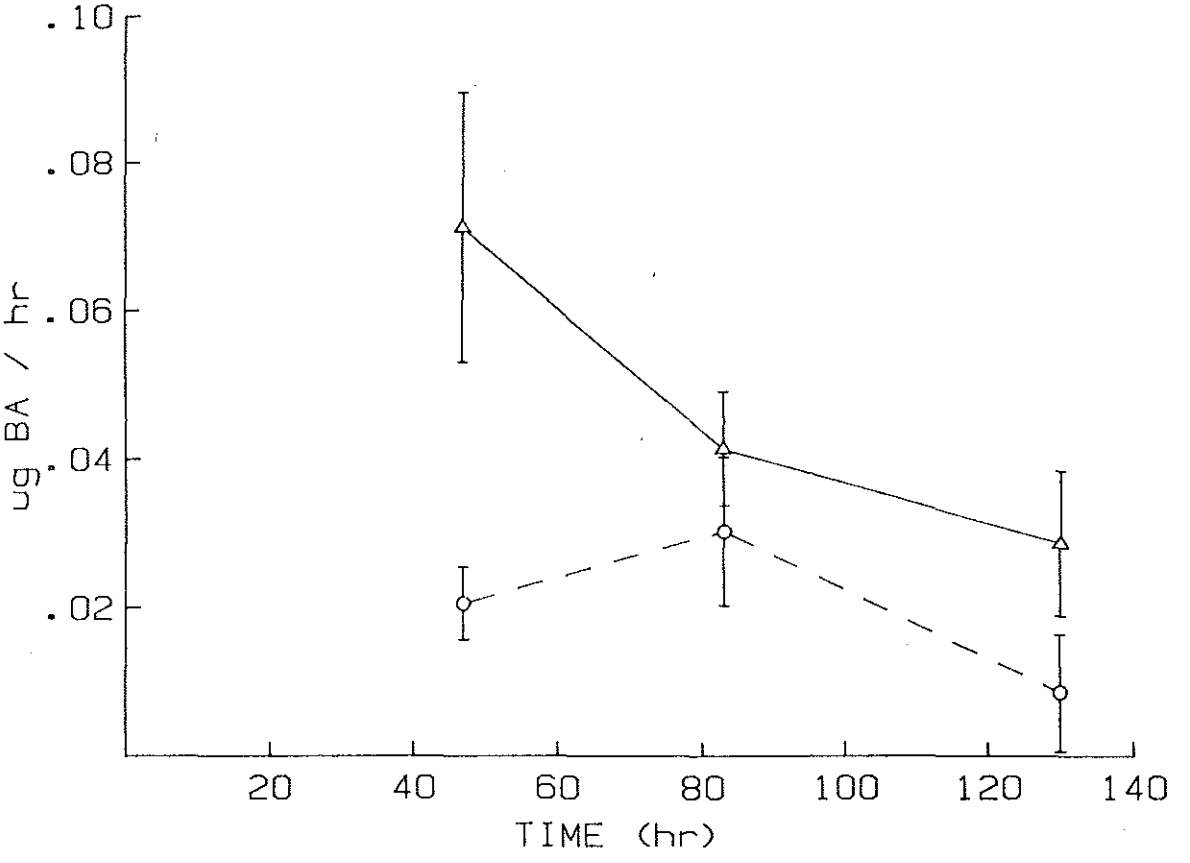


Figure 3.4: BA flux to water column in experiment 2. Circles represent chambers without worms. Triangles represent chambers with worms. Values expressed as mean ± SE n=4.

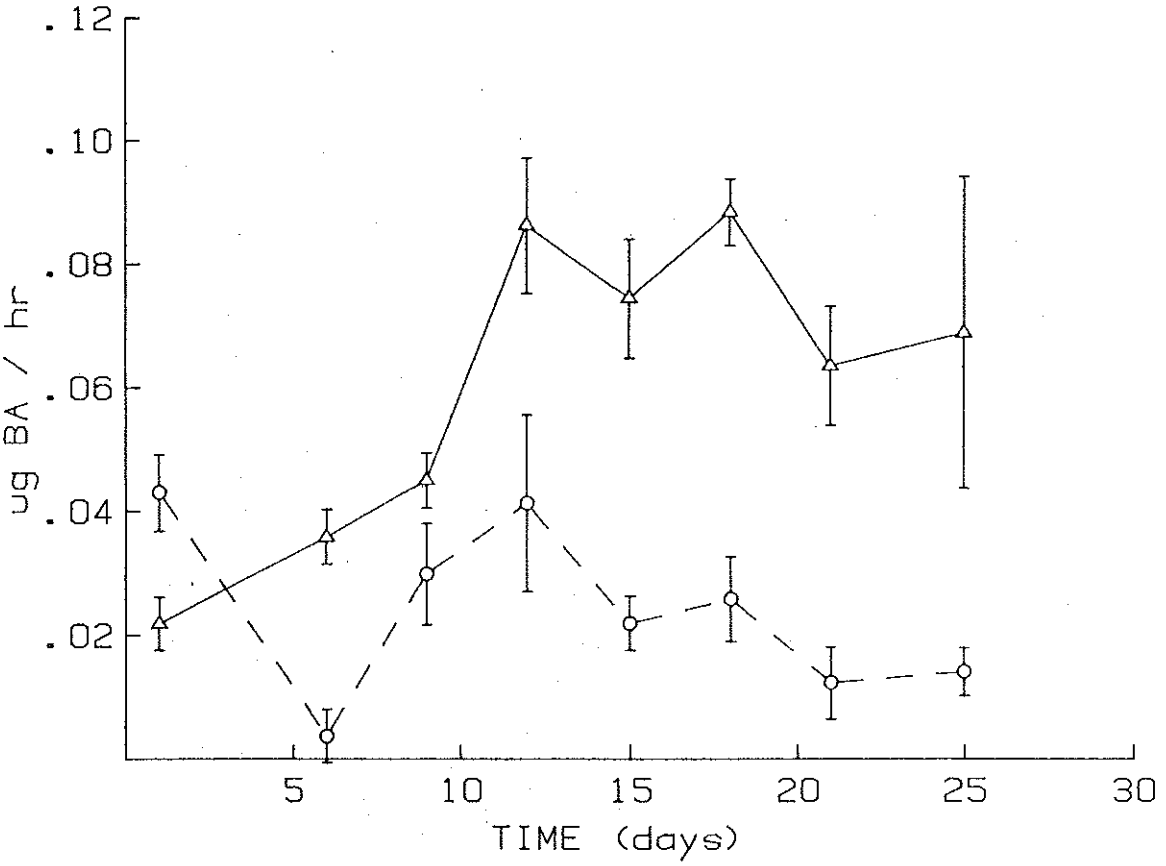


Figure 3.5: BA flux to water column in experiment 3. Circles represent chambers without worms. Triangles represent chambers with worms. Values expressed as mean ± SE n=3.

nique which was not used subsequently (See Chapter 2). The effect of BA treatment and time on flux of BA into the water column in experiments 2 and 3 were compared using two-way ANOVA with replication. Experiment 1 was not tested due to lack of equal numbers of replicates. In both experiments most of the variation could be accounted for by treatment, although both time and the time/treatment interaction accounted for significant ($p<.05$) portions of the variability.

Linear regressions of BA flux to the water column as a function of time for each treatment were run to see if there were any consistent trends with time, by testing for significant differences from zero in the slope of the regression. In the two short term experiments there were no consistent significant effects of time on flux. However, in the 25 day experiment flux in chambers with worms increased significantly ($p<.05$) with time whereas flux in chambers without worms remained constant. The increase in flux in chambers with worms was most noticable after 9 days (Figure 3.5).

The total amount of BA removed from the sediment via the water column was estimated by integrating flux over the entire experiment assuming linear rates of change between sampling points. In chambers with worms in the short-term experiments 2.04 and 5.34 ug BA were removed via the water column, whereas in chambers without worms only 0.367 and 2.31 ug BA were removed by this route in experiments 1 and 2, respectively. In the 25 day experiment the effect of worms was more pronounced, where 34.8 vs. 1.37 ug BA were removed via the water column in chambers with and without worms. This amounts to an average increase in the removal of BA from sediments to the water column by a factor of about 4

in the 6 day experiments and 25 in the 25 day experiment due to the presence of worms.

BA mineralization to CO_2 was only detectable in the 25 day experiment with sediment-sorbed BA (Figure 3.6). The presence of worms led to significant increases in the rate of BA mineralized to CO_2. Analysis by 2-way ANOVA showed that the presence of worms accounted for most of the observed variability although time and time/treatment interactions were also significant. Rate of BA mineralization in chambers with worms increased significantly with time ($p<.005$), particularly in the latter half of the experiment, whereas rates in chambers without worms remained the same.

The total amount of mineralized BA was estimated as described above for the total amount of BA lost to seawater. At the end of the experiment 13.8 and 7.25 ug of BA had been completely mineralized to CO_2 in chambers with and without worms, respectively, over the course of the experiment. The presence of worms resulting in almost a doubling in the total amount of BA mineralized.

A mass balance of the percent of total BA recovered from each compartment in chambers from the short and long term sediment exposure experiments is presented in Table 3.5. Data represent averages of radioactivity recovered from the sediment, worms, and water column (as total radioactivity extracted from seawater and as CO_2 extracted from seawater) expressed on a whole chamber basis. The results have been presented in this way to assess the importance of the presence of worms on the overall fate of sediment-sorbed BA in this system, and to allow comparison to the fate of BA in the other experiments discussed below.

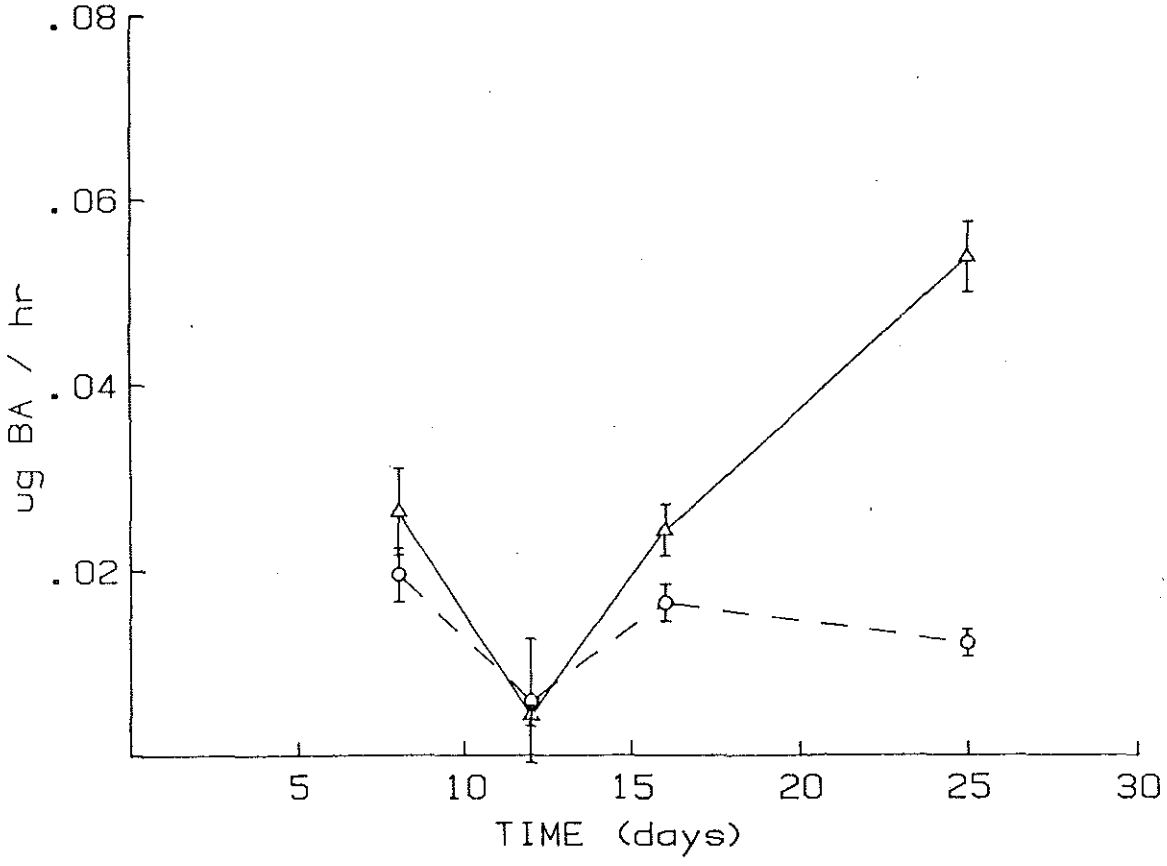


Figure 3.6: BA mineralization to carbon dioxide in experiment 3. Circles represent chambers without worms. Triangles represent chambers with worms. Values expressed as mean ± SE n=3.

Table 3.5

WHOLE CHAMBER MASS BALANCE: % OF TOTAL ACTIVITY RECOVERED AT END OF EXPERIMENTS WITH SEDIMENT-SORBED BENZ(a)ANTHRACENE (EXPERIMENTS 1,2, & 3)

	Sediment	Worms	Water Column	
			Tot ext.	CO_2
Experiment 1				
6 day exposure				
With worms	99.4	0.57	0.06	N.D.
Without worms	100.	---	0.01	N.D.
Experiment 2				
6 day exposure				
With worms	99.4	0.48	0.14	N.D.
Without worms	99.9	---	0.07	N.D.
Experiment 3				
25 day exposure				
With worms	97.3	1.13	1.15	0.46
Without worms	99.3	---	0.46	0.25

In the two 6-day experiments (exp.1&2), greater than 99% of the activity recovered was found in the sediment reservoir. Although the presence of worms had significant effects on the percent of activity in aqueous extracts of sediment and flux of BA to the water column, due to the large mass of sediment in these experiments, on a total chamber basis, the presence of worms was not significant. The difference between the percent of recovered label in the sediment averaged only 0.62% between chambers with and without worms, and 0.52% was due to activity recovered from the worms themselves. In the first two experiments, total BA recovered from worms averaged 6.1 times that removed via the seawater.

In the 25 day experiment, in chambers with worms, isotope remaining in the sediment dropped to 97.3% of total recovered, and the percentage recovered from worms was about equal to that removed via the seawater, and 2.5 times that mineralized to CO_2. In contrast, greater than 99% of activity was recovered from the sediment in chambers without worms even though a greater percentage of isotope was recovered as CO_2 and as total BA in seawater in this experiment than was in the first two experiments.

Experiment with BA added to the water column:

Mean total BA concentrations on a whole core basis are shown in Table 3.6. No significant differences were observed either between initial and final cores or between cores from chambers with or without worms. Variability observed in the BA concentration in sediment samples taken in this experiment was higher than seen in experiments with sediment-sorbed BA. The mean coefficient of variation for duplicate cores was 0.541. This increased variability probably resulted from patchy deposition of BA from the water column which was further enhanced

Table 3.6

BENZ(a)ANTHRACENE CONCENTRATIONS IN SEDIMENT SAMPLES FROM EXPERIMENT WITH BENZ(a)ANTHRACENE ADDED DIRECTLY TO THE WATER COLUMN (EXPERIMENT 4)

	T-Zero	T-Final
Total [BA] ug/gdw		
With Worms	.173 ±.019 (3)	.194 ±.036 (6)
Without Worms	.150 ±.038 (3)	.149 ±.037 (5)
% in Aqueous Extract		
With Worms	.0476 ±.0245 (3)	.539 ±.241 (3)
Without Worms	.0572 ±.0266 (3)	.0094 ±.0010 (2)

Means of total concentrations compared between T-0 and T-F for chambers with and without worms using Student's t-test.

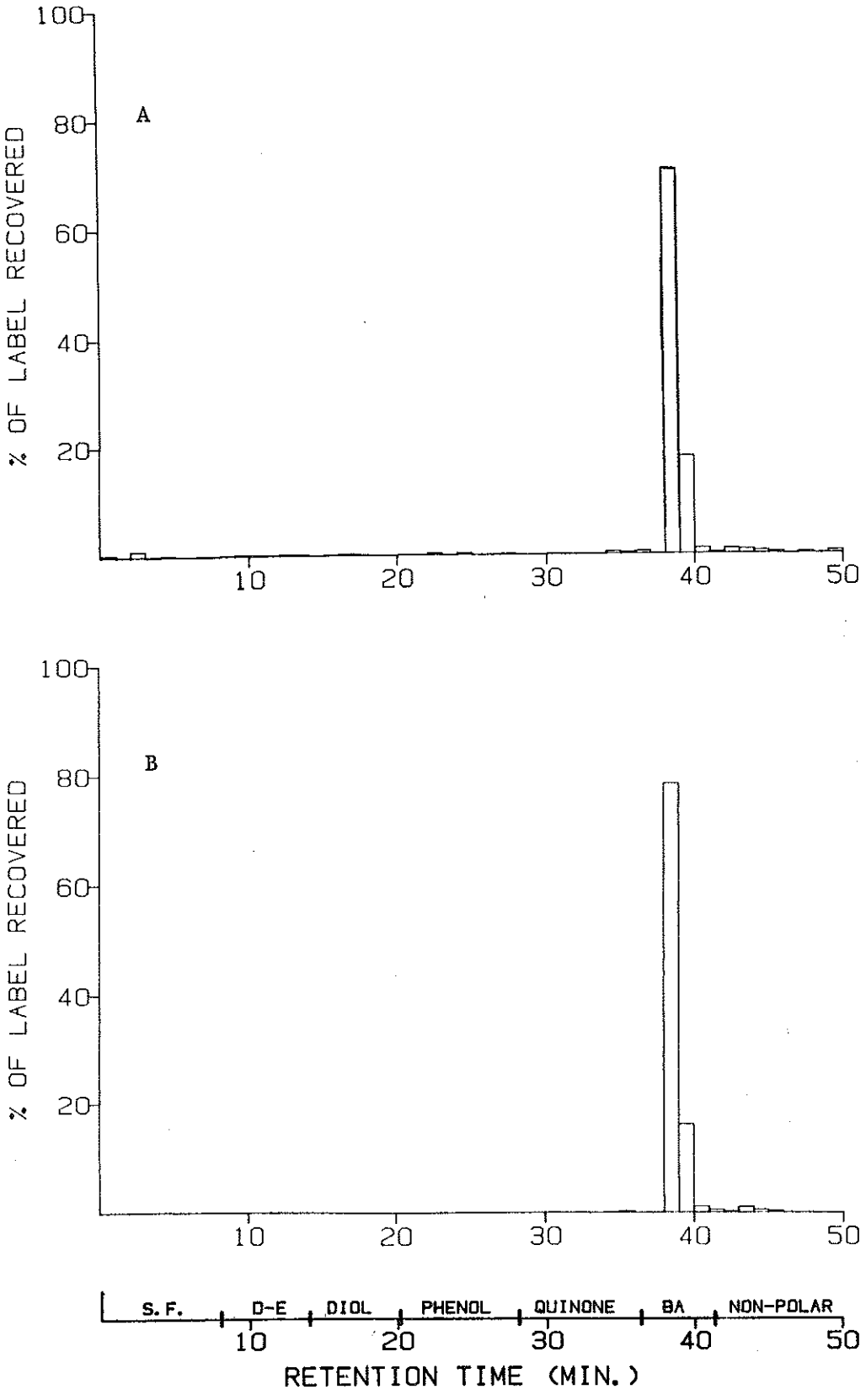
Arcsin transformations of means of % of total activity in aqueous extract were compared between all groups by ANOVA and SNK. T-F Worm ≠ all other $p<.05$.

in chambers with worms by bioturbation.

The percent of total extractable activity in the aqueous extract is also shown in Table 3.6. A significant increase in the percent of label in the aqueous extract was found in the chambers with worms at the end of the experiment. This small but detectable presence of polar BA metabolites was also evident from HPLC analysis of the sediment organic extract (Figure 3.7), although no discernible peaks were visible. Most radioactivity still chromatographed as unmetabolized BA, with 96.9$\pm$.9% of recovered label present as nonpolar compounds. This represents a significant increase in the presence of metabolized BA when compared to experiments 1,2, and 3 ($p<.001$ Mann Whitney U-test).

Since most of the BA remaining after wash-out should have been at the surface of the sediment, one set of cores from the end of the experiment was fractionated with depth prior to extraction. The ratio of BA per depth section to BA per core on both a ug/gdw and total ug basis in chambers with and without worms is shown in Table 3.7. These values were normalized to total activity recovered from each core to correct for the patchy distribution of BA in different parts of the sediment reservoir. For any given section there were significant differences in the concentration of BA present between chambers with and without worms. The presence of worms resulted in mixing BA down into the sediment. On a ug/gdw basis, only 82% of BA remained in the top cm in chambers with worms compared to 98% in chambers without worms. The effect of worms was observed at all depthes, with 11 vs. 1.2% and 7 vs. 1.3% of total label recovered from the second and bottom 3 cm of the sediment column in chambers with and without worms respectively.

Figure 3.7 ^{14}C HPLC chromatogram of organic extracts of sediment cores taken at the beginning (a) and end (b) of experiment 4. Overlay on x axis refers to retention time windows corresponding to different classes of authentic BA metabolite standards. Abreviations: SF= solvent front; D-E= diol-epoxide. Non-polar refers to all activity eluting after BA.


A
B
% OF LABEL RECOVERED
100
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20
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20
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40
50
S. F.
D-E
DIOL
PHENOL
QUINONE
BA
NON-POLAR
RETENTION TIME (MIN.)

Table 3.7

DEPTH FRACTIONATION OF RADIOACTIVITY IN SEDIMENT CORES TAKEN AT THE END OF THE EXPERIMENT WITH BENZ(a)ANTHRACENE ADDED DIRECTLY TO THE WATER COLUMN (EXERIMENT 4)

	[BA]	% of total Radioactivity Recovered
0-1 cm		
With Worms	93.12 ±3.12	86.52 ±4.83
Without Worms	99.15 ±.16	97.38 ±.51
1-2 cm		
With Worms	6.95 ±2.86	7.65 ±3.01
Without Worms	0.65 ±.21	1.08 ±.11
3 cm-Bottom		
With Worms	0.78 ±.27	5.83 ±1.86
Without Worms	0.20 ±.05	1.54 ±.60

Values presented as mean ±SE n=3, normalized to percent of activity recovered from each column.

Means between chambers with and without worms were comared using Student's t-test on arcsin transformed data. In all cases differences were significant $p<.05$.

One set of unfrozen cores were fractionated according to particle size prior to extraction (Table 3.8). As in experiment 3 most of the radiolabel was present on particles between 1 and 37 um in size with 92 % found on particles between 1 and 125 um. No obvious differences were seen between chambers with and without worms in this experiment, or between the size distribution of BA in this experiment and that found in the experiments with sediment-sorbed BA.

Concentration of BA recovered from worms exposed to BA introduced via the water column for 6 days is presented in Table 3.9. Total BA accumulated was 3.81$\pm$.36 ug/gdw, similar to that occurring after 6 days in experiments with sediment-sorbed BA (See Table 3.4). However, the mass of BA in the sediment reservoir in experiments 1 and 2, where sediments were uniformly labeled, was much larger than in this experiment, where most of the BA was in the top cm of the sediment (3540 vs. 39.7 ug). Consequently even though the concentration of BA accumulated was similar, the percentage of total BA present accumulated into worms was much higher. Table 3.9 shows the BA concentration factor in worms (ppm worm/ppm sediment) partitioned according to depth. Normalizing to concentrations in the top one cm worms accumulated 2.06$\pm$.67 times the concentration in the sediment. This represents a concentration factor 3 times higher than observed in short-term experiments with sediment-sorbed BA (See Table 3.4). The concentration factor calculated without taking into account the depth distribution was 19.6$\pm$5.5. These data suggests that worms were accumulating BA from the surface where they feed even though most of the surface area of the worm's integument was exposed to sediments containing negligible amounts of BA.

Table 3.8

SIZE FRACTIONATION OF RADIOACTIVITY IN SEDIMENT CORES TAKEN FROM THE EXPERIMENT WITH BENZ(a)ANTHRACENE ADDED DIRECTLY TO THE WATER COLUMN (EXPERIMENT 4)

% of Total Activity Recovered
Normalized to gdw

	With Worms	Without Worms
Filtrate	1.5	2.5
Gff-37 um	73.3	75.8
37-125 um	19.9	15.5
125-250 um	3.8	3.3
> 250 um	1.4	2.8

Table 3.9

BENZ(a)ANTHRACENE ACCUMULATION INTO WORM TISSUE IN EXPERIMENT WITH BENZ(a)ANTHRACENE ADDED DIRECTLY TO THE WATER COLUMN (EXPERIMENT 4)

Total Accumulated

3.81 ±.36 ug/gdw

Concentration Factors
(ug/gdw worm)/(ug/dgw sediment)

Total Core	19.6 ± .6
0-1 cm	2.06 ± .67
1-2 cm	42.8 ± 12.8
3 cm to bottom of core	175 ± 76

BA concentration in the water column during the period when chambers were in the recirculating mode after spike addition is shown in Figure 3.8. After the chambers were switched to flow through, the remainder of the spike still retained in the water column was washed out of the chambers. The washout was 98% complete within 16.5 hours, with BA flux leveling off 39 hours after the chambers were re-opened. BA flux to the water column after switching to flow-through is shown in Figure 3.9. No difference in flux rate was observed between chambers with worms and those without worms.

On the third day after the chambers were re-opened, extracts of large 250 ml water samples were pooled and analyzed for the presence of polar metabolites by HPLC. Activity in fractions collected from the HPLC are shown in Figure 3.10 for a combined extract from the chambers containing worms. Small peaks averaging a few percent of the total activity recovered were present in the region of tetrol and diol-epoxide, diol, phenol, and quinone metabolite standards. Although most of the activity (>85%) was still present as nonpolar BA, it was substantially less than that seen in sediment extracts from experiments 1,2, and 3. Unfortunately the sample from the chambers without worms was lost, so there is no way to assess the possible impact of worms on the presence of BA metabolites in the water column.

Flux of BA mineralized to CO_2 into the water column is shown in Figure 3.11. No differences were seen between rates in chambers with worms and those without worms. Rates were undetectable 15 hours after the start of flow through conditions and increased throughout the rest of the experiment, although they were always much lower than rates observed

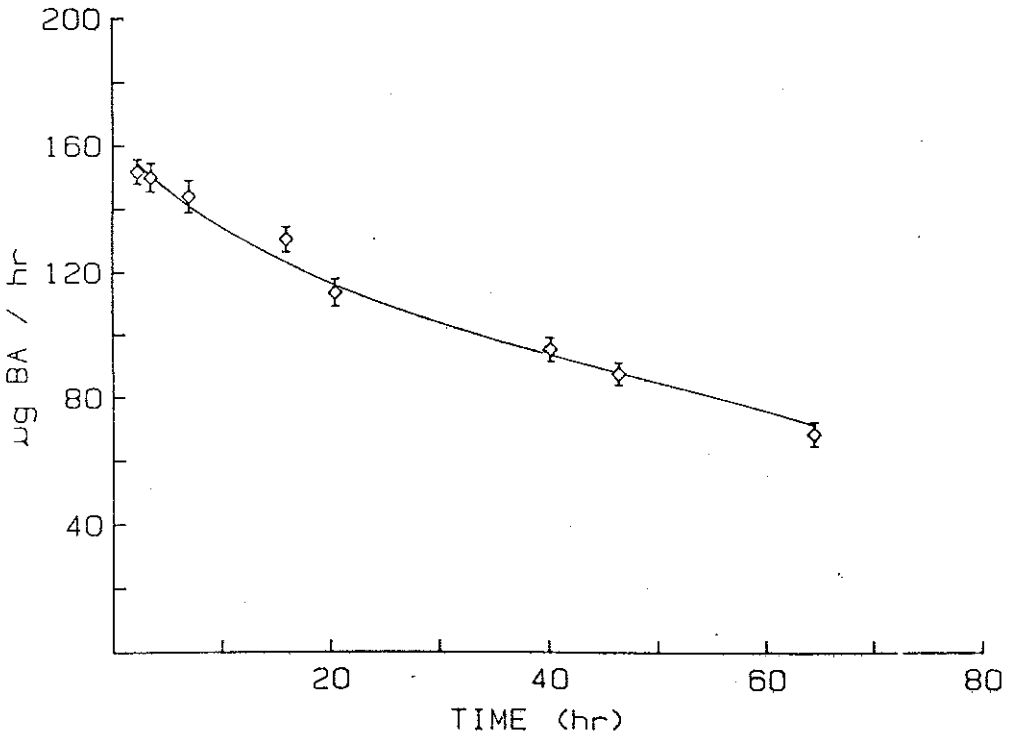


Figure 3.8 Initial removal rate of BA from the water column during the recycle mode in experiment 4. Values expressed as mean ± SE for all chambers n=6.

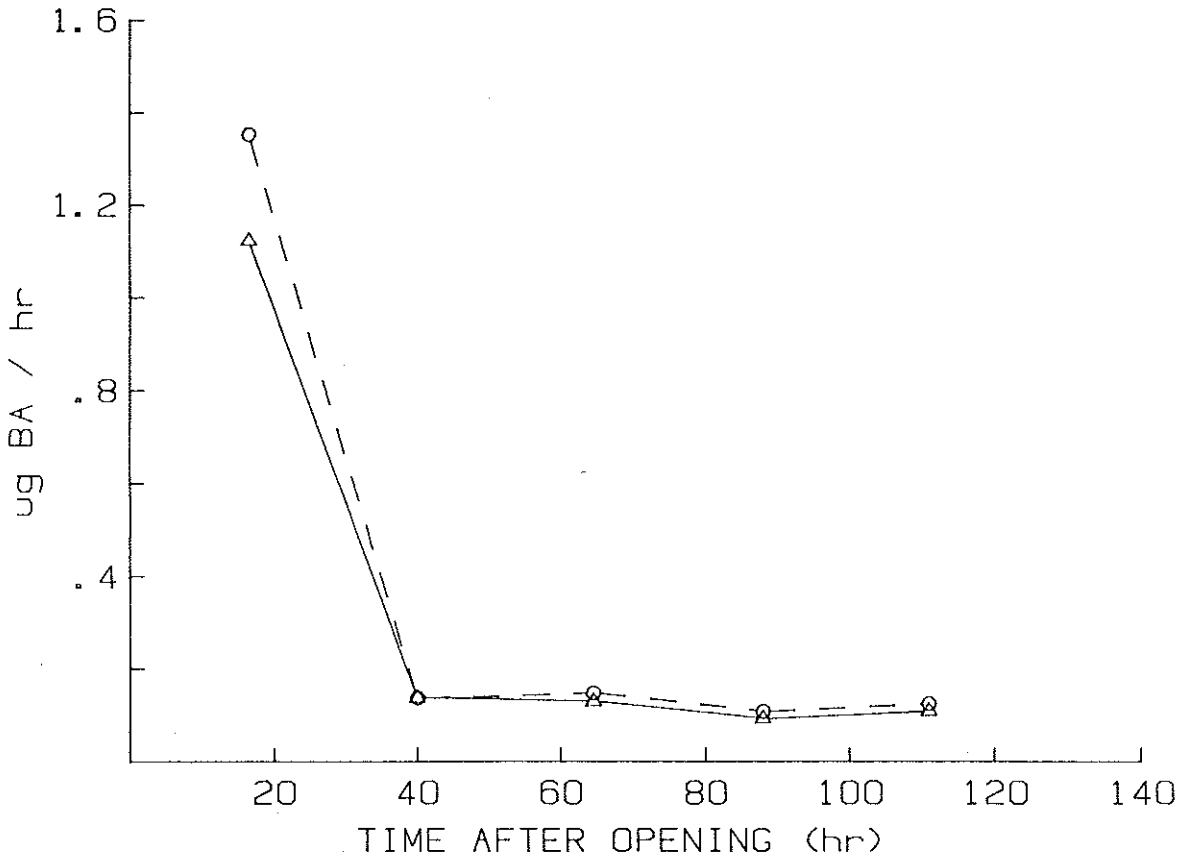


Figure 3.9: BA flux to water column in experiment 4 after reinitiation of flow-through circulation. Circles represent chambers without worms. Triangles represent chambers with worms. Values represent radioactivity in samples pooled from 3 replicate chambers.

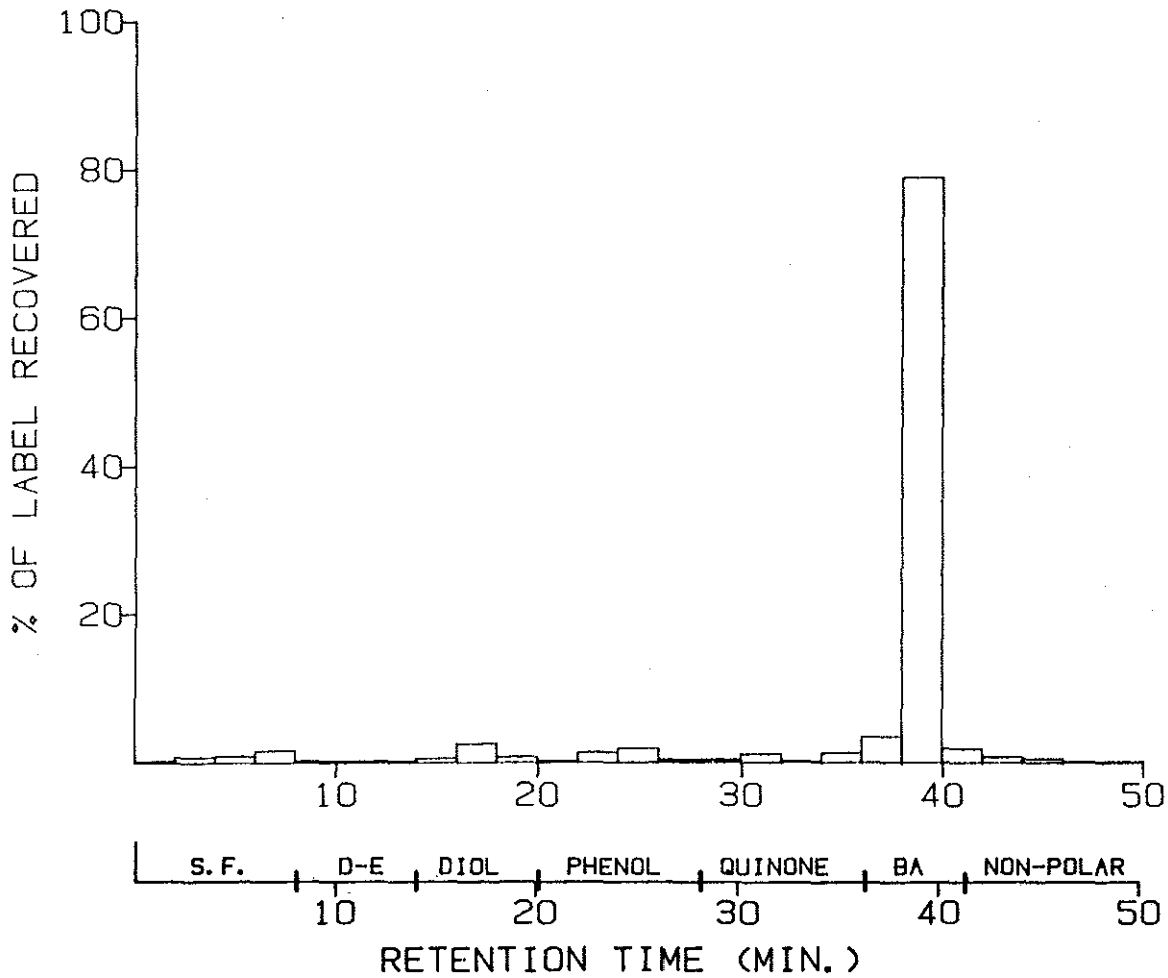


Figure 3.10: ^{14}C HPLC chromatogram of organic extract of water sample from experiment 4. Overlay on x axis refers to retention time windows corresponding to different classes of authentic BA metabolite standards. Abreviations: SF= solvent front; D-E= diol-epoxide. Non-polar refers to all activity eluting after BA.

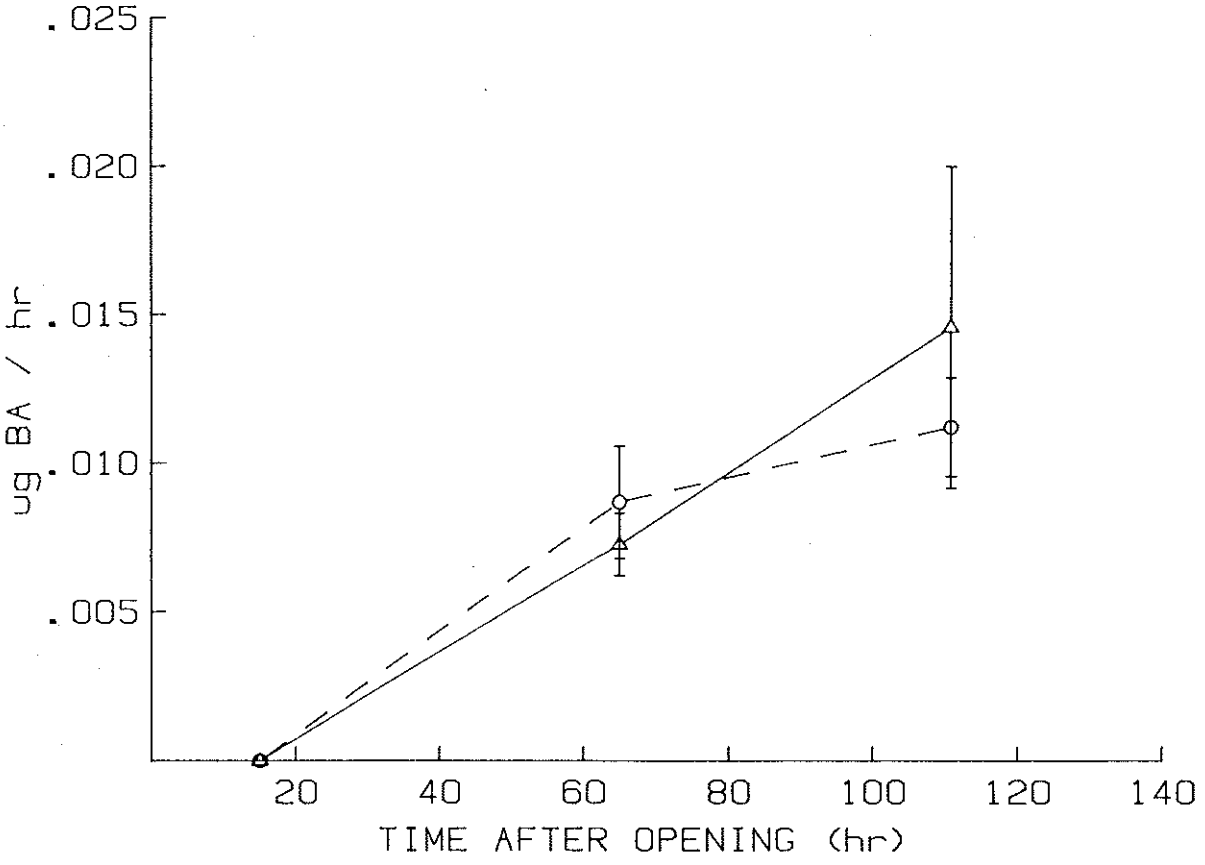


Figure 3.11: BA mineralization to carbon dioxide after initiation of flow-through conditions in experiment 4. Circles represent chambers without worms. Triangles represent chambers with worms. Values expressed as mean ± SE n=3.

in the 25 day experiment with sediment-sorbed BA (See Figure 3.6). Variability in mineralization rates was large, coefficients of variation ranging from 62 to 97%. However, even with such large variability the change in flux rate with time was statistically different than zero ($p<.01$) both for chambers with and chambers without worms.

Calculating a mass balance of BA in this experiment was more complicated than in experiments with sediment-sorbed BA. Since BA activity was not uniformly distributed throughout the sediment column, the total mass of BA recovered from the sediment was calculated by taking the average total recovered per core and multiplying by the number of cores it would take to completely sample the sediment surface. Because BA flux to the seawater did not change with time after washout was complete, total removal of BA to the water column was calculated by taking the average rate over the last 4 days and multiplying by the length of exposure. No attempt was made to include BA left over from the initial spike that was washed out of the chambers after resumption of flow-though conditions in mass balance calculations.

The mass balance of BA for this experiment, calculated as described above, is presented in Table 3.10. In the chambers with worms only 72% of BA recovered was in the sediment. Approximately 13% was recovered from the worms or removed via total extractable label in the water column, and only 0.75% was lost to the water column mineralized to CO_2. In chambers without worms similar proportions of total radioactivity recovered were found in the sediment and as mineralized to CO_2, although 24% of total activity was removed via the water column. The actual amounts of BA removed via the water column were very similar for chambers with

Table 3.10

WHOLE CHAMBER MASS BALANCE: TOTAL RADIOACTIVITY AND % OF TOTAL RECOVERED AT END OF EXPERIMENT WITH BENZ(a)ANTHRACENE ADDED DIRECTLY TO THE WATER COLUMN (EXPERIMENT 4)

	Sediment	Worms	Water Column Tot ext.	CO_2
ug Total Benz(a)anthracene Recovered (BA plus metabolites)				
Worms	71.7	12.7	13.0	.738
Without worms	47.4	---	14.3	.740
% of Total Radio-activity Recovered				
Worms	73.1	12.9	13.2	.750
Without worms	75.9	---	22.9	1.18

and without worms (13.0 vs 14.3 ug). However, the presence of the worms as a BA reservoir in the chambers with worms, and a slightly, but not significantly, lower amount of BA recovered from sediment in chambers without worms, resulted in a higher percentage of total recovered attributed to flux into the water column. After washout, the average BA flux to the water column was 0.2 ug/hr, as compared to 0.005 ug/hr as an average for the same length of time in the experiments with sediment sorbed BA, even though concentration of BA at the surface was lower in this experiment. This elevated flux indicates that BA loosely associated with the surface floc is more available for desorption than BA previously bound to sediment particles.

Experiment with BA labeled food:

All chambers contained worms in this experiment. Each chamber received a single dose of 4 pieces of labeled food which was placed at the sediment surface in different parts of the chamber. In all chambers, within four hours, all but one piece of food had been eaten. The last piece was consumed sometime between 6 and 14 hours after addition.

All chambers received control sediments, so no initial cores were taken in this experiment. Concentrations of BA extracted from individual sediment cores taken 96 hours after the addition of labeled food are given in Table 3.11. Concentrations were very low, averaging only 0.780 ng/gdw, and were highly variable ranging over one order of magnitude In contrast to the other experiments, 9.1% of the total activity recovered from the sediment was found in the aqueous extract. Activity in the aqueous extract was also highly variable ranging from 0.52 to 21.2% of the total recovered per core. Replicate cores from each chamber had an

Table 3.11

BENZ(a)ANTHRACENE RECOVERED FROM INDIVIDUAL SEDIMENT CORES IN EXPERIMENT WITH BENZ(a)ANTHRACENE LABELED FOOD (EXPERIMENT 5)

TOTAL CONCENTRATION AND % OF TOTAL IN AQUEOUS EXTRACT

ng BA/gdw	% in Aqueous Extract
.329	11.75
.282	16.10
.508	10.29
.767	0.52
.730	3.05
2.457	21.16
.410	1.04
Mean = .780	Mean = 9.13
SE = .285	SE = 3.00

average coefficient of variation of 45% for total activity and 114% for the percent of total in the aqueous extract. Unfortunately total activity of the sediment extracts was insufficient to allow analysis for polar metabolites on HPLC.

The concentration of BA recovered from worms taken from each chamber 96 hours after feeding was 0.517±.033 ug/gdw. Based on relative concentrations, the worm/food concentration factor was only 0.028, but the total amount of BA in worm tissue accounted for 83% of the labeled BA added to the chamber. The ratio between the BA concentration in worms and sediments was 663.

BA flux to the water column is shown in Figure 3.12. The first water sample represents the first hour after addition of labeled food to the chambers. The relatively low flux observed at this point indicated that little leakage of BA from the food occurred before it was consumed. Flux was highest the day after ingestion, 2.3 ng/hr. Considering the fact that these measurements were made only once each day, the actual maximum rate may have been higher. On the second, third, and fourth day rates remained fairly constant, averaging around 2.5 times that seen right after feeding. Flux of BA to the water column were approximately an order of magnitude less than those observed in experiments with sediment-sorbed BA and two orders of magnitude lower than those observed when BA was introduced via the water column. There was no detectable flux to the water column of BA mineralized to CO_2.

The mass balance of BA in this experiment was calculated as the total amount recovered, as a percentage of total activity recovered and as a percentage of the spike added to each chamber (Table 3.12). After

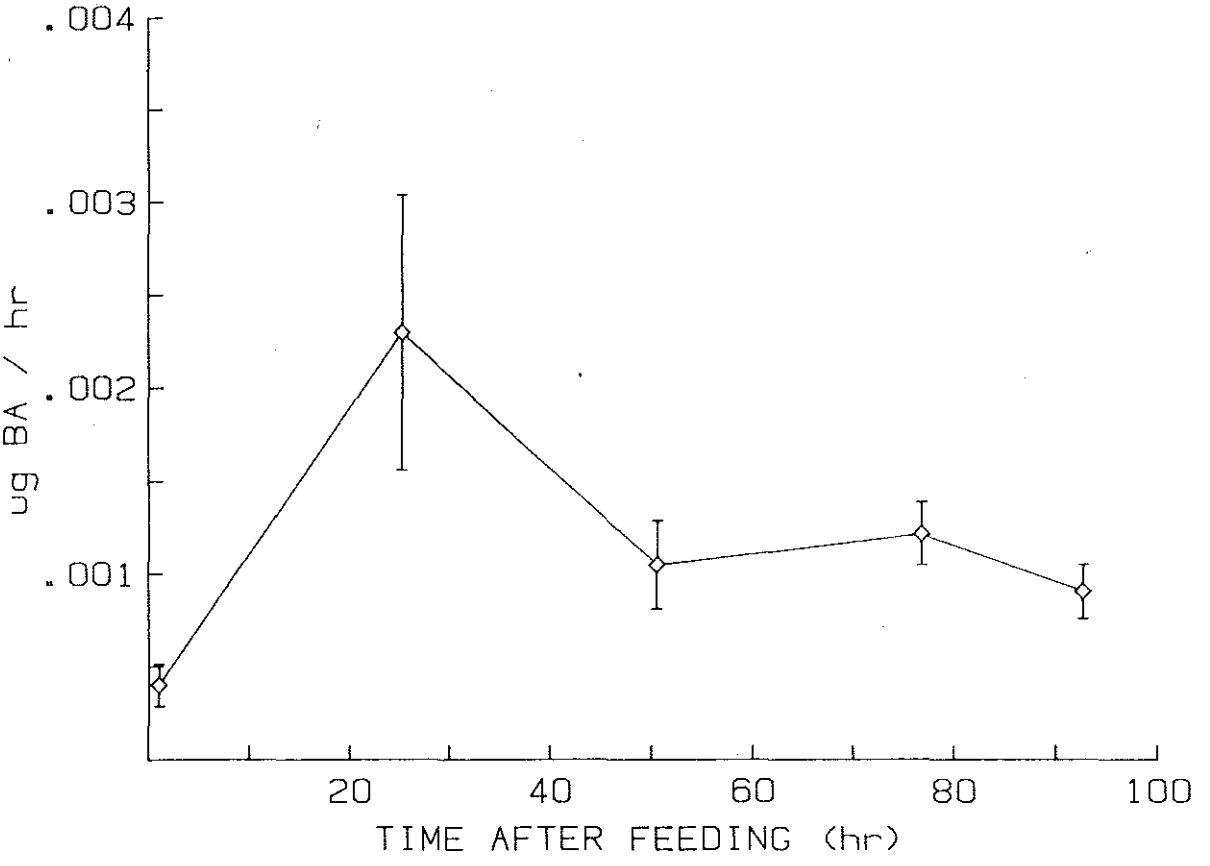


Figure 3.12: BA flux to water column in experiment 5 after introduction of labeled food. Values expressed as mean $\pm$ SE n=4.

Table 3.12

WHOLE CHAMBER MASS BALANCE: TOTAL ACTIVITY RECOVERED AT THE END OF EXPERIMENT WITH BENZ(a)ANTHRACENE ADDED IN LABELED FOOD (EXPERIMENT 5)

	Sediment	Worms	Water Column Tot ext.	Water Column CO_2
ug Total BA Recovered (BA plux metabolites)	.301	2.14	.122	N.D.
% of Total Recovered	11.8	83.5	4.76	0
% of Total Added	11.6	82.3	4.69	0

2.60 ug Benz(a)anthracene added
98.5% recovered

96 hours, 82.3% of the spike added was recovered from the worms, with 4.7% removed via the water column, and 11.6% recovered from the sediment. Over 98% of BA added to the chambers was recovered indicating that the worms did indeed consume all food offered and that the chambers were sampled effectively.

Discussion:

The presence of worms had significant effects on the fate of BA in the benthic microcosms investigated in these experiments. In experiments with sediment-sorbed BA, flux from the sediment to the water column was always higher in chambers with worms than in chambers without worms, and this effect intensified with time. Increased flux of BA out of sediments due to the presence of worms could have been caused by several processes. Worm burrows increase the surface area of the sediment exposed to the water column and thereby provide more area for diffusion. Irrigation activities of the worms for respiration flush the burrows and maintain a concentration gradient favoring BA flux out of the sediment. Subsurface worm activity also increases oxygen and nutrient supplies to sediment at depth which could also increase microbial mineralization of BA. In addition, excretion of water soluble metabolites by the worms would also augment the flux of BA from the sediments.

In the short-term experiments there was no evidence of microbial BA mineralization, so it is unlikely that microbes were responsible for the increased flux in these experiments. More likely, the increased flux was mediated by a combination of worm burrow activity and excretion of BA metabolites. Assuming that as a minimum, subsurface burrows must have been large enough to contain the worms present in each chamber, worm

burrows increased the surface area of the sediment water interface by a factor of approximately 2 to 3 in the chambers used in this study. Additionally, using a conservative estimate of burrow irrigation rates for benthic organisms ventilating just for respiratory purposes of 2.5 ml/g-hr (calculated from Davis, 1979), the water in burrows would be replaced twice an hour. Based on these calculations, it is surprising that worms did not increase BA flux even more than was observed.

The increase in BA flux from the sediment reported here is similar to that reported by Karichkoff and Morris (1985) who looked at the effect of tubifex worms on release of chlorinated hydrocarbons from sediment. They found that worms increased the net upward mobility by a factor of 20 by completely mixing the 10 cm bioturbated zone. However, they also found that packaging sediment into fecal pellets retarded desorption. The net effect of worm reworking was to increase net flux out of the sediment by a factor of 6 over that seen by diffusion alone. The enhanced flux of hydrocarbons from the sediment reported by Karichkoff and Morris were due to the complete mixing of the bioturbated zone by a small oligochaete. In contrast, the enhanced release of BA from the sediment mitigated by Nereis was due to construction and irrigation of discrete burrows.

In the longer-termed experiment with sediment-sorbed BA in addition to flux mediated by burrow construction and irrigation, enhancement of flux may have been mediated in part by microbial activity. Both BA mineralization rate and flux to the water column increased in chambers with worms after 12 days. BA mineralized to CO_2 would not be detected by solvent extraction of BA from the water column. The co-occurrence of

increased rates of $^{14}CO_2$ production and total extractable ^{14}C observed in the water column could have been fortuitous, or could have been due to incomplete mineralization of BA leading to an increase in water soluble metabolites.

Supplementation of diffusive flux of BA out of the sediment by the excretion of water soluble BA conjugates produced by worms is supported by two lines of evidence. Worms from both the short- and long-term sediment exposure experiments contained significant amounts of water soluble BA metabolites (See Chapter 4). The formation of conjugated metabolites is thought to be the primary pathway for PAH excretion in organisms with competent MFO systems. The increase in the percentage of activity seen in the aqueous extracts of sediments taken from chambers with worms supports the premise that worms were excreting some water soluble metabolites. No increase in water soluble activity in sediment cores taken from chambers without worms was observed. In addition the percentage of activity in aqueous sediment extracts was more pronounced in the longer-termed sediment exposure experiments, as was the percentage of activity recovered from the worms.

Although the presence of worms significantly increased the rate of removal of BA from sediments in experiments with sediment-sorbed BA, due to the large excess of sediment in the chambers as compared to worms (~200 X), on a whole chamber basis worms did not have a large effect on bulk removal of BA. Using the rates of CO_2 mineralization and flux to seawater observed in the 25 day experiment and assuming they remained constant, the residence time of BA in chambers with worms would be approximately 4 years as opposed to 9.5 years for chambers without worms.

Gardner et al. (1979) looked at the impact of a small polychaete Capitella sp. on the removal of anthracene, fluoranthene, BA, and BP mixed into sediments. In 60 days they found increased removal of PAH from sediments in the presence of worms, and that the effects were more pronounced with larger PAH. Capitella increased the rate of BA removal by a factor of 1.3 to 1.8 times depending on sediment type. They hypothesized that the increased removal was due to either worm or microbial metabolism of BA, but, they did not measure either process directly. In contrast Augenfeld et al. (1982) did not find the presence of the deposit-feeding polychaete Abarenicola to have significant effects on decreasing sediment concentrations of BP, chrysene, and phenanthrene, in 60 day benthic chamber experiments. The mass of sediment was approximately 1000 times greater than that of the worms in their study, so the effect of worms may have been more difficult to detect.

In the experiments where BA was added directly to the water column, the presence of worms had a different effect. Neither the flux of BA out of sediment nor microbial mineralization of BA to CO_2 were affected by the presence of worms. Since almost all of the activity was present at the sediment-water interface, flux of BA through worm burrows should not have the same importance as when bulk sediments were labeled. However, worms did produce a net downward mixing of BA into the sediment. The reduction in flux out of the sediment due to burial may have been compensated by increased flux of BA due to burrow irrigation or excretion of water soluble metabolites.

Regardless of mode of introduction BA was available for accumulation by Nereis virens. The bioavailability of sediment-sorbed and ingested BA

contrasts with an earlier report by Rossi (1977) who found PAH in these forms unavailable for uptake by the worm Neanthes arenaceodentata. Other investigations with worms have reported PAH and PCB accumulation from sediment and dietary sources (Augenfeld et al., 1982; Lyes et al., 1979; Courtney and Langston, 1978; Goerke, 1979; Roesijadi et al., 1978; Fowler et al., 1978); as have investigations with other organisms such as fish (Varanasi and Gmur, 1981, Varanasi et al., 1979; Palmork and Solbakken, 1981), midge larvae (Leversee et al., 1982), blue crab larvae (Lee et al., 1976), and the accumulation of PCBs in larval bivalves (Dobroski and Epifanio, 1980).

Quantifying the efficiency of different modes of uptake is difficult. An accurate determination requires either comparison of accumulation when all components of the systems are at equilibrium, or detailed analysis of the kinetics of uptake, metabolism, and depuration. Since it can take months for benthic organisms such as deposit feeders to reach equilibrium concentrations with hydrophobic compounds such as the larger PAH and PCBs (Roesijadi et al., 1978; Fowler et al., 1978), and determination of detailed uptake and depuration kinetics was beyond the capabilities of the experimental system available, a compromise approach was used. Bioaccumulation from different sources was compared after similar exposure periods.

On the basis of percent of total BA accumulated into worms, dietary BA incorporated into digestible food was more available than BA added to the water column, which was more available than BA sorbed to bulk sediments. The percentage of total activity in the chambers recovered from worms was 83% when worms were fed BA labeled food, 13% when BA had been

added to the water column, and less than 1% when worms were exposed to a sediment reservoir uniformly labeled with BA. However, due to the large differential in the total amount of BA available to worms in experiments using these different methods of introduction, this comparison is not entirely valid. The concentration factors between water, sediment, and worms observed here are not directly comparable to those reported by other studies because no attempt was made in this study to ascertain if equilibrium conditions had been reached. However, worm/sediment concentration factors for worms exposed to BA in the short-term experiments (1,2, & 4) can be compared. In short-term exposures to sediment-sorbed BA (exp.1&2), worms only accumulated BA to 0.6 times the concentration seen in bulk sediments. In comparison, in the short-term water column exposure experiment (exp.3), worms accumulated BA to a greater extent. Even when normalized to the highest sediment concentrations found in the top centimeter of the sediment, the worm/sediment concentration of BA was greater than 2. On a whole core basis the accumulation factor increased to 17. These ratios indicate that BA introduced directly to the water column, most of which was transfered to the sediment-water interface via mixing and adsorption during the recycle mode, was more available for worm accumulation than BA already sorbed to sediment particles.

Making comparisons based on concentration factors between experiments with sediment-sorbed BA or BA added to the water column and the experiment with BA labeled food are problematic. In the first two cases, the worms were exposed to a large pool of BA, but they probably only came in direct contact with a small portion of that present. In the feeding experiment, once worms ate the food, the entire dose was within the worm.

Nevertheless the extremely high efficiency with which Nereis accumulated BA from food (>83%) suggests that of the different modes of introduction investigated in this study, BA in ingested food was most available for accumulation.

This finding is particularly interesting when compared to a recent field study by Malins et al. (1985). Bile samples from English sole collected from different parts of Puget Sound showed flourescence spectra indicating the presence of PAH metabolites. Although PAH concentrations in the sediment where some of these fish were collected were low, PAH concentrations in the stomach contents (predominantly polychaete worms) of the fish were high. Due to the mobility of these fish, it is impossible to say where they accumulated the PAH. Nevertheless, these results strongly indicate that diet may be an important source for PAH accumulation in the field, and support the conclusions of this study that dietary transfer of PAH may be an important process.

On a whole chamber basis, the mode of introduction had dramatic effects on what happened to BA. Over the time course of a month, most of the sediment-sorbed BA remained in the sediment. Removal by accumulation into worms, flux to seawater, or microbial mineralization was small compared with the total mass of BA present. As discussed above, the presence of worms enhanced removal, but in the short time scale of these experiments, the overall effect was small.

When BA became associated with the sediment-water interface after being spiked to a recirculating water column, in addition to being more available for accumulation by worms, it was much more available for removal via diffusive flux to seawater and microbial mineralization to

CO_2 regardless of whether or not worms were present. In this experiment approximately 1% of total BA recovered was mineralized to CO_2, and 13 to 23% was lost to the water column. In comparison, rates of BA mineralization were undectable and only 0.1% of total radioactivity was lost to the water column in week-long experiments with sediment-sorbed BA. A substantial portion of these effects may be due to the deposition of BA at the sediment-water interface, an area of enhanced microbial activity and diffusive flux. Nevertheless, the increased concentration factors exhibited by worms in this experiment suggest that BA may not be as tightly bound to sediments when introduced in this way. These findings are in agreement with studies indicating negligible microbial mineralization of sediment-sorbed PAH with more than 3 rings (Herbes and Schwall, 1979, and Gardner et al., 1979). The results presented here are also in agreement with two previous investigations of BA added to the water column in large benthic microcosms (Hinga et al., 1981; and McElroy et al., 1982, Appendix 1), reporting microbial mineralization of BA.

Summary:

The results of this investigation emphasize the interactive effects of biological, chemical, and physical processes on the fate of chemicals in the benthos. The presence of the burrowing polychaete Nereis virens and the mode of BA introduction had significant effects on the fate of BA in benthic microcosms. BA loosely associated with particles at the sediment-water interface was relatively labile, and was more available for accumulation and metabolism by Nereis, mineralization by microbes, and diffusive flux to the water column than BA sorbed to bulk sediments. The presence of worms also had a positive effect on microbial mineraliza-

tion of BA. BA in a protein-based food was most efficiently accumulated by Nereis, whereas sediment-sorbed BA was least available for accumulation.

CHAPTER 4:
IN VIVO METABOLISM OF BENZ(a)ANTHRACENE BY Nereis virens

Results:

Distribution of accumulated activity into metabolite classes:

Concentrations and the percent of total activity accumulated into different metabolite classes are shown in Table 4.1. Nereis was able to extensively metabolize accumulated BA regardless of the mode of introduction. Only 2 to 23% of radioactivity recovered from worms was unmetabolized BA. Due to the presence of unmetabolized BA in sediment in the intestines of these worms when they were collected, in experiments 1-4 the percent of total activity in worms reported as BA is probably an overestimate by up to a few percent.

High pressure liquid chromatography of worm organic extracts revealed a small percentage of radiolabel eluting after BA. Although the identity of this fraction is unknown, chromatographic conditions suggest that it is nonpolar and probably larger than BA. This fraction accounted for only a small percentage of recovered isotope in each experiment, so it will not be discused further. However, previous work on in vivo metabolism of BA by the worm Nephty incisa indicated that incorporation of radiolabel into this unknown fraction can be significant (See Appendix 1).

The distributions of parent BA and metabolite classes in each experiment are given in Tables 4.1 and 4.2, and displayed in Figures 4.1 and 4.2. Arcsin transformations of the percentages were compared by ANOVA and SNK. Brackets at the bottom of each histogram join classes that are not significantly different from each other ($p>.05$).

The size of each metabolite pool as a proportion of total is plotted

Table 4.1

DISTRIBUTION OF RADIOACTIVITY RECOVERED FROM WORMS
PERCENTAGE OF RECOVERED RADIOACTIVITY IN MAJOR METABOLITE CLASSES

	% of Total Label Recovered				
	BA	Polar Met.	Conj. Met.	Unext.	Non-pol. Lipid
Experiments with sediment-sorbed BA					
6 day exposures	23.0 ± 4.2	11.0 ± 2.4	31.7 ± 2.1	32.9 ± 2.1	1.36 ±0.63
25 day exposure	4.47 ±0.94	6.48 ±2.98	35.1 ± 1.4	52.6 ± 2.4	1.25 ±0.50
Experiment with BA added to the water column					
6 day exposure	8.80 ±0.67	5.52 ±0.04	45.9 ± 1.6	38.1 ± 0.8	1.65 ±0.33
Experiement with BA labeled food					
4 day exposure	2.11 ±0.43	7.68 ±0.52	48.1 ± 1.2	40.8 ± 0.9	1.35 ±0.16

BA = benz(a)anthracene
Polar Met. = polar metabolites
Conj. Met. = conjugated metabolites
Unext. = unextractable label
Non-pol. Lipid = lipid extractable, nonpolar label

Values expressed as mean ± SE

Values for BA in experiment 1-4 may be slight overestimates due to unmetabolized BA on sediment in gut of worm. Gut contents can account for 3% of body weight.

Results of statistical analysis presented with data in Figures 4.1-4.3.

Table 4.2

DISTRIBUTION OF RADIOACTIVITY RECOVERED FROM WORMS CONCENTRATIONS IN MAJOR METABOLITE CLASSES

ug/gdw

	BA	Polar Met.	Conj. Met.	Unext.	Non-pol. Lipid
Experiments with sediment-sorbed BA					
6 day exposures	1.22 ±0.26	.585 ±.137	1.65 ±0.07	1.61 ±0.03	.073 ±.036
25 day exposure	.590 ±.134	.836 ±.369	4.59 ±0.15	6.89 ±0.55	.166 ±.066
Experiment with BA added to the water column					
6 day exposure	.333 ±.031	.210 ±.018	1.75 ±0.18	1.45 ±0.15	.062 ±.009
Experiement with BA labeled food					
4 day exposure	.0107 ±.0016	.0399 ±.0044	.249 ±.021	.210 ±.011	.007 ±.001

BA = benz(a)anthracene
Polar Met. = polar metabolites
Conj. Met. = conjugated metabolites
Unext. = unextractable label
Non-pol. Lipid = lipid extractable, nonpolar label

Values expressed as mean ± SE

Values for BA in experiment 1-4 may be slight overestimates due to unmetabolized BA on sediment in gut of worm. Gut contents can account for 3% of body weight.

Results of statistical analysis presented with data in Figures 4.1-4.3.

for each experiment in Figure 4.1. The majority of total radioactivity recovered was found in the conjugated metabolite and unextractble fractions in all experiments. The relative distribution of metabolite classes was the same in the last 3 experiments. Only in the short-term experiments with sediment-sorbed BA (exp. 1&2) was a distinctly different pattern seen, where the percentage left in the worms as parent compound was approximately equal to that present as conjugated metabolites and as unextractable activity.

Figure 4.2 shows the same data grouped according to metabolite class, illustrating the patterns in percent of total activity accumulated in each class between experiments. In short-term exposure to BA, the percent of parent compound remaining is highest in experiments with sediment-sorbed BA and lowest in the experiment with BA labeled food, with an intermediate value observed when BA was added directly to the water column. The percent of total remaining as BA after long-term exposure to sediment-sorbed BA was similar to that seen in the short-term experiments with labeled food and BA added to the water column. The percent of total activity recovered in the polar metabolite fraction was not significantly different in any experiment.

The percent of activity recovered as conjugated metabolites was similar after both short- and long-term exposure to sediment-sorbed BA. Conjugates in the short-term experiments, where BA was added to the water column or when BA was added in labeled food, represented a much higher percentage of total activity. The percent of total activity in the unextractable fraction was highest after long-term exposure to sediment-sorbed BA, lowest after short-term exposure to BA., and approximately

Figure 4.1: Distribution of recovered radiolabel for each type of experiment, comparing different metabolite classes. Values expressed as mean $\pm$ SE of the percent of total radioactivity recovered in each metabolite class. Bars connecting different groups indicate no significant difference ($p>0.05$). Means of arcsin transformed data compared by ANOVA and SNK.

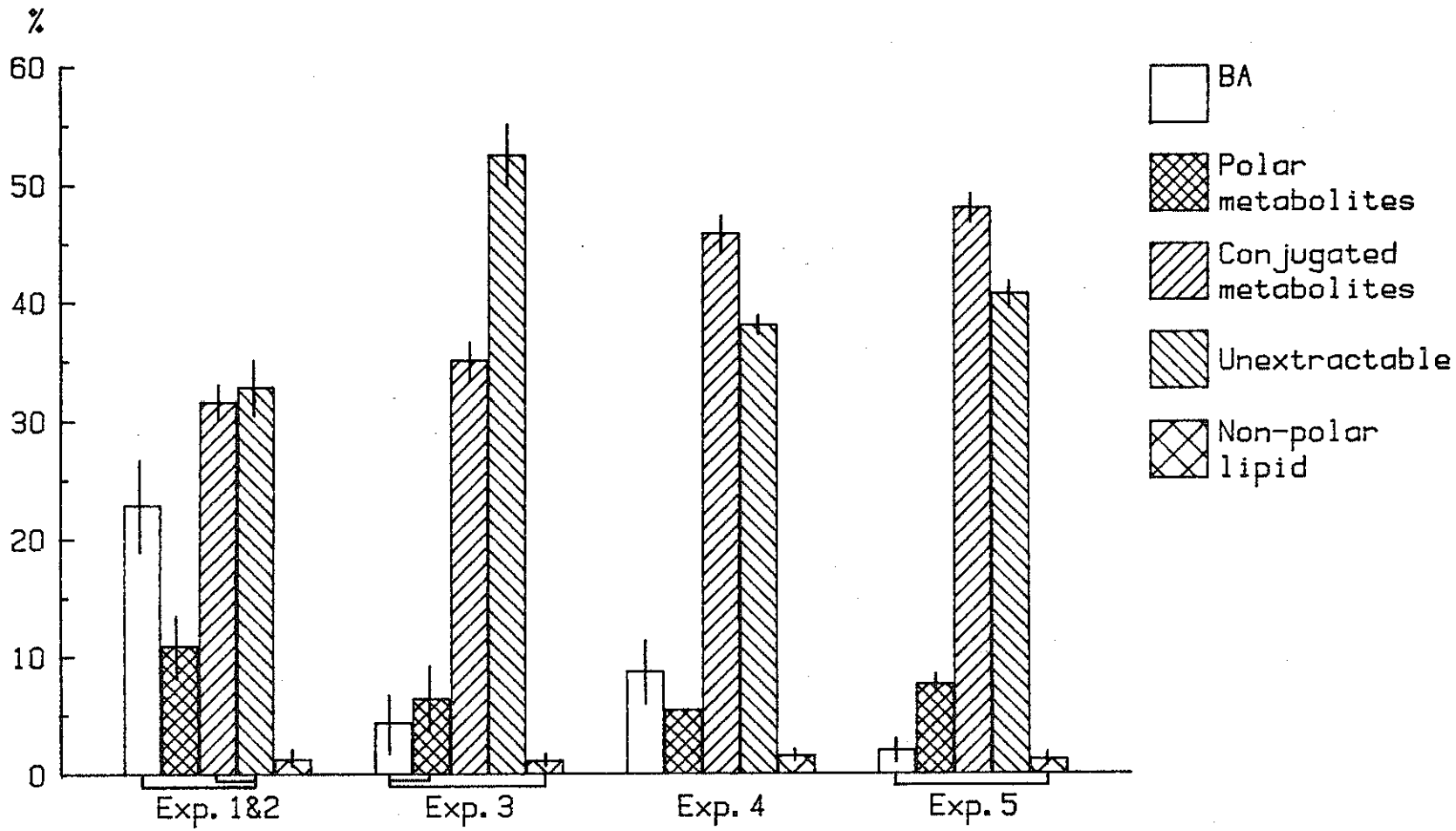
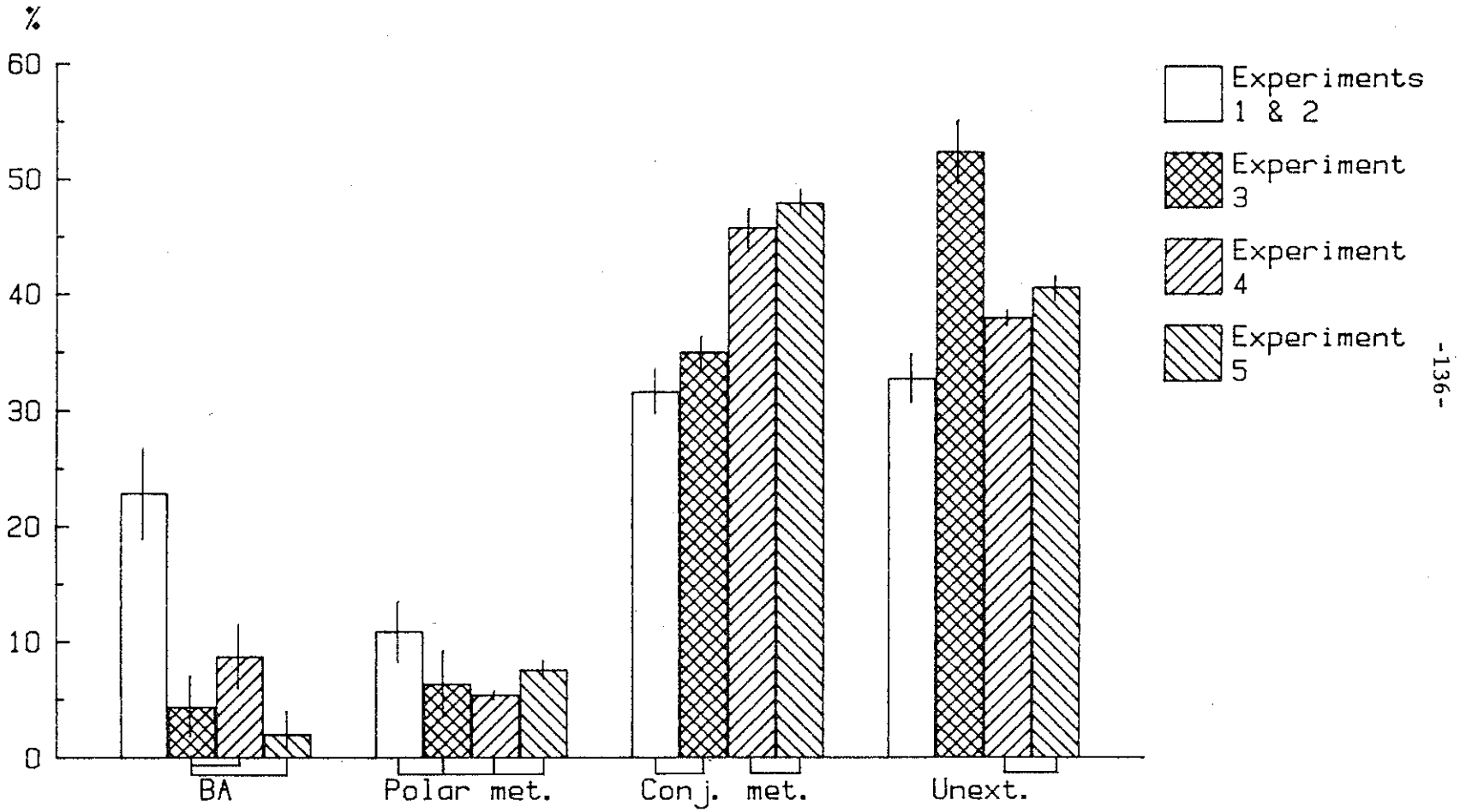

%
60
50
40
30
20
10
0
BA
Polar metabolites
Conjugated metabolites
Unextractable
Non-polar lipid
Exp. 1&2
Exp. 3
Exp. 4
Exp. 5

Figure 4.2: Distribution of recovered radiolabel in different metabolite classes compared between experiment. Values expressed as mean ± SE of the percent of total radioactivity recovered in each metabolite class. Bars connecting different groups indicate no significant difference ($p>0.05$). Means of arcsin transformed data compared by ANOVA and SNK.


%
60
50
40
30
20
10
0
BA
Polar met.
Conj. met.
Unext.
Experiments 1 & 2
Experiment 3
Experiment 4
Experiment 5

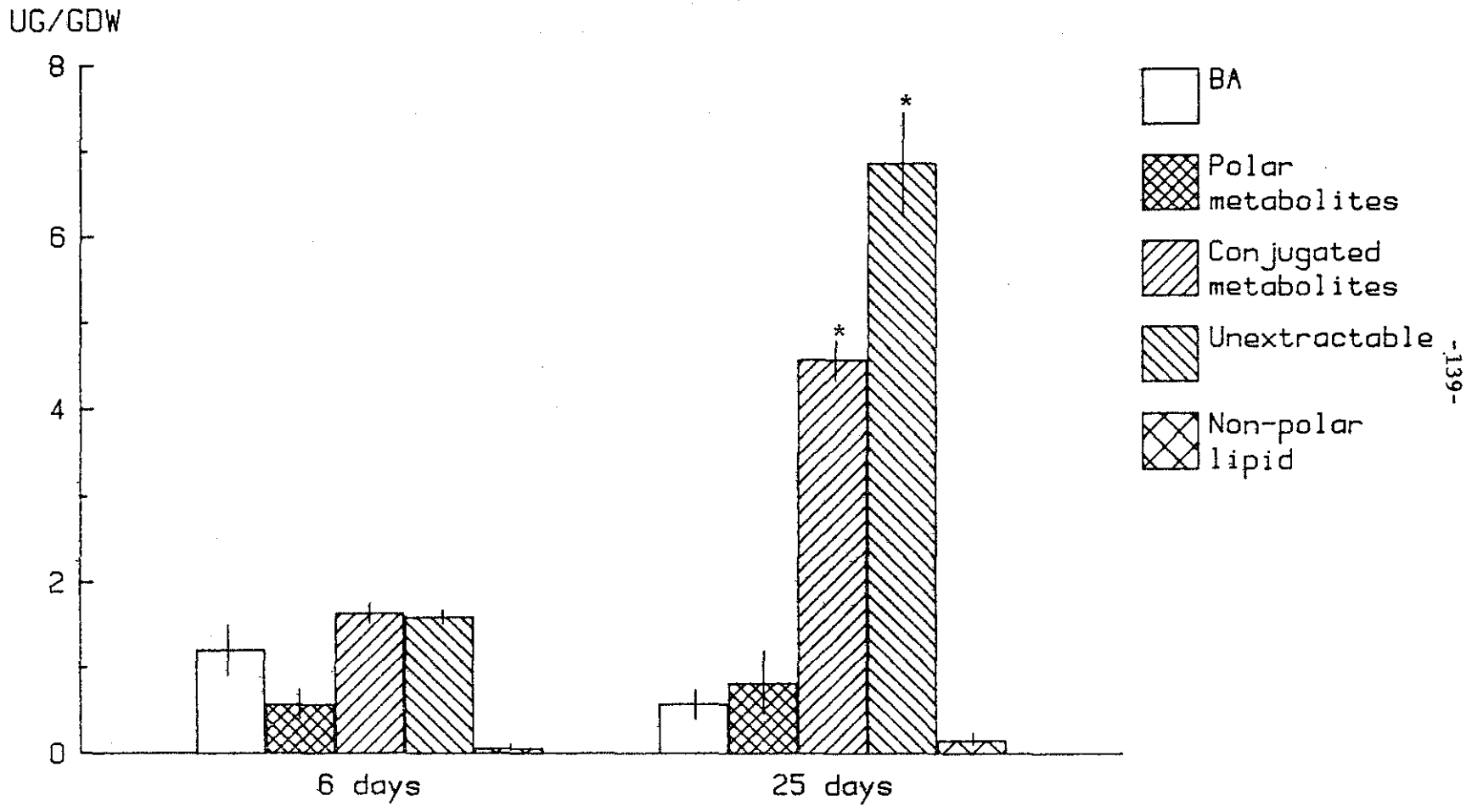
equal in experiments where BA was added either to the water column or in the food.

In the short- and long-term experiments with sediment-sorbed BA, worms were continuously exposed to similar concentrations of BA. Therefore comparisons between the absolute amounts accumulated into each of the metabolite classes can be considered. Figure 4.3 shows total accumulation into each metabolite class in ug/gdw for the 6 and 25 days experiments. Although the percentage recovered as BA decreased with length of exposure (see Figure 4.2), the absolute amount remained the same. Both the percentage of total and the absolute amounts of polar metabolites were not affected by length of exposure. In contrast, concentration of conjugated metabolites and of activity in the unextractable fraction increased dramatically with time. The concentration of conjugates increased by a factor of 2.8, while concentration in the unextractable fraction increased by a factor of 4.3. Although the percentage of total activity in these two pools were equal in the short-term experiments (See Figure 4.1), the concentration in the unextractable fraction was significantly higher than that in the conjugate pool after 25 days.

Separation of polar metabolites in organic extract by reverse phase HPLC:

Organic extracts of worm tissue were analyzed by reverse phase HPLC to separate BA from polar metabolic products. Due to the complex mixture of compounds in addition to BA and BA metabolic products present in the organic extract, UV absorbance was of little use in identifying polar metabolites (Figure 4.4). Attempts to clean-up worm organic extracts using Waters Assoc. C_{18} or silica Sep-Pak cartridges were unsuccessful.

Figure 4.3: Total incorporation into different metabolite classes in experiments with sediment-sorbed BA. Values expressed as mean ± SE. Means for each metabolite class compared between 6 and 25 day experiments using Student's T-test. * indicates significant difference ($p<0.05$).


UG/GDW
8
6
4
2
0
6 days
25 days
*
*
BA
Polar metabolites
Conjugated metabolites
Unextractable
Non-polar lipid

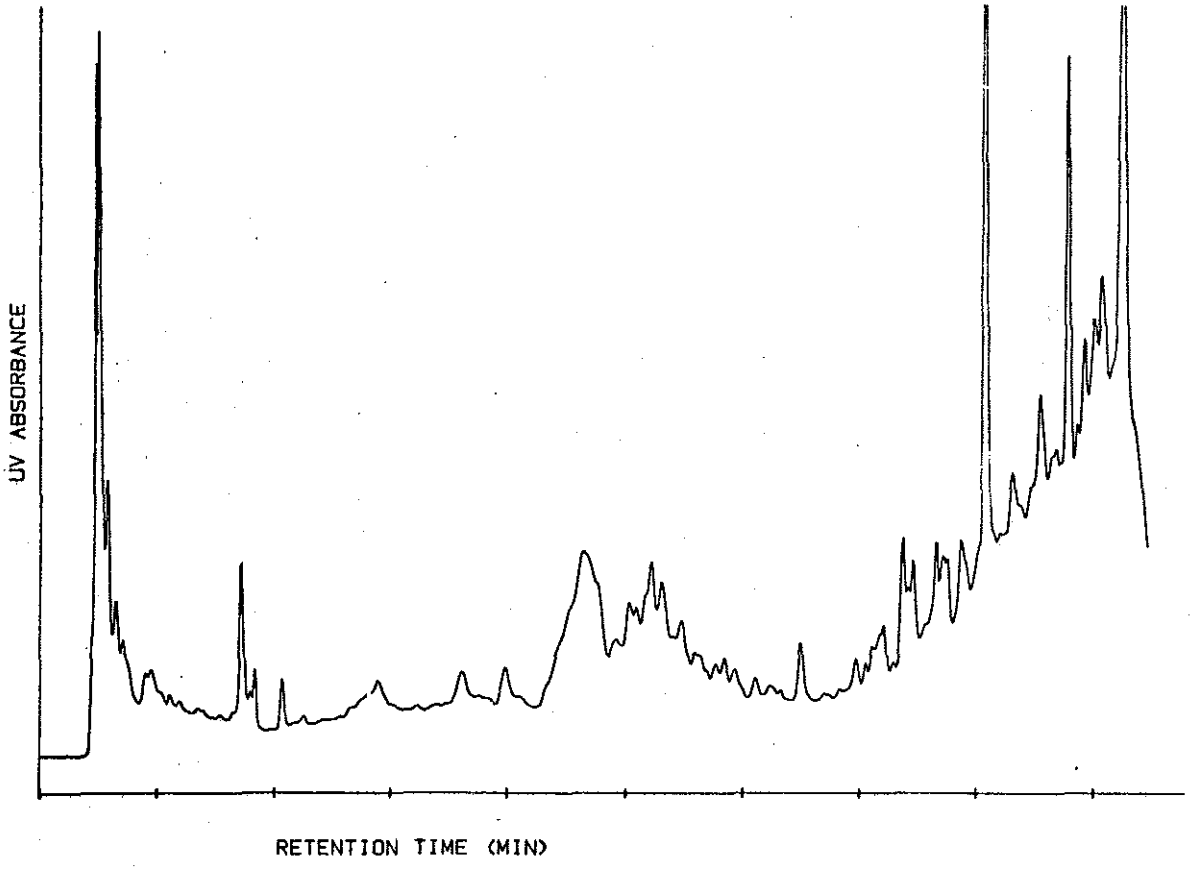
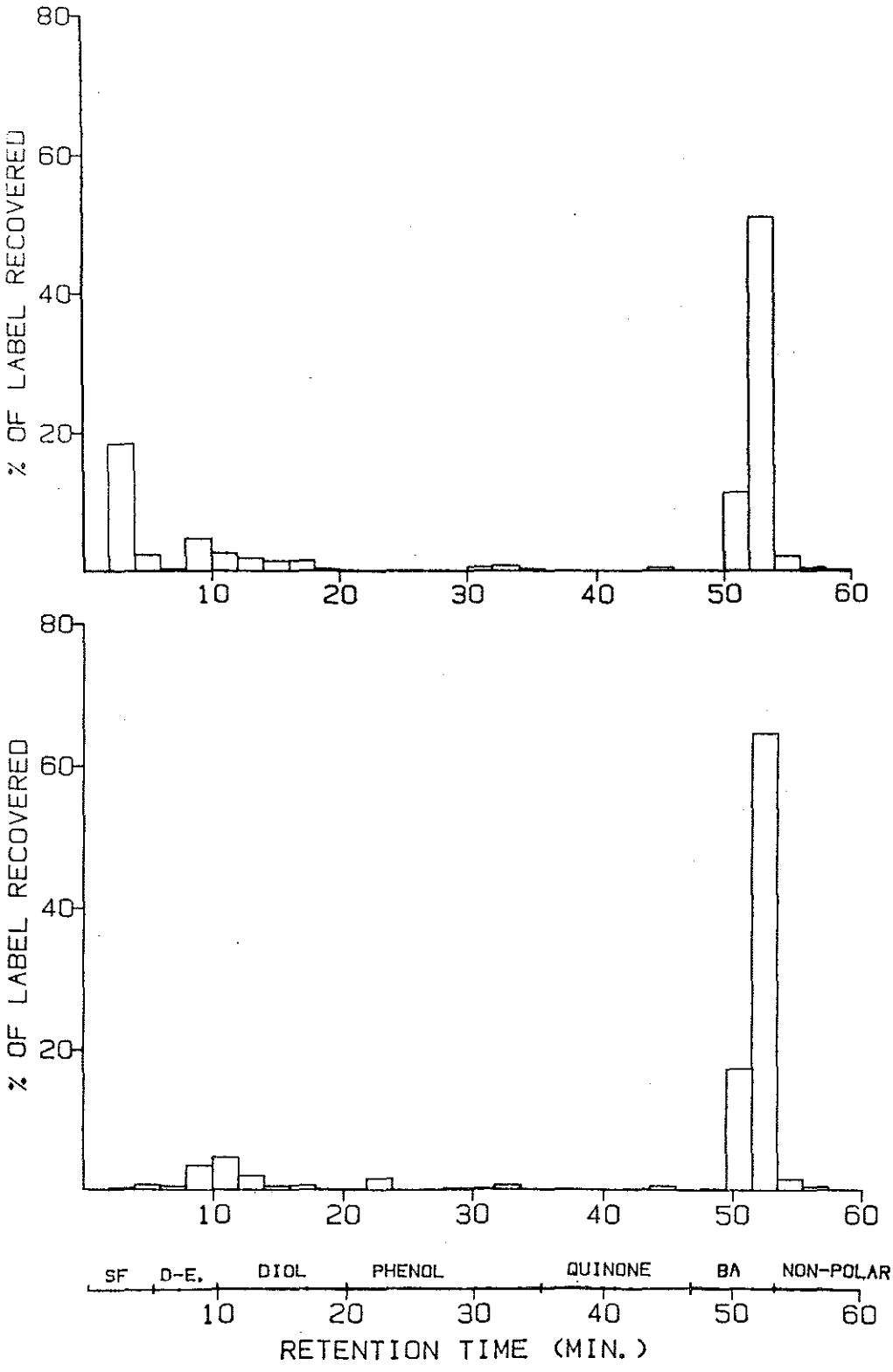


Figure 4.4: UV HPLC chromatogram of worm organic extract

Due to the wide range in polarity of polar metabolites and parent BA, it was not possible to significantly reduce either the polar or non-polar UV absorbing contaminants without also removing radiolabeled compounds of interest. To avoid missing unknown peaks, fractions of the HPLC eluant were collected for the entire run and ^{14}C activity analyzed by LSC. Authentic standards were available from the NCI for some polar BA metabolites. A chromatogram of representative classes of metabolite standards is shown in Figure 2.10. Co-injection of standards and worm extract indicated that the presence of extract did not alter retention time of standards, thus identification of polar metabolites was based on retention time.

The chromatograms generated from the ^{14}C activity collected in fractions seperated by HPLC (Figures 4.5-4.9) are also complex. Due to the high lipid content of the worm organic extracts, not much material could be injected on the HPLC. Frequently there was a smearing of activity with broad humps in various portions of the chromatogram rather than in discrete peaks. Part of this smearing may have been due to overloading the column, and some was probably due to the fact that relatively long (1 or 2 minute) fractions were collected that most likely split some peaks and joined others. Identification of specific metabolites was further hampered by the availability of only some of the possible polar metabolites, and co-elution of others. Therefore radioactivity separated using HPLC was only identified to the level of metabolite class. Intervals of the chromatogram were assigned to several classes of metabolites based on the retention times of all available standards. These retention time windows have been overlayed on the x axis of each chromatogram.

Figure 4.5: ^{14}C HPLC chromatograms of worm organic extracts from two chambers in experiment 1. Overlay on x axis refers to retention time windows corresponding to different classes of authentic BA metabolite standards. Abreviations: SF= solvent front; D-E= diol-epoxide. Non-polar refers to all activity eluting after BA.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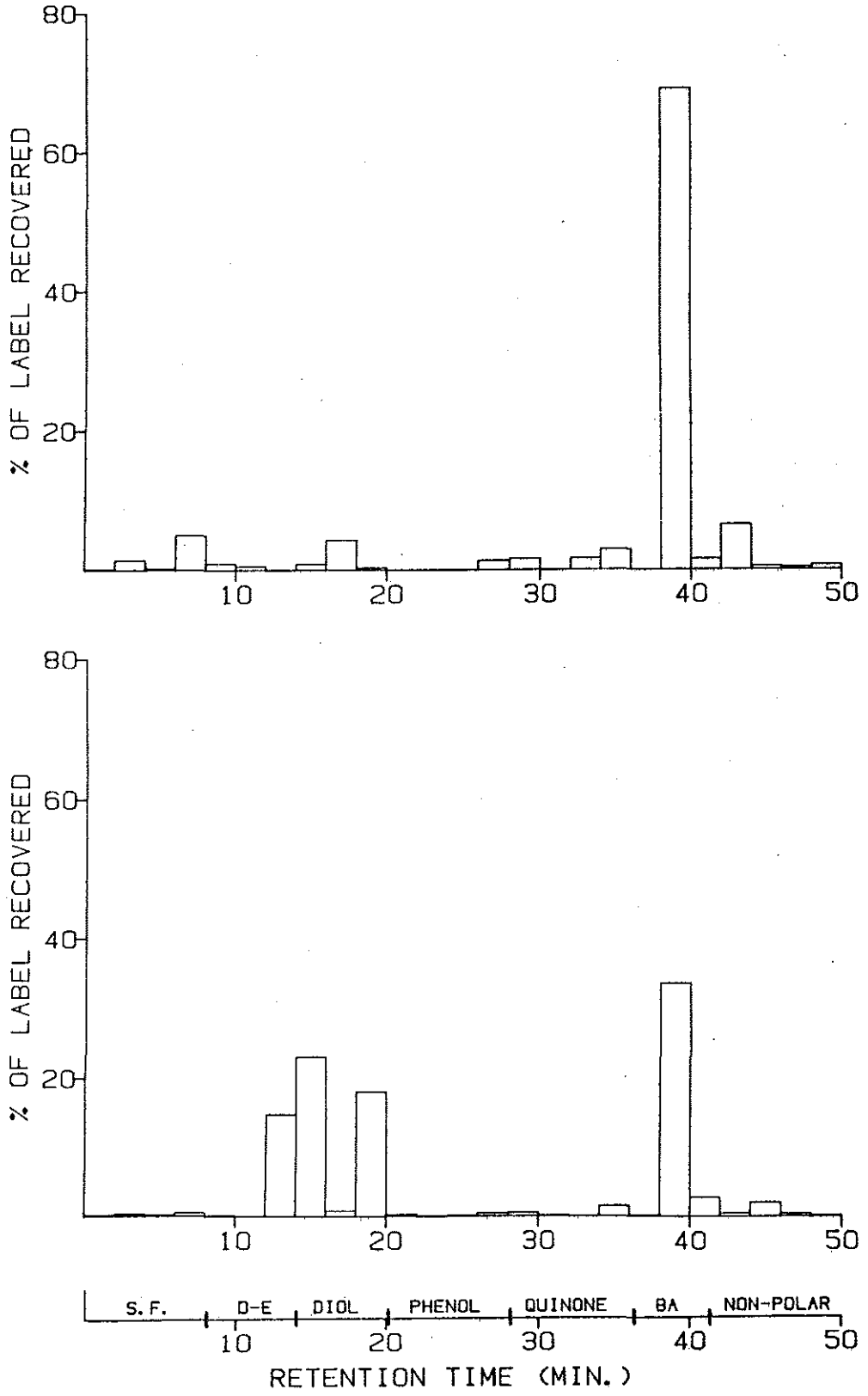
RETENTION TIME (MIN.)

Figure 4.6: ^{14}C HPLC chromatograms of worm organic extracts from two chambers in experiment 2. Overlay on x axis refers to retention time windows corresponding to different classes of authentic BA metabolite standards. Abreviations: SF= solvent front; D-E= diol-epoxide. Non-polar refers to all activity eluting after BA.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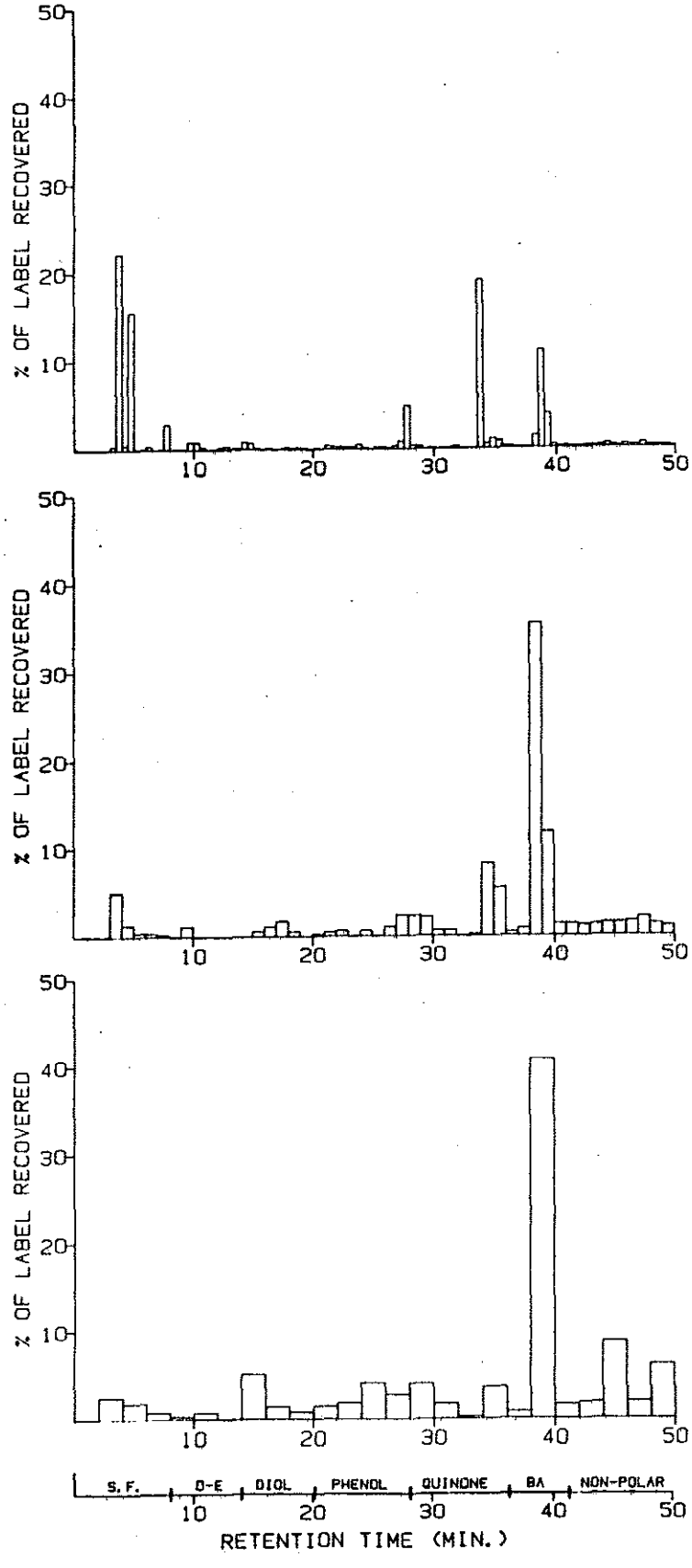
RETENTION TIME (MIN.)

Figure 4.7: ^{14}C HPLC chromatograms of worm organic extracts from all chambers experiment 3. Overlay on x axis refers to retention time windows corresponding to different classes of authentic BA metabolite standards. Abreviations: SF= solvent front; D-E= diol-epoxide. Non-polar refers to all activity eluting after BA.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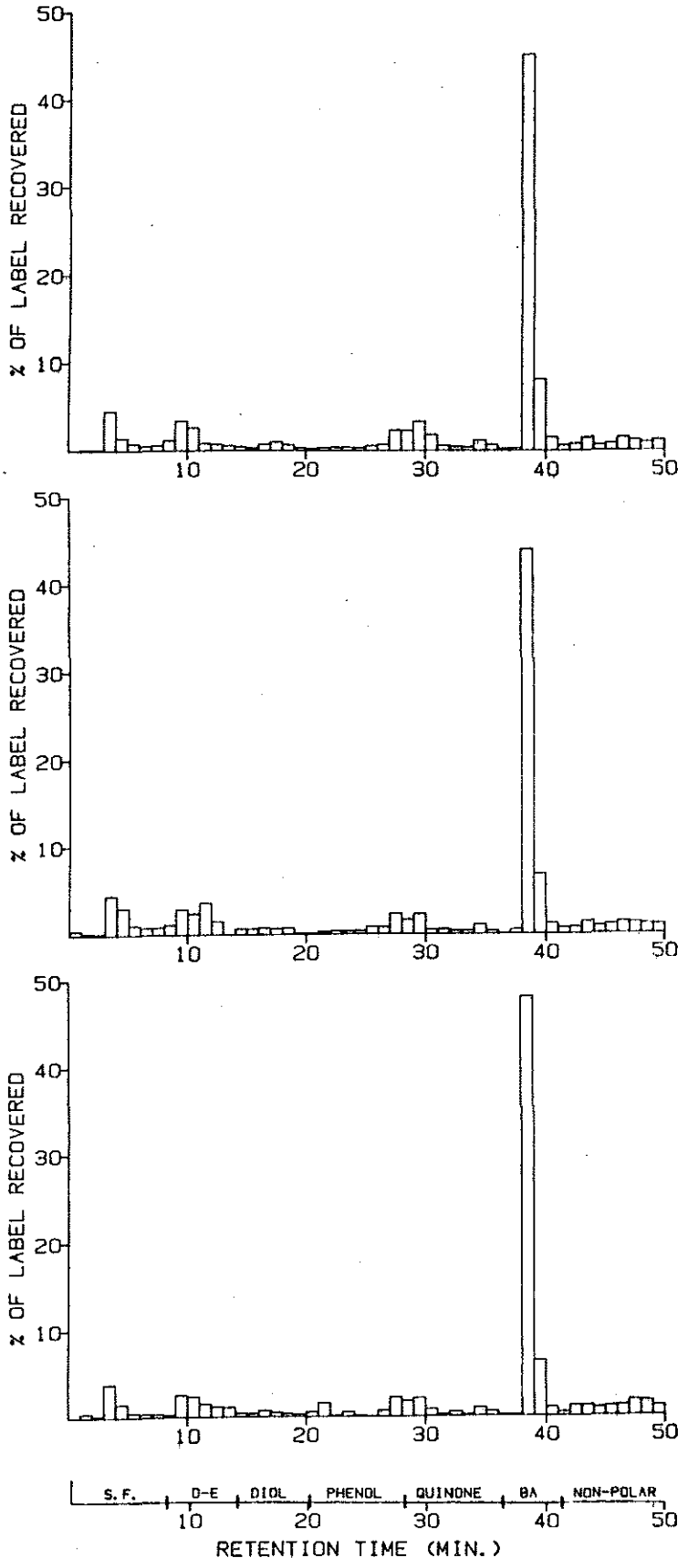
RETENTION TIME (MIN.)

Figure 4.8: ^{14}C HPLC chromatograms of worm organic extracts from all chambers in experiment 4. Overlay on x axis refers to retention time windows corresponding to different classes of authentic BA metabolite standards. Abreviations: SF= solvent front; D-E= diol-epoxide. Non-polar refers to all activity eluting after BA.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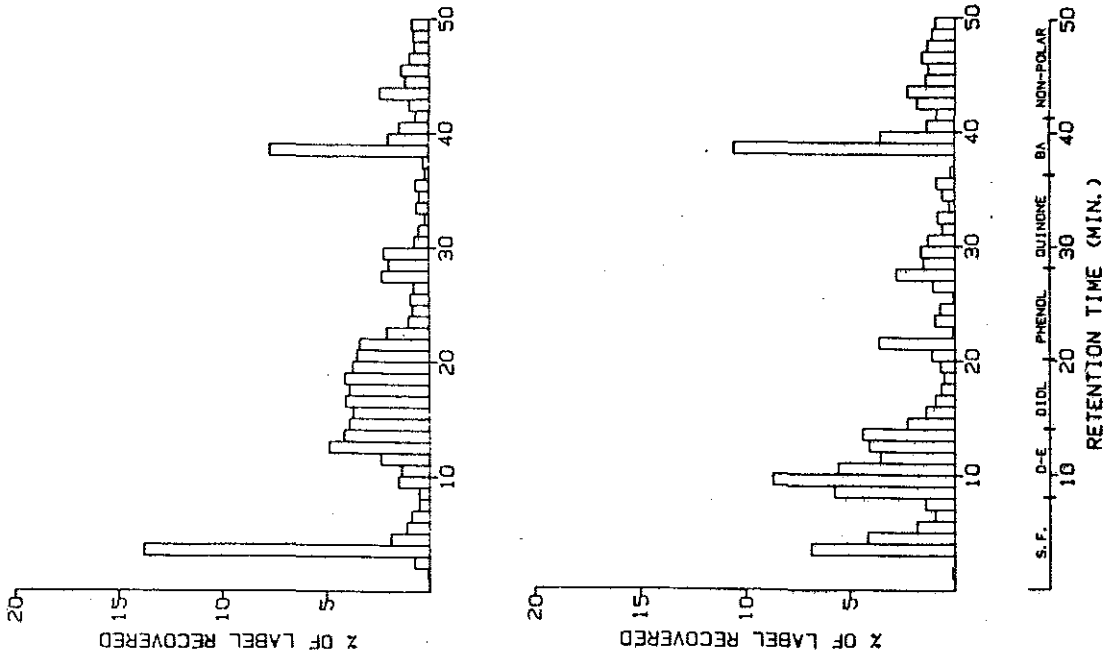
NON-POLAR
RETENTION TIME (MIN.)

Figure 4.9: ^{14}C HPLC chromatograms of worm organic extracts from all chambers in experiment 5. Overlay on x axis refers to retention time windows corresponding to different classes of authentic BA metabolite standards. Abreviations: SF= solvent front; D-E= diol-epoxide. Non-polar refers to all activity eluting after BA.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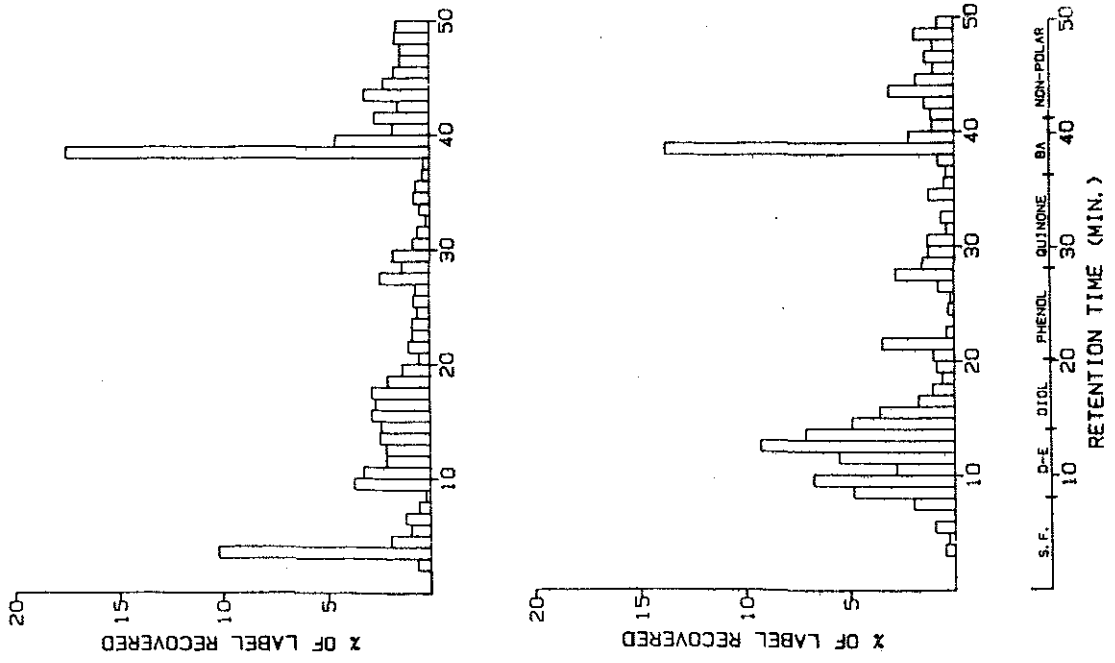
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RETENTION TIME (MIN.)

Radioactivity recovered in these intervals from each experiment is compiled in Table 4.3.

The patterns of polar metabolites separated by HPLC appear to be distinctly different between experiments. In all experiments the majority of radiolabel in the organic extract was present as unmetabolized BA. The short term experiments with sediment-sorbed BA had the highest proportions of activity chromatographing as unmetabolized BA, whereas the short-term experiments with BA labeled food had the lowest. A noticable peak in label eluting in the diol-epoxide/tetrol range was observed in the experiment with labeled food. Due to the imprecise nature of class identification, further dissection of these results would be presumptuous.

Enzymatic analysis of glucuronide and sulfate conjugates:

The proportion of activity in the aqueous extract that was present as glucuronide or sulfate conjugates was measured in experiments 3,4, and 5, and is listed in Table 4.4. Only 6% or less of total activity in the aqueous extract was found to be glucuronide or sulfate conjugates. Values for this fraction were similar in the long-term experiment with sediment-sorbed BA and the experiment with BA added to the water column. Less than half that amount was found in the experiment with BA labeled food. Organic extracts of the aqueous extract after enzymatic treatment, were analyzed by HPLC. Due to the low radioactivity in these extracts, it was necessary to pool fractions from replicate chambers within each experiment before chromatographic analysis. Chromatograms of organic extracts treated with conjugate cleaving enzymes and blanks which were incubated without enzymes are shown in Figures 4.9-4.12. It appears that

Table 4.3

DISTRIBUTION OF RADIOACTIVITY RECOVERED FROM WORMS: METABOLITE CLASSES IN ORGANIC EXTRACT SEPARATED BY REVERSE PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY

Metabolite Class	Experiment			
	1 & 2	3	4	5
Solvent front	6.8 ±5.6	18.8 ±13.0	8.2 ±0.7	13.4 ±3.4
Tetrols & Diol-epoxides	5.3 ±2.1	2.4 ±0.6	10.1 ±1.2	24.1 ±5.8
Diols	12.4 ± 1.7	3.8 ±1.7	2.9 ±0.2	14.0 ± 3.4
Phenols	0.8 ±0.2	7.6 ±1.3	5.5 ±0.3	10.4 ± 1.5
Quinones	0.7 ±0.7	17.3 ± 4.3	7.9 ±1.3	7.1 ±0.2
Parent BA	74.7 ± 4.7	38.5 ±10.1	55.1 ± 0.8	18.8 ± 3.8
Lipid Nonpolar	0.8 ±0.8	11.6 ±5.4	10.5 ±1.3	12.1 ±1.2

Experiment Key
1&2: 6 day exposure to sediment-sorbed BA
3: 25 day exposure to sediment-sorbed BA
4: 6 day exposure to BA added to the water column
5: 4 day exposure to BA in diet

Values expressed as means ± SE.

Metabolite class assignment based on comparisons to retention times of authentic standards.

Solvent front category refers to isotope eluting within the solvent front.

Lipid-nonpolar category refers to isotope eluting after BA

Table 4.4

DETERMINATION OF GLUCURONIDE AND SULFATE CONJUGATES IN AQUEOUS EXTRACTS OF WORM TISSUE

	% Total Radioactivity in Aqueous Extract
Experiment 3 25 day exposure to sediment-sorbed BA	6.03 ± 1.53 (3)
Experiment 4 6 day exposure to BA added to the water column	5.66 ± 0.25 (3)
Experiment 5 4 day exposure to BA labeled diet	2.25 ± 0.32 (4)

Values expressed as mean ± SE (n)

nonenzymatic hydrolysis of conjugated metabolites in the blank extracts was substantial. Nonenzymatic hydrolysis probably resulted from the lengthy evaporation and incubation at elevated temperatures used in this procedure. Comparisons between chromatograms of extracts of analysis blanks with extracts of samples receiving enzymes, suggest that some phenolic, quinone, and diol metabolites were liberated by enzymatic treatment.

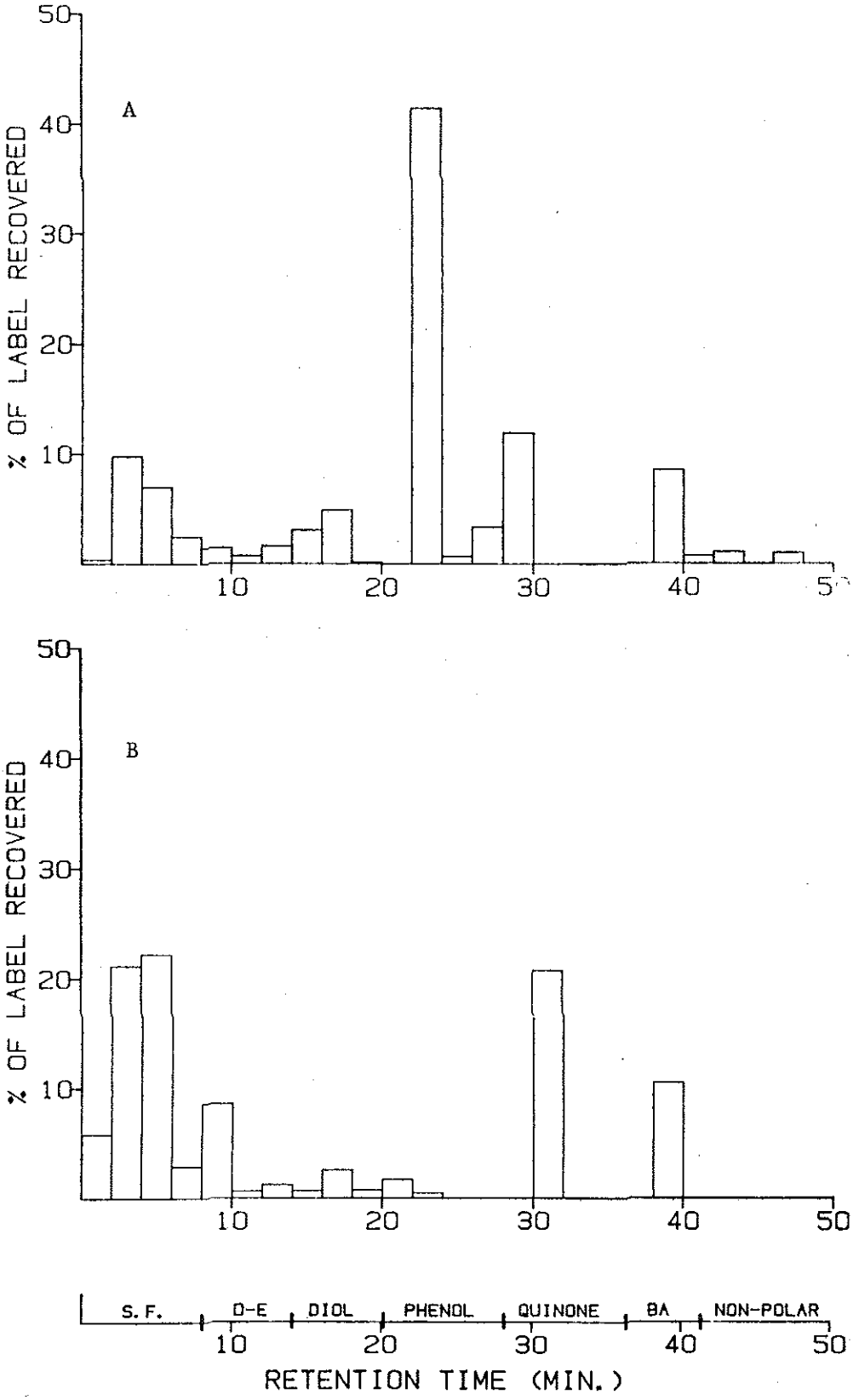
Discussion:

Rapid *in vivo* metabolism of BA by *Nereis virens*

The results of this investigation clearly demonstrate that *Nereis virens* is able to metabolize BA. In as little as 96 hours after ingesting food containing BA, only 2% of activity remaining in the worm was in the form of unmetabolized parent compound. These results are consistent with similar findings that PAH can be rapidly metabolized in a few other invertebrates including the blue crab *Callinectes sapidus* (Lee et al., 1976), the diptera larvae *Chironomus* (Leversee et al., 1982), larval shrimp (Sanborn and Malins, 1977), and the sea urchin *Strongelocentrotus drobachiensis* (Malins and Roubal, 1982), and in many different marine and freshwater teleosts (See review by Malins and Hodgkins, 1981).

If only unmetabolized BA had been analyzed in the present study, measurements of total BA accumulated would have been underestimated by as much as 98%. In organisms which are known to rapidly metabolize PAH such as fish, the discrepancy between accumulated parent PAH and total activity might be even higher. Lu et al. (1977), clearly demonstrated the importance of including metabolite analysis in studies on accumulation of PAH. Accumulation of unmetabolized BP in fish could only be demonstrated

Figure 4.10: ^{14}C HPLC chromatograms of organic extracts after enzymatic cleavage of glucuronide and sulfate conjugates in experiment 3. (a) sample with enzyme (b) blank. Overlay on x axis refers to retention time windows corresponding to different classes of authentic BA metabolite standards. Abreviations: SF= solvent front; D-E= diol-epoxide. Non-polar refers to all activity eluting after BA.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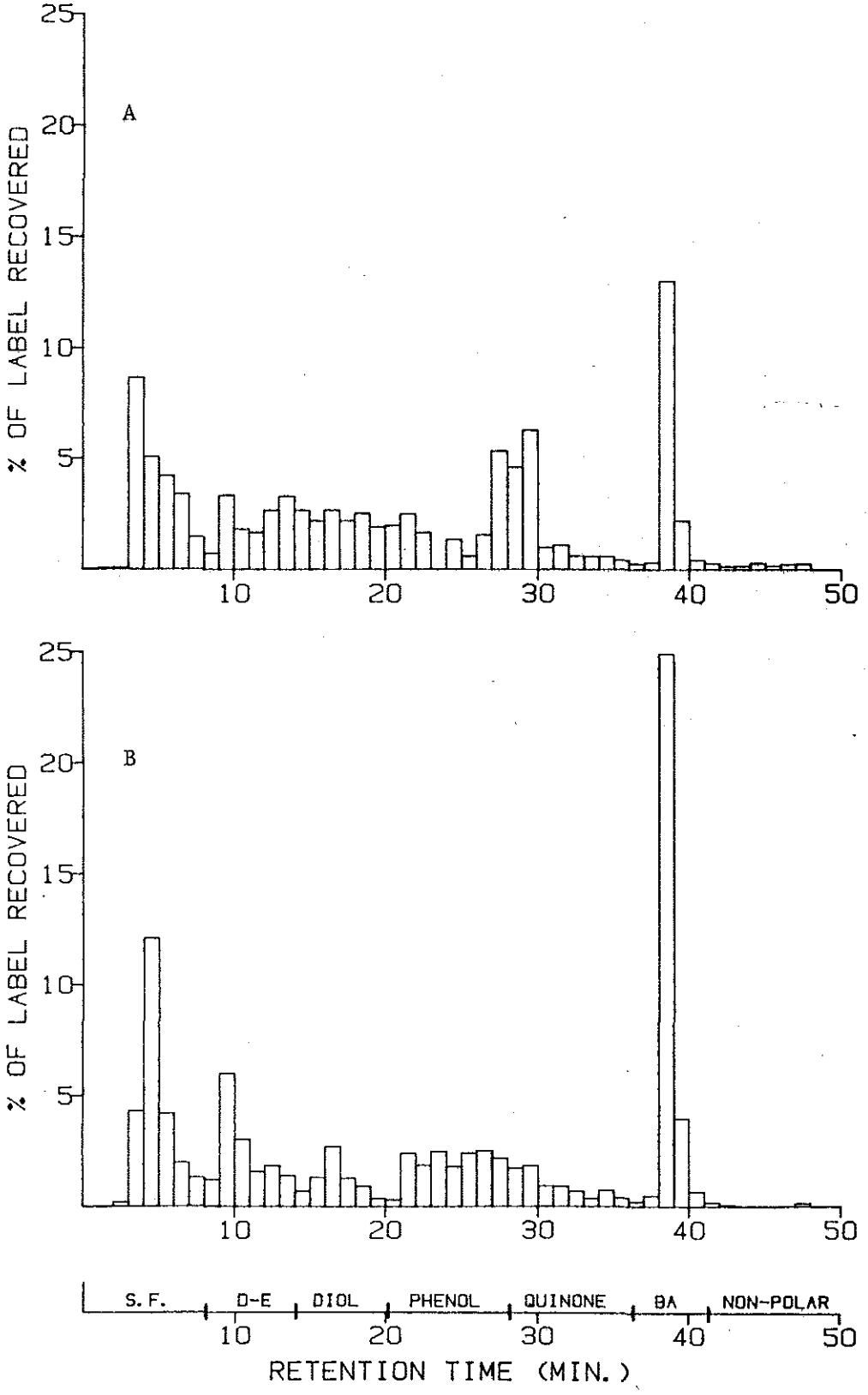
NON-POLAR
RETENTION TIME (MIN.)

Figure 4.11: ^{14}C HPLC chromatograms of organic extracts after enzymatic cleavage of glucuronide and sulfate conjugates in experiment 4. (a) sample with enzyme (b) blank. Overlay on x axis refers to retention time windows corresponding to different classes of authentic BA metabolite standards. Abreviations: SF= solvent front; D-E= diol-epoxide. Non-polar refers to all activity eluting after BA.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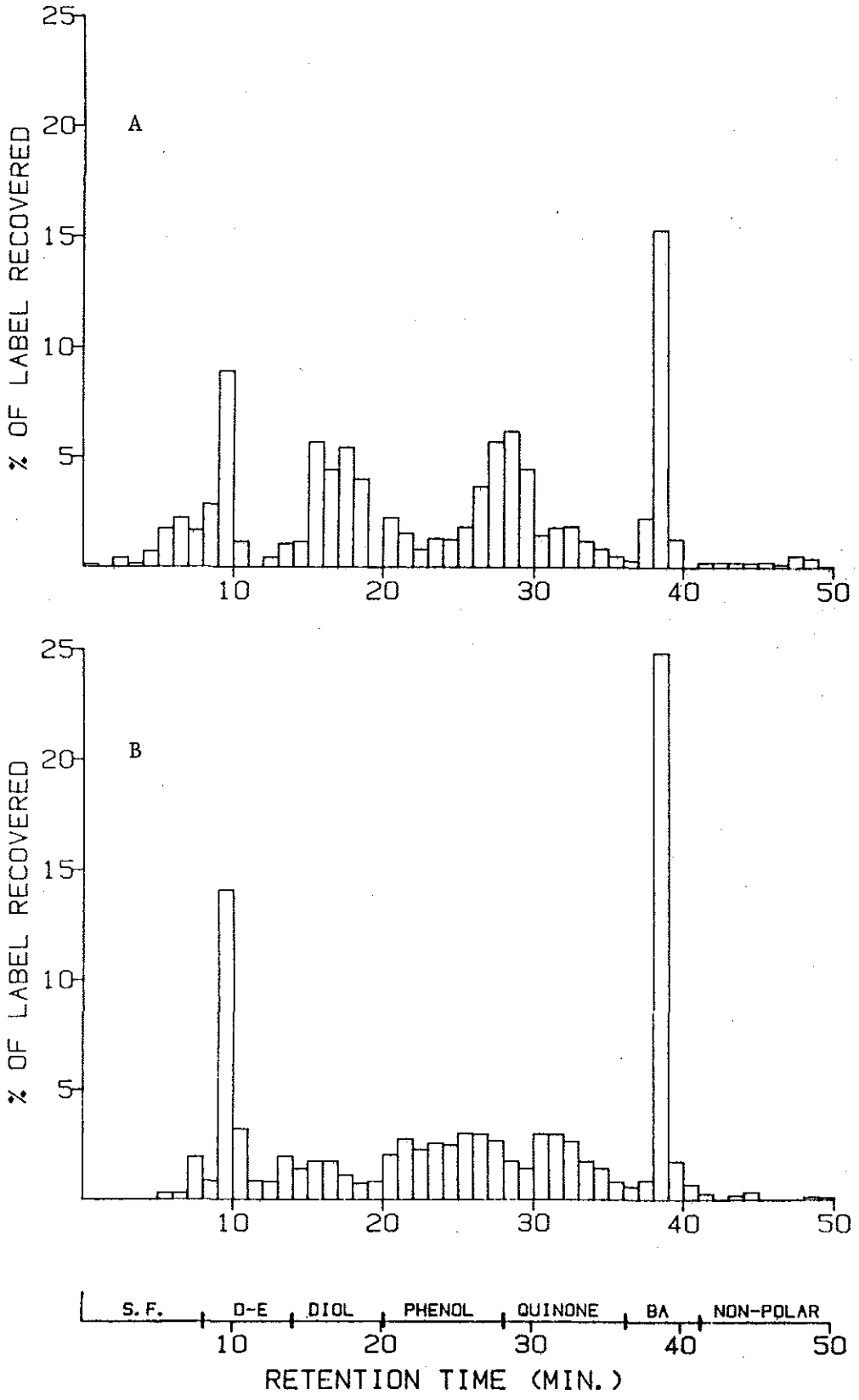
NON-POLAR
RETENTION TIME (MIN.)

Figure 4.12: ^{14}C HPLC chromatograms of organic extracts after enzymatic cleavage of glucuronide and sulfate conjugates in experiment 5. (a) sample with enzyme (b) blank. Overlay on x axis refers to retention time windows corresponding to different classes of authentic BA metabolite standards. Abreviations: SF= solvent front; D-E= diol-epoxide. Non-polar refers to all activity eluting after BA.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NON-POLAR
RETENTION TIME (MIN.)

in the presence of the MFO inhibitor piperonyl butoxide. In multispecies model ecosystems, activity in all organisms capable of PAH metabolism was found primarily in an unextractable form after only one day of exposure. Addition of the MFO inhibitor increased activity in less metabolized fractions. Leversee et al. (1982) found bioconcentration factors based on total activity to be more than three times those based on the parent compound alone in Chironomus riparius.

The present results are in apparent contrast with those of Augenfeld et al. (1982), who reported no evidence of solvent extractable polar metabolites in tissues of the polychaete Neanthes arenaceodentata exposed for 60 days to sediment-sorbed radiolabeled BP, chrysene, or phenanthrene. They did observe that from 2 to 22% of isotope recovered from the worms was not solvent extractable, and acknowledged that this portion of recovered label was probably not due to parent compound. Unfortunately Augenfeld et al. did not quantify isotope in the aqueous worm extract, which was found here to account for a major portion to accumulated activity. In addition, it is not clear from their report if the HPLC procedure they employed would have resolved polar metabolites from parent BA. Because of differences between techniques, it is difficult to say whether the results of the present study on in vivo PAH metabolism in Nereis virens are in conflict or agreement with the data reported by Augenfeld et al. for a closely related species Neanthes arenaceodentata.

Effect of time and mode of introduction on degree and type of metabolites formed:

Length of exposure and mode of introduction significantly affected both the extent to which BA was metabolized and the relative percentages of different metabolites formed. In experiments with sediment-sorbed BA

absolute amounts of activity in the conjugate and unextractable pools increased with length of exposure. Comparing experiments with different methods of introduction, but similar lengths of exposure, parent BA was most extensively metabolized when presented in labeled food, and metabolized the least when accumulated from uniformly labeled sediments. The general trend, looking at all metabolic fractions, was for BA to be most extensively metabolized when accumulated from a protein based diet. BA added to the water column and secondarily adsorbed to surface sediments demonstrated intermediate availability for metabolism. BA sorbed to bulk sediments, prior to placement in the experimental chambers was least available for metabolism. The same trend was observed in the availability of BA for accumulation by worms (See Chapter 3).

These results support the premise that PAH from different sources, or accomodated in different matrices are not equally available for metabolism by marine organisms. Investigations with other species also support this conclusion. Lu et al. (1977) reported patterns of BP metabolites in fish to be different when the parent compound was introduced to the water or when fish encountered BP through the food chain. Lu found higher biomagnification in fish from the food chain than from direct uptake from the water column. This is interesting, considering that the prey contained substantial amounts of BP metabolic products including those that were unextractable. Sanborn and Malins (1977) found that *in vivo* metabolism of naphthalene by larval spot shrimp was affected by complexation of the PAH with bovine serum albumin. Corner et al. (1976) reported differences in accumulation and metabolism of naphthalene from the water column or from dietary sources by the copepod *Calanus*.

Naphthalene was more available for accumulation and less rapidly depurated when introduced in the diet than when added to the water column. However, Corner found that only 10% of the activity accumulated from the diet was in the form of polar metabolites. This is in contrast to results of an earlier study by Lee (1975) who found several dissolved PAH to be accumulated and extensively metabolized by Calanus. Varanasi et al. (1979) found naphthalene to be more extensively metabolized when adsorbed through the diet than when injected intraperitoneally in two species of flat fish.

Although the results of these studies are consistent with the results of this present investigation, they are not directly comparable as type of PAH, method of dosing, and analytical methods were all different. In addition, the contribution of photo or microbial degradation to the production of PAH metabolites can be substantial, and were not assessed in these investigations. Nevertheless these studies do indicate that accumulation and metabolism of PAH encountered in the diet can be substantial in several marine organisms. The present study clearly demonstrates that PAH in the diet are also efficiently accumulated and metabolized by the polychaete Nereis virens.

This investigation was not directed at the kinetics of BA metabolism by Nereis. However, the absolute proportions of activity in the different metabolite fractions in the short and long-term experiments with sediments-sorbed BA give some indication of the relative rates for the formation of these classes. The concentration of parent BA or of polar lipid soluble metabolites in the worms does not increase with time. However, the concentration of conjugated metabolites and of metabolites

in the bound fraction increase dramatically with time. Similar differences are seen in the patterns between the short-term experiments with sediment-sorbed BA and the experiments where BA was more available (exp. 4 & 5). These data show that neither unmetabolized BA nor polar metabolites produced via the MFO system accumulate relative to other metabolite fractions, suggesting that initial uptake and metabolism is rapid.

Transformation into conjugated metabolites, and incorporation into cellular constituents as major fates of accumulated BA in *Nereis virens*

Thirty-two to forty-eight percent of accumulated activity was found as water soluble conjugated metabolites in these experiments. The rapid formation of conjugated metabolites observed in *Nereis* is consistent with reports of rapid conjugate formation in other aquatic organisms. Herbes and Rissi (1978) found 21% of the total label in *Daphnia* in the aqueous extract after 168 hours of exposure to ^{14}C-anthracene. Water soluble metabolites were found to account for up to 100% of the total accumulated activity in some organs of several marine animals also exposed to ^{14}C-anthracene by Solbakken and Palmork, (1981).

In contrast to work by other investigators, enzymatic analysis using B-glucuronidase and aryl-sulfatase indicated that glucuronide and sulfate conjugates comprised only a maximum of 6% of total water soluble metabolites in *Nereis*. Malins and Roubal (1982) found sulfate conjugates of dimethylnaphthalene to comprise up to 90% of extractable activity in the gonads and digestive tract of the sea urchin *Strongelocentrotus drobachiensis*. In the English sole Varanasi and Gmur found that a minimum of 30% of total metabolites of naphthalene or BP were present as glucuronide conjugates. The same trends are seen in mammals. In isolated rat hepa-

tocytes typically 5 to 20% of BP conjugates were glucuronides (Jones et al., 1978).

Another major class of conjugated metabolites are those bound to glutathione which can lead to mercapturic acid formation. Reduced glutathione is present in all cells of higher organisms (Lehninger, 1975), and glutathione S-transferases have been measured in a wide variety of marine organisms (James et al., 1979a). The presence of glutathione conjugates is often inferred by difference (i.e. by the lack of other forms of conjugates). It is possible that water soluble metabolites in Nereis were conjugated to glutathione. Reactive electrophiles can also bind directly to glutathione S-transferase. This scavenging pathway provides an effective means of sequestering reactive electrophiles and lipophilic compounds (Jakoby and Habig, 1980). The large percentage of accumulated radioactivity present in an unextractable form is not inconsistent with this hypothesis, as both products (glutathione conjugates and macromolecular adducts) result from the same highly reactive epoxide intermediate (See Fig. 1.1).

In the first four experiments in this study, worms were in an environment containing unmetabolized BA throughout the experiment. Therefore, even though they were metabolizing BA, they were continuously exposed to unmetabolized BA. Consequently the patterns of accumulated radioactivity observed represent some sort of steady state between accumulation, metabolism, and excretion. In the last experiment worms were given a single dose of BA labeled food. The persistence of metabolic products of all types in tissue 4 days after feeding indicates that although some of these compounds were water soluble, they were not immed-

iately excreted from the worm's body. In addition, 83% of the entire dose added to the chambers was recovered from the worms. This indicates that at most 17% of the accumulated dose could have been excreted. The increase in conjugated metabolites over time in experiments where worms were exposed to a continuous supply of unmetabolized BA also supports the conclusion that water soluble BA metabolites have an appreciable residence time in Nereis.

The persistence of PAH metabolic products relative to parent compound has been reported for a number of marine organisms. In studies of BP accumulation by Chironomus, Leversee et al. (1982) reported that while total activity continued to increase for 8 hours, the percentage as unmetabolized BP leveled off after 1 hour. Rossi and Anderson (1978) reported depuration of metabolized naphthalene was slower than that of the parent compound in the worm Neanthes arenaceodentata. Similar findings have been reported for BP in the blue crab Callinectes (Lee et al., 1976) and in three species of fish (Lee et al., 1972b); for naphthalene in the rainbow trout (Varanasi et al., 1978); and for the chlorinated hydrocarbon lindane in Nereis virens (Goerke and Ernst, 1980).

Incorporation into cellular macromolecules appears to be the major fate of BA accumulated by Nereis virens. Varanasi et al. (1981) reported binding to DNA and protein as the major fate of BP force fed to juvenile English sole. Lu et al. (1977) in their studies on the fate of ^{14}C-BP in model ecosystems also found the majority of radioactivity recovered from organisms possessing MFO activity was unextractable. Incoroporation of accumulated radiolabeled PAH into invertebrate exoskeletons has been observed in several studies (Lee et al., 1976; Leversee et al., 1982; and

Malins and Roubal, 1982). In concert these studies indicate that incorporation of a major percentage of accumulated PAH into relatively stable cellular macromolecules may be a widespread phenomenon in marine organisms.

Summary:

These results demonstrate that Nereis virens is capable of rapid in vivo metabolism of BA in the benthos. A possitive correlation was observed between the degree to which accumulated BA was metabolized and efficiency for accumulation of BA introduced in different ways. It appears that excretion of water soluble BA conjugates in Nereis is not as rapid as initial metabolism, and that incorporation into cellular macromolecules is a major fate of accumulated BA.

CHAPTER 5:
PHYSIOLOGICAL EFFECTS OF BENZ(a)ANTHRACENE EXPOSURE ON *Nereis virens*

Results:

Biochemical indices:

Aryl hydrocarbon hydroxylase (AHH) activity was measured *in vitro* in microsomes prepared from homogenates of whole worms removed from the experimental chambers. This assay was done for two reasons: (1) to see if exposure to BA led to increased ability in the worms to metabolize PAH, and (2) as a check on the PAH metabolizing ability of the different groups of worms used throughout this study. Benzo(a)pyrene (BA) was used as a substrate for determining AHH activity.

Table 5.1 shows BP hydroxylase activity in microsomes prepared from whole worms used in these experiments. The rates were highly variable and quite low, ranging from 0.68 to 2.60 pmol/mg-min with an average coefficient of variation of 34%. No substantial or consistent differences were observed between control and exposed groups in any experiment or between worms used in different experiments. These results provide no evidence for induction of the MFO system by exposure to BA in these experiments. The similarity between rates observed in different experiments also indicates that all worms used in these experiments had approximately the same ability to metabolize PAH.

Concentrations and ratios of adenylate nucleotides extracted from worm tissue in each experiment are given in Table 5.2. In all experiments except No. 5 where the animals were fed, worms exposed to BA showed trends for lower ATP concentrations and higher ADP and AMP concentrations. The concentration of total adenylates showed no consistent trends

Table 5.1

BENZO(a)PYRENE HYDROXYLASE ACTIVITY IN MICROSOMES PREPARED FROM *Nereis virens* HOMOGENATES

	pmol/mg-min	
	Control Worms	Exposed Worms
Experiments with Sediment-sorbed BA		
6 day exposure	0.89±.25	2.19±.18
6 day exposure	0.68±.27	0.78±.17
25 day exposure	1.04±.05	1.24±.37
Experiment with BA Added to the Water Column		
6 day exposure	2.60±.20	1.12±.38
Experiment with BA Labeled Food		
4 day exposure	1.60±.20	1.67±.22

Values expressed as mean ±1 SE n=3

Assays run in triplicate on microsomes pooled from 3 replicate chambers for each experiment.

Table 5.2
ADENYLATE NUCLEOTIDE POOLS AND RATIOS IN WORMS TISSUE

		Experiment 1	2	3	4	5
Concentrations						
ug/gww						
ATP	Control	1.58 ±.09	2.20 ±.13 *	2.40 ±.16	2.45 ±.72 *	4.55 ±.45
	Exposed	1.49 ±.11	1.77 ±.09	2.30 ±.13	1.79 ±.20	4.75 ±.54
ADP	Control	.815 ±.062 *	1.02 ±.57	1.13 ±.13	.873 ±.072	1.32 ±.13
	Exposed	1.04 ±.07	1.02 ±.09	1.34 ±.16	1.08 ±.17	1.02 ±.19
AMP	Control	.320 ±.034	.225 ±.020	.272 ±.030	.224 ±.023	.334 ±.057
	Exposed	.355 ±.064	.282 ±.032	.285 ±.037	.247 ±.052	.306 ±.057
A_T	Control	2.72 ±.12	3.50 ±.14 *	3.88 ±.13	3.59 ±.22	3.54 ±.38
	Exposed	2.86 ±.16	3.04 ±.15	4.93 ±.26	3.12 ±.37	5.19 ±.63
Ratios						
EC	Control	.731 ±.016	.784 ±.012 *	.784 ±.019	.816 ±.009 *	.819 ±.014
	Exposed	.706 ±.013	.746 ±.016	.762 ±.014	.754 ±.019	.848 ±.014
ADP/ATP	Control	.533 ±.045 *	.480 ±.037 *	.504 ±.095	358 ±.026 *	353 ±.036
	Exposed	.749 ±.086	.598 ±.057	.595 ±.077	.604 ±.071	.255 ±.029
AMP/ATP	Control	.215 ±.028	.110 ±.014 *	.113 ±.016	.090 ±.009 *	.092 ±.012
	Exposed	.230 ±.027	.170 ±.054	.125 ±.018	.138 ±.023	.081 ±.012

Values expressed as mean ± SE. Square root transformations of ratios were done prior to statistical analysis. Means compared using Student's t-test. * indicates significant difference ($p<.05$).

between experiments. Even though changes in the concentrations of individual nucleotides were consistent, these differences were not consistently statistically significant due to high variability between concentrations in different animals. AMP concentrations were never significantly different between exposed and control animals.

Energy charge (EC) was depressed in worms exposed to BA in all experiments except No. 5 where the animals were fed. Again, these trends were only significant in two out of four cases. The ADP/ATP ratio was found to be significantly higher in exposed animals in all of the short-term experiments but No. 5. Although the ADP/ATP ratio was not significantly different in the long-term exposure to sediment-sorbed BA, the magnitude of the difference between control and exposed groups was the same for this experiment as it was in two short-term experiments where the differences were found to be statistically significant. The AMP/ATP ratio was also higher in exposed animals in all experiments but No. 5, but this difference was only statistically significantly different in the second experiment with sediment-sorbed BA and in the water column exposure experiment. In the experiment with BA labeled food, opposite trends were observed for all indices, with control animals having lower ATP and higher ADP and AMP concentrations, and lower EC and higher ADP/ATP and AMP/ATP ratios than exposed animals. These differences were never significant even though the sample size was larger in this experiment. The total dose of BA to the chambers, or even the accumulated dose, in the experiment with labeled food was more than an order of magnitude less than observed in the other experiments. Therefore it is not surprising that no differences between control and exposed groups were observed.

Concentration of ATP, ADP, total adenylates, and the ADP/ATP ratio in control animals used in experiment 5 were significantly different from values obtained from animals used in the other experiments (See Table 5.3). These differences indicate that the metabolic potential of recently fed animals was different from animals that had been starved for a period of days to weeks, which is not an unexpected result. Significant differences were also seen in total adenylate concentrations and EC and AMP/ATP ratios between control worms in the first experiment and all others. These differences indicate that natural variability in all these indices, both concentrations of individual nucleotides and their ratios, is high. Analysis of variance indicated statistically significant differences between values for all indices between control worms used in each experiment. These differences may be due to dietary status, as mentioned above, seasonal or reproductive status, or just natural variability. Regardless of cause, variations in adenylate nucleotide concentrations and ratios of this magnitude make using absolute values of these indices as indicators of environmental stress equivocal.

Whole animal physiologic indices:

Whole chamber oxygen consumption and ammonia production were measured periodically during each experiment (Figures 5.1-5.5). Rates were highly variable between replicate chambers and with time. Analysis by two-way ANOVA comparing the effect of treatment and time (Tables 5.4 & 5.5), showed that only in the 25 day sediment exposure experiment were significant differences due to treatment observed in oxygen consumption rate between control and exposed chambers. Both oxygen consumption rates and ammonia excretion rates were strongly affected by time, although no

Table 5.3

SUMMARY OF NEWMAN-KEULS (SNK) ANALYSES FOR DIFFERENCES IN ADENYLATE NUCLEOTIDE CONCENTRATIONS AND RATIOS BETWEEN MEANS FROM CONTROL WORMS USED IN DIFFERENT EXPERIMENTS

Concentrations ug/gww	Experiment					
ATP	1	2	3	4	5	$p<.001$
ADP	1	2	3	4	5	$p<.01$
AMP	1	2	3	4	5	
A_T	1*	2	3	4	5**	*$p<.05$ **$p<.001$
Ratios						
EC	1	2	3	4	5	$p<.05$
ADP/ATP	1	2	3	4	5	$p<.05$
AMP/ATP	1	2	3	4	5	$p<.001$

Line joining groups indicates they are not statistically different

A_T = ATP+ADP+AMP

$$EC = \frac{1/2(ADP)+ATP}{A_T}$$

Experiment 1&2: 6 day exposure to sediment-sorbed BA
Ke 3: 25 day exposure to sediment-sorbed BA
4: 6 day exposure to BA added to the water column
5: 4 day exposure to BA in diet

Figure 5.1: Whole chamber oxygen consumption, ammonia production, and the O/N ratio in experiment 1. Circles represnt control chambers. Triangles represent exposed chambers. Values expressed as mean $\pm$ SE n=3.

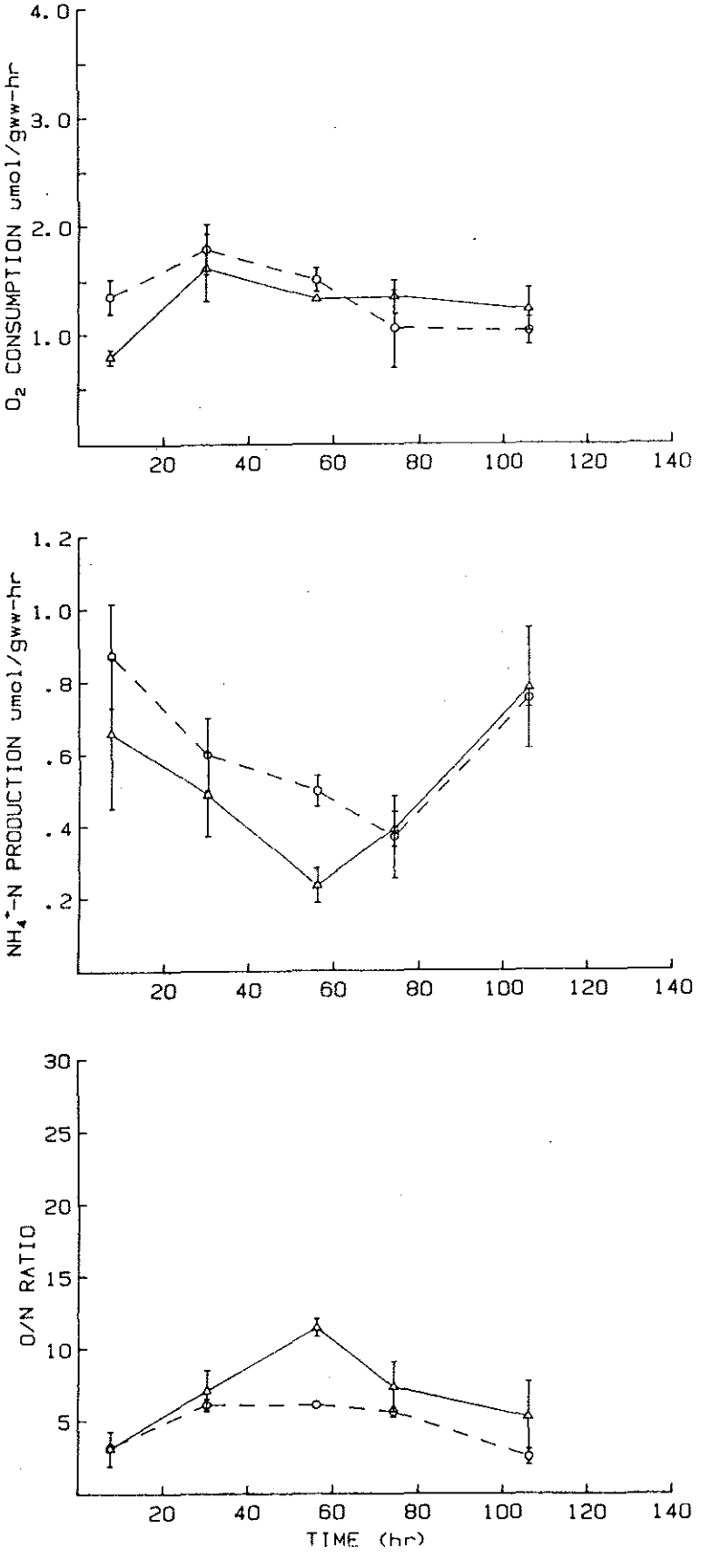
4.0
3.0
2.0
1.0
O_2 CONSUMPTION umol/gww-hr
1.2
1.0
.8
.6
.4
.2
NH_4^+-N PRODUCTION umol/gww-hr
30
25
20
15
10
5
O/N RATIO
20
40
60
80
100
120
140
TIME (hr)

Figure 5.2: Whole chamber oxygen consumption, ammonia production, and the O/N ratio in experiment 2. Circles represnt control chambers. Triangles represent exposed chambers. Values expressed as mean $\pm$ SE n=3.

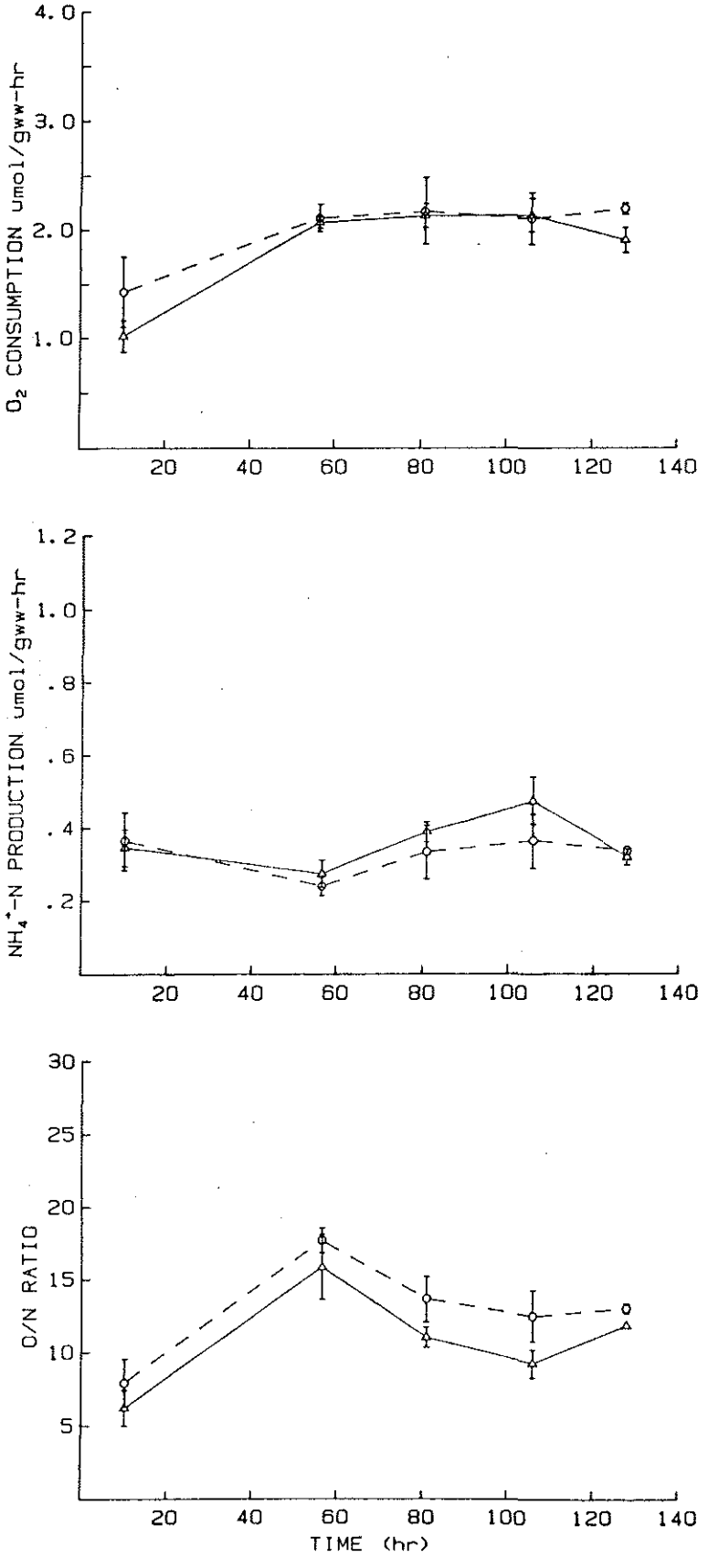
4.0
3.0
2.0
1.0
O_2 CONSUMPTION umol/gww-hr
1.2
1.0
.8
.6
.4
.2
NH_4^+-N PRODUCTION umol/gww-hr
30
25
20
15
10
5
O/N RATIO
20
40
60
80
100
120
140
TIME (hr)

Figure 5.3: Whole chamber oxygen consumption, ammonia production, and the O/N ratio in experiment 3. Circles represnt control chambers. Triangles represent exposed chambers. Values expressed as mean $\pm$ SE n=3.

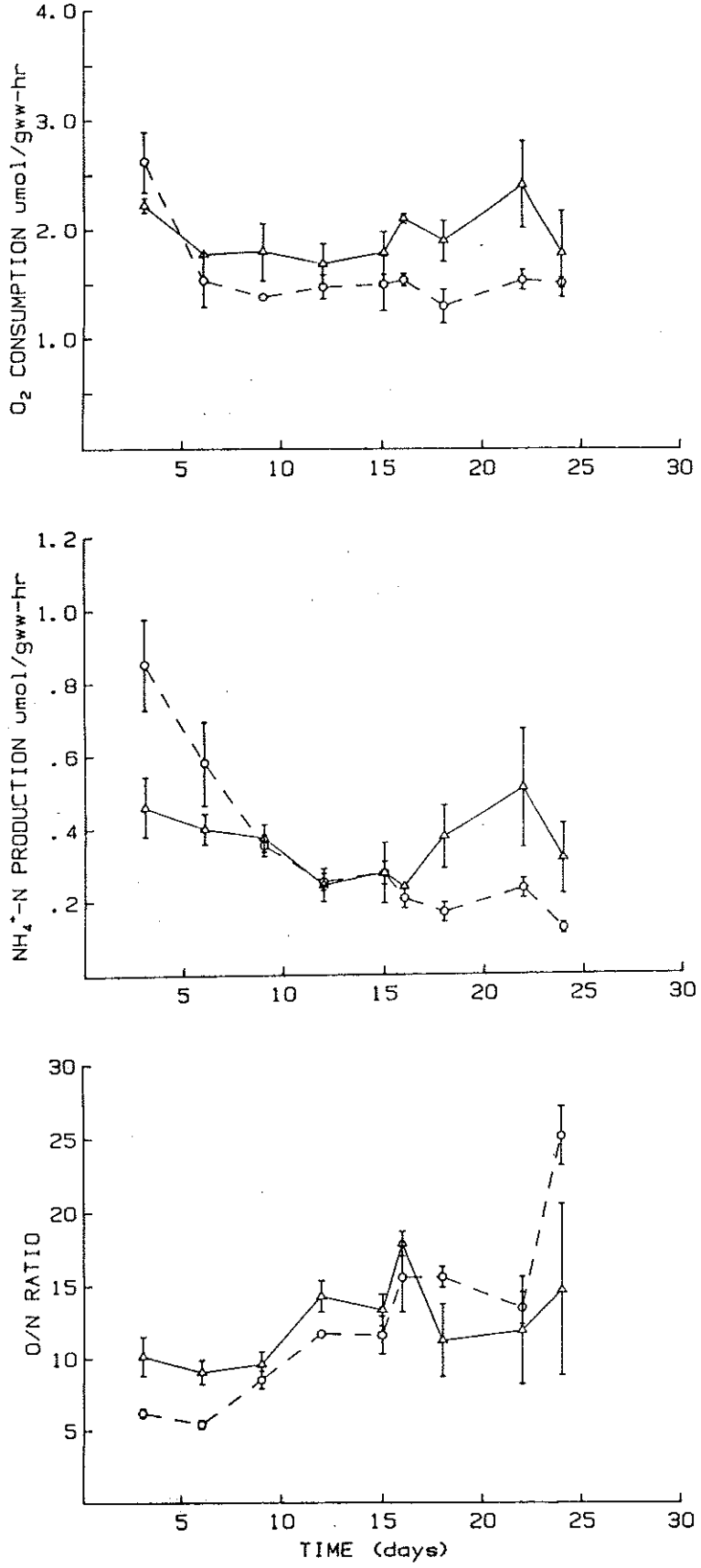
4.0
3.0
2.0
1.0
O_2 CONSUMPTION umol/gww-hr
5
10
15
20
25
30
1.2
1.0
.8
.6
.4
.2
NH_4^+-N PRODUCTION umol/gww-hr
5
10
15
20
25
30
30
25
20
15
10
5
O/N RATIO
5
10
15
20
25
30
TIME (days)

Figure 5.4: Whole chamber oxygen consumption, ammonia production, and the O/N ratio in experiment 4. Circles represnt control chambers. Triangles represent exposed chambers. Values expressed as mean $\pm$ SE n=3.

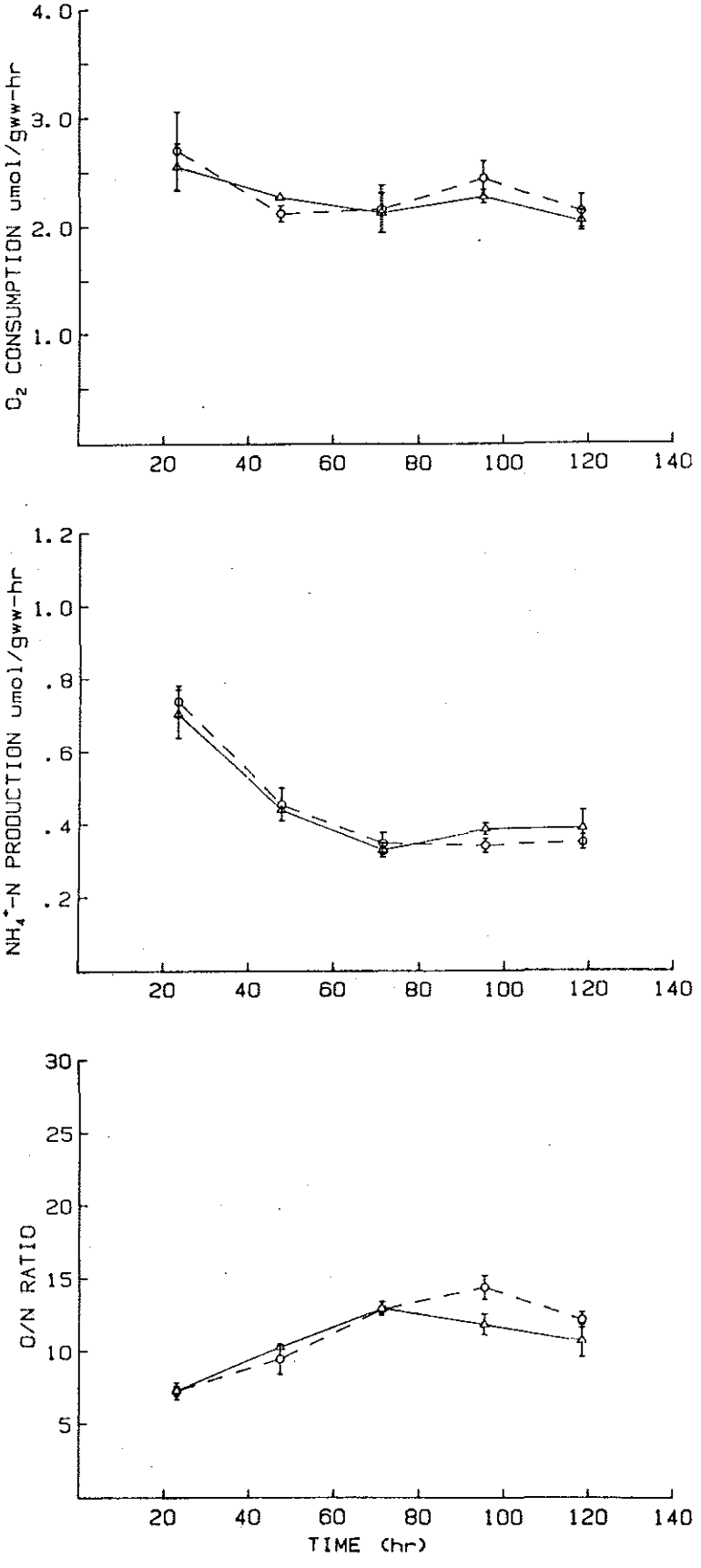
4.0
3.0
2.0
1.0
O_2 CONSUMPTION umol/gww-hr
1.2
1.0
.8
.6
.4
.2
NH_4^+-N PRODUCTION umol/gww-hr
30
25
20
15
10
5
O/N RATIO
20
40
60
80
100
120
140
TIME (hr)

Figure 5.5: Whole chamber oxygen consumption, ammonia production, and the O/N ratio in experiment 5. Circles represnt control chambers. Triangles represent chambers with BA. Values expressed as mean $\pm$ SE n=4.

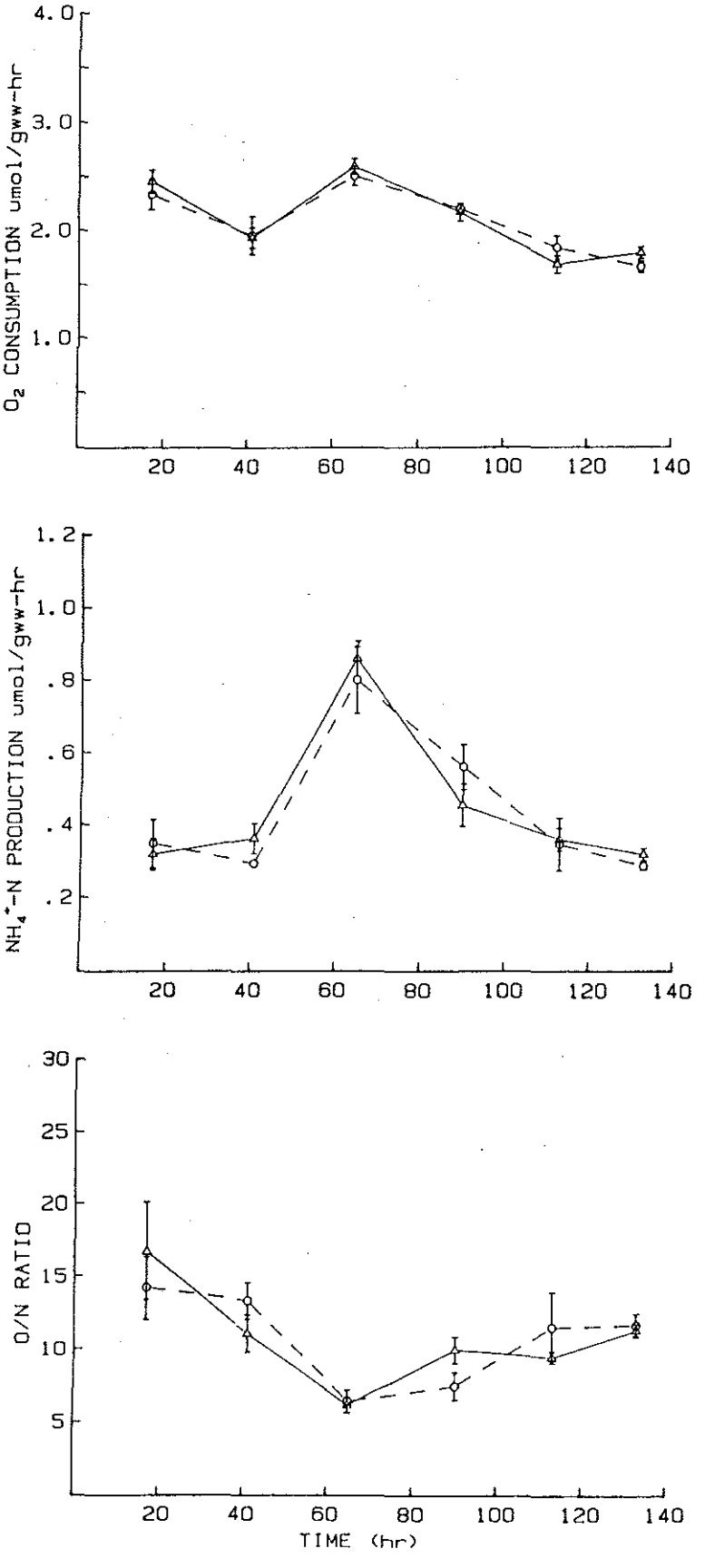
4.0
3.0
2.0
1.0
O_2 CONSUMPTION umol/gww-hr
1.2
1.0
.8
.6
.4
.2
NH_4^+-N PRODUCTION umol/gww-hr
30
25
20
15
10
5
O/N RATIO
20
40
60
80
100
120
140
TIME (hr)

Table 5.4

SUMMARY OF TWO-WAY ANALYSIS OF VARIANCE ON THE EFFECTS OF BENZ(a)ANTHRACENE EXPOSURE AND TIME ON WHOLE CHAMBER OXYGEN CONSUMPTION

	Exposure	Time	Interaction
Exposure to Sediment-sorbed BA			
6 days	ND	*	ND
6 days	ND	*	ND
25 days	*	*	ND
Experiment with BA Added to the Water Column			
6 days	ND	ND	ND
Experiment with BA Labeled Food			
4 days	ND	*	ND

* Control chambers significantly different from exposed chambers ($p<.05$)

ND No difference

Table 5.5

SUMMARY OF TWO-WAY ANALYSIS OF VARIANCE ON THE EFFECTS OF BENZ(a)ANTHRACENE EXPOSURE AND TIME ON WHOLE CHAMBER AMMONIA PRODUCTION

	Exposure	Time	Interaction
Exppsure to Sediment-sorbed BA			
6 days	ND	*	ND
6 days	ND	ND	ND
25 days	ND	*	*
Experiment with BA Added to the Water Column			
6 days	ND	*	ND
Experiment with BA Labeled Food			
4 days	ND	*	ND

* Control chambers significantly different from exposed chambers ($p<.05$)

ND No difference

obvious trends with time were observed. In experiment 3 an interactive effect between time and treatment was observed.

Oxygen consumption and ammonia production were also measured on chambers without worms in experiments 1-5. Rates were generally low and constant over time accounting for 29 and 20%, respectively, of rates seen in chambers with worms. The physical and biological characteristics of chambers without worms did not represent realistic controls for microbial respiration in chambers with worms, thus worm chamber oxygen consumption and ammonia production rates were not corrected for microbial input. Respiration and ammonia production rates for chambers with worms include both worm and microbial contributions, and therefore should not be construed to be absolute values for worms alone, but comparative estimates between treated and control animals.

One caveat to the generally low and consistent ammonia production rates seen in chambers without worms was observed in the long-term experiment with sediment-sorbed BA. In this experiment a large increase in ammonia production was observed in chambers without worms beginning on day 6, where ammonia production was equal to or exceeded that seen in chambers with worms. This increase in ammonia production could have been due to several factors. Although none of the chambers appeared to be anoxic, either by visual inspection or analysis of redox potential, it is likely that sediments in chambers with worms were more oxidized than unbioturbated sediment. Therefore, although not measurable, there may have been patches of reducing environments were bacterial ammonia release might have occurred. The discrepancy between ammonia production in chambers with and without worms may also have been due to increased

uptake of dissolved ammonia by microbes in chambers with worms. This possibility is supported by the elevated $^{14}CO_2$ flux seen in chambers with worms in this experiment (See Chapter 3).

The atomic ratio of oxygen consumed to nitrogen excreted, calculated here from the oxygen consumption and ammonia excretion rates measured, gives an indication of the relative reliance on carbohydrate and lipid vs. protein reserves used for energy (Mayzaud, 1973). Considering the variability in oxygen consumption rates and ammonia excretion rates, it is not surprising that no large differences were seen between control and exposed chambers (See Figures 5.1-5.5). Only at the end of the 25 day experiment with sediment-sorbed BA (Fig. 5.3) did O/N ratios for control chambers appear to diverge from those for exposed chambers. Analysis of variance indicated that there was a statistically significant effect of time and a significant time/treatment interactive effect in this experiment, although the effect of treatment alone was not significantly different. The relative decrease in O/N seen in exposed chambers indicates that worms exposed to BA were relying more heavily on protein reserves than control worms.

No consistent significant changes in weight were observed between control and exposed worms (Table 5.6). In the long-term experiment with sediment-sorbed BA there was a trend for increased weight loss in exposed worms, but the significance level of the difference was only 94%. The large variability in weights is primarily due to the necessity to pool worm weights from each chamber, there being no way to identify individual worms.

Discussion:

Table 5.6

WEIGHT CHANGE OF *Nereis virens* DURING EXPERIMENTS

		Weight Change Initial-Final gww	Initial/Final Weights
Experiments with Sediment-sorbed BA			
6 days	Control	.938±.263	.910
	Exposed	-.167±.280	1.01
6 days	Control	-.216±.082	1.01
	Exposed	-.869±.444	1.05
25 days	Control	2.032±.132	.874
	Exposed	2.834±.496	.814
Experiment with BA Added to the Water Column			
6 days	Control	-.314±.108	1.02
	Exposed	-.017±.085	1.00
Experiment with BA Labeled Food			
4 days	Control	-.119±.365	1.01
	Exposed	-.050±.085	1.00

Values expressed as mean ±SE Exp. 1-4 n=3, Exp.5 n=4

In general the physiological effects of BA on Nereis virens observed in these experiments were minimal. The dose of this single PAH of less than 10 ppm in the sediment is similar to concentrations seen in sediments receiving substantial petroleum input from urban run-off (Readman et al., 1982). However, on a total hydrocarbon basis, even with the added BA, toxicant levels were not significantly higher than those encountered in relatively clean coastal sediments (Youngblood and Blumer, 1975). Lethal concentrations for the WSF of fuel oil for polychaete worms have been estimated to be on the order of 1 to 20 ppm in water (Carr and Reish, 1977), so it is not surprising that dramatic effects were not observed in this investigation. However, the subtle effects seen in adenylate nucleotide pools in as little as 6 days with whole animal physiological effects beginning to become apparent after a period of weeks, suggest that the multilevel approach used in this investigation has utility in identifying sublethal bioenergetic perturbations. Whether or not effects at this level would lead to significant whole animal or population level effects would require experimentation on a much longer time scale.

Activity of the mixed function oxygenase system measured using BP as a substrate in a microsomal preparation made from whole worm homogenates averaged only 1.38 pmol/mg-min for control and exposed groups in all experiments. Although this rate is low in comparison to rates reported for AHH activity in teleost fish (Stegeman, 1979), it is comparable to a value of 2.5 $\pm$0.8 pmol/mg-min reported for crude Nereis virens homogenates by Lee et al. (1981). Analysis of the whole worm results in a large dilution of intestinal tissue, where the majority of MFO activity in

Nereis virens has been reported (Lee et al., 1979). AHH activity in microsomes prepared from intestinal tissues of Nereis virens taken from the same group of worms used form this study averaged 88.9 ±1.8 pmole/mg-min (n=7) (McElroy, unpublished data); a much more respectable rate. Unfortunately, using just intestinal tissue for the MFO assay was prohibited by the necessity of also using worm tissue for analysis of adenylate nucleotide pools and BA accumulation.

In investigations of MFO activity in intestinal microsomes, Lee has reported increases in BP hydroxylase activity 48 hours after feeding Nereis clams contaminated with BA (Lee et al., 1979). The actual dose delivered to the worms was not determined, but potentially could have been much higher than exposures in this experiment. Subsequent experiments by Lee failed to detect significant increases in BP hydroxylase activity in Nereis given food contaminated with extremely high concentrations (1 mg/g) of BP continuously for a period of four to eight weeks (Lee et al., 1981). However, BP hydroxylase activity and P-450 concentrations in Nereis collected from an oiled environment were significantly higher than in worms collected from a control site in Maine. These results suggest that the indication of induction observed in the field may have been due to factors other than PAH exposure, that induction of the MFO system in Nereis virens by PAH may require either exposure to very high levels, or that the induction process is extremely slow, requiring a period of months. Although the question of induction of the MFO system in Nereis virens remains equivocal, the absence of evidence for induction in these experiments can be used to support the similar abilities of worms used in different experiments to metabolize BA.

Decreases in energy charge reported here after short-term exposure to BA are consistent with other studies showing decreases in energy with sublethal stress such as exposure to nickel in Mytilus edulis (Zaroogian et al., 1982), stress due to filtration of algal cultures (Jewson and Dokulil, 1982), changes in metabolism during temperature acclimation in trout (Walesby and Johnston, 1980), anoxia in Spartina alterniflora roots (Mendelson, 1981), exposure in Fundulus grandis to low pH water (MacFarlane, 1981), anoxia in sea anemones (Ellington, 1981), tubificid worms (Schottler, 1978), and Arenicola marina (Surholt, 1977). Adenylate nucleotide ratios have also been implicated in regulation of glutamate dehydrogenase activity in brackish water clams (Matsushima and Kado, 1983). However, a similar number of studies have reported no consistent change in energy charge in response to temperature adaptation in sea anemones (Walsh and Somero, 1981), in response to zinc in lobster (Haya et al., 1983), in isopods rendered moribund by exposure to toluene (Skjoldal and Bakke, 1978), temperature stress on diatoms (Falkowski, 1977), and after starvation in crayfish (Dickson and Giesy, 1982). Since very different methods were used to analyze nucleotides in these experiments it is difficult to compare these results as various methods are better suited for different organisms (Karl and Holm-Hansen, 1978). Based on these results the general applicability of energy change is somewhat limited and probably very species and process dependent.

The results of this present study support the use of adenylate nucleotide ratios as a relative index of metabolic perturbation only when comparing groups in carefully controled laboratory exposures. The results also suggest that this measurement can be useful in signalling

changes in the metabolic potential of an organism before effects are noticed at the whole animal level. Oxygen consumption and ammonia production rates measured here on Nereis virens exposed to BA were highly variable, and were only significantly different from controls in the longer-term experiment with sediment-sorbed BA where a significant increase in oxygen consumption, a time dependent increase in ammonia production, and a decrease in the O/N ratio were observed. Similar observations have been reported for oxygen consumption and ammonia excretion in larval lobsters exposed to oil/water dispersions of crude oil (Capuzzo et al., 1984). These results indicate that after prolonged exposure to BA, Nereis expended more energy maintaining basal metabolism, and showed an increased reliance on protein reserves than control worms.

Significant differences were not observed in growth rate in these experiments. However, it is likely that the trend observed for increased weight loss in exposed animals would have become significant with time. Carr and Neff (1984) reported significant differences between glycogen content of Nereis virens collected from petroleum contaminated and control environments in Maine. They found similar results in laboratory experiments in response to starvation or long term exposure to pentachlorophenol (Carr and Neff, 1981).

The variability in oxygen consumption rates, and ammonia excretion rates observed here are probably largely due to intermittent burrow irrigation and respiratory activities in these worms, a phenomenon well documented in burrowing organisms in general (Mangum, 1964) and in Nereis virens in particular (Scott, 1976). Making these measurements on whole chambers containing 4 animals with a water column residence time of 2.3

hours would tend to produce a time-averaged rate which should have smoothed some of the individual and time related variability. However, microbial activity probably added variability to these measurements. Even with these caveats, oxygen consumption and ammonia production rates measured here compared well with those reported for Nereis virens by Kay and Brayfield (1973) and Scott (1976). Additionally, the increase in both oxygen consumption and particularly ammonia producton seen after feeding in experiment 5 was exactly what would be expected after consumption of a protein rich meal (Lied and Braaten, 1984, Jobling and Davis, 1980), validating use of these microcosms as metabolic chambers.

It is possible that BA exposure caused changes in the worm irrigation or burrowing rate as has been observed in other polychaetes exposed to oiled sediments (Gordon et al., 1978, Augenfeld et al., 1980-81). Quantitative measurement of either irrigation rate or burrowing rate were not possible in this experiment, but visual observation of worm position in the sediment, fecal pellet production, and the number of burrow openings indicated no obvious differences between control and exposed groups. If exposed worms were irrigating their burrows at reduced rates, reduced oxygen consumption rates should have been observed. The enhancement of oxygen consumption in chambers with worms in the long-term experiment suggests that suppression of burrow irrigation due to BA exposure was not occurring.

Summary:

In these experiments Nereis virens accumulated and metabolized up to ppm levels of BA, yet biochemical and physiological measurments traditionally associated with stress were only minimally affected. It appears

that at least for the short term Nereis was capable of adapting to exposure to BA at these concentrations, or that BA is not particularly detrimental to Nereis. The small increases in respiration and ammonia excretion observed in the 25 day exposure experiments suggests that with time exposure to BA at this level may have deliterious effects.

CHAPTER 6:
SUMMARY

The benthos is a complex environment where biological, physical and chemical processes are continually interactive. In this study of the fates and effects of PAH in the benthos, an interdisciplinary approach was undertaken, focusing on interrelations between the source of PAH to the benthos, PAH metabolism, and animal/chemical/sediment interactions. Although the results are separated into chapters dealing with the overall fate of BA in the system, in vivo BA metabolism by Nereis virens, and the physiological effects of BA exposure on Nereis virens, from an experimental standpoint, much information would have been been lost if any part of this investigation had been undertaken in isolation.

Many studies on the effects of pollutant compounds are weakened by the lack of detailed chemical characterization of the dose and its bioavailability. In many chemical studies on the fate of pollutant compounds, biological activity is evoked to take care of distributions that can not be explained purely by chemical or physical processes. In both cases, interpretations are limited by the absence of interdisciplinary data. Therefore, although the complexity of the experimental system made analysis and interpretation difficult, the results from this study provide biogeochemical and physiological information on both the fate and effects of a single PAH in an intact benthic system.

The results presented in Chapter 3 demonstrated that the mode of introduction had significant effects on the fate of BA in the experimental chambers. BA sorbed onto particles in the entire sediment reservoir was relatively refractory, with concentrations remaining unchanged from the start to finish of experiments lasting from 6 to 25 days. On the

other hand, BA added to the water column which became associated with the sediment-water interface, was much more labile. A significant portion of activity originally deposited at the sediment-water interface was removed via flux to the water column in 6 days. In addition, small but detectable rates of microbial mineralization were observed in this experiment as compared to undetectable rates observed in short-term experiments with sediments uniformly labeled with BA.

The presence of worms also had significant effects on the fate of BA in these chambers. In experiments with the sediment reservoir labeled with BA, the flux of BA to the water column was significantly higher in chambers with worms. Increased flux in chambers with worms was observed in both the short- and long-term exposure experiments, and the effect intensified with time. The presence of worms also increased microbial mineralization of BA. It was concluded that the effect of worms on flux rate was primarily due to their tubicolous lifestyle. In the experiment where BA was added to the water column, the presence of worms had no net effect on flux of BA sorbed to the sediment surface out into the water column. In this case the activity of worms resulted in mixing BA at the sediment-water interface down into the sediment reservoir.

Nereis was capable of accumulating BA, although efficiency of uptake was strongly affected by how the chemical was introduced. Bioavailability is very difficult to assess. However, consistent trends became apparent when both relative accumulation as a percentage of total available, and concentration ratios between the worms and the source of BA were considered. Under these experimental conditions, BA incorporated into a protein-based diet was most available, while BA sorbed to the bulk

sediment reservoir was least available for accumulation by Nereis.

Analysis of BA metabolites produced in vivo, as discussed in Chapter 4, clearly shows that most BA accumulated was rapidly metabolized to more polar metabolic products. A minimum of 2% and a maximum of 23% of accumulated BA remained as unmetabolized parent compound. In all but the short-term experiments with sediment-sorbed BA, most accumulated activity was present as either water soluble conjugated metabolites, or in an unextractable fraction that probably represented BA metabolites covalently bound to cellular macromolecules. PAH metabolite fractions are routinely missed by analytical procedures commonly utilized in hydrocarbon investigations. These results indicate that most of the literature on accumulated levels of PAH in marine organisms capable of PAH metabolism severely underestimate total accumulation and total exposure.

Mode of introduction also affected the degree to which accumulated BA was metabolized. The proportion of total BA-derived radioactivity accumulated and remaining as parent compound was inversely proportional to the efficiency of accumulation. In addition, the relative proportions of specific metabolite pools and the pattern of polar metabolites formed were different in experiments with different modes of exposure. Since different metabolic pools have widely different biological and chemical reactivity, these results indicate that not only are PAH from different sources disproportionately available for accumulation by benthic organisms, but in addition, longterm effects produced by the presence of these various metabolites may be quite different.

The high percentage of accumulated activity in the conjugate and unextractable pool relative to that retained as parent BA or polar meta-

bolites implies some interesting conclusions about *in vivo* BA metabolism in *Nereis*. Firstly, these results suggest that primary metabolism to polar metabolites is a rapid process, and that polar metabolites are further transformed as rapidly as they are produced. Secondly, the build up of metabolic products in the later two pools suggests that excretion of water soluble metabolites is relatively slow, and that binding to cellular macromolecules is a major endproduct of BA accumulation. The high percentage of accumulated activity in these two pools, and the evidence suggesting minimal formation of sulfate and glucuronide conjugates, suggest that BA metabolism in *Nereis* is dominated by the highly reactivie epoxide intermediate leading to the formation of macromolecular adducts and possibly glutathione conjugates preferentially over the formation of less reactive metabolites such as phenols, sulfate and glucuronide conjugates, metabolites that can be readily excreted. As binding to cellular macromolecules such as DNA, RNA, and protein is considered a toxification pathway, this finding suggests that PAH metabolism in *Nereis* may have delitereous consequences. Alternatively, binding to structural macromolecules such may provide a means to sequester xenobiotic substances in a manner analogous to metallothionein binding of metals.

Physiological effects of BA exposure to *Nereis virens* were minimal. Although the worms readily accumulated and metabolized up to ppm levels of BA, the single consistent quantifiable result was subtle perturbations in adenylate nucleotide pools. Only after a period of weeks of constant exposure to sediment-sorbed BA did changes in oxygen consumption and ammonia excretion begin to become evident. These results support the idea that *Nereis virens* as a relatively tolerant organism. These results

are also not inconsistent with the conclusion that over the short term, BA or its metabolites are not particularly toxic. They also indicate that attempts to measure physiological responses to low level stress should be of longer duration. Additionally, the effects observed would have been more powerful if they had been generated in response to a wide enough range concentrations to demonstrate some sort of dose repsonse curve. A great deal of natural variability was observed between control groups of worms both within and between experiments in all indices measured. These results also point out the fact that due to natural variability in physiological indices, particularly in invertebrates which are capable of tolerating wide fluctuations in their biochemical and physiological state, it is essential to use large numbers of organisms with closely matched controls when attempting to differentiate the effect of any kind of perturbation from normal variability.

The conclusions of the experiments discussed above describe the fate and metabolism of BA in controlled microcosms containing fine sediments and high densities of Nereis virens. Nevertheless, some of the conclusions of this study can be extrapolated to describe factors important to the fate and metabolism of PAH in the benthos. This study demonstrated that not all PAH reaching the benthos are equally available for bioaccumulation and metabolism, or removal by purely physical forces. Although PAH in a form similar to those generated by fossil fuel combustion could not be investigated in this study, the data and relative bioavailability of sediment-sorbed vs. initially dissolved PAH suggests that PAH released by combustion would be even less available for accumulation and metabolism. PAH in the water column which becomes associated with particulates

at the sediment water interface is likely to have a much shorter residence time than PAH buried in the sediment, even though buried PAH may still be in the bioturbated zone. The presence of infaunal organisms in environments with buried PAH are likely to enhance their removal, primarily due to bioturbation and irrigation activities, rather than metabolism. Although microbial mineralization of the larger (>3 ring) PAH occurs, this process will probably be insignificant relative to other processes acting to remove PAH.

PAH accumulated by marine organisms are likely to be metabolized quickly by any organism with a functional MFO system. Even though PAH metabolites are more polar than the unmetabolized parent compound, they have an appreciable residence time in the organism. Retention of BA metabolic products in Nereis and the efficiency of uptake and metabolism of BA introduced as food, as observed here, indicates that dietary transfer of PAH in the benthos is an important process. Dietary accumulation of PAH would provide a rapid vector for the transfer of PAH in benthic reserves to highly mobile fish and human consumers.

Results from the physiological effects portion of this work do not bear directly on the fate of PAH in the benthos. However, the absence of dramatic effects due to exposure to PAH or from the production and accumulation of metabolic products in Nereis virens suggests that, at least for the relatively short duration of these experiments, the physiological adaptive capacity of this worm was not exceeded by exposure to a single PAH, BA. The natural variability observed between control groups both within and between experiments in the absolute values of indices measured also points out the problems inherent with making these measurments on

field populations and using absolute values as indices of environmental stress.

These results suggest several avenues of further research. More specific information on *in vivo* metabolism of PAH by *Nereis* and other organisms is warrented. To assess to importance of the bound fraction of accumulated activity in organisms, it will be necessary to first determine what type of cellular macromolecules are involved. Secondly, kinetic experiments are needed to determine the speed of formation and persistence of these adducts. An attempt should be made to determine if *Nereis* really does preferentially form conjugates with glutathione. Also it would be interesting to know what other benthic organisms are capable of rapid *in vivo* metabolism of PAH. This information would be of interest to regulatory agencies which are increasingly looking at residues of toxic substances in benthic organisms as an index of bioavailability and exposure of marine communities to contaminated sediments. Further work on routes of uptake for substances like PAH by inaunal organisms would be interesting, particularly addressing dietary vs. body surface pathways. Additionally, the question of bioavailability of PAH liberated by combustion vs petroleum source PAH still needs to be addressed.

CONCLUSIONS

1. The majority of BA accumulated by Nereis was metabolized, regardless of how BA was introduced.

2. The primary fate of accumulated BA in Nereis is incorporation into macromolecular components.

3. Nereis accumulated and metabolized BA most efficiently from labeled food in comparison to BA previously sorbed to sediment particles.

4. BA loosely associated with particles at the sediment-water interface was much more labile than BA sorbed to the entire sediment reservoir. It was accumulated and metabolized more rapidly by Nereis, was removed more rapidly via diffusion, and was mineralized more readily by microbes.

5. The presence of worms had significant effects on the distribution and removal of PAH from the benthos.

6. Under the conditions of these experiments, Nereis virens was only minimaly affected by accumulation and metabolism of up to ppm concentrations of BA.

APPENIDX 1
BIOGEOCHEMISTRY OF BENZ(a)ANTHRACENE IN RECIRCULATING BENTHIC MICROCOSMS

Introduction:

This study was the final portion of a larger investigation on the biogeochemistry of petroleum components at the sediment-water interface under the direction of J.W. Farrington and J.M. Teal. Two recirculating benthic microcosms were used to look at the metabolism of ^{14}C-labeled benz(a)anthracene (BA) at the sediment-water interface. This experiment was a collaborative effort between J.W. Farrington, J.M. Teal, B.W. Tripp, and myself. Appendix 1 provides a brief description of the experiment and results obtained.

Methods:

Methods used in this investigation were esscentially the same as those described in Chapter 2 with the following exceptions. Two 230 l recirculating benthic chambers (Winget, 1978) each containing a 0.25 m^2 sediment box were used in this experiment. Sediments were collect from Buzzards Bay (See Chapter 2 for location and method), frozen, and then placed in the sediment trays of the chambers. Seawater was recirculated over the sediment reservoir for approximately two weeks prior to addition of the spike. ^{14}C-BA was added in a carrier mixture of aromatic hydrocarbons the total dose calculated to be the same as that used by Hinga et al. (1981) in their investigation of BA metabolism in the MERL microcosms at URI. After spike addition, sediment cores, and water samples were collected periodically over 42 days for analysis of extractable ^{14}C-labeled compounds in the water column and sediment cores, and for $^{14}CO_2$ in the water column. The sediment reservoir was divided into two com-

partments by a metal partition placed parallel to the direction of flow. On day 17 approximately 40 Nephtys incisa were placed in one portion of each chamber. The worms were retrieved at the end of the experiment. Extraction of sediment and worm tissue was done using tetrahydrofuran, ethylacetate, and methanol following methods described in Hinga et al. (1981).

Results and Discussion:

BA added to the water column quickly became associated with suspended particulates. Over the coarse of the experiment, concentration of BA in suspended particulate decreaed steadily (Figure A-1). Due to the small amount of radioactivity in the water column, it was not possible to analyze water samples for polar BA metabolites. Microbial mineralization of BA to carbon dioxide was observed with concentrations of $^{14}CO_2$ increasing logarithmically over the first 18 days, then remaining relatively constant for the remainder of the experiment (Figure A-2). The kinetics of BA mineralization to CO_2 were very similar to those observed by Hinga et al.(1981) in the much larger MERL microcosms.

Most of the isotope added to the chambers quickly became associated with the sediment reservoir. After 42 days, 98% of radioisotope recovered from the chambers was found in the sediment, with more than 97% located in the top 2 cm (Table A-1). Figures A-4, A-5,and A-6 show radio- and UV chromatograms of HPLC analysis of organic extracts of representative sediment samples taken throughout the experiment. For comparison, figure A-3 shows the UV chromatogram of representative authentic BA metabolite standards. Unfortunately the specific activity of isotope in these samples was too low to say much about production of

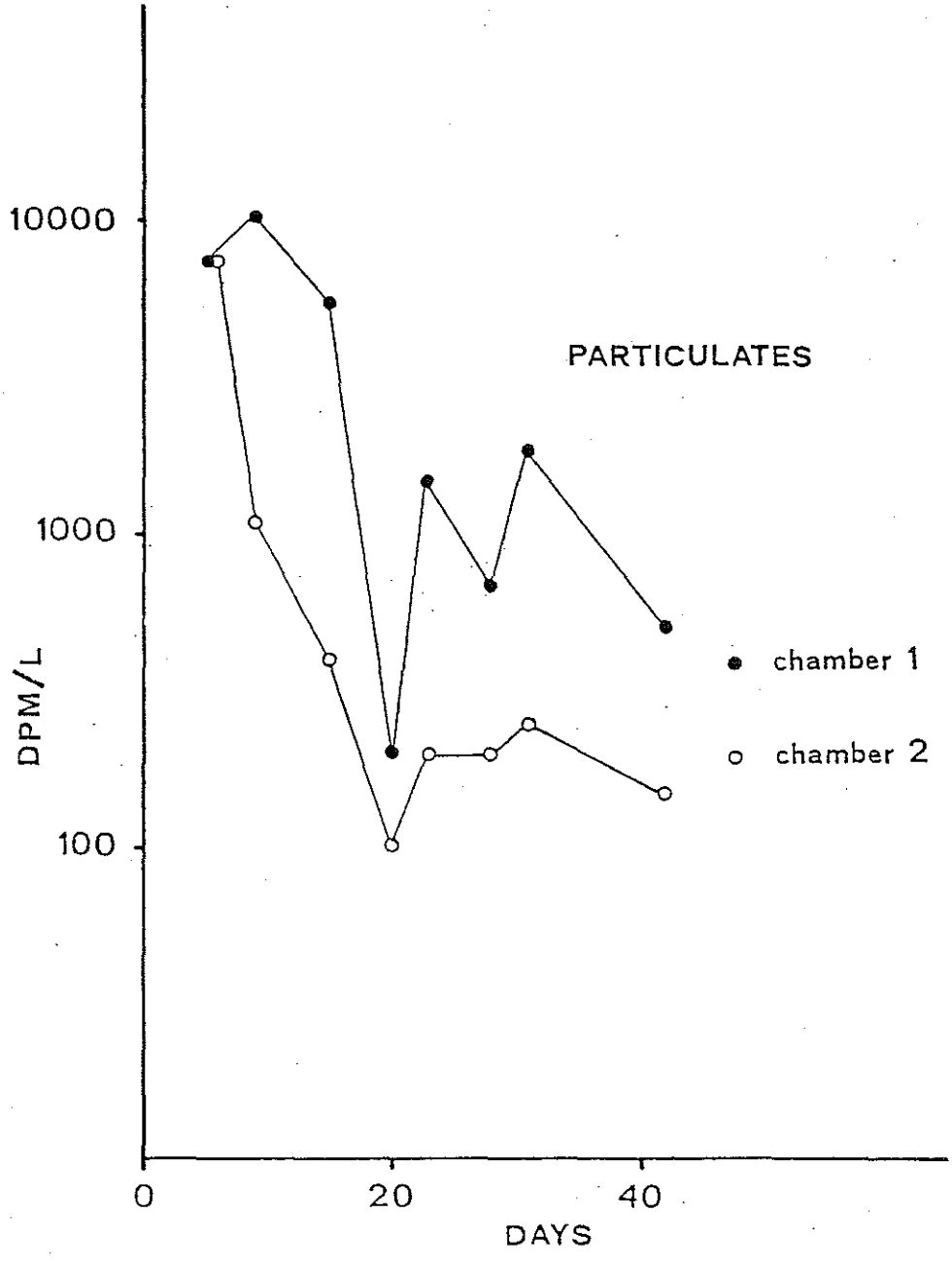

Figure A-1: Radioactivity recovered on suspended particulates.

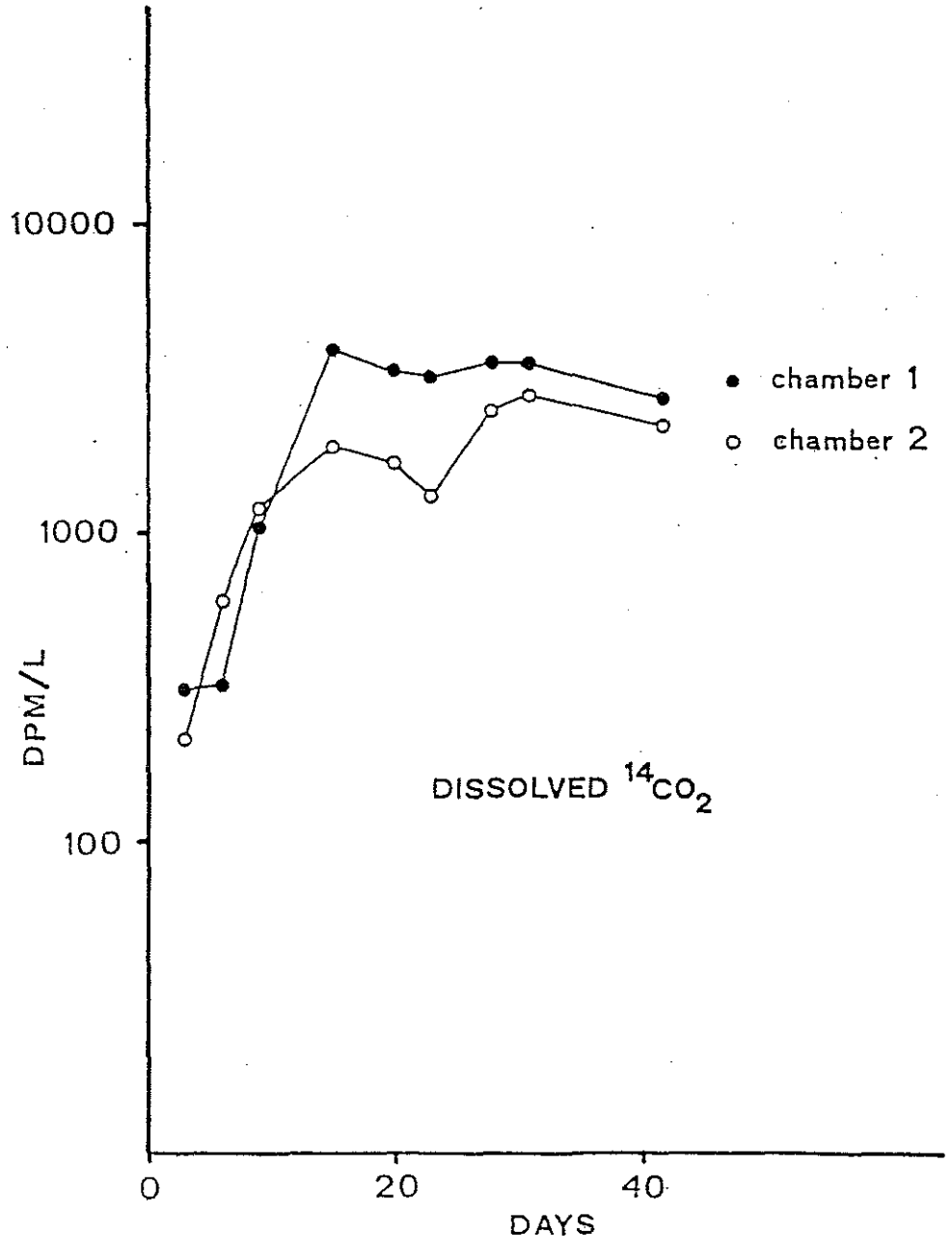

Figure A-2: Concentration of $^{14}CO_2$ in the water column.

Table A-1:

DISTRIBUTION OF ^{14}C AT END OF 42 DAY EXPERIMENT

	PERCENT OF ^{14}C-BENZANTHRACENE RECOVERED					
	CHAMBER 1			CHAMBER 2		
SEDIMENT	A	B	A+B	A	B	A+B
0-1 cm	37.29	48.82	86.12	56.14	24.40	80.73
1-2 cm	4.96	5.20	10.19	1.10	15.50	16.62
2-4 cm	1.14	0.34	1.48	0.17	0.54	0.71
TOTAL			97.79			98.06
WORMS			0.01			0.02
SUSPENDED PARTICULATES			0.28			0.09
DISSOLVED CO_2			1.48			1.52
DISSOLVED			0.47			0.30
TOTAL DPM RECOVERED			4.25×10^7			3.42×10^7
PERCENT OF ^{14}C-BENZANTHRACENE ORIGINALLY ADDED TO CHAMBER			54.1			43.4

A = WORMS ABSENT

B = WORMS PRESENT

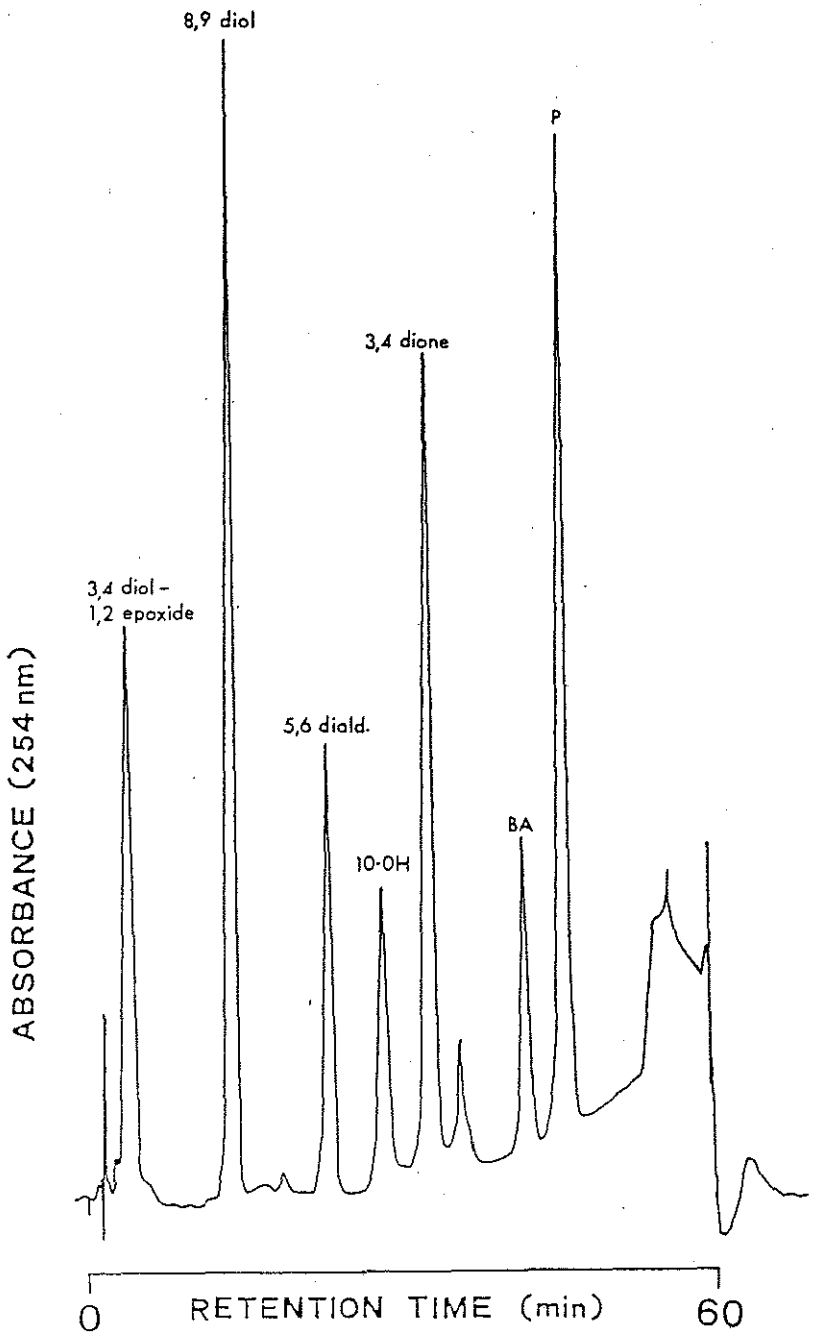

Figure A-3:
UV chromatogram of metabolite standards.
P= perylene, BA= benz(a)anthracene.

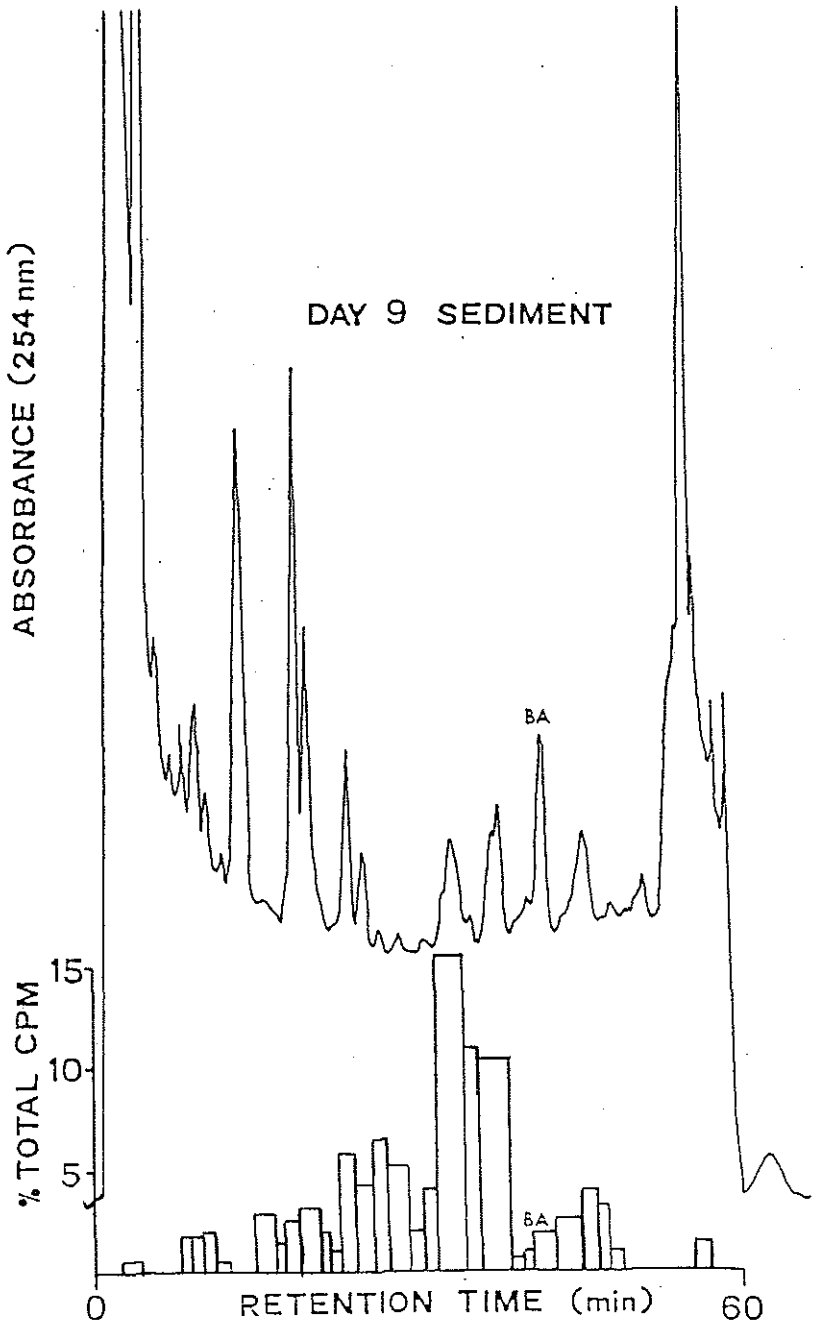

Figure A-4: HPLC chromatogram of organic extract of sediment collected on day 9.

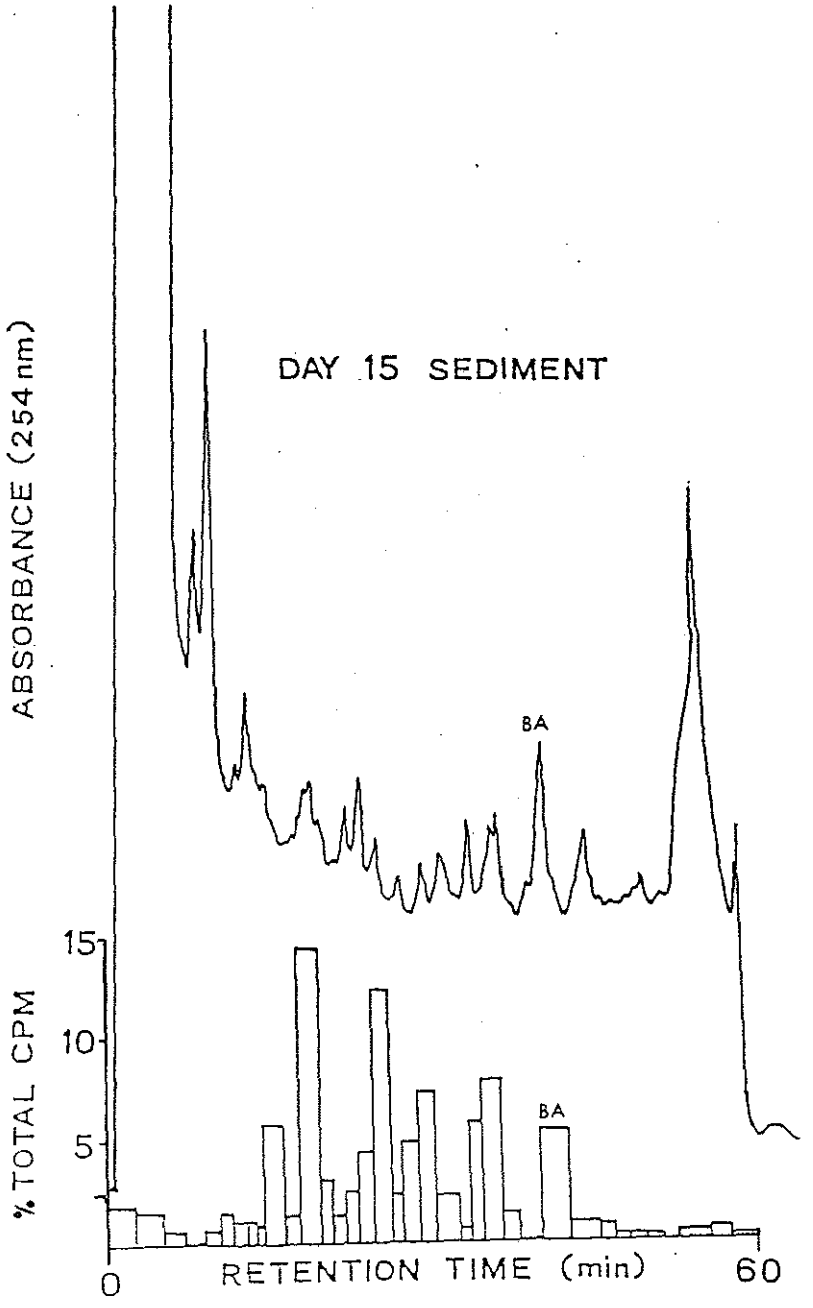

Figure A-5: HPLC chromatogram of organic extract of sediment collected on day 15.

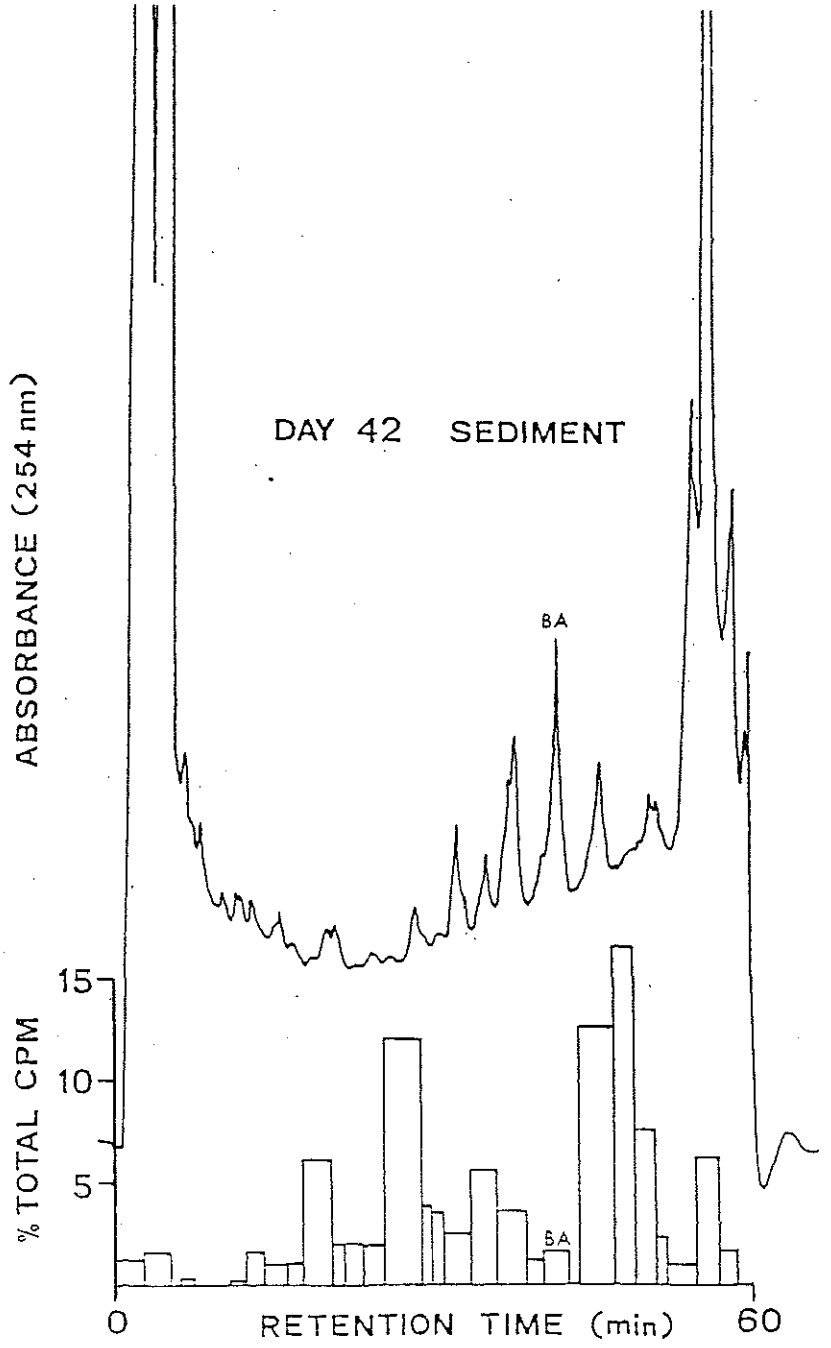

Figure A-6: HPLC chromatogram of organic extract of sediment collected on day 42.

specific metabolites. As early as nine days after spiking the isotope to the chambers, most of the BA in sediments had been metabolized. Analysis of a group of polychaetes Nephtys incisa exposed to BA in the chambers revealed patterns of metabolite formation similar to those seen in the sediment (Figures A-7 & A-8).

Table A-2 summarizes the distribution of radioactivity separated using reverse phase HPLC summed into three general classes. In all samples analyzed, most radioactivity recovered was in the form of polar metabolites. At the end of the experiment, only a few percent of radioactivity in both worm and sediment samples was unmetabolized parent compound. In almost all extracts analyzed, a significant portion of radiolabel eluted after BA using reverse phase HPLC. The proportion of total activity recovered in this class seemed to increase with time. Preliminary data using gel filtration chromatography suggested that this activity was associated with compounds of a higher molecular weight than BA (McElroy, unpub. data). The reverse phase and gel filtration data collectively suggest that this activity represents BA or BA metabolites that have been incorporated into larger molecules with non-polar characteristics.

Only 50% of the isotope spiked to these chambers was recovered in by our sampling procedure (Table A-1). A large portion of the surface area of these microcosms was involved in the water recirculation system, and could not be sampled. It is possible that particulate matter and microorganisms associated with these surfaces sequestered a significant portion of the unrecovered radioactivity.

The presense of worms had no measurable effect on the distribution

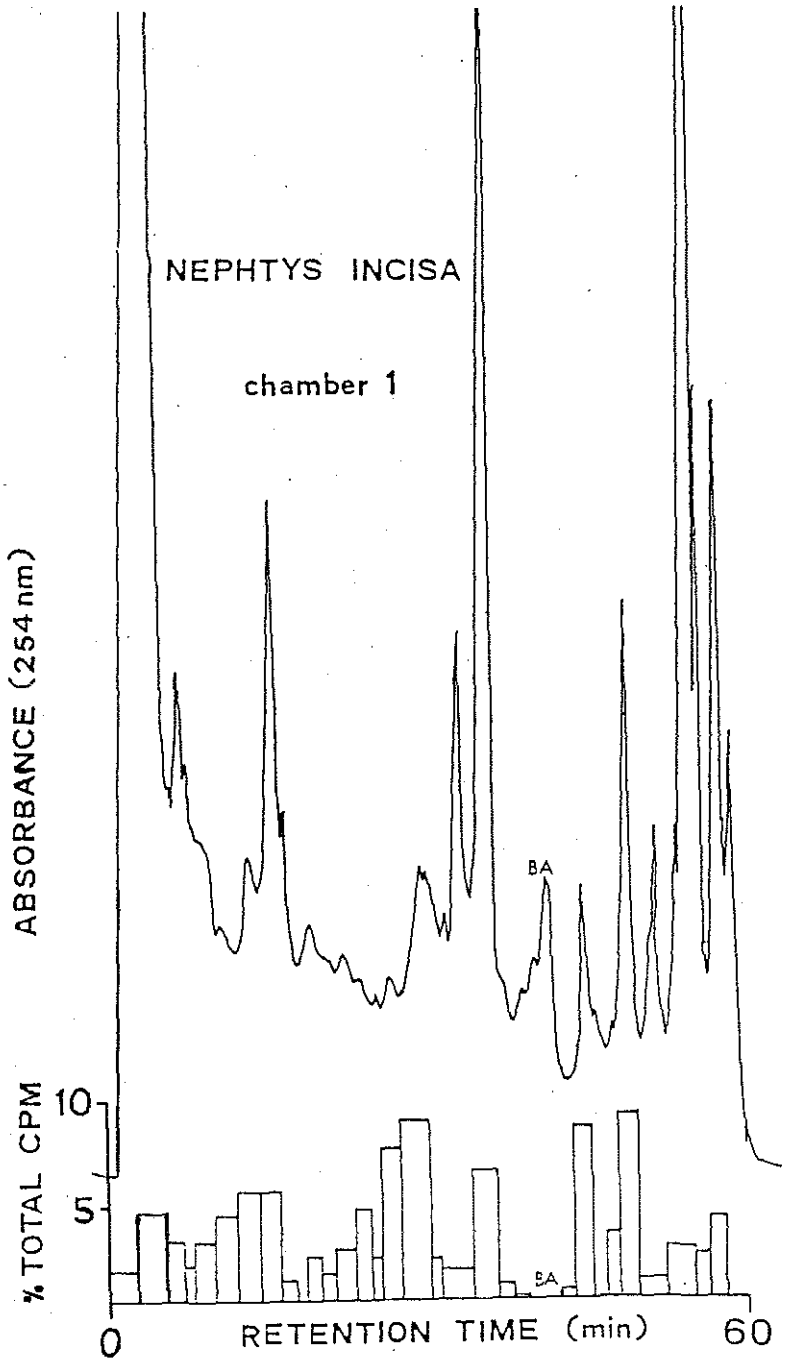

Figure A-7: HPLC of organic extract of Nephtys incisa from chamber 1.

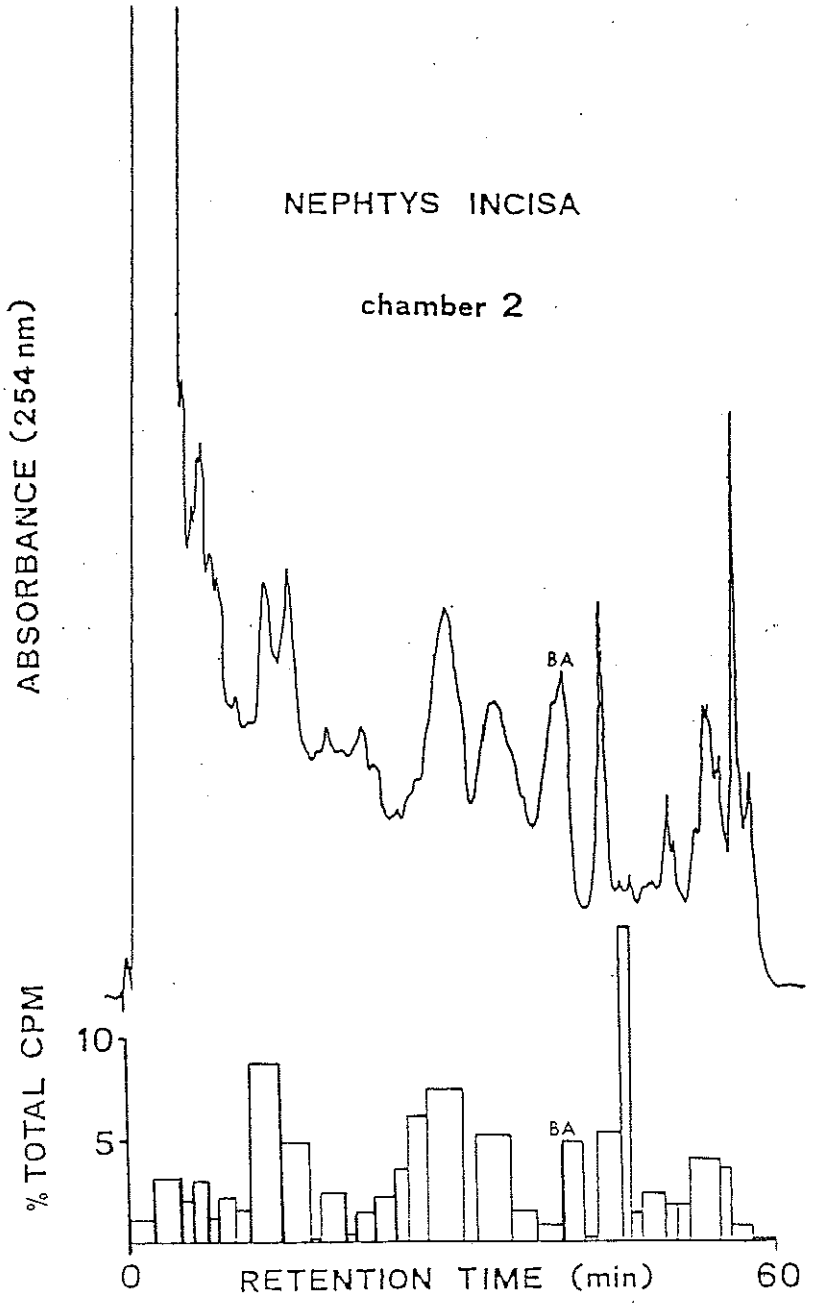

Figure A-8: HPLC of organic extract of Nephtys incisa from chamber 2.

Table A-2:

DISTRIBUTION OF ^{14}C-LABELED FRACTIONS FROM SURFACE SEDIMENT AND WORM TISSUE SEPARATED USING REVERSE PHASE HPLC

	% OF ^{14}C RECOVERED		
	BENZANTHRACENE	MORE POLAR	LESS POLAR
DAY 42			
SEDIMENT CHAMBER 1A	2	70	28
SEDIMENT CHAMBER 1B	3	50	48
SEDIMENT CHAMBER 2A	3	65	32
WORMS CHAMBER 1	ND	69	31
WORMS CHAMBER 2	6	57	37
DAY 23			
SEDIMENT CHAMBER 2A	11	69	20
DAY 15			
SEDIMENT CHAMBER 1	6	90	4
SEDIMENT CHAMBER 2	31	61	8
DAY 9			
SEDIMENT CHAMBER 1	4	84	12

A = WORMS ABSENT

B = WORMS PRESENT

or degree of metabolism of BA in the sediments (See Table A-1 & A-2).

Summary:

This experiment was a preliminary attempt to investigate the metabolic fate of BA in a relatively controled benthic system containing a large burrowing polychaete. The results clearly show that substantial metabolism of BA occurred rapidly, in a matter of days. Although metabolites were recovered from the worm Nephtys incisa it was not possible to to determine if the metabolites were produced in the worms, or if the worms accumulated them directly from the sediment. Microbial mineralization of BA was observed. It was not possible to determine from these data whether mineralization occurred in the water column or at the sediment surface.

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