SUBLETHAL EFFECTS OF PETROLEUM HYDROCARBONS AND TRACE METALS, INCLUDING BIOTRANSFORMATIONS, AS REFLECTED BY MORPHOLOGICAL, CHEMICAL, PHYSIOLOGICAL, AND BEHAVIORAL INDICES

by

Donald C. Malins, Harold O. Hodgins,
Usha Varanasi, Sin-Lam Chan, Bruce B. McCain,
Douglas D. Weber, and Donald W, Brown

Other Contributing Authors:

Tracy K. Collier, Leroy C. Folmar, William D. Gronland,
Edward H. Gruger, Jr., Joyce W. Hawkes, William L. Reichert,
William T. Roubal, Herbert R. Sanborn, Michael H. Schiewe,
and John E. Stein

Environmental Conservation Division
Northwest and Alaska Fisheries Center
National Marine Fisheries Service, NOAA
2725 Montlake Boulevard East
Seattle, Washington 98112

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1. SUMMARY OF OBJECTIVES, CONCLUSIONS, AND IMPLICATIONS WITH RESPECT TO OCS OIL AND GAS DEVELOPMENT

1.1 Summary of Objectives

The overall objectives of this program were to assess the potential effects of petroleum and petroleum-related activities on marine organisms indigenous to Alaskan waters. The principal objectives addressed were to:

(a) Determine the uptake of polycyclic aromatic hydrocarbons by salmonids and pleuronectids exposed to these compounds in sediment, water or via their diet.

(b) Study the metabolism of aromatic hydrocarbons by fish and evaluate the potential of their metabolizes for interacting with DNA and other cellular constituents.

(c) Evaluate the uptake, disposition and toxicity of petroleum hydrocarbons in larval and adult invertebrates.

(d) Determine the activities of enzymes (aryl hydrocarbon mono-oxygenases) that metabolize aromatic hydrocarbons in a variety of aquatic species.

(e) Study the factors (e.g., temperature. routes and length of exposure) that-influence the uptake, metabolism and disposition of petroleum hydrocarbons in marine fishes.

(f) Study the uptake and deputation of toxic trace metals by salmon and flatfish.

(g) Determine the effects of petroleum hydrocarbons and themical dispersants on disease resistance and host defense mechanisms of marine fish and shellfish.

(h) Evaluate pathological effects that can result from exposing adult and juvenile flatfish to sediments contaminated with petroleum.

(i) Assess the cytopathological changes in marine fish resulting from exposure to petroleum hydrocarbons.

(j) Evaluate the avoidance, homing, and predator-prey behavior of salmon exposed to water-borne hydrocarbons, and the avoidance of oil-contaminated sediment by juvenile flatfish.

(k) Determine the effect of ingested and water-borne crude oil on reproductive success and/or the early developmental stages of salmonids, flatfish, smelt, and invertebrates.

(1) Assess the effects of water-borne hydrocarbons on the chemosensory-mediated behavior of invertebrates.

1.2 Summary of Conclusions

The conclusions of this program are summarized according to discipline; chemistry, pathology, and behavior and physiology.

1.2.1 Chemistry

Marine fish and invertebrates accumulate a broad spectrum of aromatic hydrocarbons when exposed to these compounds in water, sediment, or via force feeding or intraperitoneal injection. In fish, the mode of exposure to aromatic hydrocarbons can, markedly influence the extent of accumulation of parent hydrocarbons and their metabolizes as well as the types of metabolizes formed. Also, increasing the number of benzenoid rings or the degree of alkyl substitution of aromatic hydrocarbons results in increased accumulation of these compounds by fish.

Measurement of aryl hydrocarbon monooxygenase activities show that most invertebrates and vertebrates investigated were capable of metabolizing aromatic hydrocarbons.

Regardless of species, mode of exposure or structure of hydrocarbon, results show that metabolic products are retained in the tissues of animals for a longer time than are the parent hydrocarbons.

Fish brain appears to accumulate mainly parent aromatic hydrocarbons (e.g., naphthalene), whereas other sites, such as liver and bile, contained primarily conjugated and nonconjugated metabolizes of aromatic hydrocarbons.

Lowering the environmental temperature increased both concentrations and resident times of naphthalene in the tissues of fish perorally exposed to naphthalene. Lowering the temperature also decreased the bioconversion of naphthalene and altered the proportions of individual metabolizes in the tissues.

The metabolite profiles of naphthalene in both adult and larval shrimp were similar to those reported for fish, and demonstrate that the early developmental stages of shrimp have the enzyme systems necessary for converting naphthalene to both conjugated and nonconjugated metabolizes. In addition, exposure to low levels (1-100 ppb) of naphthalene in seawater impaired fertilization and early embryonic development of mollusc larvae, and survival of crustacea larvae.

The liver enzymes of pleuronectids convert benzo(a)pyrene into reactive intermediates (such as epoxides) that bind to DNA and proteins. Such interactions are known to damage critical cellular constituents in mammals.

Studies with both aromatic hydrocarbons and metals demonstrate that the skin and epidermal mucus of fish are involved in both the uptake and discharge of these compounds.

Salmonid and pleuronectid fish accumulated water-borne lead and cadmium; the metals persisted in many organs and tissues for weeks after the exposure was terminated. Cadmium was preferentially bound by low molecular weight proteins in liver cytosol; high accumulation of lead was found in the brains of fish.

Food chain transfer of aromatic hydrocarbons was demonstrated. Sea urchins feeding on 2,6-dimethylnaphthalene-exposed algae accumulate and metabolize this hydrocarbon.

1.2.2 Pathology

Juvenile and adult flatfish and adult spot shrimp exposed to oil-contaminated sediment did not show an altered resistance to bacterial infection. Juvenile salmon exposed to seawater-accommodated crude oil for 2 weeks, as well as salmon and trout perorally exposed to crude oil for up to 10 months, also showed no demonstrable changes in disease resistance or evidence of immune dysfunction. However, preliminary tests suggested an adverse effect of a petroleum dispersant (Corexit 9527) on disease resistance of salmon.

Three species of flatfish exposed to oil-contaminated sediments for up to 4 months differed substantially in the degree to which they accumulated parent aromatic hydrocarbons. Pathological changes occurred in the livers of all flatfish species tested, but similar abnormalities were frequently observed in controls. In addition, the physiochemical characteristics of sediment greatly influenced its retention of petroleum. Retention of aromatic hydrocarbons was more than 10-fold greater in high-silt than in high-sand sediment.

Ultrastructural changes in the liver and lens tissues of adult trout occurred after high doses of petroleum hydrocarbons were administered perorally for 2-12 months. Also, both salmon and flatfish exposed to waterborne hydrocarbons exhibited gill lesions characterized by loss of surface cells.

Exposing embryonic smelt and flatfish to low ppb concentrations of seawater-accommodated crude oil resulted in high mortality at hatching. The eye and brain of exposed smelt embryos appeared to be target organs and, in the later phases of embryonic development, exhibited extensive necrocytosis. In flatfish there was evidence of disruption in both epithelial cell mitochondria and in the olfactory epitheliums.

1.2.3 Behavior and Physiology

At low (15-150) ppb concentrations of waterborne hydrocarbons, spot shrimp overt feeding behavior and the sea urchin pedicellarial defense response were reduced by half. At these hydrocarbon concentrations nudibranchs failed to locate mating conspecifics and suffered impaired reproduction and embryological abnormalities. in addition, less than 10% of the smelt eggs exposed throughout embryogenesis hatched; of the larvae that hatched, only 10% survived. Flatfish embryos exposed to

80 ppb developed into normal larvae, but embryos exposed to more than 130 ppb hatched into dead or grossly abnormal larvae.

At waterborne hydrocarbon concentrations of 150-500 ppb there was a significantly increased consumption of exposed sea urchins by starfish predators and of exposed salmon fry by salmon predators. Salmon predation decreased sharply after the predators were exposed to oil for 3 or more days. Exposing salmon eggs throughout embryo and alevin development at these hydrocarbon concentrations resulted in a 400% increase (compared to controls) in mortality; exposure as either embryos or alevins alone increased mortality by 100-150% over that of controls.

Exposing adult salmon to 1-2 ppm of aromatic hydrocarbons caused a 3 day delay in return from offshore in seawater to their "home" stream; concentrations of 2-3 ppm inhibited upstream spawning migration. However, short term exposure to a 4 ppm concentration did not discernibly affect adult salmon olfactory perception. Also, exposing adult salmon to 40 ppm of freshwater-accommodated crude oil did not alter their homing capability in freshwater or their rate of return.

Juvenile flatfish did not consistently avoid oil-sediment mixtures containing 8,000-10,000 ppm total hydrocarbons, and maturing trout fed large amounts of crude oil (1,000 ppm added to food) for 7 months did not show statistically significant changes in their hatching success.

1.3 Implications with Respect to OCS Oil and Gas Development

Research findings from this program have clear implications with respect to petroleum effects on aquatic species and consequently to OCS oil and gas development. Most of the studies were designed as laboratory experiments with emphasis on exposures of aquatic organisms in flowing-seawater tanks. Controlled studies with experimental designs of the type reported here are indispensable parts of a total program directed at understanding effects of petroleum on the marine environment. The degree to which laboratory results can be directly applied to natural events remains a considerable problem. However, lacking the opportunity for testing target species directly under natural conditions, models, such as those used in present studies, and representative test situations, must be applied.

1.3.1 Chemistry

The results of studies exposing a variety of fish species to ppb concentrations of metals and aromatic hydrocarbons imply that low levels of both types of compounds arising from petroleum operations could result in substantial metal and hydrocarbon accumulation in fish. This is particularly notable for flatfish, which show a striking ability to accumulate both types of pollutants. Also, the tendency of fish to accumulate considerable amounts of the metabolic products of aromatic hydrocarbons [e.g., metabolizes of benzene, naphthalenes, anthracene,

benzo(a)pyrene] is a cause for concern because of the toxicity ascribed to certain metabolizes in other animal experiments. These studies have clearly established that aromatic hydrocarbons are converted to a variety of oxidized products by marine organisms and that the metabolizes tend to be retained in tissues for a longer time than the parent hydrocarbons. Thus, in assessing marine pollution, considerable bias may arise from determining only the concentrations of parent hydrocarbons in marine animals.

Results show that polycyclic aromatic hydrocarbons, such as benzo(a)-pyrene, can persist in sediment and are thus available for continual uptake by demersal fish. Benzo(a)pyrene is rapidly and extensively metabolized by flatfish into a number of mutagenic and carcinogenic compounds. The extent of metabolism and retention times of metabolizes by flatfish are considerably greater for benzo(a)pyrene than for naphthalene. Although benzo(a)pyrene is a minor component of crude oil, these factors raise serious concerns regarding benzo(a)pyrene and other high molecular weight polycyclic aromatic hydrocarbons in the marine environment.

Findings also show that lowering the water temperature resulted in an increased retention of petroleum hydrocarbons and their metabolizes in the tissues of exposed salmon and flatfish. These results suggest that fish in colder regions may accumulate particularly heavy burdens of potentially damaging xenobiotics from prolonged petroleum exposure. This finding is of major importance when considering the environmental effects of arctic and subarctic petroleum operations.

The low concentrations (1 ppb) of aromatic hydrocarbons that produce adverse effects on the fertilization and early embryonic development of molluscs indicate the incompatibility of aromatic petroleum hydrocarbons and gametes of these species in the water. This is of considerable importance because the gametes of many commercially important species of molluscs are exuded directly into the water where fertilization takes place.

It was shown that dimethyl naphthalene can be accumulated by algae and transferred to sea urchins feeding on the algae. Moreover, sea urchins and spot shrimp were shown to be capable of metabolizing aromatic hydrocarbons and retaining both metabolizes and the parent compound, which raises serious concern about the transfer of potentially toxic metabolizes through the food web.

1.3.2 Pathology

Exposing flatfish and spot shrimp to crude oil-contaminated sediments, and feeding crude oil to salmonid species, produced no demonstrable alterations in disease resistance. Preliminary testing suggested that chemical dispersants may reduce disease resistance. However, additional research to verify and expand this observation would be necessary before implications could be made.

Exposing juvenile and adult flatfish to oil-contaminated sediment resulted in pathological changes which were considered reversible. But whether flatfish exposed to similarly contaminated sediments could, under natural conditions, successfully compete for food, reproduce, escape predators, and perform other vital functions remains unknown. However, the cytopathological changes observed in surf smelt embryos (e.g., necrosis of eye and brain tissue) were severe. It was concluded these changes would clearly affect development and survival.

1.3.3 Behavior and Physiology

Behavioral studies indicate that salmon are likely to avoid acutely toxic concentrations of petroleum hydrocarbons, and migrating salmon which encounter subavoidance levels would be unlikely to suffer a detrimental effect on the physiological processes involving homing capability. An adverse effect on hatching success or survival of offspring as a result of crude oil ingestion is also unlikely. It should be noted, however, that the effect of oil exposure on other important behavioral and physiological aspects of reproduction, such as redd building, mate selection, and egg laying, were not investigated. For intertidally spawned salmon eggs, ppb concentrations of weathered crude oil resulted in a high mortality of embryos and alevins, but only when exposure encompassed a considerable portion of the early developmental stage.

Studies on predator-prey reactions indicate that salmon fry exposed to ppb concentrations of petroleum hydrocarbons for 24 hr are much more susceptible to predation than non-exposed fry. Conversely, salmon predators exposed to similar ppb hydrocarbon concentrations did not statistically significantly reduce prey consumption for at least 3 days. Thus, salmonid fry may be vulnerable to predation immediately after an oil spill, while a continued exposure may impair adult feeding.

It was concluded from these studies, however, that the overall probability of salmon encountering concentrations of petroleum capable of eliciting severely adverse effects is slight; only in unusual circumstances would substantial damage to Pacific salmonids be anticipated.

In contrast, low ppb concentrations of waterborne weathered crude oil resulted in a high mortality of flatfish and smelt embryos and larvae. Oil-exposed eggs were often ruptured, with subsequent fragmentation of the chorions and disintegration of the embryos, and affected larvae were unable to swim normally. Low hydrocarbon concentrations also had a pronounced effect on invertebrate chemosensory mediated behaviors, such as feeding, defense, and reproduction.

Therefore, from the studies concerned with the early developmental stages of fish and the chemosensory mediated behavior of invertebrates, the petroleum concentrations necessary to produce deleterious effects observed could realistically be expected to occur in the marine environment. Due to the subtle nature of behavioral changes, and the likelihood that dead eggs and abnormal larvae would sink out of the water column, it

is doubtful that these effects could be detected during field evaluation of oil contamination. The result would be, therefore, that pollutant effects would be substantially underestimated. Thus, the most useful application of this data would be through systems modeling.

Experiments concerning the effect of oiled sediment on flatfish behavior indicate that high levels of crude oil incorporated in the sediment were apparently accepted by juvenile flatfish without noticeable behavioral effects. Although there is little direct evidence from these studies that oil-contaminated sediment is detrimental to the health of juvenile and adult flatfish, it is still a reasonable assumption that long-term residence by these fish in a heavily oil-contaminated environment is not compatible with survival.

2. INTRODUCTION

The responses of marine organisms to environmental contaminants are reflected in a number of changes detectable at organismic, as well as at tissular, cellular, subcellular, and molecular levels. The general purpose of this study was to detect these petroleum-related changes in marine species and to evaluate their implications for the survival and health of the animals.

When petroleum is transported in, or obtained from, coastal or offshore areas, petroleum hydrocarbons and associated trace metals inevitably escape into the marine environment. These materials, at various levels, can produce critical damage to marine resources. Damage by crude oil components takes many forms (Blumer, M., Testimony before Subcommittee on Air and Water Pollution, Senate Comm. on Public Works, Machias, Maine, 8 Sept. 1970).

1. Direct kill of organisms through coating and asphyxiation, through contact poisoning, or through exposure to water-soluble toxic components of oil at some distance in space and time from the accident.

2. Destruction of the generally more sensitive juvenile forms of organisms.

3. Incorporation of sublethal amounts of oil and oil products into organisms resulting in reduced resistance to infection and other stresses and in failure to reproduce.

4. Destruction of the food sources of higher species.

5. Exposure to long-term poisons, e.g., carcinogens.

6. Low level interruption of any of the numerous events necessary for the feeding, migration, and propagation of marine species and for the survival of those species which stand higher in the marine food web.

7. Contamination of marine food resources, making them unfit for human consumption.

Studies by OCSEAP RU 73 were largely concerned with the indirect, long-term effects of petroleum such as those detailed in items 2, 3, 5, and 6. These effects are much more difficult to detect and evaluate than those related to acute exposures, but may over a period of time have even more serious consequences for marine biota.

3. BACKGROUND

3.1 Chemistry

With increased exploration, production, and transportation of petroleum, and the inevitability of accidental oil release, petroleum hydrocarbons have become common contaminants of the marine environments. At the time our OCSEAP research was initiated, most studies concerning the uptake and biochemical effects of oil on aquatic organisms focused on accumulation of parent hydrocarbons in whole organisms, and, to a lesser extent, in specific tissues (Lee et al. 1972, Anderson 1975, Varanasi and Malins 1977).

However, in the mid-1970's, an increasing interest developed in the enzyme systems of aquatic organisms that convert aromatic hydrocarbons to their electrophilic metabolizes (Payne 1976, Pedersen et al. 1976, Philpot et al. 1976, Gruger et al. 1977). The hepatic tissues of many aquatic organisms contain enzymes, such as aryl hydrocarbon monooxygenases (AHPI), capable of metabolizing aromatic hydrocarbons (Malins 1977, Varanasi and Malins 1977, Bend and James 1978). Some aromatic hydrocarbon metabolizes have been shown to be mutagenic and carcinogenic in mammals (Sims and Grover 1974). The early reviews clearly point out that petroleum hydrocarbons can induce or enhance AHM activity in aquatic species. Evidence is rapidly accumulating to suggest that all vertebrate marine organisms possess the AHM system; there are conflicting reports on its presence in invertebrates. In contrast, studies on the uptake and disposition of polycyclic aromatic hydrocarbons (PAH) were few and virtually no information was available concerning the extent of PAH metabolism or profiles of PAH metabolizes in either marine fish or invertebrates.

This report describes results of studies conducted to assess the uptake, metabolism, and disposition of various hydrocarbons in marine organisms, with special emphasis on tissue concentrations of hydrocarbons and their metabolizes and the types of metabolizes formed in vivo. Studies conducted by other researchers during the course of our investigations are referred to in Section 7.1.

3.2 Pathology

Effects of Petroleum on Disease Resistance

Considerable evidence indicates that petroleum hydrocarbons and associated trace metals affect host defense mechanisms in various mammals (Kripke and Weiss 1970, Keller 1973, Keller and Kovacic 1974, Stjernsward 1974, Cook et al. 1975, Keller et al. 1975, Keller and Roan 1980), and birds (Vengris and Mare 1974). In addition, a few studies suggest an immunosuppressive potential in fish (Robohm and Nitkowski 1974, O'Neill 1981). Because disease is the result of a complex interaction among the host, the pathogen, and the environment, any environmental perturbation which compromises host defense can precipitate an outbreak of disease; particularly those diseases caused by the many opportunistic bacterial pathogens.

This report presents the results of experiments undertaken to assess the effects of various exposures of crude oil on the disease resistance of commercially important species of the Northeastern Pacific Ocean, including salmonid and flatfish species, and a crustacean, the spot shrimp (Pandalus platyceros). A preliminary investigation of the effects of chemical dispersants on disease resistance is also presented.

Pathological Changes in Flatfish from Exposure to Oil-Contaminated Sediment

The considerable amount of literature which reports histopathological changes in marine fish as a result of exposure to petroleum hydrocarbons (see Malins 1982 for comprehensive review) primarily reflects exposures to waterborne hydrocarbons. These reported laboratory studies can, at best, only suggest the effects of oil exposures on bottom-dwelling fish coming in contact with contaminated sediment.

Only a few studies are available on the pathological effects of exposure to oil-contaminated sediment. In one of these, a field study, two species of flounder (Pseudopleuronectes americanus and Limanda ferruginea) were collected from control stations and from stations close to the ARGO MERCHANT oil spill; no correlation was established between petroleum from the spill and observed morphological damage (Sawyer 1978).

Early studies of the AMOCO CADIZ oil spill off the Coast of France, however, suggested a definite link between the spilled petroleum and gross pathological alterations, such as fin erosion in plaice (Pleuronectes platessa) from Aber Benoit and Aber Wrac'h (Miossec 1981). A later survey of plaice from Aber Benoit and Aber Wrac'h between 1979 and 1980 revealed fin and tail necrosis, extensive gill lesions, abdominal muscle and gastric gland degeneration, and increased concentrations of hepatic microphage centers (Haensly et al. 1982). A comparison of these findings to those from a reference area (Baie de Douarnenez and the ports of Loctudy and Ile Tudy) suggested a likely association between spilled oil and the observed biological alterations in plaice.

The studies presented in this report were designed to evaluate the possible relationships between biological anomalies and crude oil-contaminated sediment under controlled laboratory conditions.

Cytopathology

Several reviews have discussed the results of histological and ultrastructural studies of aquatic organisms exposed to environmental contaminants (Hawkes 1977, Hodgins et al. 1977, Gardner 1978, Hawkes 1980, Malins 1982). This section concentrates on studies of embryonic and larval fish, an area that warrants special attention because even low levels of petroleum seem to have a particularly deleterious effect on the early life stages. Eggs, embryos, and larvae are very susceptible to external environmental influences, for, in contrast to the adult organism, the early development stages have few, if any metabolic "resting points," a greater surface-volume ratio, fewer cells, and undeveloped or poorly developed defense and homeostatic mechanisms (LeGore 1974). Petroleum compounds capable of interacting with nucleic acids (see chemistry section of this report), or of interfering with cell migration, communication, or metabolic activity can alter normal development.

Our studies of early developmental stages describe the effects of the seawater-soluble fraction of crude oil on smelt embryos, particularly cytopathological changes in the brain and eye, and to a lesser extent, on the timing of these changes. We also examined the effects of waterborne crude oil on the morphology of flatfish larvae.

3.3 Behavior and Physiology

Chemical agents released by animals, and chemical signals in the environment itself, can influence a variety of activities: symbiosis (Ache and Davenport 1972); homing (Cook 1969); reproduction (Atema and Engstrom 1971, Kittredge et al. 1971, Ryan 1966); site selection and larval settlement (Crisp 1974); evaluation of local habitat (van Weel and Christofferson 1966, Laverack 1974); and detection of both predators and prey (Phillips 1978). The importance of chemical sensing in aquatic organisms has been long recognized, but only during the past decade has there been extensive research on the chemical communication of aquatic species and the effects that man-induced contaminants may have in interfering with this communication. There is clear evidence that oil products interfere with chemosensory-mediated behavior (Atema et al. 1973), and that aromatic hydrocarbons in particular are probably the most active petroleum components in this regard (Kittredge et al. 1974, Takahashi and Kittredge 1973). Behavioral disruptions at exposure concentrations in the low parts-per-billion (ppb) range have been noted among marine organisms as diverse as bacteria, algae, and invertebrates (Johnson 1977, Jacobson and Boylan 1973). Although disruption of invertebrate behavior may occur at low ppb hydrocarbon concentrations, vertebrate behavioral reponses and changes in activity patterns during

hydrocarbon exposure have been observed at only high ppb or low parts-per-million (ppm) concentrations (Pattern 1977).

Field observations have suggested that mobile marine organisms do not avoid areas of petroleum contamination. Cross et al. (1978) reported dead fish and crustacea subsequent to the AMOCO CADIZ incident, and MacLeod et al. (1978) cited the presence of Bunker C oil in the stomach of codfish taken near the site of the ARGO MERCHANT spill. This project is the first to report on the behavior of adult salmon exposed to waterborne hydrocarbons or flatfish exposed to oil-contaminated sediment.

Infertile gametes and teratogenic effects on progeny were demonstrated for trout exposed to DDT (Burdick et al. 1964, Macek 1968), and in flathead sole (Hippoglossoides elassodon) fed a single dose of benzo(a)pyrene (BaP) (Hose et al. 1981). The studies of trout reproduction discussed in this report represent the first known investigation of the effects on the reproductive processes of fish from long-term dietary exposure to crude oil components.

In reviews of acute toxicity and sublethal biological effects of petroleum on arctic and subarctic marine fishes, Craddock (1977) and Patten (1977) presented evidence of lethargy, loss of appetite, and alterations in schooling behavior associated with exposure to various seawater-soluble fractions of petroleum. However, no studies were reported on the influence of petroleum on predator-prey behavior which has been described as a sensitive indicator of perturbed environmental conditions (Goodyear 1972, Hatfield and Anderson 1972, Sylvester 1972, Coutant et al. 1974, Yocum and Edsall 1974, Sullivan et al. 1978, Weltering et al. 1978). The purpose of the present studies was to determine the influence of crude oil in seawater on salmonid predator-prey interactions. Coho salmon (Oncorhynchus kisutch) were chosen as predators since this species has been identified as a primary predator of juvenile salmonids in seawater (Parker 1971).

4. STUDY AREA

Host experiments were performed in the laboratories at either the Northwest and Alaska Fisheries Center (NWAFC) in Seattle, or at the NWAFC's saltwater field station at Mukilteo, Washington. Field experiments were conducted in the Puget Sound area.

Organisms used in experiments are representative of temperate, arctic, and subarctic species, and with few exceptions were either collected from Puget Sound or were indigenous anadromous fishes of the Puget Sound drainage.

5. METHODS

5.1 Chemistry

5.1.1 Accumulation and Biotransformation of Specific Aromatic Hydrocarbons in Salmonids

Fingerling coho salmon (ca 20 g; purchased from DomSea Farms, Bainbridge Island, WA) maintained in freshwater, were injected intraperitoneally (i.p.) with 2.5 µCi of ^{14}C-labeled benzene (sp. act. 25 mCi/mmole), naphthalene (NPH) (sp. act. 5mCi/mmole), or anthracene (sp. act. 23 mCi/mmole) dissolved in 0.05 ml of ethanol. The fish injected with benzene were sampled (3 fish per time point) 6 and 24 hr after injection. Anthracene and NPH-exposed fish (3 fish per time point) were sampled 24, 72, and 144 hr after the injection. Brain, liver, gallbladder, heart, muscle (flesh), and residual carcass were analyzed for parent hydrocarbons and metabolic products; tissues were added to 2-5 ml of 90% formic acid overlaid with 5-10 ml of hexane at room temperature. After 12-24 hr, a saturated solution of sodium hydroxide was added until the solution was strongly alkaline (pH>12). Hydrocarbons remained in hexane and metabolizes in the aqueous phase. (For further details see Roubal et al. 1977a.)

5.1.2 Accumulation of Petroleum Hydrocarbons by Fish Exposed to Seawater Soluble Fraction (SWSF) of Prudhoe Bay Crude Oil (PBCO)

Coho salmon (11-19g; purchased from DomSea Farms, Bainbridge Island, WA) and starry flounder (Platichthys stellatus) (32-186g; captured from Puget Sound) were exposed to a SWSF of PBCO in seawater[a], at 10"C, under continuous flow-through bioassay conditions. The apparatus used is depicted in Figure 1. The concentration of total soluble hydrocarbons in flowing seawater delivered from the solubilizer was 5 ppm as measured by capillary gas chromatography (GC). Hydrocarbons were analyzed by gas chromatography-mass spectrornetry (GC/MS). SWSF delivered from the solubilizer was diluted with seawater to produce a final hydrocarbon content of 0.9 + 0.1 ppm (Fig. 1). Coho salmon were exposed to the 0.9 ± 0.1 ppm SWSF for a 6-week period, followed by 6 weeks of holding exposed fish in oil-free seawater to evaluate deputation.

Muscle tissue of salmon was analyzed for concentrations of SWSF hydrocarbons starting one week after the beginning of the exposure period. Excised tissues were thoroughly rinsed with 3.5% saline and aliquots were digested at room temperatures in 4 N sodium hydroxide. The digests were analyzed for hydrocarbons using capillary GC. Similar analyses were made on samples of starry flounder muscle gills and liver from 10 fish. (For further details see Roubal et al. 1978.)

5.1.3 Metabolism of NPH by Coho Salmon

Four coho salmon (160 + 35g; purchased from DomSea Farms, Bainbridge Island, WA) maintained at 10° ± 0.5°C were force-fed 74.6 uCi of ^{3}H-NPH

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a Seawater salinity was 27-30°/oo in all chemistry studies.

BLOCK DIAGRAM OF OIL-IN - SEAWATER SOLUBILIZER SYSTEM

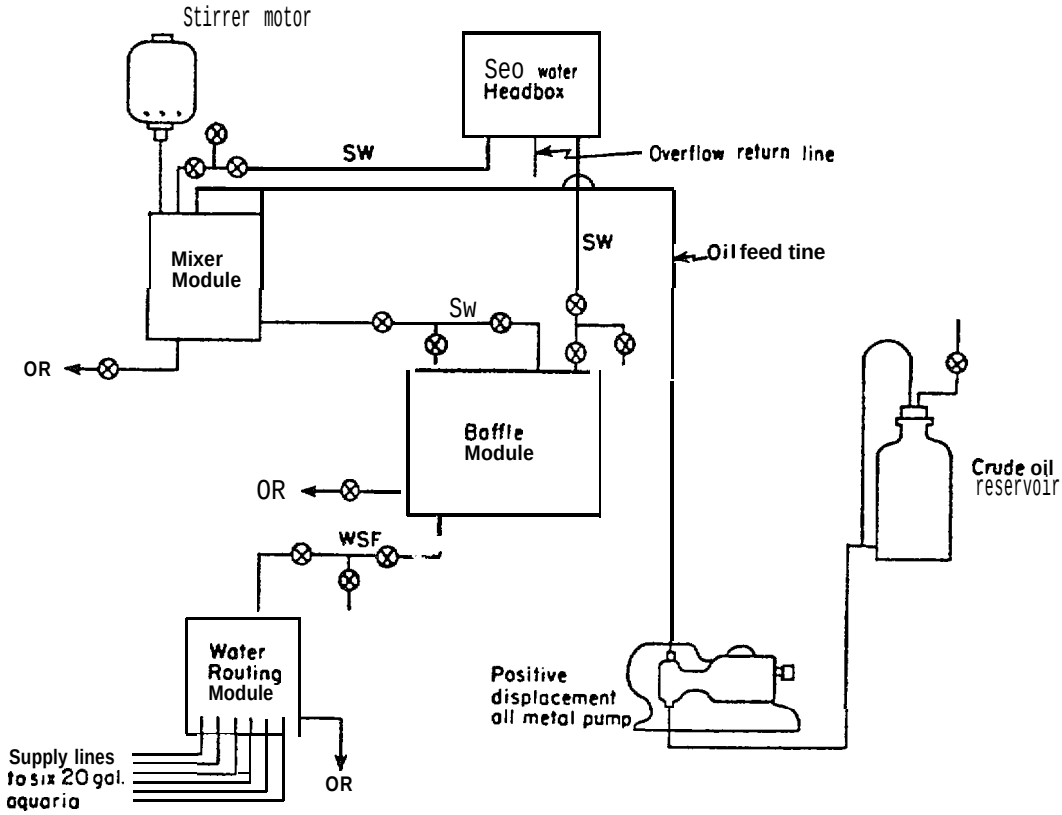

FIGURE 1. Schematic of oil-in-seawater solubilizer system used to produce the seawater soluble fraction (SWSF) of crude oil. (From Roubal et al. 1977b)

(sp. act. 85 mCi/mmole) dissolved in salmon oil. After 16 hr, the livers and gall bladders were removed and pooled for analysis. Tissues were homogenized in distilled water, saturated with sodium chloride, and proteins were precipitated with acetone. Metabolizes were extracted into ethyl acetate, the extract dried under nitrogen, and the residue was redissolved in methanol. Metabolize extracts were chromatographed by high-pressure liquid chromatography (HPLC). (For further details see Collier et al. 1978.)

5.1.4 Naphthalene and Its Metabolizes in Fish Skin and Mucus

Rainbow trout (Salmo gairdneri) (150 + 50g; purchased from Trout Lodge, Puyallup, WA), held at 8°C were either force-fed 74.6 µCi of [1,4,5,8,-^{3}H] NPH (sp. act. 83.3 mCi/mmole) dissolved in 250 µl of salmon oil or injected i.p. with 40 µl of oil containing 94.6 µCi of ^{3}H-NPH. Starry flounder (100 + 20g; captured from Puget Sound) held at 12°C were force-fed a gelatin capsule containing 87 µCi of ^{3}H-NPH (sp. act. 198 mCi/mmole) dissolved in salmon oil. Concentrations of NPH and total metabolizes in skin and mucus were determined by digestion of the sample at room temperature in hexane and 4 N NaOH. Concentration of the NPH was determined from radioactivity in the hexane layer, and concentration of total metabolizes was determined from the aqueous layer. Concentrations of NPH and its metabolizes in the skin were compared with those in the liver. (For further details see Varanasi et al. 1978 and Collier 1973.)

5.1.5 Accumulation and Biotransformation of NPH by Flatfish

Sexually immature starry flounder and rock sole (both species: 82 + 30g) were captured near the mouth of the Columbia River and at Point Puny in Puget Sound, respectively, and were maintained at experimental temperatures of 12 + 1°C in flowing seawater for a period of 2 wk prior to treatment. Fish were fed daily to satiation on a mixture of earthworms and euphausiids.

Force-feeding study. Test fish of both species were force-fed a gelatin capsule (No. 5) containing 56 µCi of [1-^{3}H]-NPH (sp. act. 198 mCi/mmole) dissolved in 25 µl of salmon oil. The fish were then placed in aquaria supplied with flowing seawater at 12°C. Three to six fish were analyzed at 24, 48, and 168 hr after the initiation of exposure. Rock sole were analyzed also at 6 wk. Fish were not fed during the first week after the initiation of NPH exposure.

Injection Study. Starry flounder were injected i.p., with 25 ul of salmon oil containing 56 µCi of the [1-^{3}H]-NPH (sp. act. 198 mCi/mmole). The fish were held at 12°C, and sampled at 24 and 168 hr after injection.

Sample collection. Epidermal mucus and skin were collected, and samples of muscle, liver, brain, gills, blood, kidney, stomach, intestine, and bile were also collected.

Analytical methods. Radioactivity associated with both NPH and total metabolizes in each tissue (~100 mg) was determined by digestion in hexane-sodium hydroxide. Dry weight of each tissue was obtained by freeze-drying and the values expressed as percent of wet weight of tissue. Lipid content of liver, muscle, and skin of starry flounder and rock sole (Lepidopsetta bilineata) were also determined.

Data were statistically analyzed using Student's t-test. Also, rates of decline of NPH and metabolize concentrations in tissues were obtained by assuming a lognormal distribution and describing the decay of concentration by y = ax-b.

NPH metabolizes were isolated from liver, muscle, skin, and bile of the exposed rock sole and starry flounder; the samples were homogenized in methanol and then extracted twice with hexane to remove NPH, followed by extraction with a mixture of boiling methylene chloride :2-propanol:water (75:25:2, v/v/v) and twice with boiling ethanol :diethyl ether (50:50, v/v) to remove metabolizes.

Individual classes of metabolizes were separated via thin-layer chromatography (TLC). Nonconjugated metabolizes were separated using a solvent system of p-dioxane:benzene:acetic acid (25:90:4, v/v/v). Conjugated metabolizes were separated by TLC using a solvent system consisting of the upper phase of 1-butanol :concentrated ammonium hydroxide:water (80:20:100, v/v/v). Nonradioactive standards added to each sample allowed visualization of individual classes of metabolizes after staining with color producing reagents.

After the determination of the position of the various metabolizes the adsorbant was scraped from the chromatograms in 5 mm bands and radioactivity in each band was determined. (For further details see Varanasi et al. 1979, 1981.)

5.1.6 Effect of Temperature on Disposition of NPH and Its Metabolizes in Fish

(a) Coho salmon (150 + 50 g; purchased from DomSea Farms, Bainbridge Island, WA) maintained at 4–and 10*C were force-fed 5.55 μCi NPH (sp. act. 5.1 mCi/mmole) to determine the effect of temperature on the amount of [1-^{14}C] NPH incorporated into selected organs. After 8 and 16 hr the fish were sampled and the brain, liver, kidney, gallbladder and gut were removed along with samples of blood, dark muscle and light muscle. To analyze for NPH and total metabolizes in the fish, the formic acid digestion/hexane extraction method was used (see Section 5.1.1).

(b) Sexually immature starry flounder from the same group of fish used in Section 5.1.5 were held at 4 + 1°C in flowing seawater for 2 wk before the experiment was conducted. 'Fish were fed daily (to satiation) a mixture of earthworms and euphausiids.

The fish were force-fed 56 μCi of [1-^{3}H]-NPH (sp. act. 198 mCi/mmole) dissolved in 25 μl of salmon oil, but were not fed thereafter. Six fish were sampled at 24 and 168 hr after force-feeding.

NPH and its metabolizes were analyzed by the same procedures outlined in Section 5.1.5. (For further details see Varanasi et al. 1981a.)

5.1.7 Uptake and Metabolism of Sediment-Associated Aromatic Hydrocarbons by Flatfish

English sole (62 ± 22 g; captured from Puget Sound) were held in flowing seawater (12.0 ± 0.5°C) and fed a diet of minced clams for two weeks. The feeding was-stopped three days prior to the initiation of experiments.

Oil-contaminated sediment (1%, v/v) was prepared as described by McCain et al. (1978) except that 3.6 mCi ^{3}H-BaP (sp. act. 0.83 mCi/mmole) and 0.14 mCi ^{14}C-NPH (sp. act. 167 mCi/mmole) were dissolved in PBCO prior to mixing with the sediment. The oil-contaminated sediment was placed in a 17-1 glass aquaria to a depth of 5-6 cm where it was allowed to stand in flowing seawater (20 l/day) for 24 hr (day 1).

Six fish were placed in the experimental tank for a 24-hr exposure on day 2, three fish were sampled on day 3, and the remaining 3 fish were placed on clean sediment in flowing seawater for 24 hr. Immediately after the first six fish were removed on day 3, five additional fish were placed in the experimental tank. These fish were exposed to the oiled sediment for 168 hr before sampling on day 10.

Samples of sediment and sediment-associated water (SAW) were taken from 2 cm below the sediment/water interface. The wide end of a glass pipette was vertically inserted into the sediment while the tip of the pipette was covered. After positioning the pipette, the tip was uncovered to allow sediment and SAW to rise within the pipette. The sample was carefully transferred to a vial and the SAW was decanted off after the suspended particles had settled.

Samples of wet sediment, unfiltered and filtered (0.45A, millipore) SAW, and samples of gill, skin, muscle, blood, liver, bile, stomach, and intestine were analyzed for total radioactivity (3H and ^{14}C).

Ethyl acetate extracts of sediment, SAW, and liver and bile--before and after enzymatic hydrolysis with β-glucuronidase or sulfatase--were analyzed by TLC for parent BaP and its metabolizes. Four solvent systems were employed for TLC analyses: Solvent system A (toluene: ethanol, 9:1, v/v) was used for separation of nonconjugated BaP metabolizes; solvent system B (Plate was developed up to 6 cm in ethyl acetate and then redeveloped in the same direction with toluene:ethanol, 100:3, v/v) was used for separation of nonconjugated NPH metabolizes, solvent system C (upper phase of concentrated ammonium hydroxi de:water:n-butanol , 10:50:40, v/v/v) was used for separation of conjugated NPH metabolizes,

and solvent system D (hexane:diethyl ether, 95:5, v/v) was used for separation of nonconjugated BaP metabolizes from BaP and liver lipids. Assessments of NPH and total NPH metabolizes in sediment, SAM, bile, and liver were also made by a solvent partitioning method using hexane and sodium hydroxide. In addition, ethyl acetate-soluble metabolizes from bile before and after enzymatic hydrolyses were analyzed by HPLC.

Protein from the aqueous phase of liver homogenates was pelleted by centrifugation, followed by extraction with acetone and diethyl ether, dryed, and then solubilized to determine radioactivity that was not extractable. (For further details see Varanasi and Gmur 1981.)

5.1.8 BaP Metabolism by English sole

English sole (Parophrys vetulus) (74 ± 17g; captured from Puget Sound), held at 12°C in flowing seawater, were force-fed ^{3}H-BaP (2 mg/kg body wt) and liver, muscle and bile were analyzed for BaP and its metabolites by methods described above (Section 5.1.7). Three fish were sampled at 8, 16, 24, 48 and 168 hr after force feeding BaP. (For further details see Varanasi and Gmur 1981b.)

5.1.9 Binding of BaP Intermediates to DNA Catalyzed by Liver Enzymes of Fish

Starry flounder (131 ± 42g; captured near the mouth of the Columbia River) and English sole (105 ± 33g; captured from Puget Sound) were injected intraperitoneally with 10 mg/kg of BaP, 3-methyl cholanthrene (MC), or PBCO, in corn oil. Control fish in these studies were untreated, because there was no detectable difference in the binding of metabolically activated ^{3}H-BaP to DNA when liver enzymes from untreated or corn oil-treated fish were used. The fish were sampled 24 hr after injection and supernatants of liver homogenates (10,000 x g) were prepared. Rat liver supernatants were prepared in the same manner. The influence of a number of parameters, such as substrate concentration, temperature, reaction time, and concentrations of cofactors were tested to obtain optimum conditions for *in vitro* binding assays. The standard reaction mixture contained: 2 mg of DNA added in 2.5 ml of 0.02 M phosphate buffer (pH 7.4); 0.75 mg NADPH added in 0.1 ml of 0.1 M EDTA (pH 7.4); and 0.2 ml of the 10,000 x g supernatant (5 mg protein). The reaction was started by adding 5 nmoles of BaP in 50 µl of ethanol. The mixture was incubated in the dark for 15 min at 25°C when fish liver supernatant was used, and at 37°C when rat liver supernatant was used. DNA was isolated from the reaction mixture by extraction with phenol saturated with phosphate buffer followed by ethanol precipitation.

BaP metabolizes were formed by incubating liver supernatants with ^{3}H-BaP under the conditions described above, without the addition of DNA. The mixture was extracted with ethyl acetate (2 x 6 ml) and radioactivity in aqueous and organic phases was determined. Separation and quantification of ethyl acetate-soluble metabolizes were carried out using both TLC and HPLC. (For further details see Varanasi and Gmur 1980 and Varanasi et al. 1980.)

5.1.10 AHM Activities in Different Species

Fish, crabs, and snails were collected during NOAA cruise No. MF-77-1 of the Miller Freernan. Livers from fish, visceral organs from crab, and whole snail were frozen and held at -60°C during transit (from Alaska to Seattle) and in the laboratory prior to analyses. The specimens were collected from January 25 to February 10, 1977, in areas northeast of Kodiak, Alaska.

Analyses of AHM activities were also carried out on livers of Pacific cod (Gadus macrocephalus) which were found to have pseudobranchial tumors. The fish were collected during a Miller Freeman cruise in Alaska, and were part of another OCSEAP project (Research Unit 332).

Specific activities of AHM were measured using ^{3}H-BaP as the substrate for the AI-IN. The procedures employed were a modification of those by DePierre et al. (1975). The temperature and pH of the AHN assay mixtures were optimized for the fish (i.e., 25°C and pH 7.5) and assays were performed with NADPH (tetrasodium salt; Sigma Chemical Co.) rather than with a NADPH-generating system.

A typical assay reaction in 2.1 ml contained 0.67 mM NADPH, 1.4 mM $MgCl_2$, 50 ul of enzyme source (25 mg protein/ml), 20 ul of an acetone solution of 3.2 mM tritiated benzo(a)pyrene (0.96 µCi), and 60 mM Tris HCl buffer (pI-l 7.5). The enzyme source was a 20% (wt/vol) homogenate of tissue (e.g., liver) in cold 0.25 M sucrose solution that was separated as a supernatant fraction from cellular debris by centrifugation at 9,000x9 (or 10,000xg) for 20 min. Duplicate reaction mixtures (in open culture tubes under subdued light) were shaken for 10 min at 25° before initiation of reactions by the addition of the ^{3}H-BaP. Incubation time was 20 min. During work up, two hexane extractions were employed, in contrast to a single extraction according to DePierre et al. (1975). This procedure resulted in better agreement among assays and less variation in blanks.

Additional analyses of hepatic AHM activities were conducted with the use of NPH as the substrate (Nilsson et al. 1976). Protein contents of the enzyme sources were determined by the method of Lowry et al. (1951).

5.1.11 Uptake, Metabolism and Toxicity of Hydrocarbons in Invertebrates

(a) Sperm and eggs from artificially spawned mussels, Mytilus edul is, (col lected from Puget Sound) were placed in 400 ml of seawater containing ^{3}H-NPH (sp. act. 198 mCi/mmole) at concentrations of 100, 10, and 1 ppm and in control seawater at 11 ± 1°C. Each test and control condition was run in triplicate. Samples of eggs and/or larvae were removed from each container at 0.5, 1, 2, 3, 6, 12, and 24 hr, and were preserved in 5% buffered formalin solution. Water samples were taken at each time period for determinations of the NPH concentration. Specimens were counted and the developmental stages identified.

(b) Sperm and eggs of oysters, Crassostrea gigas, (collected from Puget Sound) were separately exposed to a seawater solution of ^{3}H-NPH (sp. act. 198 mCi/mmole) at two concentrations (10 and 1 ppb)

for 15 min after which the complementing gametes were added. In addition, non-exposed gametes of both sexes were introduced into seawater containing NPH at concentrations of 10, 1, and 0.1 ppb and one control in uncontaminated seawater. Larval samples were taken at intervals of up to 48 hr and were preserved in 5% buffered formalin. Water samples were taken at each time point to determine the concentration of NPH. The eggs and/or larvae in each sample were counted and the developmental stages identified.

(c) One-year old spot shrimp (collected from Puget Sound) were exposed in flow-through aquaria for 7 days to the SWSF of PBCO at an average concentration of 110 ppb as determined by GC. The SWSF was obtained from the solubilizer (Fig. 1). The animals were washed, extracted, and the extracts were analyzed for accumulated hydrocarbons by GC/MS. Abdomens were separated from thoracic segments and analyzed separately. (For further details see Sanborn and Malins 1980.)

(d) Newly metamorphosed larval stages of spot shrimp and Dungeness crab, Cancer magister, (collected from Puget Sound) were exposed in flowing seawater (10 ± 1°C) to 8-12 ppb of [1-^{14}C] NPH (sp. act. 5 mCi/mmole) or [1-^{14}C] NPH complexed with bovine serum albumin (BSA). The spot shrimp and Dungeness crab were hatched in the laboratory from ovigerous females. The shrimp larvae hatched each day were held in separate holding tanks and fed brine shrimp. Exposure periods varied from 12 to 24 hr and deputation studies were carried out for periods of up to 132 hr. Larvae were examined for both [1-^{14}C] NPH and its metabolizes. Total radioactivity in the animals was determined, and total metabolizes of [1-^{14}C] NPH were determined by employing formic acid/hexane extractions (see Section 5.1.1). (For further details see Sanborn and Malins 1978.)

(e) Adult spot shrimp were placed in an 80 ppb seawater (10 +1°C) solution of ^{3}H- (sp. act. 198 mCi/mmole) and ^{14}C- (sp. act. 3.67 mCi/mole) labeled NPH; the ^{3}H/^{14}C ratio was 48:1. The concentration was maintained under flow-through conditions. The animals were removed after 10 hr, washed, the thorax and abdomen separated, and the tissues analyzed by HPLC. (For further details see Sanborn and Malins 1980.)

(f) Stage I spot shrimp larvae that had been hatched in the laboratory were exposed to an 18 ppb seawater solution of ^{3}H-NPH (sp. act. 198 mCi/mmole) at 10°-12°C. After 10 hr the larvae were washed, weighed, extracted and analyzed for NPH metabolizes by HPLC.

5.1.12 Food Chain Transfer of 2,6-Dimethylnaphthalene (2,6-DMN) to Sea Urchins via Algae

The marine algae, Fucus distichus, and the green sea urchin (Strongylocentrotus droebachiensis) were collected local ly. The Fucus was exposed to 2,6-DMN as follows: Two hundred grams of the wet algae was rinsed in seawater and then introduced-into an 68 1 aquarium containing seawater and 10 mCi of [2,6-^{3}H]-DMN (sp. act. 2 Ci/mmole) in 0.5 ml of ethanol. Samples of the seaweed were removed at 5 hr intervals over a period of 35 hr, rinsed well, divided into aliquots, then frozen at -20°C until analyzed for incorporated tritium.

A fresh batch of the seaweed was harvested and added to an aquarium containing the ^{3}H-2,6-DMN. After 25 hr, the time of maximum tritium-incorporation, the exposed Fucus was removed, rinsed well in seawater, and then placed in an aquarium containing 6 sea urchins (170 ± 4g). A new batch of the 2,6-DMN-treated Fucus was added 24 hr later to the sea urchin aquarium after removal of unconsumed Fucus. This sequence of presenting exposed Fucus to the sea urchins at 24 hr intervals was repeated throughout the 14 day experiment.

After three days of feeding on the 2,6-DMN-treated Fucus, 3 sea urchins were removed, rinsed, and frozen at -20°C until analyzed. The remaining 3 sea urchins were removed after 14 days of feeding on the treated Fucus and handled similarly.

The exoskeleton of sea urchins was pulverized and extrdcted with hot methanol and filtered. Aliquots were analyzed for tritium by scintillation counting. Half-gram portions of the powdered exoskeleton residue remaining after methanol extraction were also digested in tissue solubilizer and then assayed for tritium.

Fucus samples and aliquots of homogenized gonadal and digestive tract were analyzed for 2,6-DMN by digestion in hexane-sodium hydroxide. Metabolizes of 2,6-DMN were analyzed by extracting soft tissues with hot methanol-diethyl ether, followed by TLC of the extracts. Sulfate fractions were removed from TLC plates and extracted with hot methanol. After removal, the sulfate fractions were treated with aryl sulfatase at 37°C in buffer (pH 5.0, 0.2 M acetate). The digests were extracted with ethyl acetate and analyzed by TLC. The 3- and 4-hydroxy derivatives of 2,6-DMN, isolated as a single band, were resolved on a TLC plate developed 5 times with toluene. (For further details see Malins and Roubal 1982.)

5.1.13 Biological Fate of Metals in Fish

Exposure conditions for metals studies

Water-immersion Studies. Coho salmon (2U0 ± 20 g; obtained from National Marine Fisheries Service, Manchester, WA) and starry flounder (3U ± 15 g; captured from near the mouth of the Columbia River) were held at experimental temperatures of 4° or 10"C for a period of two weeks, and then exposed to either seawater-borne lead-210 or cadmium-109 (150 ppb) under partial flow-through conditions.

The fish were exposed to either lead or cadmium for a period of two weeks at each temperature then three or four fish per exposure group were sampled. At the end of the two-week period, remaining fish were placed in control seawater for deputation and sampled after 7 and 37 days. A group of control fish of similar weight was kept under identical conditions in control seawater. Concentrations of lead and cadmium in control seawater were less than 5 and 2 ppb, respectively.

Injection Studies. Coho salmon (80 + 5 g) were injected intravenously (i. v.) with 32 ± 2 µg of metals as either-lead nitrate mixed with ^{210}Pb or cadmium chloride mixed with ^{109}Cd dissolved in 250 µl of tris buffer (pH 7.2). A similar experiment was carried out with fish which were exposed to 150 ppb of nonradioactive cadmium or lead for 2 wk prior to iv. injection of radiolabeled metals to determine if prior exposure to metals caused any alterations in radioactivity associated with metal binding proteins.

Analytical methods for metals studies.

Samples of mucus, skin (with scales), scales, skin, blood, gills, liver, and kidney were obtained and concentrations of ^{210}Pb or ^{109}Cd were determined in these tissues by liquid scintillation spectrometry. Concentrations of total lead or cadmium in these tissues, expressed on a wet weight basis, were obtained from ratios of ^{210}Pb/Pb and ^{109}Cd/Cd in stock solutions used in the exposures. Concentrations of nonradioactive lead and cadmium in seawater and fish tissues were determined by Laucks Analytical Laboratories (Seattle, Washington).

Samples of liver and kidney of fish from the injection study were homogenized and cytosol obtained. Three samples were chromatographed on a column packed with Sephadex G-75 superfine. The radioactivity in the eluted fractions was determined to assess the distribution of metals bound to various protein fractions. The protein concentration in each eluant was determined by a modified method of Lowry et al. (1951). (For further details see Varanasi and Markey 1978 and Reichert et al. 1979.)

5.2 Pathology

5.2.1 Effects of Petroleum on Disease Resistance

Acquisition, Handling, and Exposure of Test Animals. Test animals for disease resistance studies were obtained from the following sources: juvenile coho salmon were from the Issaquah Hatchery of the Washington Department of Fisheries, from the Willard National Fish Hatchery, Cook, Washington and from Sashin Creek, Little Port Walter, Alaska. The Sashin Creek fish were received as eyed eggs and reared at the Northwest and Alaska Fisheries Center (NWAFC), Seattle, Washington. Juvenile rainbow trout were from a stock maintained at the NWAFC and were the progeny of fish originally obtained from the Spokane Hatchery of the Washington Department of Game. Adult flatfish were captured by otter trawl and juvenile flatfish collected by beach seine in Puget Sound; adult spot shrimp were collected by pot fishing in Puget Sound.

All experimental animals were held under laboratory conditions for at least 2 wk before the start of testing. The salmonid fish were fed an Oregon moist pellet (OMP) diet at 2% of their body weight, and the flatfish were fed a mixture of minced clams and krill. Spot shrimp were maintained on a diet of fish offal.

Several different methods were used to expose fish and crustacea to petroleum hydrocarbons. Salmonids were exposed both via the diet, by mixing OMP with PBCO as described by Hodgins et al. (1977), and via flow-through exposure to the SWSF of PBCO (Roubal et al. 1977b). Flatfish and spot shrimp were exposed to PBCO or Cook Inlet crude oil (CICO) by maintenance on oil-contaminated sediment (McCain et al. 1978). Sediment, water, and selected tissues from test animals were analyzed for total extractable petroleum hydrocarbons (TEPH) by the methods described by Malins et al. (1980).

In vivo Assays of Immunocompetence.

(a) Throughout these studies oil-exposed animals were compared to controls for their ability to survive a laboratory challenge by the marine fish pathogen Vibrio anguillarum. Several different bacterial isolates were used, the taxonomic identity of each was confirmed by conventional cultural and biochemical tests, and by deoxyribonucleic acid hybridization.

As needed, bacteria were cultivated overnight in either Trypticase soy broth (BBL) or brain heart infusion (Difco) on a reciprocal shaker at room temperature (ca 23°C). Both media were supplemented with an additional 10 my/l NaCl. Ten-fold serial dilutions were prepared in 0.15 M NaCl and the numbers of viable bacteria were estimated by standard spread-plate technique. For challenge, fish were transferred from the exposure facilities at the Mukilteo Field Station to the Disease Isolation Laboratory at the NWAFC. Groups of 5 to 20 test animals were placed in individual 38 1 aquaria containing aerated seawater (salinity 26-30°/00) or freshwater maintained at the exposure temperature (10-15°C). Fish were challenged with selected test concentrations of bacteria by either i.p. injection (flatfish) or by subcutaneous injection at the posterior insertion of the dorsal fin (salmonids). Shrimp were challenged by injection of bacteria at the suture separating the thorax and abdomen on the dorsal surface. Aquaria were checked daily for mortalities for a minimum of 10 d after challenge. Tissues from dead fish and shrimp were cultured for bacteria; only in those cases in which V. anguillarum was reisolated in pure culture were the deaths attributed to the bacterial challenge.

LD_{50} values (i.e., the number of bacteria that kill 50% of the animals) and their 95% confidence intervals (C.I.) were calculated by logit analysis (Cox 1970). The LD_{50} values were statistically compared using a method similar to that described by Litchfield and Wilcoxon (1949). The 95% C.I. for the difference between any two LD_{50} values was calculated and the hypothesis that the two values were equal was rejected (α=0.05) if this C.I. did not contain the point zero. In those cases in which inadequate partial kills prevented calculation of an LD_{50} by the logit procedure, the method of Reed and Muench (1938) was used. In addition, statistical comparisons between the percent mortality occurring in oil-exposed and control groups challenged with the same number of bacteria were made using Chi-square analyses.

(b) An additional test was conducted to assess the effect of oil exposure on the adaptive immune response of fish. Groups of PBCO-exposed and control rainbow trout were vaccinated with a heat-killed vaccine prepared from V. anguillarum and subsequently challenged with varying concentrations of the living organisms.

In vitro Assays of Immunocompetence.

(a) Numbers of antibody-forming cells in anterior kidney and splenic tissues of PBCO-exposed and control rainbow trout were determined by a modified Jerne plaque assay (Chiller et al. 1969). Fish were immunized against trinitrophenol conjugated with lipopolysaccharide (LPS) from Escherichia coli B. Plaque formation was assayed on a lawn of sheep red blood cells (SRBC) which were coated with LPS, in a soft agarose matrix. Rainbow trout sera, frozen and thawed one time, were used as a source of complement.

(b) Serum agglutinating antibody levels were compared between oil-exposed and control fish by the microdilution technique (Microtiter, Cooke Engineering Company). Fish were immunized against V. anguillarum and 21 d later 2-fold serial dilutions of serum were tested for specific agglutinins. Fish were held at 15°C to facilitate antibody formation.

(c) For polyclonal lymphoid cell activation assays, peripheral blood leukocytes from oil-exposed and control fish were incubated with purified protein derivative (PPD) prepared from the tubercle bacillus. Activity was quantified by measurement of plaque-forming ability on lawns of SRBC's in agarose.

(d) The degree of mitogenic stimulation was compared between PBCO-exposed and control fish under assay conditions previously described in detail by Etlinger et al. (1976). Briefly, leukocyte cultures prepared from splenic tissue were assayed for lymphocyte proliferation following incubation in the presence of the plant-derived mitogenic substance concanavalin A (Con A). Stimulation was quantified by measurement of the incorporation of ^{3}H-thymidine in the proliferating cellular DNA.

(e) Early in the course of these in vitro investigations of immunocompetence it was noted that the oil-exposed rainbow trout had reduced spleen sizes compared to those of the controls. Since antibody-forming leukocytes have been previously identified as one of the principal cellular components of the spleen (Chiller et al. 1969), this condition was further examined. Spleen-weight to body-weight ratios were measured and cellular composition with respect to total numbers of erythrocytes and leukocytes-thrombocytes were determined. These same hematological parameters were also determined for anterior kidney tissue homogenates and peripheral blood.

Effects of Corexit 9527 on Disease Resistance. Juvenile coho salmon were exposed for 30 min at 15 +_ ° to 30 ppm (v/v) Corexit 9527 in seawater containing various concentrations of V. anguillarum. Control fish were similarly exposed in seawater containing either bacteria or

Corexit only. Fish were then transferred to individual 38 1 aquaria containing aerated fresh seawater and mortality was monitored for 10 d. All dead fish were examined by bacterial culture and death was considered to be due to V. anguillarum only when the bacterium was reisolated in pure culture.

5.2.2 Pathological Changes in Flatfish from Exposure to Oil-Contaminated Sediment

Acquisition and Handling of Test Animals. English sole, rock sole, or starry flounder were captured with otter trawl or beach seine from either the mouth of the Columbia River or in Puget Sound. Fish were transported to Mukilteo, Washington and held for 7-14 days prior to use. All fish were determined to be actively feeding before experiments were initiated. Two types of sediment (either high-silt or high-sand content) contaminated with 0.2, 0.5, or 1.0% (v/v) PBCO were tested; both juvenile and adult stages were examined and exposures ranged from 2 wk to 4 mo. The general approach for all experiments was similar and is outlined below.

Experimental Design. Fish were randomly assigned to a test or control group, weighed, measured, and cold-branded (Fujihara and Nakatani 1967) for individual identification. Prior to each test, samples for hematology and histopathology were collected from 6 to 10 control fish. Hematological tests included hematocrit, hemoglobin, .and total red blood cell and leucocyte counts using standard techniques as described by Blaxhall and Daisley (1973). Tissue samples (gill, skin, fin, intestine, kidney, liver, and spleen) were placed in phosphate-buffered formalin. In addition, samples of liver, skin and muscle were placed either in glass vials or aluminum foil and stored at -20°C for subsequent chemical analysis. Sediment samples (400 g) for analysis of TEPH were collected and stored at -20°C.

At intervals of 1 to 4 wk, depending on the length of the experiment, sediment samples and samples of 3 to 6 test and control fish were collected using the same procedures. The remaining fish were weighed and measured and returned to the aquaria.

Fish were fed to satiation, twice daily five days per week, a mixture of clams, euphausids, and live earthworms. Tanks were monitored daily for mortality. Water flow in the 200 1 test and control aquaria was maintained at 3 l/rein; average water temperature, 8.5°C; average salinity, 27 $O/_{oo}$; light was maintained on a 12L:12D schedule.

Sediment was collected at 2 sites near Sequim, Washington. The high-sand type (approximately 99% of particles >0.07 mm diameter) was from Port Williams; the high-silt type (48% of particles <0.07 mm), from a lagoon adjacent to the Battelle Northwest Laboratory. PBCO and sediment were mixed together for 3(J min in fiberglass-lined cement mixer at concentrations of 0.2, 0.5, or 1.0% v/v.

Both control and test apparatuses were made of fiberglass-lined plywood containers set inside larger fiberglass tanks (Fig. 2). The design allowed water circulation through the sediment. Sediment alone or sediment mixed with oil was layered at a thickness of 5 cm in the containers and the tanks flushed with sea water for 24 hr prior to use.

Histological Procedures. For light microscopic examination, preserved tissues were embedded in paraffin and sectioned at 5 µm following the procedures outlined by Preece (1972). As necessary, gills and bones were decalcified using a commercial decalcification solution (Scientific Products, Redmond, WA) prior to processing. Paraffin sections were routinely stained with Mayer's hematoxylin and eosin-phloxine (Luna 1968). As stained tissues from each fish were examined microscopically, the presence of parasites and descriptions of all observed lesions were recorded. For further characterization of the components of specific lesions, additional sections were stained with May-Grunwald Giemsa for RNA and uNA; Masson's trichrome for collagen; Periodic acid-Schiff (PAS) for glycogen, mucin and basement membrane; Brown and Brenn's Gram stain for bacteria; Congo red for amyloid; Laqueur's method for alcoholic hyalin; Ziehl-Neelsen method for acid-fast bacteria; Gomori 's iron reaction for iron pigments; or with the Armed Forces Institute of Pathology method for lipofuscin and ceroid (Thompson 1966, Luna 1968, Preece 1972).

All sectioned tissues were examined using a blind system. Each fish was assigned a number when necropsied, then prior to microscopic examination the fish was assigned a random identification number. Histopathologists examining tissue sections had available only information on the species, length, weight, sex, and the presence of grossly visible lesions. All slides from an individual specimen were first screened for abnormal tissues, and those sections with obvious lesions were segregated for more extensive examination.

Chemical Analysis. Hydrocarbon analyses of sediment and fish tissue were performed using a modification of procedures described by MacLeod et al. (1977) which involved gravimetric determination of total extractable hydrocarbons and GC for alkanes and arenes. By subtracting the concentration of total extractable materials in the control sediment from the level of extractable hydrocarbons in the oil-contaminated-sediment, the value of total extractable petroleum hydrocarbons (TEPH) was determined. Some data presented are defined as selected aromatic hydrocarbons (SAH), which is a sum of the major individual aromatic hydrocarbons.

5.2.3 Cytopathology

A variety of life stages of flatfish, salmonids, and smelt were exposed to crude oil. The design and exposure regimes for each experiment is described in Section 5.3.2 and 5.3.3 e and f.

All tissues were fixed prior to microscopic examination in 0.75% glutaraldehyde, 3% formalin, 0.5% acrolein in ().1 M sodium cacodylate

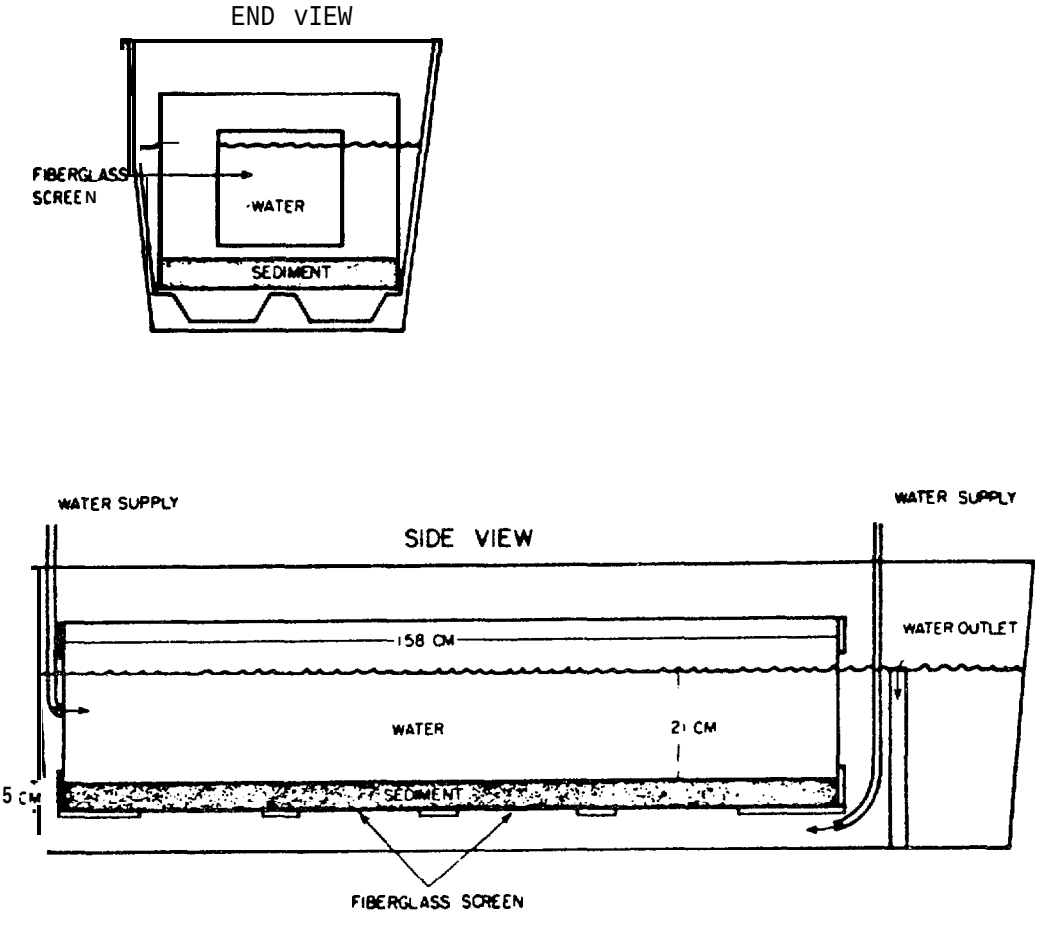

FIGURE 2. Diagram of aquaria used in experiments 'involving exposure of flatfish to oil-contamined sediments. (From McCain et al. 1978)

buffer with 0.02% $CaCl_2.H_2O$, and 5.5% sucrose (Hawkes 1974). The tissues designated for light microscopy (LM) or for transmission electron microscopy (TEM) were post-fixed for 1 1/2 hours in 1% osmium tetroxide in the same buffer, dehydrated in an ethanol series, and embedded in plastic. For LM, sections were cut at either 1.0 or 0.5 µm. The 1.0 urn sections were stained with Richardson's mixture or with a PAS reagent (Nevalainen et al. 1972); the 0.5 um sections were stained with toluidine blue or a trichrome (MacKay and Mead 1970). For TEM, sections were cut with a diamond knife and stained with lead citrate and uranyl acetate and examined with a Philips EM-3(J1 electron microscope. For scanning microscopy (SEM), samples were dehydrated after the initial fixation, critically point dried, coated with gold-palladium, and examined with an AMR-1000 scanning electron microscope.

5.3 Behavior and Physiology

5.3.1 Chemical Analysis of Water, Sediment and Tissue

Water and Sediment Analysis. Water samples for hydrocarbon analysis were collected in 100 or 300 ml screw cap glass bottles with Teflon(R)-lined lids. The samples were usually extracted on the day of collection; however, if they were to be processed later, 3 ml of concentrated hydrochloric acid was added to eliminate microbial activity and the samples were stored at 2°C.

Two methods of extraction and analysis were used. In initial experiments, a recovery standard was added to the 100-M1 water samples and extracted using 5 ml of carbon disulfide. The organic phase was separated and evaporated to 0.8 ml, a GC internal standard added, and 8 µl of the extract was gas chromatographed on a l-m long by 2-mm ID glass column packed with 0.2% carbowax 1500 on 59/80 mesh carbopack C (Supelco, Belleforte, PA). Nitrogen was used as a carrier gas with a flow rate of 46 ml/min and temperature programming was from 40 to 215°C at 15°C/min.

In later studies, water samples were collected in 300 ml bottles containing 12 ml of methylene chloride. Immediately after collection, the samples were shaken for 3 min to partition hydrocarbons into the methylene chloride and the samples were stored at 2°C. At the time of processing, recovery standards were added and the water samples were extracted three times with a total of 24 ml of methylene chloride.- The extract was dried with sodium sulfate and reduced to 0.8 ml in a concentrator tube. GC internal standards were added and the extracts analyzed by glass capillary-gas chromatography (characteristics described by MacLeod et al. 1977). The limit of detection for individual aromatic compounds in the water sample was 1 ppb.

For both GC methods employed, identities of hydrocarbon components were confirmed by mass spectral analysis (for description] of equipment and characteristics see Malins et al. 1980), and hydrocarbon concentration was determined from the total area under the chromatogram. All data were corrected for extraction efficiency except where expressly stated.

For specific analysis of benzene, 1 ml of benzene-seawater mixture was placed in a 2 ml GC vial, capped, and allowed to stand for 18 to 20 hr. One ml of air was then removed from the vial headspace and injected directly into a GC. These results were compared with results of benzene standards prepared by injection of µl amounts of benzene-methanol solutions into GC vials containing 1 ml of seawater, which were then processed in the same manner.[1]

Extraction and analysis of petroleum hydrocarbons in sediment followed a procedure described by Brown et al. (1979).

Tissue Analysis. Analytical procedures for tissue analysis on trout followed methods of Warner (1976) utilizing alkaline digestion, solvent extraction, and silica gel chromatography. To reduce losses of volatile compounds of PBCO, the alkaline digestion procedure was modified by adding 6 ml of 4 N NaOH to the 10 g of sample, and the sample digested at 30°C for a minimum of 16 hr.

Silica gel chromatography Fraction 3 from the modified Warner method, containing triaromatic compounds, was concentrated to 2.0 ml and analyzed using an Aminco-Bowman spectrofluorometer (American Instrument Company, Silver Spring, Maryland). Dilute solutions of PBCO (0.1 µg/ml to 10.0 µg/ml in methylene chloride:petroleum ether [20:80 v/v] were used as standards for the spectrofluorometric quantitation of the samples. The maximum excitation wavelength and maximum emission wavelength for PBCO were found to be 262 nm and 364 nm, respectively.

Chemical analysis of surf smelt embryos, salmon liver, and salmon brain tissues for petroleum hydrocarbons was conducted using methods described by Malins et al. (1980).

5.3.2 Preparation of Oil-Water Mixtures

Four flow-through systems introduced petroleum hydrocarbons into water:

(a) The system developed by Roubal et al. (1977b) provided a SWSF of crude oil composed almost entirely of monocyclic aromatic hydrocarbons (Roubal et al. 1978). For several studies a mixture of hydrocarbons modeled on the SWSF of fresh PBCO was formulated, and contained 95% (by weight) of those hydrocarbons detected in the SWSF (Table 1).

(b) A method of introducing the "model" hydrocarbon mixture into water was devised using a water jet eductor (Schutte and Koerting, Cornwells Heights, PA, type 264, 1/2 in, capacity ratio of 0.36). Water was passed through the eductor at a flow rate of 16 l/rein, and a pressure of 5.5 kg/cm^2. The hydrocarbon mixture and individual

[1] An alternative method for analysis of benzene, especially when benzene was a component of the total SWSF, was to use carbon disulfide as an extracting solvent. Carbon disulfide is not detected by GC and thus does not mask the GC response to benzene.

TABLE 1. Composition of mixture of monocyclic aromatic hydrocarbons modeling the SWSF of PBCO.

Hydrocarbon	% by weight in mixture
toluene	57.0
o-xylene	16.2
benzene	7.9
l,2,4-trirnethylbenzene	7.3
p-xylene	4.8
m-xylene	4.8
ethyl benzene	2 0

components were introduced into the vacuum port of the eductor by a calibrated, continuous flow, syringe pump; the reduced pressure in the eductor port vaporized the hydrocarbon mixture prior to its mixing with water. The effectiveness of the eductor in solubilizing the mixture of monocyclic hydrocarbons in the water was determiend by GC analysis of replicate water samples taken at 2(I min intervals with known injection rates of the model mixture set on the calibrated syringe pump. Since the solubilizing system was found to precisely introduce hydrocarbons into water (Fig. 3), the concentrations of hydrocarbons present during testing were often based on calculated values.

(c) The SWSF of crude oil was also produced by a flow-through apparatus similar to that described by Nunes and Benville (1978). Crude oil was continuously pumped (1 ml/min) onto the surface of seawater in a 40-cm-diameter glass carboy. A flow of 4 l/min of seawater at a constant head penetrated a dispersion plate and dripped through the oil layer. The resultant SWSF was continuously removed from the bottom of the carboy and delivered to exposure tanks.

(d) Weathered oil was prepared in a wave machine that subjected fresh PBCO or CICO to mixing with seawater by wave action, exposure to sunlight, and loss of volatile components through evaporative processes (Fig. 4). A paddle hinged at the bottom was attached to an electric motor which produced a steady wave periodicity of 48/rein. At the opposite end from the paddle an artificial beach was added to dampen wave action and simulate water-accommodated oil passing down through the gravel of a churn salmon spawning redd. Coarse gravel (80%, 1 to 5 cm in diameter, remainder fine) was spread 25 cm deep over a perforated

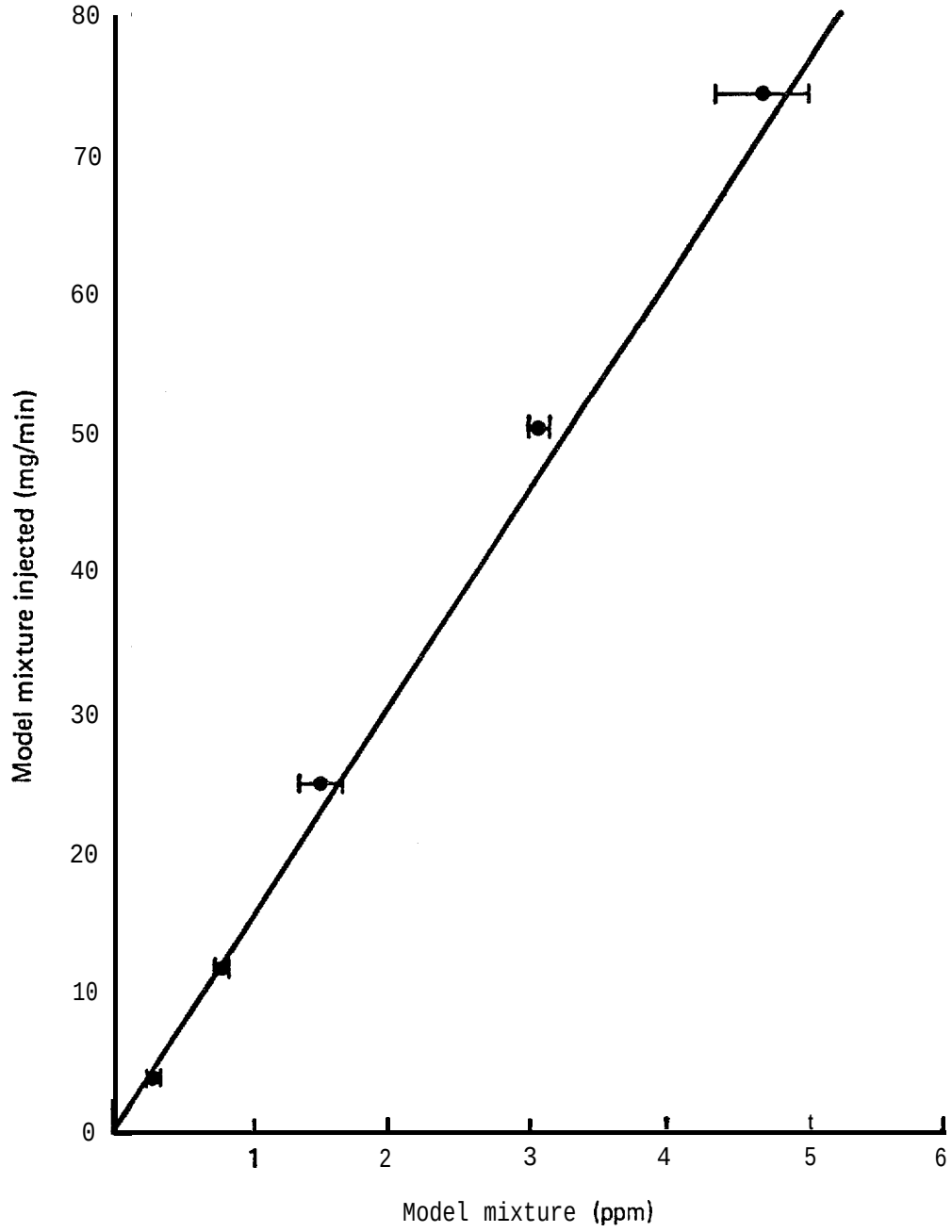

FIGURE 3. Amount of aromatic hydrocarbon mixture injected into the solubilizing system and ppm of mixture present in the water as calculated from water flow (line) and by GC analysis (circles, average GC value). Horizontal bars represent standard error of GC values. (From Maynard and Weber 1981)

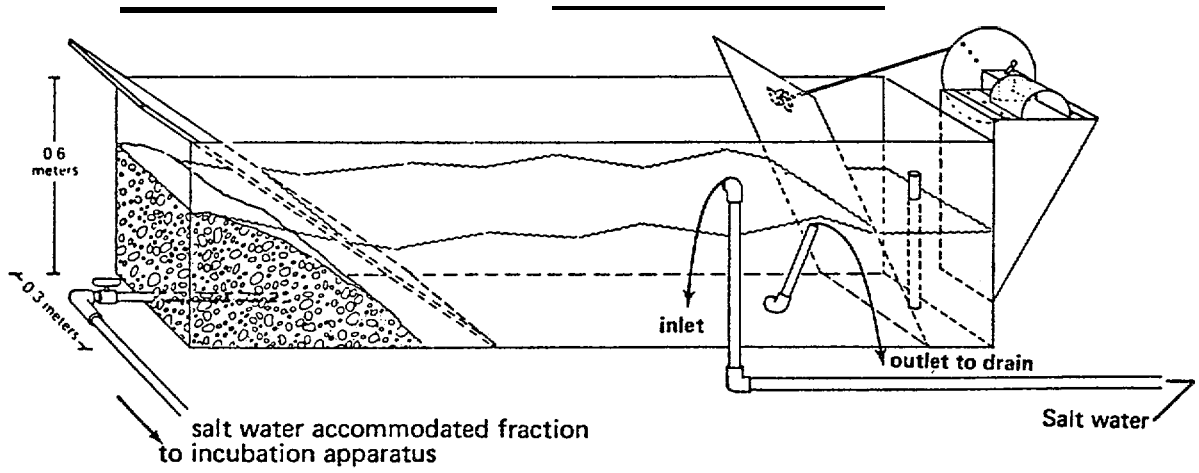

FIGURE 4. Apparatus for weathering of crude oil.

pipe. Over this gravel beach was placed a removable baffle which isolated the gravel from the oil during the weathering process, thus preventing oil from coming in contact with the "beach" before weathering was completed.

Six-hundred and eighty ml of fresh PBCU or CICO were layered on the water surface (91 $\mu l/cm^2$) in the wave machine resulting in an initial oil concentration of 4,000 ppm. During the weathering process, there was a continuous flow of seawater (27-30 /oo salinity) through the wave machine with a water replacement time of once per hour. Water overflow was removed 25 cm beneath the water's surface via an adjustable standpipe in order to maintain the surface oil slick. After 30-48 hr of flow-through operation and wave action, the oil was defined as weathered.[1] For some experiments the salinity of the incoming water was then reduced (to 16-24 /00) and the baffle removed, allowing the

[1] Weathering of PBCU and CICU is defined here as a loss of volatile components generally those hydrocarbons with boiling points less thdn 210°C and representative of the naphtha fraction (See Table 53, Section 6.3.1[e]). Presumably the principal processes in this loss are a combination of dissolution and evaporation. The term seawater-accommodated fraction (SWAF) denotes water removed from under the oil slick. In water samples collected, there was often a Tyndall effect indicating that along with the soluble petroleum hydrocarbon components there were some oil particles dispersed in the water column.

oil contaminated water to come in contact with the gravel. The SWAF was then drawn off through the gravel and delivered to the exposure apparatus. In other experiments the gravel in the wave machine was replaced with an inclined plane similar to the removable baffle shown in Figure 4, and the perforated pipe for SWAF delivery extended through the incline.

5.3.3 Vertebrate Studies

Behavior of Pacific salmon exposed to petroleum hydrocarbons

(a) Olfactory disruption in juvenile coho salmon. Two series of electrophysiological studies were conducted on juvenile coho salmon ($\bar{x}$ length" 20 cm). The first series, using salmon reared in seawater, used standard experimental procedures and recording techniques to monitor the electroencephalographic (EEG) response from the olfactory bulb (tiara 1973, Bodznik 1975). Amino acid stimulants (L-serine, L-alanine, and L-methionine at 10^{-3}M) and individual aromatic hydrocarbons were dissolved in seawater filtered to 5 um. Extracts of PBCO were prepared by hand shaking 100 ul of oil with 50 ml of filtered saltwater for 5 min. After the mixture stood for 1 h, the SWAF was drawn off from beneath the slick. Concentrations of undiluted stock solutions of individual aromatic hydrocarbons and the SWAF were determined by GC.[1]

The second series, using coho reared and maintained in freshwater, used EEG to assess detection and disruption. Control water and water used to make L-serine solutions were obtained from the same source as that used in producing a water-"model" hydrocarbon mixture. The "model" hydrocarbon mixture (Table 1) was introduced into the water using a syringe pump and water jet eductor. Hydrocarbon solutions were placed in Teflon wash bottles and replenished at least every 30 min. To assess detection of aromatic hydrocarbons the olfactory bulb EEG response to different hydrocarbon concentrations introduced in the nares was obtained from a minimum of ten, 5-10 sec rinses with solutions containing 1.9, 2.8, or 3.7 ppm of the aromatic hydrocarbon mixture. Olfactory disruption as a result of aromatic hydrocarbon exposure was evaluated by recording the EEG response to 10^{-3} M L-serine solution both before and after rinsing the nares continuously for 5-10 see, 10 rein, or 20 min with a solution containing 4.0 ppm of the aromatic hydrocarbon mixture. The EEG responses were quantified by measuring the area under the integrated EEG signal for the first 2 through 6 sec following stimulation of the nares. Relative changes in amplitude of the EEG response was analyzed using an unpaired t-test for detection experiments and a matched pair t-test for disruption experiments.

(b) Migratory and Homing Behavior in Adult Salmon. One field study evaluated whether salmon avoid their borne stream when petroleum hydrocarbons are present in the water, Two others were designed to

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[1] A single 20 ml aliquot of each stock solution was extracted with 5 ml of carbon disulfide and processed using procedures described in the first part of Section 5.3.1.

determine if short-term exposure to petroleum components had an effect on a salmon's homing capability.

Avoidance reaction. The study site was located on Chambers Creek, southern Puget Sound, Washington. One kilometer from the mouth of the creek is a tidewater dam having a central spillway, ladders on each side and trapping facilities at the upstream head of each ladder. The average discharge of the creek during the period of study (October-November, 1978) was 1.8 m^3/sec with approximately 12% of the water passing through the west fish ladder, 28% passing through the east ladder, and the remainder passing over the spillway. Water temperature was 9-10"C. The mixture of monocyclic aromatic hydrocarbons introduced into the water contained the following percentages of individual components by volume: benzene, 7.9%; toluene, 57.0%; ethyl benzene, 7.0%; m- arid p-xylene, 23.3%; and o-xylene, 4.8%. This mixture closely approximated the water-soluble fraction of PBCO in its relative proportions of aromatic hydrocarbons (Table 1).

To generate the water-hydrocarbon mixture, water from the dam impoundment was pumped through a water-jet eductor at a flow rate of 12 l/rein and a pressure of 6.7 kg/cm^2. The hydrocarbon mixture was introduced into the vacuum port of the eductor at flow rates of 3 to 105 ml/min. The eductor vacuum served as a pump; the hydrocarbon flow was regulated by a calibrated metering valve. From the eductor the water-hydrocarbon mixture was piped to the top of the west fish ladder (the east ladder was left untouched) where a diffuser pipe, with its outlet 0.6 m below the water surface, provided dispersion of the water-hydrocarbon mixture across the width of the ladder with further mixing taking place in the water turbulence in the ladder.

Prior to an avoidance test, the salmon in the traps at the head of each ladder were counted and placed upstream; water depth in the top step of each ladder was measured and the flow was calculated using the Francis, sharp-crested, weir formula (King 1954). The duration of each test varied from 4.5 to 22 hr, depending upon the numbers of migrating fish, stream discharge and tidal fluctuation.

At the end of each test, the amount of hydrocarbons used was measured; duplicate water samples for chemical analysis were taken from the top of the ladder into which the hydrocarbons were introduced, and from the middle of the ladder as tidal height permitted (a water sample was also collected from the top of the opposite ladder serving as control); and the numbers of fish in each trap were counted by species and placed upstream.

Disruption of homing capability. Two studies concerning the effect of petroleum exposure on homing capability were similar in experimental design (capture-exposure-transport-rel ease) but differed in species used, petroleum components, and geographical location.

In the autumn of 1976 adult male chinook salmon were collected from the homing pond of the School of Fisheries, University of Washington, Seattle (Fig. 5). The fish were divided into control and experimental

groups, lightly anesthetized with tricaine methanesulfonate, identified with individually coded spaghetti tags inserted under the dorsal fin, and each group was placed in a 2,400-1 flow-through circular holding tank supplied with 16 l/rein of fresh water. Water delivered to the test (oil exposure) tank was first passed through a sealed 38-1 glass mixing chamber containing glass baffles to extend the duration of oil-water contact. PBCO was metered into the mixing chamber by a calibrated, continuous-flow, syringe pump. The fresh water-accommodated fraction (FWAF) was introduced at the bottom of the test tank and drained at the surface via a central stand pipe, thus producing a distribution of crude oil throughout the water column. The calculated concentrations of PBCO introduced in the water ranged from 0.5 to 40.6 ppm; an oil film and a Tyndall effect were evident during all trials. Concentrated HCl was added to the water samples collected for hydrocarbon analysis; the samples were then sealed and allowed to sit for 24 hr. Any oil film on the surface was removed prior to extraction. Thus, the FWAF concentrations determined by GC are considerably less than the calculated amount of crude oil introduced, and more closely reflect that fraction which is actually freshwater-soluble.[1]

After 14-18 hr of oil exposure, the fish were transported 7 km downstream and released. Three times a week all the fish in the University of Washington homing pond were examined and tagged salmon were recovered. A third group of 18 chinook salmon had their nares occluded with vaseline-saturated cotton. The olfactory-occluded fish were treated identically to controls and were released in equal numbers along with control and oil-exposed salmon during the first three tests.

A second series of experiments was conducted in the autumn of 1977. Returning salmon were trapped at the head of a tidewater fish ladder located on Tulalip Creek in northern Puget Sound. The trapped jack coho salmon (males which mature and return to spawn after one ocean growing season) were divided into control and oil-exposed groups, tagged using the same procedure as described for chinook salmon, and each group was placed in a 600 1 holding tank with a water inflow of 16 l/rein. Water was pumped into the tanks from the bottom of the fish ladder, and the salinity varied with tidal fluctuations. Treated fish were exposed to a mixture of monocyclic aromatic hydrocarbons representati"ve of the SWSF of PBCO (Table 1). This hydrocarbon mixture wasinjected into the water using a syringe pump and water Jet eductor. The water-hydrocarbon mixture was introduced into the bottom of the circular test tank, and drained at the surface via a central standpipe.

The concentration of aromatic hydrocarbons in the water was calculated from the measured amounts of aromatic hydrocarbon mixture injected per minute and water flow rate. In addition, water samples for GC analysis were taken from test and control tanks at the middle and just prior to termination of the exposure period. After 8-22 hr

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[1] Some oil which was accommodated at the time of sampling probably equilibrated with the water in the sample bottle during storage; thus, the reported GC concentrations are considered maximum values.

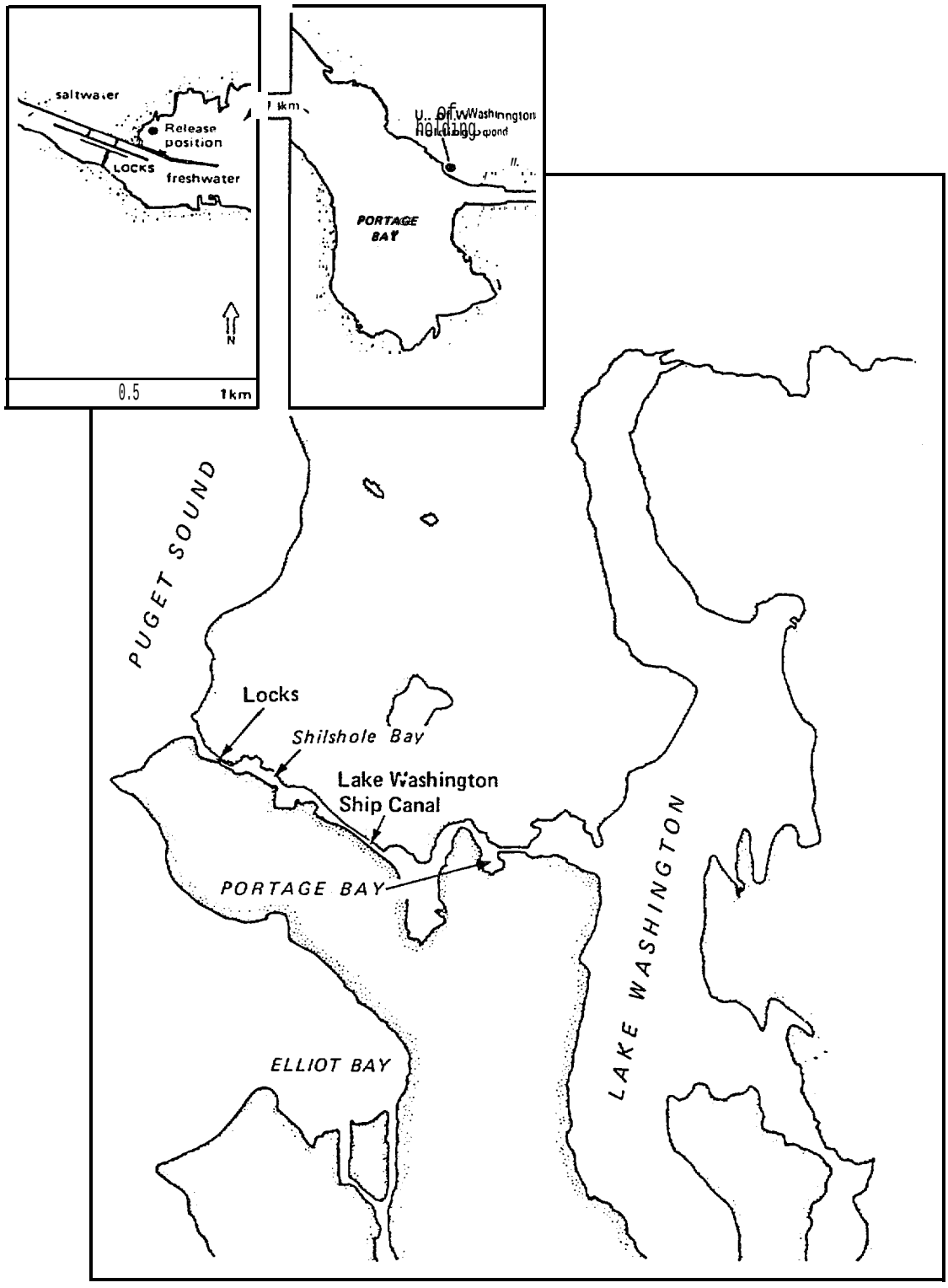

FIGURE 5. Location of adult chinook salmon study and detail of release and recovery sites.

exposure, both control and treated fish were transported and released at one of two marine sites located 1.6 or 4.7 km from the "home" stream. The Tulalip Creek trap and fish ladder were monitored daily and the tagged fish recovered.

(c) Predator-Prey Behavior. Two series of experiments were designed: the first tested oil-exposed prey; the second, oil-exposed predators. In both series the SWSF of CICO was prepared using a solubilizer similar to that described by Nunes and Benville (1978). Water samples were collected twice weekly from the exposure tanks and analyzed for total hydrocarbon content by GC. For both studies the salinity was 26.5 + 1.3 ‰($\bar{x}$ + sd) and temperature 13.4 ± 0.7°C.

For the oil-exposed prey experiments, chum salmon (O. keta) fry were obtained from the Washington State Department of Fisheries Hatchery at Hoodsport, Washington. The predators were 2 yr old coho salmon from the Department of Fisheries Hatchery at Issaquah. They were obtained as fertile eggs, hatched and reared in freshwater at the NWAFC, Seattle, to an age of 1 year and then in seawater at the Mukilteo Facility to an age of 2-plus years and an average length of 35.0 cm.

Three to 4 days before an experiment, test and control fry were distinctively marked by cold-branding (Fujihara and Nakatani 1967). The coho salmon predators were trained to feed on chum fry for at least one month before testing, and were always starved 48 hr before being used.

Test fry were exposed to the SWSF of CICO for periods of 24, 48, 72, and 96 hr while holding control fry under similar conditions for identical lengths of time. Ten test and 10 control fry were then introduced simultaneously into an observation tank containing 3 coho predators. After approximately one-half of the fry were consumed, the predation was halted and the numbers of test and control prey remaining were determined. Fifty percent predation usually took place in a few minutes but was never allowed to extend beyond 2 hr.

For the oil-exposed predator studies, fish were obtained from the following sources: adult coho salmon from a stock reared at the NWAFC and rainbow trout fry from Trout Lodge, Tacoma, Washington. The trout fry were maintained (fed to satiation) on a diet of OMP. The coho predators were maintained on OMP until four months prior to the experiments, then fed exclusively on live chum salmon or rainbow trout fry. Although natural predation by coho salmon on Salmo gairdneri in seawater is unlikely, we concluded after preliminary trials that this combination provided a viable experimental design for a laboratory evaluation.

Two 170 l rectangular fiberglass tanks were used as the exposure and control tanks; circular 950 l fiberglass tanks were used for the predation tests. Seawater inflow rates to the circular tanks provided a 95% exchange within 24 hr.

The coho salmon predators were randomly divided into two groups" of 21 fish each. The group designated as the test group was exposed to the SWSF of CICO. All test fish were exposed (together) to the oil for a period of 17 days, with predator evaluations occurring after 3, 10, and 17 days of exposure. Each of the test groups was further divided into subgroups of 3 fish each for the predator evaluations, so that there were 7 replicates for each group at each of the 3 evaluation periods. Each fish was cold-branded (Fujihara and Naktani 1967) for identification and maintained in the same subgroup throughout the study, with the following exceptions: three mortalities occurred in the control group (one fish each from Subgroups 1, 3, and 6), just prior to the final testing period. The 2 surviving fish from Subgroup 1 were redistributed to Subgroups 3 and 6 to maintain the proper population of 3, which were essential to stimulate feeding behavior. The predators were not fed between evaluation periods.

Predators were transferred from the control or exposure tanks to the predator evaluation tanks at 4 pm on the day prior to testing. At 9 am on the day of testing 10 rainbow trout fry were transferred without acclimation into a tank containing one of the 7 predator subgroups (3 adult coho salmon); the number of prey surviving after 10 minutes was recorded. Surviving prey were discarded. Predators were returned to their respective control or oil-exposure tanks immediately after the predator-prey evaluations. At the termination of the experiment, the brains and livers of 3 control (Subgroup 4) and 6 oil-exposed fish (Subgroups 1 and 6) were analyzed for hydrocarbon content.

(d) Avoidance Behavior of Flatfish Exposed to Oil-Contaminated Sediment

English sole (0-1 yr, 45-120 mm length) were tested in the choice apparatus shown in Figure 6. The choice apparatus was a 61 x 152 cm box with a water depth of 20 cm controlled by standpipes at each end. The box contained two identical 3,400 cm2 trays, each filled with 15 1 of sediment to a depth of 3.5 cm. A perforated pipe running the width of the box and located 2 cm above the water surface provided seawater at 12 l/rein; half of the flow was directed toward each outlet. Studies in which dye was introduced in the water column showed that this water flow configuration provided negligible mixing of water between sides of the test apparatus. When the trays contained sediment, the water volume of the test apparatus was 113 1 with a water replacement flow of 6 times/hr. Seawater temperature averaged 10"C, and salinity averaged 29 $^{o}/_{oo}$. Light intensity at the water surface was uniform (200 lux) and maintained on a 10L:14D schedule.

High-sand content sediment was collected from a beach near Sequim, Washington (Port Williams) - an area known to have low levels of petroleum contamination in previous assays, and for which sediment characteristics (particle size, organic carbon, and metal content) have been determined (McCain et al. 1978). Juvenile English sole were collected in Puget Sound with a beach seine.

Control tests were conducted to determine the movement of flatfish in the choice apparatus when both trays contained uncontaminated sediment. The uncontaminated sediment was rinsed with flowing seawater in the test apparatus for 4 to 24 hr and then 20 juvenile English sole were released on the sediment of one tray; sides for release were alternated between tests. After 19 to 21 hr in the test apparatus, the fish were fed diced clams introduced simultaneously on each tray for either 15 min or to satiation, whichever occurred first. Three hours later the water was turned off and a screen inserted between the two sediment-containing trays. The standpipes were removed and the water level lowered to 5 cm depth. The fish in each side were removed with a dip net, counted, measured, visually checked for stomach fullness, and either the test was terminated or the fish were returned to the side from which they were taken and the process repeated the next day.

Prior to testing flatfish avoidance of oil, the sediment in one tray (used in previous control test) was removed and mixed with either 375 or 750 ml of PBCO in a cement mixer for 30 min to give an initial oil concentration of either 2.5 or 5% (v/v). After rinsing the oiled sediment for 4 to 24 hr, 20 juvenile English sole were released on the tray containing uncontaminated sediment. The same feeding and counting procedure was followed as described for control tests. In addition, at the termination of each test, or at time of counting if the test was continued, a sample of sediment, sediment-associated water, and above-sediment water were collected from each side for GC analysis, and/or for gravimetric determination of TEPH. The sediment sample consisted of a 150 g composite obtained from the surface of each tray by inserting a 50 g capacity corer 2.0 cm deep. For sampling of sediment-associated water, an open ended glass tube (1.7 x 25 cm) was vertically inserted 2 cm into the sediment while holding the upper end of the tube closed. After positioning, the upper end was opened and 50 ml of water rising within the tube was removed with a syringe. Prior to chemical analysis of the sediment-associated water for petroleum hydrocarbons, the samples were centrifuged at 2000 g for 10 min to remove suspended particulate. Above sediment water was taken by submerging and filling a 315 ml glass sample bottle containing 3 ml of concentrated hydrochloric acid.

The biological data were analyzed using two by two contingency table analysis.

(e) Effect of Petroleum Hydrocarbons on Development of Embryos and Larvae

The experimental design for exposure of early development stages of salmon, flatfish, and smelt to petroleum was dependent largely upon the fishes' spawning characteristics and the conditions under which embryogenesis occurs. The beginning of each of the following 3 subsections briefly describes the fishes' natural spawning behavior.

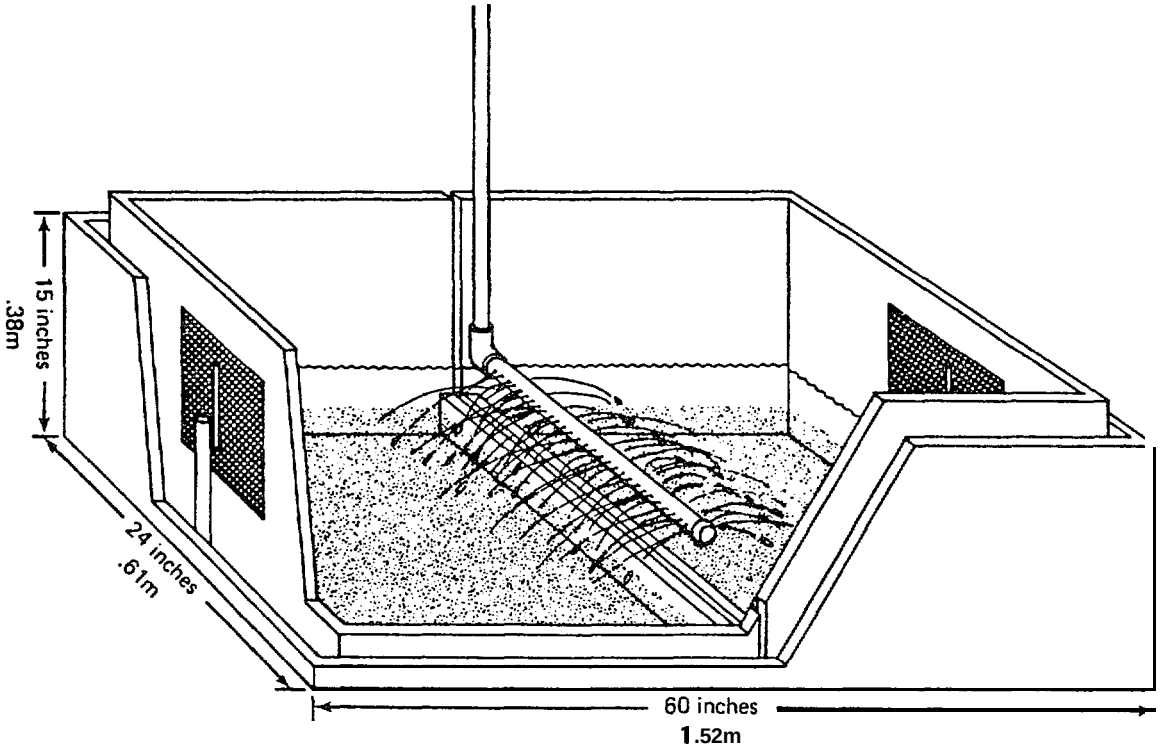

FIGURE 6. Choice apparatus used intesting avoidance of juvenile English sole to oil-contaminated sediment.

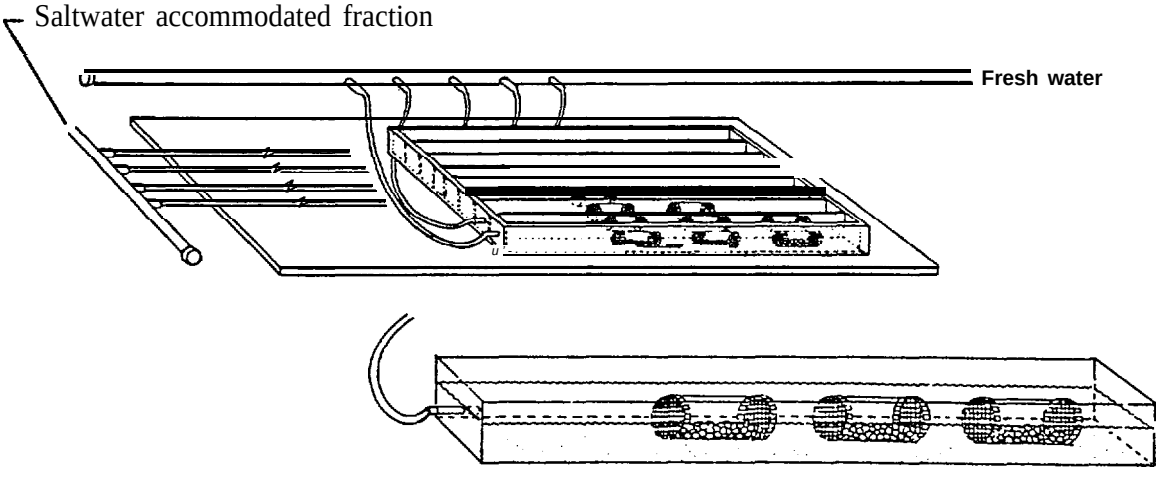

FIGURE 7. Chum salmon egg incubation apparatus with delivery of brackish SWAF of weathered PBCU from the wave generator (Fig. 4) for 3 hr/day and delivery of fresh water for 21 hr/day.

(1) Chum Salmon. Chum salmon frequently spawn in tidal areas at at the mouths of streams (Neave 1966, Bakkala 1970). Eggs are deposited in redds and then covered with gravel as a result of upstream redd digging and stream flow. At least one high tide a day inundates the redds with salt and brackish water; the length of exposure to water of high salinity depends upon redd location. After approximately 50-90 days (development rate is a function of water temperature) the eggs hatch; however, the salmon alevins remain in the gravel for another 30-50 days before emerging and migrating.

Chum salmon eggs were obtained from the U.S. Fish and Wildlife Service National Fish Hatchery at Quilcene, Washington. Immediately after fertilization, the eggs were transported to the Mukilteo laboratory. Subsequent sampling from control groups indicated that 97.5% of the salmon eggs were fertilized. One day after fertilization, approximately 70 chum salmon eggs were placed in each of 56 (30 x 75 cm) glass cylinders, and both ends covered with Teflon netting. A glass tray was divided longitudinally into eight troughs and pea gravel was layered 2.5 cm deep on the bottom. Seven cylinders of eggs were placed horizontally into each trough (Fig. 7).

Eggs were exposed 3 hr/day to oil-contaminated brackish water (16-24 o/oo salinity, and 4.5-10.2 "C ambient temperature) at a flow rate of 400 ml/min per trough. For 21 hr/day the eggs received fresh dechlorinated water (5.5 to 10.5°C ambient temperature) at the same flow rate for a water replacement of 8 times per hr. Exposure to oil-contaminated water occurred 4 days per week for 16 consecutive weeks. On the other 3 days eggs and alevins received uncontaminated brackish water for 3 hr/day. The oil exposure conditions (by group) designated for each of the 8 troughs, are expressed diagrammatically in Figure 8.

(2) Flatfish. Sand sole and English sole are pleuronectid flatfish with pelagic eggs and larvae. Flatfish eggs are released on or near the bottom; after about one day, depending upon depth of spawning, the eggs rise to float near the water's surface (Ketchen 1956, Alderdice and Forrester 1971). Eggs hatch after approximately one week and the larvae emerge relatively undeveloped.

Gravid English sole and sand sole were obtained by trawling in Puget Sound. Eggs were stripped from ripe females and fertilized immediately with sperm from a ripe male on board the trawling vessel. Fertilization success and subsequent viability in flatfish eggs was high (approximately 90%) as indicated by cell cap formation. Twenty-four hrs after fertilization (early cell-cap stage) approximately 350 to 700 flatfish eggs were introduced into 1,000 and 2,000 ml separator funnels containing 900 and 1800 ml, respectively, of uncontaminated seawater or the SWAF of weathered PBCO obtained from the wave machine. The funnels were attached to an air supply through the bottom and the water bubbled slowly, thus creating a current in the funnel which kept the eggs in suspension. Funnels were submerged in a water bath with 10"C flowing seawater.

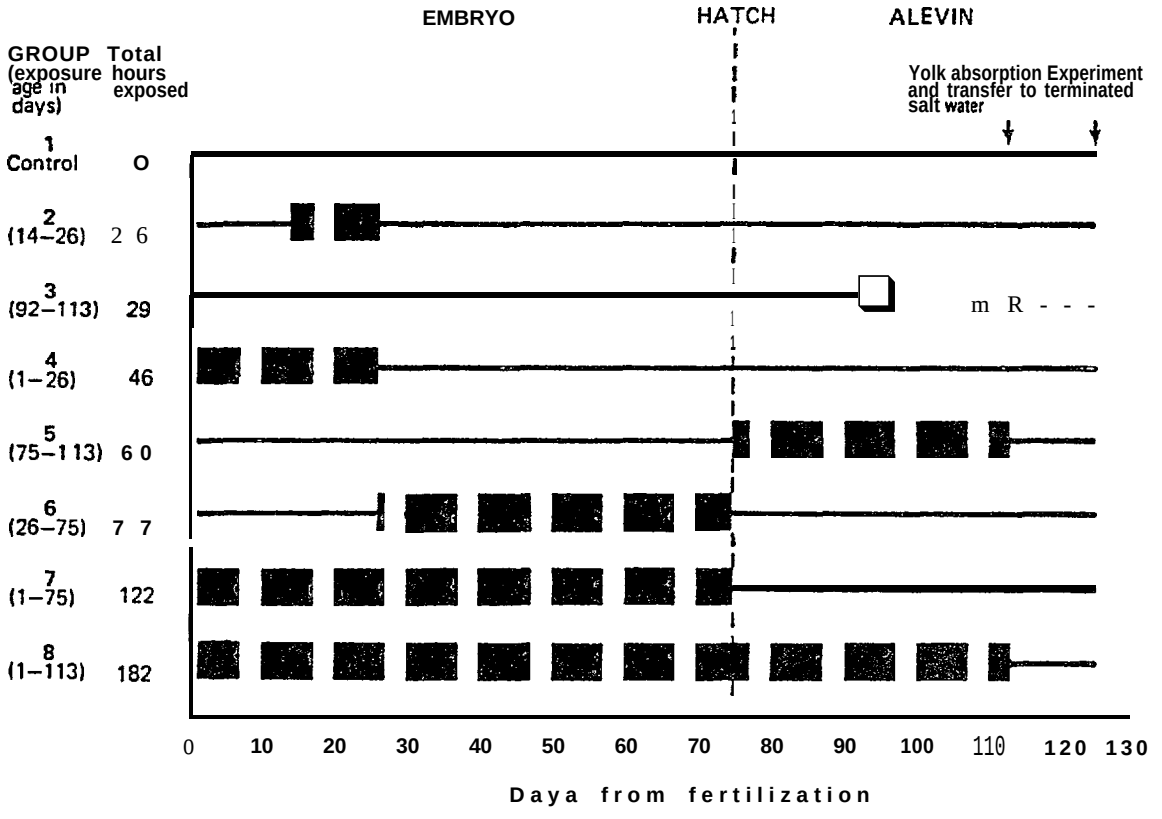

FIGURE 8. Exposure groups of churn salmon embryos and alevins showing duration and stage of exposure. Solid bars represent weekly exposure periods of 3 hr/day, 4 day/wk.

(3) Surf Smelt. Surf smelt are found along the Pacific coast from California to the Aleutian Islands. They spawn intertidally on coarse sand-pea gravel beaches, the adhesive eggs being submerged intermittently at high tide until the embryos hatch as well-developed larvae. Duration of embryonic development (8 to 30 days) depends upon both water and air temperature (Yap-Chiongco 1941, Loosanoff 1937, Pentilla 1978), and hatching is promoted by wave agitation at completion of embryogenesis (Misitano 1977).

Surf smelt eggs were collected from either the intertidal area of Hood Canal, Puget Sound, or from spawning females from Hood Canal. All surf smelt eggs were visually checked at the initiation of each experiment and only those with viable embryos were used in the tests. Four to six days after fertilization, 400 to 500 eggs were placed in each of four square-sided baskets (7 x 14 x 14 cm with 570-µm mesh Teflon netting on the bottom) containing 2.5 cm of fine gravel (2-8 mm diameter) (Fig. 9). The baskets were submerged 3 hr/day throughout the incubation period in the SWAF of weathered CICO with a flow rate of 750 ml/min to each basket. The SWAF from the wave generator was introduced into a diluter and three concentrations used in exposure:

(1) undiluted (100%) SWAF from the wave machine, (2) 1/2 diluted (50%) SWAF, and (3) 3/4 diluted (25%) SWAF. Exposure of surf smelt eggs to the SWAF of CICO was repeated once in the month of November and once in December of 197'9, at ambient water temperatures of 8.8 to 11.5°C and air temperatures ranging from -2.2 to 16.7°C.

(4) Water Samples for Hydrocarbons. During the 16wk exposure period of chum salmon embryos and alevins to the SWAF of weathered PBCO, water samples were collected, 4 days/week after 30, 54, 78 and 102 hr of weathering and analyzed for total hydrocarbons. At 102 hr the gravel and wave machine were cleaned, the cleaned gravel replaced, fresh oil added, and the sampling schedule repeated.

Flatfish embryos were exposed to the SWAF of PBCO which was first weathered for 48 hr in the wave machine. (During oi 1 weathering the seawater temperature was 7.8 to 9"C, and salinity was 27 to 300/00.) Water samples for chemical analysis were taken at the initiation of the tests, after 3 days of exposure, and again after hatching (day 8). Water samples were collected by transferring the contents of the incubation funnel into a beaker. The water was then siphoned from the beaker through a screened cylinder to prevent passage of embryos and/or larvae. At midincubation the SWAF was replaced with a portion of the original SWAF which had been refrigerated at 2°C in a sealed glass bottle with a Teflon-lined lid.

In experiments concerned with the early developmental stages of surf smelt, CICO was continuously weathered throughout each 3 wk test. Water samples for chemical analysis were collected daily from the trough containing undiluted SWAF, and 35 samples were analyzed for total hydrocarbons.

In the above experiments, the concentration of extractable material in control water averaged 2.2 + 1.1 ($\bar{x}$ + SD, N=10) ppb; none of this extracted material was identified as being of petroleum origin. The hydrocarbon concentrations were not corrected for extraction efficiency which averaged 67% + 9 ($\bar{x}$ + SD, N=34), 80% + 6 ($\bar{x}$ + SD, N=16), and 84% + 6 ($\bar{x}$ + SD, N=35) for-data associated with experiments on salmon, flatfish, and smelt, respectively.

Data were analyzed using chi-square tests, robust locally weighted regression (Cleveland 1979), and predictive sample reuse (Geisser and Eddy 1979). The latter analysis is designed to determine within- and between-group differences, and to select the correct model ≥95% of the time.

(f) Effect of Crude Oil on Salmonid Reproductive Success

Fish used were 3-year-old rainbow trout of Cape Cod strain, obtained in June, 1975 from the Washington State Department of Game Hatchery in Spokane. At the beginning of the study the fish measured 41 to 53 cm in fork length and weighed 1.0 to 1.8 kg. The fish were randomly placed in approximately equal numbers in one or the other of two adjacent circular fiberglas tanks (1.8 m diameter) continuously supplied with dechlorinated water at 30 l/rein; water depth was 0.8 m. Water temperature was 11 + 1°C and artificial light was maintained at a natural light: dark cycle.

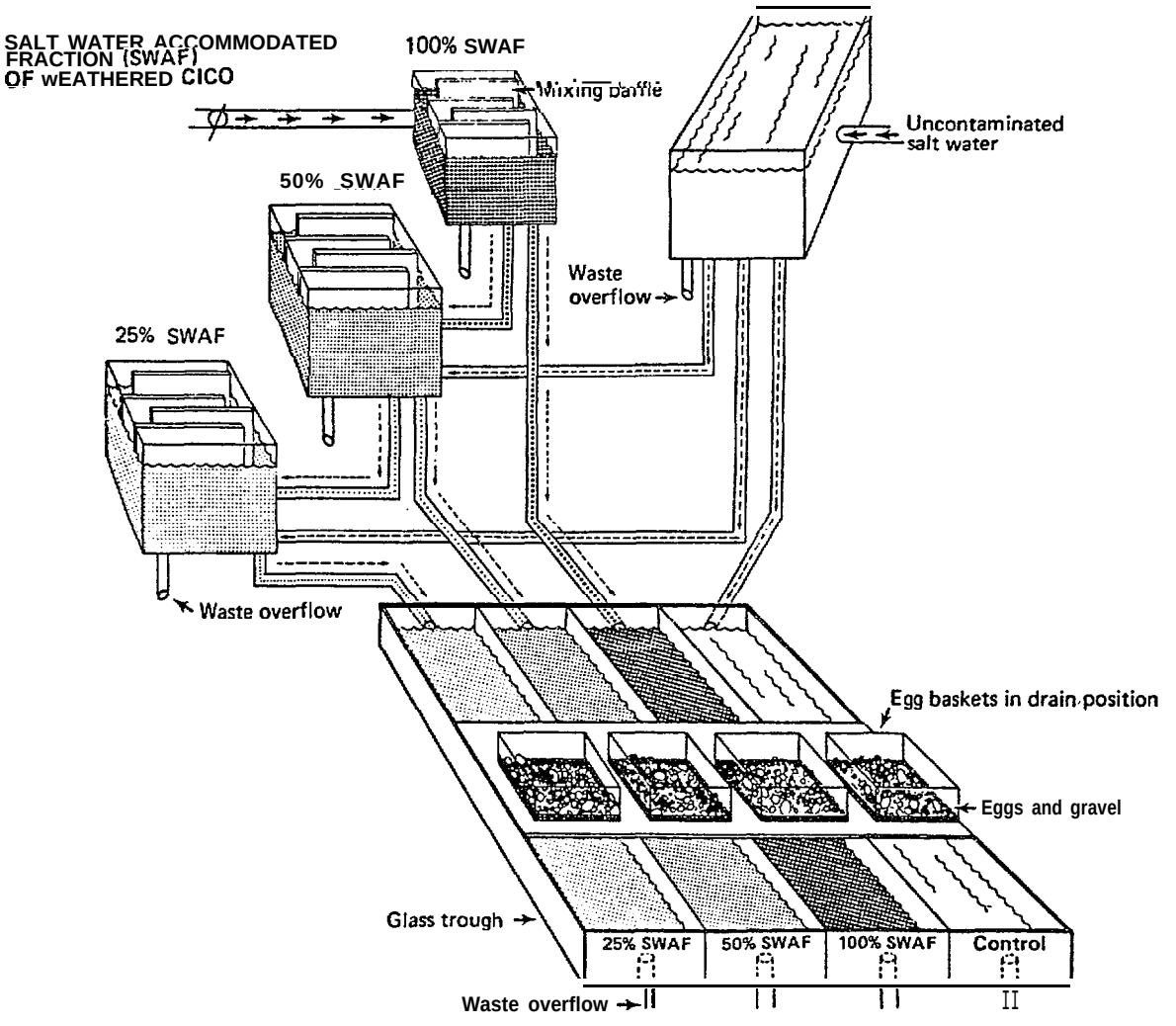

FIGURE 9. Surf smelt embryo exposure apparatus with delivery of the SWAF of weathered CICO from the wave generator (Fig. 4).

Petroleum-coated food (1 part oil :1,000 parts food, by weight) was routinely prepared in the following manner: Two kg of 1/4-inch diameter Oregon moist pellets were placed in a 4 liter glass beaker. Two g of PBCO were mixed with 148 ml of FREON ') TF solvent (trichlorotrifluoroethane) and poured over the food. The food and oil were thoroughly mixed and the food was spread over porcelain-covered metal trays for 90 min of air drying in a fume hood. The food was then weighed into daily aliquots, sealed in plastic bags, and frozen until used. Food for control experiments were prepared identically except that the crude oil was omitted. Fish were fed these diets at an approximate rate of 2% (wet weight of food) of body weight each workday between July, 1975 and August, 1976.

In late November 1975, all fish were examined for degree of maturity. Subsequent biweekly and then weekly examinations were made. As females ripened they were spawned and eggs were fertilized using standard trout-

culture methods (Leitritz 1959). Ripe males were consistently available for the duration of the spawning period from January through February 1976. All eggs from control fish were divided into equal aliquots and one aliquot was fertilized with sperm from an oil-exposed test male and the other with sperm from one control male. Ten of the oil-exposed females were similarly treated; eggs from the remaining oil-exposed females were fertilized with sperm from oil-exposed males only. A total of 31 test and 10 control crosses were made. Eggs were incubated at 7° to 9°C. Mortality data were collected through the yolk-sac absorption stage and statistically analyzed using the Mann-Whitney modification of Wilcoxon's sum of ranks test (Langley 1971).

At the time of spawning, samples of adult tissues and eggs were collected and frozen for later aria"lysis of petroleum hydrocarbons.

5.3.4 Invertebrate Studies

Effect of Petroleum Hydrocarbons on Sea Urchin Defense Behavior

Seawater-soluble fractions of PBCO were prepared according to the flow-through method of Roubal et al. (1977b). Solutions containing single aromatic hydrocarbons were prepared by a modified method described by Nunes and Benville (1978). In the latter, filtered seawater dripped into a glass reservoir at a constant head from a perforated bucket. Hydrocarbons were introduced continuously near the top of the glass reservoir via a calibrated, repeating, syringe pump and the seawater-hydrocarbon mixture was drawn off at the bottom of the reservoir and delivered to a 2-1 glass exposure beaker (for details see Johnson 1979). The flow rate of the seawater-hydrocarbon mixture through the exposure beaker was 200 ml/min; average salinity was 28 $O/_{oo}$; average water temperature was 10"C.

The reported SWSF hydrocarbon concentrations produced by PBCO are the summed concentrations of the monocyclic aromatic compounds. A gas chromatogram representative of the SWSF of PBCO is given in Figure 10, and represents the type of data used in determining the concentration of hydrocarbons present in exposure water for all experiments with invertebrates. The concentration of each component was found to be similar to that reported in detail by Roubal et al. (1978) with the monocyclic aromatic hydrocarbons accounting for 95% of the total SWSF; the remainder consisted of naphthalenes and cyclohexanes.

Urchins are preyed upon by starfish, including the sunflower starfish (Pycnopodia helianthoides; Mauzey et al. 1968), and when presented with a chemical stimulus from their starfish predator, the sea urchins exhibit a defensive response that is consistent and repeatable. The starfish can move faster than the urchin, but the urchin, if overtaken, possesses a compliment of globiferous pedicellariae that are activated by a water soluble exudate from the starfish (Phillips 1978). These globiferous pedicellaria are distributed liberally over the aboral surface of urchins, and a typical 3 cm diameter urchin may possess 100 or more. Each pedicellaria articulates on a peduncle and has three jaws, each tipped with a hollow tooth capable of delivering

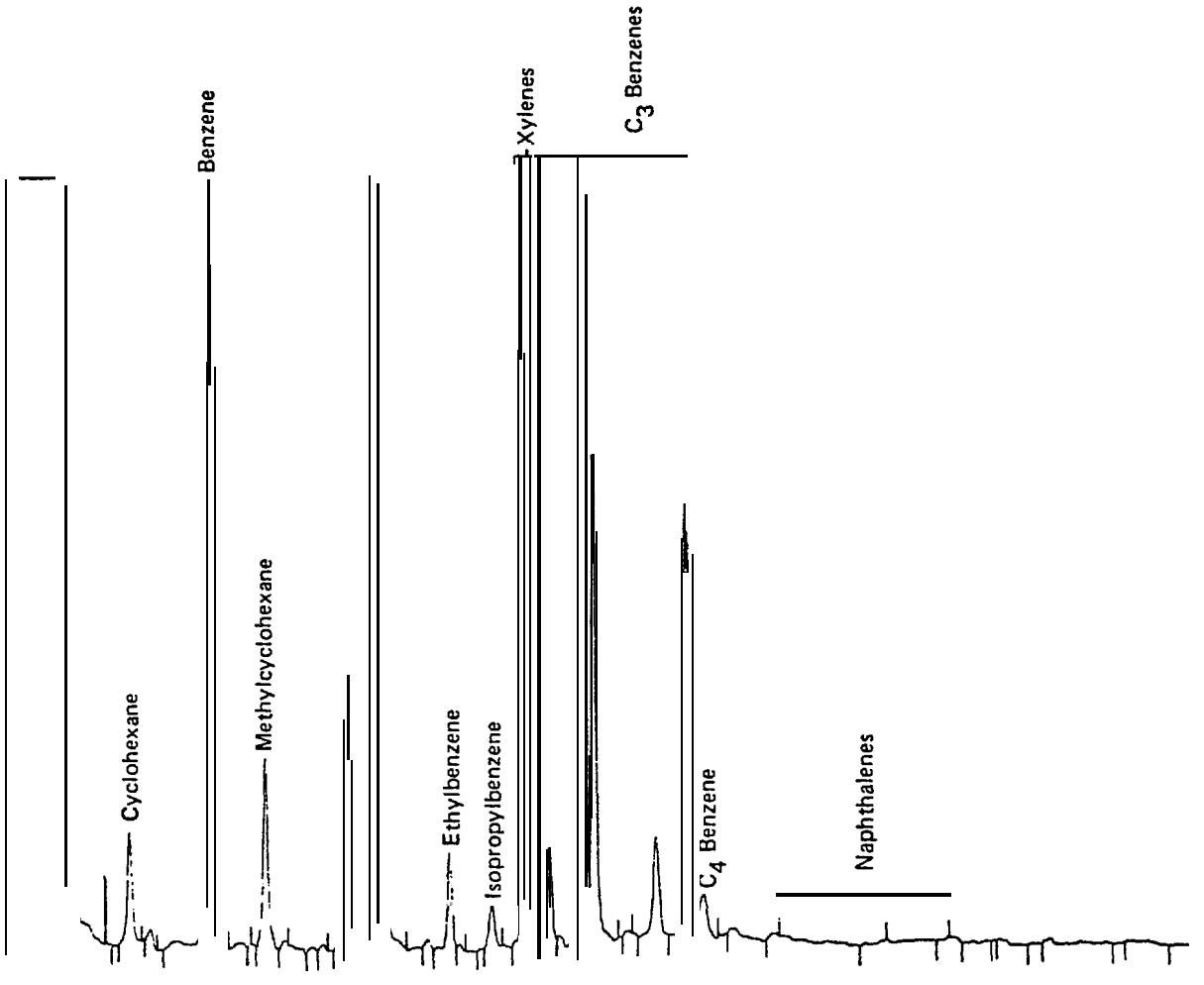

FIGURE 10. Gas chromatogram of the SWSF of PBCO.

venom (Campbell 1976). When stimulated with starfish exudate the pedicellariae rise to a position perpendicular to the test (exoskeleton) and the jaws open to a 180° angle. If a pedicellaria were to contact a tube food of Pycnopodia, the jaws would close, inject venom, and product? a rapid (though not necessarily total) withdrawal of the starfish.

Starfish exudate was prepared by placing a Pycnopodia in its equal volume of 5-µm-prefiltered seawater for 1 hr. The resulting exudate was refiltered (to 5 urn), tested for efficacy in activating pedicellariae, then frozen in aliquots. Before each test a thawed aliquot was kept at ambient water temperature, which ranged seasonally from 7-14°C.

Prior to behavioral testing, sea urchins (2.5 to 3.5 cm test diameter) were exposed to hydrocarbons by one of two methods. Most exposures consisted of holding 5 urchins for 24 hr in 2-l flow-through exposure

beakers containing the SWSF of PBCO or single aromatic hydrocarbons, and then placing them individually in 100-ml beakers containing 40 ml of the same hydrocarbon solution. The urchins were then left undisturbed for approximately 10 min before stimulation with starfish exudate. The second method was to expose the urchins individually in the 100-ml beakers for only 10 min before testing. Following exposure, 1 ml of starfish exudate was slowly administered with a syringe in a circular motion over each urchin. The defense reponse assay consisted of counting the number of pedicellariae that opened to 180°.

If 75 or more pedicellariae responded to starfish stimulus, the response was considered total (100%); if less than 75 opened, a partial response was recorded (0-99%). For each test, the average response of at least 5 hydrocarbon treated urchins was divided by the average response of at least 5 controls to yield a response index for each treatment. This method of analysis takes into consideration variations in response of controls for each group tested.

Reproductive Behavior of Dorid Nudibranchs Exposed to Petroleum Hydrocarbons

(a) Chemosensory disruption. Mature dorid nudibranchs were collected from the seawater system at the Mukilteo Biological Station. The adult nudibranchs were exposed to the SWSF of PBCO for 24 hr in 2-1 flow-through aquaria with a seawater flow of 250 ml/min and a temperature and salinity of 13.6 + 0.6°C ($\bar{x}$ + range) and 27.0 + 2°/oo, respectively. The SWSF was produced by a method described by Roubal et al. (1977 b); to obtain different hydrocarbon concentrations in each aquarium the SWSF flows were diluted by mixing with seawater, Two water samples were collected from each aquarium for GC analysis of hydrocarbon concentration.

Following SWSF exposure, the nudibranchs were assayed in "untreated" seawater for chemotatic behavioral response.[1] Individual nudibranchs were placed in the bottom arm of a "Y" choice chamber and allowed to move into either the "stimulus" arm or the non-stimulus ("blank") arm. (For detai 1s of testing apparatus see Malins et al. 1978 or Johnson 1979.) The "stimulus" arm contained four reproductive nudibranchs. The "blank" arm was of identical construction, but did not contain nudibranchs. Clean seawater filtered to 5 µm supplied each arm of the choice chamber at a flow rate of 60 ml/min. The "stimulus" and "blank" chambers were alternated and thoroughly washed between tests to remove mucus. A positive response denoted movement toward either chamber; "no choice" meant the nudibranchs did not make a definite movement into either arm within 10 min from the start of the test.

The percentage moving toward the "stimulus" chamber for the SWSF exposed group of nudibranchs was statistically compared with the control percentage using an arc sin transformation test for equality of two percentages (Sokal and Rohlf 1969).

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[1] The reproductive behavior of the nudibranch is thought to be initiated by a sex pheromone as has been implicated in mediating reproductive aggregations in other gastropod (Dinter 1975, Audesirk 1977).

(b) Embryological development. The exposure system was identical to that used above in testing chemosensory disruption of dorid nudibranchs. There was, however, a difference in determining the petroleum hydrocarbon concentration in each aquarium. Over the 18-day exposure period four water samples for GC analysis were taken from the oil solubilizer head box. Hydrocarbon concentration in each aquarium was calculated from the average SWSF concentration in the head box and the amount of SWSF diluted with untreated seawater.

Four groups of 20 mature nudibranchs of approximately equal size (total weight 2.1 g for each group) were placed in 4-separate 2-1 flow-through aquaria. The first egg masses laid by the dorid nudibranchs were collected and placed in holding containers within each exposure aquaria; replicate egg aliquots of 2-3 mm3 from the center of each egg mass were taken daily 5 days per week until hatching was complete or the experiment terminated. The eggs were fixed in buffered preservative (Hawkes 1974, with the addition of 17 g of synthetic seawater per liter of buffer to attain 800 mOsm). In addition, eggs laid each following day were collected, weighed, and then discarded.

Embryonic development was studied using light microscopy. Eggs in aliquot samples were counted and each normal egg assigned to one of the following 7 categories: One-cell; two-cell; blastula; gastrula; early velure; late velure; and shell.[1] Abnormal eggs were categorized as: moderate abnormality (a recognizable embryo but malformed, or with extra cellular material in the capsule) or severe abnormality (no recognizable embryo and often no capsule present). Photomicrographs of the normal and abnormal categories listed above are given in reports by Malins et al. (1978) and/or Mumaw (1978).

Feeding Behavior of Shrimp Exposed to Petroleum Hydrocarbons

Spot shrimp were caught in commercial shrimp pots at depths of 100 to 120 m in Puget Sound. Following capture the shrimp were maintained in 1200-1 flow-through tanks at ambient seawater temperature of 10 to 12°C and a salinity of 27 to 30 °/oo. Three days prior to testing, individual shrimp were moved to 3-1 glass exposure tanks, each with a flow rate of 300 ml/min. The shrimp were not fed for 3 days prior to exposure or during the 6 day exposure period. The number of shrimp tested and the number of observations made using 4 SWSF concentrations of PBCO are given in Table 2.

To obtain different concentrations of hydrocarbons in exposures, the SWSF from an oil solubilizer (Roubal et al. 1977b) was diluted just prior to its entry into the test chambers while maintaining a total flow of 300 ml/min (Fig. 11). The shrimp chambers were enclosed in black plastic and observations on feeding behavior were made through one-way mirrors. Each data point consisted of three, 3-rein observations of background activity, response to seawater control, and response to a 1:10 dilution of artificial squid extract (Mackie 1973). Seawater

[1] Developmental stages used here are described in detail in several reports involving nudibranch development (McGowan and Pratt 1954, Perron and Turner 1977, Thompson 1960, 1962, 1967).

TABLE 2. Number of shrimp tested and number of tests conducted on feeding behavior in relation to concentrations of the SWSF of PBCO.

Hydrocarbon concentration		Number of shrimp tested	Number of tests
ppb ($\bar{x} \pm sd$)	N		
Control		6	36
11 ($\pm$1)	2	3	18
18 ($\pm$3)	4	9	54
40 ($\pm$12)	2	4	24
575 ($\pm$485)	7	3	7

a. Based on GC data of water taken from shrimp test chamber.

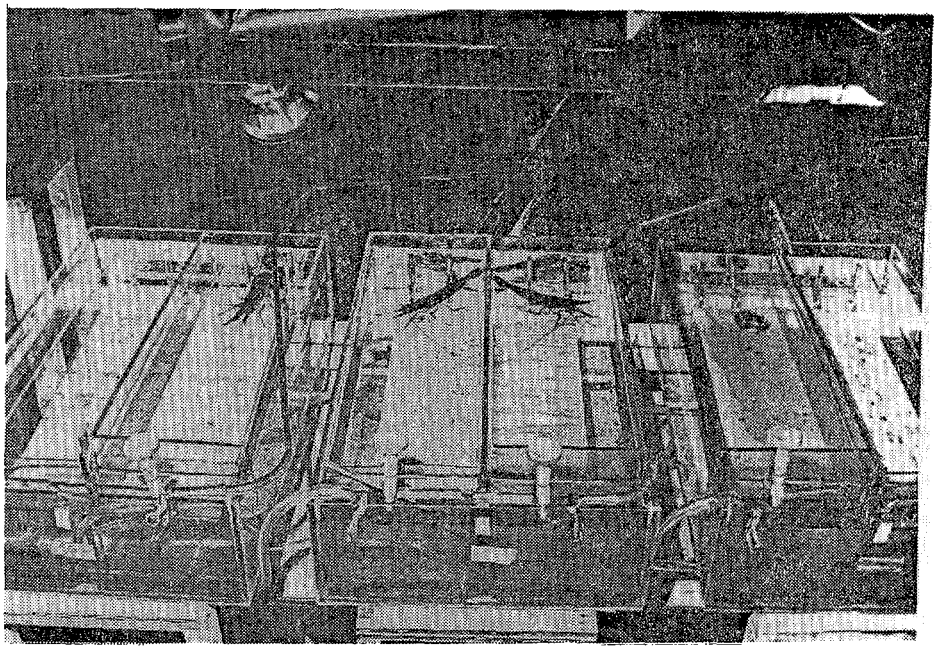


FIGURE 11. Glass chambers for testing effect of the SWAF of PBCO on the feeding response of spot shrimp. Valves and mixing box in foreground of each chamber are for adjusting flow rate and SWAF concentration.

control and squid extract stimulants were introduced at the upstream end of the test chamber at a flow rate of 10 ml/min.

Two behavioral indices were selected for evaluating feeding responses to a given stimulant[1] (1) Perepod probing--Probing the substrate with the first perepods was recorded as the number of probing bouts during a test; this activity is indicative of higher order searching behavior. (2) Contact with stimulant inlet--Defined as grasping or actively picking at the inlet with chelae or mouth parts; this behavior was considered representative of maximum feeding response.

6. RESULTS

6.1 Chemistry

6.1.1 Uptake and Biotransformation of Specific Hydrocarbons in Salmonids

Coho salmon injected with radiolabeled benzene, NPH, and anthracene differentially retained these hydrocarbons in liver, brain and muscle as indicated by the data in Figure 12, Table 3. In the tissues examined, the percent of administered dose at all time periods was the lowest in fish exposed to benzene and the highest in those exposed to anthracene (Table 3).

The data in Table 3 also show that the administered hydrocarbons were metabolized by coho salmon and metabolic products were present in the liver and gall bladder. However, in the brain most of the recoverable radioactivity was in the form of the parent hydrocarbons 24 hr (>98%) after injection.

To delineate the nature of metabolic products, solvent extracts of various tissues were analyzed by TLC. The results revealed a wide spectrum of metabolic products in various proportions in the brain, liver, gall bladder, heart, and flesh of fish at 24 hr after administration of NPH. Naphthol, a dihydrodiol derivative of NPH and their conjugates (e.g. , glucuronides, sulfates and mercapturic acids) were detected.

6.1.2 Accumulation of Petroleum Hydrocarbons by Fish Exposed to SWSF of PBCO

The hydrocarbon composition of SWSF of PBCO is given in Table 4 which shows the presence of mono and diaromatic hydrocarbons. Data are presented in Table 5 for the accumulation of hydrocarbons in coho salmon exposed to a SWSF of PBCO equal to 0.8 ppm of aromatic hydrocarbons in flow-through seawater. After 1 wk of exposure, no hydrocarbons representative of the SWSF were detected in muscle tissue. Exposures from 2 to 6 wk, however, resulted in the accumulations of significant

[1] Three additional behaviors were also recorded but not presented here; antennular flicks, antennular cleaning, and forward movement. For each of these behaviors there was often a response to seawater control stimulus which resulted in inconclusive data. For details of these observations and results see Malins et al. (1977) and/or Miller (1980).

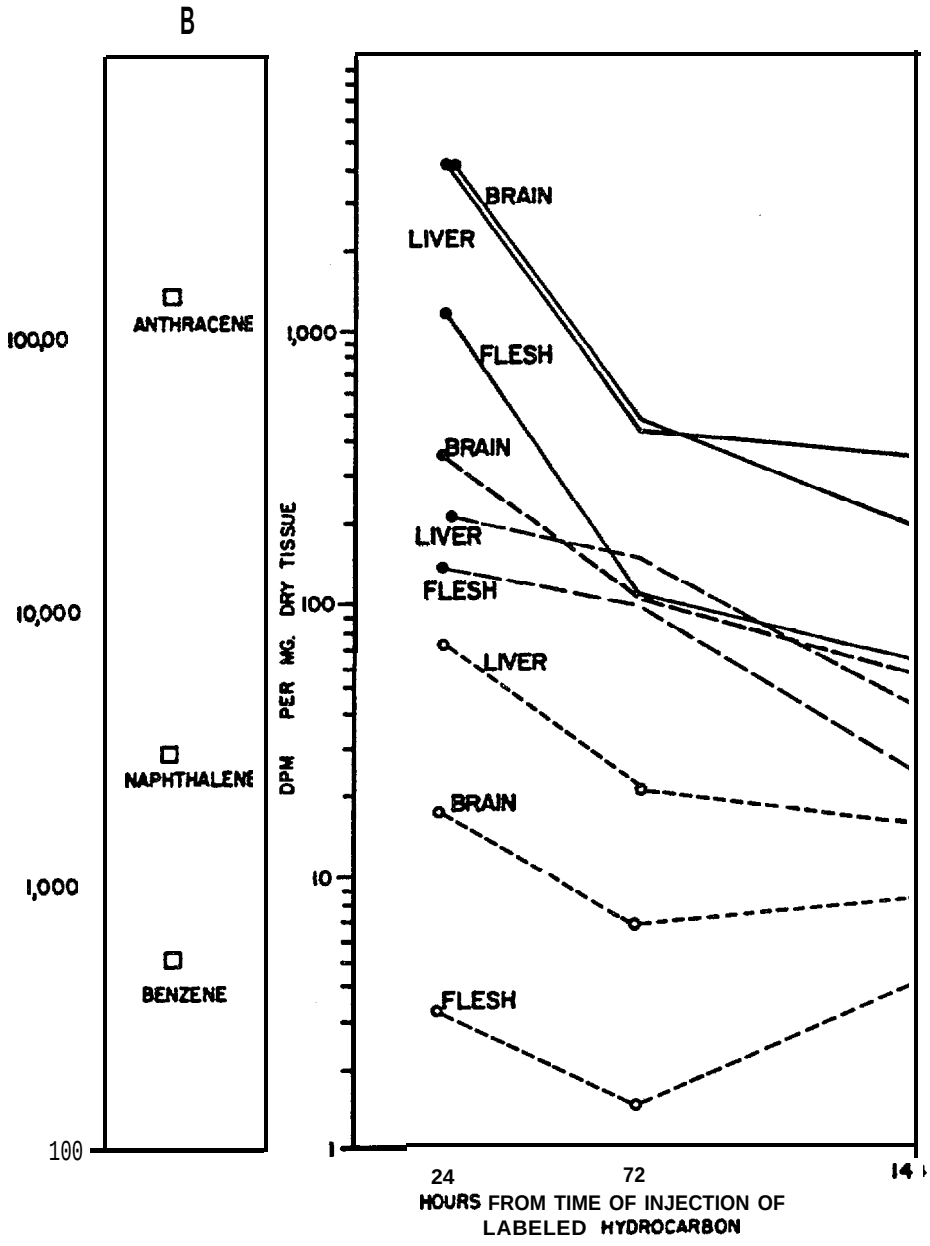


FIGURE 12. Radioactivity in tissues of coho salmon receiving ^{14}C-labeled hydrocarbons (2.5 uCi) by intraperitoneal injection. A, anthracene radioactivity (———), naphthalene radioactivity (-----) and benzene radioactivity (-----); B, 24-hr values for radioactivity in the gall bladder of injected fish. Total carbon-14 in organs as % of administered dose is given in Table 3. (From Roubal et al. 1977a)

Table 3 *Distribution of hydrocarbons, total aromatic metabolizes and total carbon-14 in organs of coho salmon receiving hydrocarbons by intraperitoneal injection*

Hydrocarbon	Time after injection (hr)	Brain: pCi radioactivity (x1000) for whole organ	Brain: Total carbon-14 in organ (% administered dose)	Brain: Un[b] (%)	Brain: Met (%)	Liver: pCi radioactivity (x1000) for whole organ	Liver: Total carbon-14 in organ (% administered dose)	Liver: Un (%)	Liver: Met (%)	Gall bladder: pCi radioactivity x1000) for whole organ	Gall bladder: Total carbon-14 in organ (% administered dose)	Gall bladder: Un (%)	Gall bladder: Met (%)	Flesh: pCi radioactivity (x1000) for whole organ	Flesh: Total carbon-14 in organ (% administered dose)	Flesh: Un (%)	Flesh: Met (%)	Carcass: pCi radioactivity (x100) for whole organ	Carcass: Total carbon-14 in organ (% administered dose)	Carcass: Un (%)	Carcass: Met (%)
Benzene	6	0.14	0.0028	98	2	1.2	0.024	68	32	0.3	0.006	37	63	3.3	0.066	100	0	310	6.2	100	0
	24	0.018	0,00036	100	0	0.380	.00076	58	42	0.07	0.0014	29	71	0.3	0.006	100	0	11	0.22	100	0
Naphthalene	24	3.0	0.060	98	2	4.6	0.092	87	13	13	0.26	28	72	83	1.70	96	4	770	15.4	99	1
	72	0.71	0,142	76	24	2.1	0.042	5	5	4	5	-	c	-	-	–	4	2	0.84	86	14
	144	0.49	0.010	80	20	0.910	.018	5	9	4	1	-	-	-	-	2	0	0.40	87	13	260
Anthracene	24	39	0.78	98	2	60	1.20	36	64	400	8.0	10	90	580	11.6	87	13	1700	34	97	3
	72	2.7	0.054	96	4	8.8	0.18	4	2	5	8	-	-	-	-	6	8	1.36	99	1	2s00
	144	1.4	0.028	91	9	7.5	0.15	3	1	6	9	-	-	-	-	3	9	0.78	54	46	1000

[a] Heart data: Although heart was analyzed, levels of radioactivity in benzene-injected fish were too 10 w to be statistically significant and were not reported. Twenty-four hr after injection of naphthalene, 97% of the radioactivity was represented by naphthalene, and the total radioactivity was 1000 pCi; later measurements were not taken. Ninety-six percent of the recovered radioactivity in the heart of anthracene-injected fish was attributed to anthracene after 24 hr; the total radioactivity was 4000 pCi. Measurements at later times were not taken

[b] Percentages shown are percentages of the total tissue radioactivity represented by parent hydrocarbons (unmetabolized; Un) and their total aromatic metabolites (Met)

'Analysis not performed

(From Roubal et al. 1977a)

Table 4 Hydrocarbon **content** of flow-through exposure water

Hydrocarbons	Concentration' (ppm) (Mean ± std. deviation
Cyclohexane	0.02 ± 0.002
Benzene	**0.04 ± 0.006**
Toluene	**0.4 ± 0.05**
Ethylbenzene	0.005 ± 0.002
***m*-Xylene**	**0.2 ± 0.003**
o- and ***p*-Xylenes**	0.07 ± 0.03
G-Substituted **benzenes**	0,03 ± 0.005
G-Substituted **benzenes**	0.01 ± 0.003
C_3-Substituted benzenes	**trace[c]**
Naphthalene	0.003 ± 0.002
1-Methylnaphthalene	0.003 ± 0.001
2-Methylnaphthalene	0.003 ± 0.002
G-Substituted **naphthalenes**	0.01
G-Substituted **naphthalenes**	0.005

[a] Values corrected for losses during workup

[b] Content of the hydrocarbons, **cyclohexane** through **naphthalene** was determined seven times during exposure. Values reported for the **methylnaphthalenes** are averages for two analyses. Single values **are** reported for ~-and G-substituted **naphthalenes.** Hydrocarbon content of flow-through water (≃ 0.8 ppm at the beginning of **solubilizer operation)** was only slightly greater (≃ 1.0 ppm) after five **weeks** of continuous **operation**

[c] ≃ 0.001 pp_m **was the lower limit** of **hydrocarbon** detection

(From Roubal et al. 1978)

amounts of substituted and **unsubstituted** benzenes and naphtha lenes (Table 5). After 5 wk of exposure, which was the time of maximum hydrocarbon accumulation in salmon, the **bioconcentration** factors (concentration of hydrocarbons in tissue/concentration of hydrocarbons in water) in muscle for C3-substituted **benzenes,** NPH, 1- MN, 2-MN, **C_2-substituted** NPH, and **C_3-substituted** NPH were 50, 80, 190, 130, 85, and 140, respectively. The **C_4-** and **C_5-substituted** benzene fraction of SWSF was the most prominent fraction in muscle throughout the exposures. After 5 wk of exposure, the latter amounted to 5.5 ppm, or a **bioconcentration** factor of 550 for **C_4-** and **C_5-substituted benzenes** in muscle tissue. When fish were exposed for 6 wk and transferred to clean seawater for 1 wk, the aromatic hydrocarbons were not found in muscle.

Tables 5 and 6 present data on hydrocarbons accumulated in muscle, liver, and **gil** 1s of starry flounder exposed to a SWSF of **PBCO.** In contrast, to salmon, starry flounder was found to have considerable concentrations of aromatic hydrocarbons in tissues after 1 wk exposure. **Bioconcentration** of hydrocarbons in flounder muscle was greater after 1 wk than after 2 wk of exposure. The **C_4-** and **C_5-substituted** benzene fraction of SWSF was concentrated 10 times more in muscle of flounder than in salmon muscle after 2 wk of exposure.

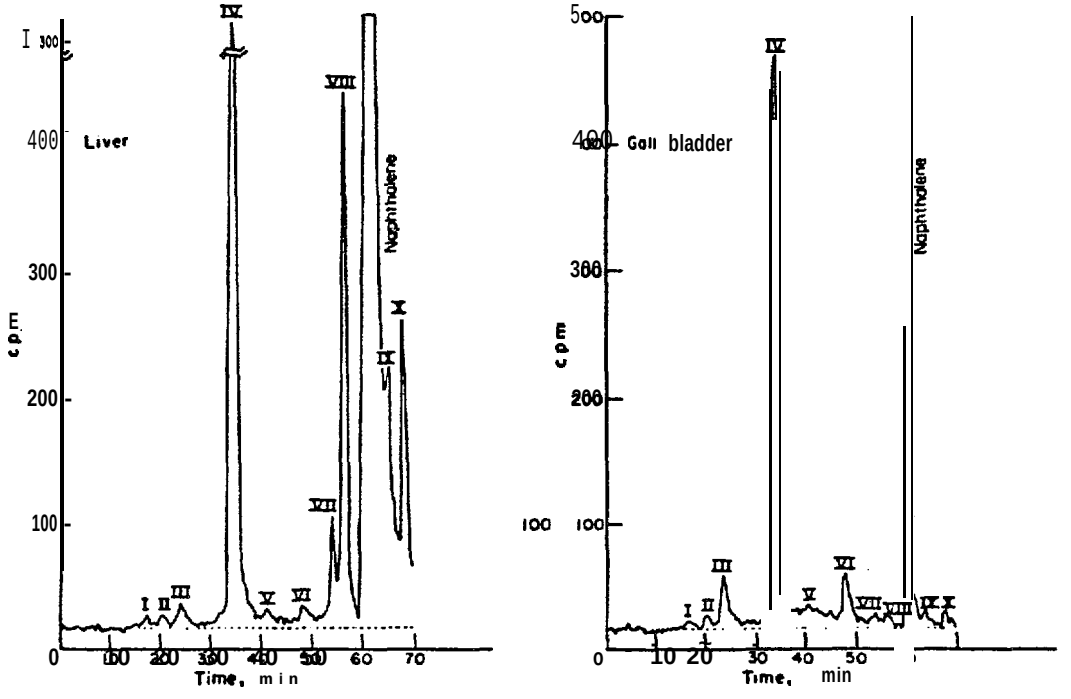


FIGURE 13. HPLC profiles of derivatives of naphthalene in liver and gall bladder of coho salmon 16 hr after force-feeding of ^{3}H-naphthalene. Profiles represent single analysis of pooled tissues from 4 fish. Identification and quantification of peaks are given in Table 7: (---) represents background level of 16 cpm; dpm = cpm/0.46. (From Collier et al. 1978)

found in the gall bladder as opposed to the liver. However, the liver had a higher organ burden of dihydrodiol than did the gall bladder. These data represent the first use of high pressure liquid chromatography (HPLC) for analyzing aromatic hydrocarbon metabolizes in fish.

6.1.4 NPH and Its Metabolizes in Fish Skin and Mucus

Skin. Skin of rainbow trout contained maximum concentrations of NPH (322 ppb) and of its metabolizes (17 ppb) 24 hr after the fish were force fed 3H-NPH. The concentrations of both NPH and its metabolizes subsequently declined to 10.3 and 2.5 ppb, respectively, at 168 hr (Table 8). The data on changes in concentrations of NPH and its metabolizes with time in the skin were statistically treated and both curves fit a lognormal probability distribution function (Table 8). Statistical treatment of the data revealed that the rates of change in concentrations of NPH and its metabolizes in the skin of the force-fed fish were significantly different ($P<0.05$). Up to 24 hr after the treatment, NPH concentration increased more rapidly than that of the metabolizes (Table 8). Subsequently, NPH concentration declined more rapidly so that

Table 7 Metabolites present in liver and gall bladder of coho salmon 16 hr after feeding of ^{3}H-naphthalene

Peak	Compound	Liver pmoles/g% Dry wt	Liver Admin dose (x 10^{-3})	Gall bladder pmoles/g% Dry wt	Gall bladder Admin dose (x 10^{-3})
I*		0.38	0.022	2.9	0.026
II	Glucuronide	2.0	0.11	20	0.18
III	Sulfate	1.7	0.097	21	0.19
IV	Dihydrodiol	110	6.1	200	1.7
V	Glucoside	0.88	0.050	4.6	0.040
VI	1-Naphthol	2.0	0.11	30	0.27
VII*		3.9	0.22	3.7	0.033
VIII*		17	0.94	3.4	0.030
IX*		15	0.83	3.2	0.028
X*		11	0.61	3.5	0.031

* Uncorrected for extraction efficiency.

(From Collier et al. 1978)

at 168 hr, NPH metabolites represented a larger portion (17.6%) of the total radioactivity in the skin than at 24 hr (4.4%) (Table 9).

To assess the importance of fish skin in the disposition of hydrocarbons, concentrations of NPH and its metabolizes in skin were compared with corresponding values for liver, which is an active site for hydrocarbon metabolism. At 16 hr after force-feeding, the concentration of NPH in skin was 46% of the liver concentration. The ratio (tritium in skin/tritium in liver) for NPH remained more or less constant thereafter. This ratio for the metabolizes remained about the same throughout the experiment. The maximum value of percent-administered dose in the skin was 0.87 at 24 hr. The skin contained only 0.03% of the administered dose at 168 hr after the treatment (Table 9).

Skin of starry flounder contained 68 ppb of NPH and 25 ppb of its metabolizes at 24 hr after the fish were force fed ^{3}H-NPH (Table 10). At 24 hr, 0.4% of the administered dose was present in skin and at 168 hr, 0.02% remained. When comparing these data with those obtained for rainbow trout, it should be noted that starry flounder were kept at 12°C and rainbow trout were maintained at 8°C. The difference in temperature may have an important effect on the levels of total radioactivity found in skin. At 168 hr, 78% and 17.6%, respectively, of the total radioactivities in the skin of starry flounder and rainbow trout (Table 9) were attributable to the metabolites.

Rainbow trout held at 8°C were injected i.p. with 3H-NPH; 4 hr after injection the skin contained 114 ppb of NPH and 6.2 ppb of the metabolizes at (Table 11). The maximum concentration of NPH, 244 ppb, was reached at 16 hr following the injection, and subsequently declined to 19.1 ppb at 168 hr. The concentration of metabolizes continued to increase-in the skin reaching a maximum value of 14.4 ppb at 48 hr and then decreasing to 3.8 ppb at 168 hr.

TABLE 8

CONCENTRATIONS OF NAPHTHALENE AND ITS METABOLIZES IN SKIN AND EPIDERMAL MUCUS OF RAINBOW TROUT (*Salmo gairdneri*) EXPOSED TO NAPHTHALENE VIA FORCE FEEDING[a,b]

Time elapsed after treatment (hr)	Naphthalene		Metabolizes[c]	
	(dpm/mg)	(ppb)	(dpm/mg)	(ppb)
Skin				
4	51.3 ± 18.3 (10)	35.0	6.1 ± 1.8(12)	4.7
16	249.8 ± 29.8 (12)	172.9	20.0 ± 2.0(16)	15.6
24	465.2 ± 46.1 (7)	321.9	21.3 ± 1.8 (7)	16.6
48	109.1 ± 18.7 (13)	75.5	8.0 ± 1.4 (13)	6.2
168	14.9 ± 1.1 (11)	10.3	3.2 ± 0.6 (9)	2.5
Mucus				
4	2.9 ± 1.0 (4)	2.0[0.011	89.8 ± 7.6 (3)	69.9 [0.46]
16	18.7 ± 3.1 (4)	12.9 [0.09]	107.0 ± 23.4 (4)	83.3 [0.551
24	64.4 ± 53.9 (2)	44.6 [0.29]	167.0 ± 7.6 (4)	130.0 [0.89]
48	65.1 ± 33.2 (4)	45.0 [0.30]	15 Lo ± 39.9 (4)	117.6 [0.78]
168	42.2 ± 30.2 (4)	29.2 [0.19]	51.3 ± 31.4 (4)	41.2 [0.27]

[a] Fish were force fed 74.6 μCi of 1,4,5,8-[^{3}H]naphthalene and samples were taken from four or five fish at each time interval. Concentration of both naphthalene and metabolites in water was less than 0.01 ppb at all times.

[b] Mean ± SE; values in parentheses represent number of individual measurements including duplicate measurements on samples from the same fish. All concentrations are given on dry weight basis. Because epidermal mucus contained as much as 99.34% water, concentrations in brackets are also given on wet weight basis.

[c] Concentration of metabolizes was calculated using molecular weight of naphthol. The data fitted log normal distribution and the pdf is given by:

$$f(x; \alpha, \mu, \sigma) = \frac{1}{(x - \mu)\sigma\sqrt{2\pi}} \exp - \frac{1}{2\sigma^2} [\ln (x - \alpha) - \mu]^2,$$

where α is a location parameter, μ is a scale parameter, and σ is a shape parameter. For example, the data for skin in this expeiment Yielded parametric values of: α = 5, μ = 4.064, and u = 1.738 (naphthalene); a = 1, μ = 1.766, and σ = 1.371(metabolites).

(From Varanasi et al. 1978)

These data (Table 11) also fit a lognormal distribut' on probabi 1 i ty function; the rates of change in concentration of NPH and its metabolizes in the skin of injected fish were significantly different ($P<0.001$). As in the force-feeding study, both the rates of increase as well as subsequent rate of decline of NPH concentration in the sk' n of the injected fish were greater than the respective rates of increase and decline of metabol i tes. Hence, the proportion of the metabolizes increased steadily and comprised as much as 15.1% of the total radioactivity in the skin of fish at 168 hr (Table 9).

Starry flounder held at 12°C were injected with 3H-NPH, and skin contained 70 ppb of NPH and 15 ppb of the metabolizes at 24 hr after

TABLE 9

VARIOUS PARAMETERS SHOWING PATTERNS OF ACCUMULATION AND RELEASE OF NAPHTHALENE AND ITS METABOLITES IN SKIN OF RAINBOW TROUT (*S. gairdneri*) EXPOSED TO NAPHTHALENE

Mode of exposure[a]	Time (hr)	Total radioactivity in skin (μCi)	Relative percentage		Administered dose in total skin (%)	^{3}H in skin/^{3}H in liver[c]	
			Naphthalene	Metabolizes		Naphthalene	Metabolites
Force feeding	4	0.06	89.4	10.6	0.08	0.05 ± 0.01 (4)	0.09 ± 0.02 (4)
	16	0.31	92.6	7.4	0.41	0.46 ± 0.12(4)	0.09 ± 0.02 (4)
	24	0.65	95.6	4.4	0.87	0.52 ± 0.10(3)	0.17 ± 0.06 (3)
	48	0.16	93.2	6.8	0.21	0.51 ± 0.10(4)	0.20 ± 0.08 (4)
	168	0.03	82.4	17.6	0.03	0.51 ± 0.12 (4)	0.10 ± 0.01 (3)
Injection	4	0.20	9s.4	4.6	0.21	0.13 ± 0.05 (3)	0.10 ± 0.03 (3)
	16	0.43	97.2	2.8	0.45	0.38 ± 0.08 (3)	0.25 ± 0.15 (3)
	24	0.38	95.4	4.6	0.40	0.34 ± 0.12 (3)	0.2610.03 (3)
	48	0.22	90.0	10.0	0.23	0.14 ± 0.04 (2)	0.29 ± 0.10 (3)
	168	0.04	84.9	15.1	0.04	0.19 ± 0.06 (2)	0.12 ± 0.03 (2)

[a] Details of exposure conditions are given in the foot notes to Tables 8 and 11.

[b] Relative percent of naphthalene and metabolites are based on disintegrations per minute per milligram values given in Tables 8 and 11.

[c] Values for radioactivity (disintegrations per minute per milligram dry weight) associated with naphthalene and metabolite fractions of each skin sample were divided by corresponding values for the liver of the same fish. Numbers represent mean ± SE of three or four ratios. Values in parentheses indicate number of individual fish.

(From Varanasi et al. 1978)

TABLE 10. Concentrations of naphthalene (NPH) and its metabolizes in skin and epidermal mucus of starry flounder (P. stellatus) exposed to NPH via force-feeding.[a]

Time elapsed after treatment (hr)	Skin		Mucus	
	NPH ppb	Metabolizes ppb	NPH ppb	Metabolizes ppb
4	30+15[b]	24+3	1 0+4	41+10
24	68+40	25+7	14+7	30+ 8
48	15+ 3	20+4	3+1	12+ 3
168	2+ 1	7+1	1+0.5	4+ 1

[a] Fish were fed 56 μg of ^{3}H-NPH at 12+1°C.
[b] X+S.D.

TABLE 11

CONCENTRATIONS OF NAPHTHALENE AND ITS METABOLITES IN SKIN AND EPIDERMAL Mucus OF RAINBOW TROUT (*Salmo gairdneri*) EXPOSED TO NAPHTHALENE VIA INTRAPERITONEAL INJECTION[a,b]

Time elapsed after treatment (hr)	Naphthalene		Metabolites[c]	
	(dpm/mg)	(ppb)	(dpm/mg)	(ppb)
Skin				
4	164.5 ± 98.5 (3)	113.8	7.9 ± 0.5 (3)	6.2
16	352.6 ± 2,7 (3)	244.0	9.9 ± 1.4 (3)	7.7
24	306.4 ±151.0 (3)	212.0	14.7 ± 0.2 (3)	11.4
48	167.8 ± 28.9 (2)	116.1	18.5 ± 3.6 (3)	14.4
168	27.6 ± 13.5 (3)	19.1	4.9 ± 0.4 (3)	3.8
Mucus				
4	18.2 ± 9.3 (3)	12.6	173.7 ± 49.6 (2)	135.2
16	4.8 ± 4.8 (3)	5.4	90.8 ± 20.6 (3)	70.7
24	2.3 ± 2.3 (3)	1.6	110.3 ± [1.0(3)	85.8
48	nd (3)[d]	nd	270.5 ± 85.9 (3)	210,5
168	nd (3)	nd	nd (2)	nd

[a] 1,4,5,8-[^{3}H]naphthalene (94.6 μCi) was injected ip in each fish and samples were taken at designated time intervals from a total of three fish. The concentration of both naphthalene and metabolizes in water was less than 0.01 ppb at all times.
[b] Mean ± SE; values in parentheses represent number of individual measurements. All measurements are given on dry weight basis.
[c] Concentration of metabolites was calculated using molecular weight of naphthol.
[d] None detected.

(From Varanasi et al. 1978)

TABLE 12. Concentrations of naphthalene (NPH) and its metabolizes in skin and epidermal mucus of starry flounder (P. stellatus) exposed to NPH via intraperitoneal injection.[a]

Time elapsed after treatment (hr)	SKIN		MUCUS	
	NPH ppb	Metabolites ppb	NPH ppb	Metabolites ppb
24	70+35[b]	1 5+3	1.2+ 1	60+41
168	11+ 3	4+0.4	ND[c]	ND

a Fish were injected with 75 µg of ^{3}H-NPH at 12°+1°C.
b $\bar{x}$+S.D.
c ND = not detected.

exposure and 11 ppb of NPH and 4 ppb of the metabolizes at 168 hr (Table 12).

Mucus. Results in Table 8 show that at each sampling time, epidermal mucus from rainbow trout fed NPH contained considerably larger concentrations of metabolites than parent hydrocarbon. Epidermal mucus is generated when epithelial mucin released by the mucus cells comes in contact with surrounding water; therefore, concentrations of aromatic compounds calculated on a dry weight basis would more closely approximate the actual concentrations of these compounds in epithelial mucin. However, because epidermal mucus of rainbow trout contained more than 99% water, concentrations of NPH and its metabolizes are also given on a wet weight basis in Table 8. Concentrations of both NPH and its metabolizes in the mucus initially increased and reached maximum values 24 hr after the force feeding. The data fit lognormal distribution, and using a one-tailed F-test of homogeneity of variance, it was calculated that rates of changes in concentration of NPH and metabolizes in the mucus of the force-fed fish were not significantly different (Table 8).

Epidermal mucus of starry flounder contained 14 ppb (dry weight) of NPH and 30 ppb of the metabolizes 24 hr after the fish were fed NPH. During the entire exposure period, the mucus contained higher concentrations of the metabolizes compared to NPH (Table 10).

Epidermal mucus of the rainbow trout in the injection study contained larger concentrations of metabolizes than NPH at each sampling period. NPH was not detected in the test fish after 24 hr. Metabolites were present in mucus for the first 48 hr, but were not detected at 168 hr (Table 11). The data in Tables 8 and 11 reveal that there was no correlation between either relative proportions or rates of change of concentrations of NPH and metabolizes in skin and mucus of the test fish.

6.1.5 Accumulation and Biotransformation of NPH by Flatfish

Force-feeding Study (Starry Flounder) at 12°C. Tissues of starry flounder contained considerable concentrations of both NPH and its metabolic products 24 hr after force-feeding NPH (Fig. 14). Maxima in concentrations of both the hydrocarbon and its metabolic products occurred during the first 48 hr, then began to decline in all tissues except bile (Fig. 14). Statistical treatment of these data shows that the rates of decline of NPH concentrations in liver, skin, and blood were significantly greater than the respective rates of decline in metabolize concentrations (Fig. 14). At 168 hr, all sites examined, except brain, contained considerably more radioactivity associated with metabolic products than with NPH (Fig. 14; Table 13); more than 70% of the total radioactivity in blood and liver was associated with the metabolic products. Throughout the experiment, radioactivity in bile of starry flounder was largely due to metabolizes.

Force-feeding Study (Rock Sole) at 12°C. Comparison of the data in Figures 14 and 15 revealed that the radioactivity in all tissues of rock sole was considerably greater than the radioactivity in the corresponding tissues of starry flounder at 24 hr. Moreover, values for NPH and metabolize concentrations in tissues of rock sole were significantly ($P<0.01$) higher than the corresponding values for starry flounder at 168 hr. Liver, skin, and muscle of starry flounder contained 11.6, 3.0, and 1.7 g of lipid, respectively, per 100 g of wet tissue; whereas, liver, skin, and muscle of rock sole contained 4.3, 1.5, and 1.0 g of lipid, respectively, per 100 g of wet tissue.

Differences between rates of decline in NPH and the metabolize concentrations in liver, blood, and skin of rock sole were also measured. As with starry flounder, the rate of decline in NPH concentration in each tissue was greater than the rate of decline in metabolize concentration (Fig. 15). It was also determined that the extent of decline of NPH in each of the tissues was greater for starry flounder than for rock sole. From 24 to 168 hr, there was 12.4- and 2.3-fold decrease, respectively, in NPH and metabolize concentrations in the livers of rock sole; corresponding values for starry flounder were 63.0 and 2.6.

Stomach and intestine of rock sole contained more radioactivity than those of starry flounder at both 24 and 168 hr. At 24 hr, stomach and intestine of the rock sole contained 0.3 and 6.0%, respectively, of the administered dose, whereas the corresponding values for starry flounder were 0.2 and 1.5.

Because considerable radioactivity was present in the rock sole after 1 wk, some fish were sampled at 6 wk after the treatment. Gills and kidney contained considerable concentrations of metabolic products, whereas bile and liver contained smaller concentrations, and in the other tissues examined radioactivity was barely detectable (Fig. 15).

Injection Study (Starry Flounder) at 12*C. At 24 hr after an i.p. injection of ^{3}H-NPH, livers of starry flounder contained considerably higher concentrations of NPH than did livers of fish receiving the same dose of NPH via force-feeding (Figs. 14 and 16). At 168 hr, livers of fish in the injection study contained 16 times as much NPH and 1.2 times as much metabolizes as livers of the force-fed fish. For a period of 24 to 168 hr after the treatment, the decrease in the NPH concentration in the liver of fish in the injection study was 9.3-fold and in the feeding study it was 63-fold; the decline in the metabolize concentrations were 1.1 and 2.6, respectively (Figs. 14 and 16).

6.1.6 Effect of Environmental Temperature on Disposition of NPH and Its Metabolizes in Fish

Studies to determine the effect of temperature on the amount of ^{14}C-NPH incorporated into selected organs of coho salmon are presented in Table 14. The results of this work show that 16 hr after force feeding, fish maintained at 4*C have statistically greater concentrations of NPH than fish maintained at 10"C ($P<0.05$). For example, in the brain there was 2 times as much NPH at 4*C as at 10"C, while in the liver at 4*C there was 6 times as much as at 10"C.

Starry flounder held at 4°C had 1.6 to 15 times greater tissue concentrations of NPH at 24 hr post exposure than in corresponding tissues of fish held at 12°C (Table 15). The largest differences were observed for liver, stomach, and intestine. At 24 hr, large amounts of NPH (>30% of the administered dose) were present in the gastrointestinal (GI) tracts of the fish at 4°C, whereas less than 3% of the dose was retained in the GI tracts of fish at 12*C. Tissue concentrations of NPH at 4*C and 12°C were much lower at 168 hr than at 24 hr; however, the decline in NPH concentrations from 24 to 168 hr was much greater at 12*C than that at 4*C resulting in even larger differences between NPH concentrations in most tissues of the two groups of fish at 168 hr (Table 15).

FIGURE 14. Concentrations of naphthalene and its metabolizes in tissues of starry flounder, at 24, 48, and 168 hr after the feeding of 56 uCi of 1-^{3}H-naphthalene. Values are expressed as mean + se. (for number of fish tested at each time period, see Table 13). Values of dry weight of tissues are given below as percent of the wet weight of tissue: stomach (22%), blood (14%), liver (25%), skin (33%), brain (18%), muscle (21%), gills (17%), kidney (18??), bile (12%), intestine (22%), and epidermal mucus (2.8%). Rates of decline in concentrations of naphthalene (N) and total metabolizes (M) were calculated using the equation y = ax-b. Values for regression coefficient b were for liver b(N) = 1.845, b(M) = 0.404; for blood b(N) = 1.671, b(n) = ().837; and for skin b(N) = 1.499, b(M) = 0.616. (From Varanasi et al. 1979)

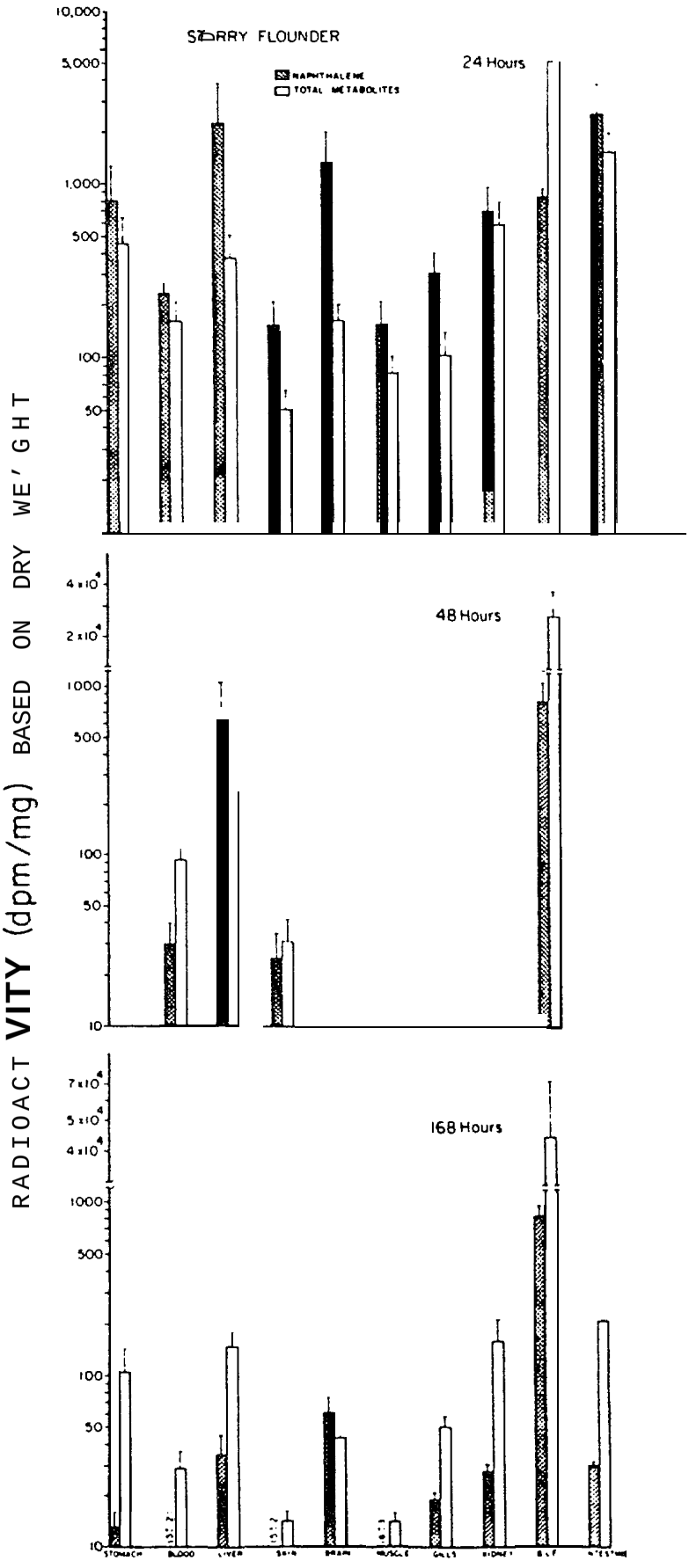

STARRY FLOUNDER
24 Hours
NAPHTHALENE
TOTAL METABOLITES
48 Hours
168 Hours
RADIOACTIVITY (dpm/mg) BASED ON DRY WEIGHT

Table 13 Total Radioactivity (Expressed as % Administered Dose) and Distribution of Naphthalene and its Metabolites in Pleuronectids Exposed to ^{3}H-Naphthalene

Treatment	Species and # of samples	Time after treatment (hr)	Stomach % dose	Stomach N[a]	Stomach M[a]	Liver % dose	Liver N	Liver M	Skin % dose	Skin N	Skin M	Brain % dose	Brain N	Brain M	Muscle % dose	Muscle N	Muscle M	Bile % dose	Bile N	Bile M	Intestine % dose	Intestine N	Intestine M
Force-feeding	R.S.[b] 6	24	0.30[c] *0.06	55	45	1.8 *1.5	84	16	1.0 ±0.6	73	27	0.06 ±0.004	85	15	3.4 ±0.5	68	32	1.7 ±0.6	5	95	6.0 ±3.2	63	37
	6	4 8	—[d]	—	—	0.4 ±0.3	62	38	0.5 ±0.4	57	43	—	—	—	—	—	—	2.2 ±1.6	2	98	—	—	—
	6	168	0.03 *0.01	29	71	0.12 ±0.05	49	51	0.08 ± 0.02	50	50	0.01 ±0.001	74	26	0.3 ±0.04	57	43	1.4 ±0.8	I	99	0.2 ±0.05	65	35
Force-feeding	S.F. 4	24	0.20 ±0. 10	64	36	007 *0.5	85	15	.0,4 ±0.2	75	25	0.04 ±0.02	89	11	1.3 ±0.4	65	35	0.2 ±0.2	14	86	1.s ±0.8	62	38
	4	4 s	—	—	.	0.3 ±0.5	76	24	0 . 1 1 4 5 5 5 *0.04	—	—	—	—	—	—	—	0 . 9 20.4		3	97	—	—	—
	4	168	0.05 ±0.02	5	95	0.02 ±0.01	19	81	0.02 ±0.01	26	74	0.003 ±0.001	59	41	0.09 ±0.02	27	73	0.9 ±0.4	2	98	0.19 ±0.06	9	91
ip injection	S.F. 3	24	—	—	—	3.6 *0.4	97	3	0 . 3 8 8 4 1 6 ±0. 12	—	—	—	—	—	—	—	—	0.49 ±0.04	7	93	—	—	—
	3	168	—	—	.	0.4 ±0.1	76	24	0 . 0 6 7 9 2 1 ±0.01	—	—	—	—	—	—	—	—	2.3 ±0.09	1		9 9	-	- -

[a] Relative % of N = naphthalene and M = told metabolites.
[b] R.S. = Rock sole; S.F. = Starry flounder.
[c] Mean ± S.E.
[d] Not done.

(From Varanasi et al. 1979)

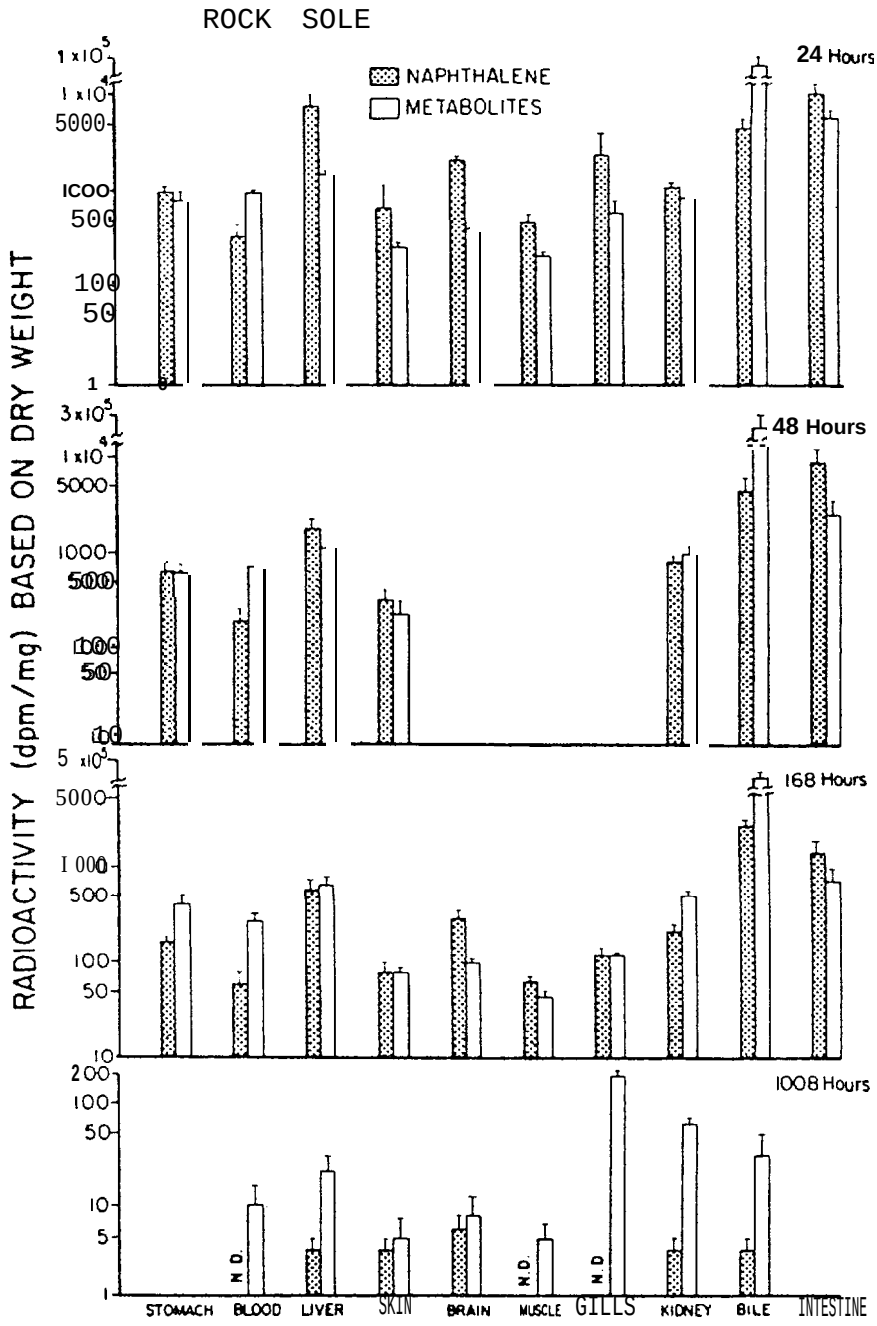


FIGURE 15. Concentrations of naphthalene and its metabolizes in tissues of rock sole at 24, 48, 168, and 1008 hr after the feeding of 56 uCi of 1-^{3}H-napthalene. Other pertinent details are given in Figure 14 and Table 13. Regression coefficient for rates of decline of naphthalene N and total metabolizes M was for liver, b(N) = 1.999, b(M) = ().530, for skin, b(N) =0.771, b(M) = 0.558; and for blood, b(N) = 0.812, b(M) = 0.601. N.D. = not detected. (From Varanasi et al. 1979)

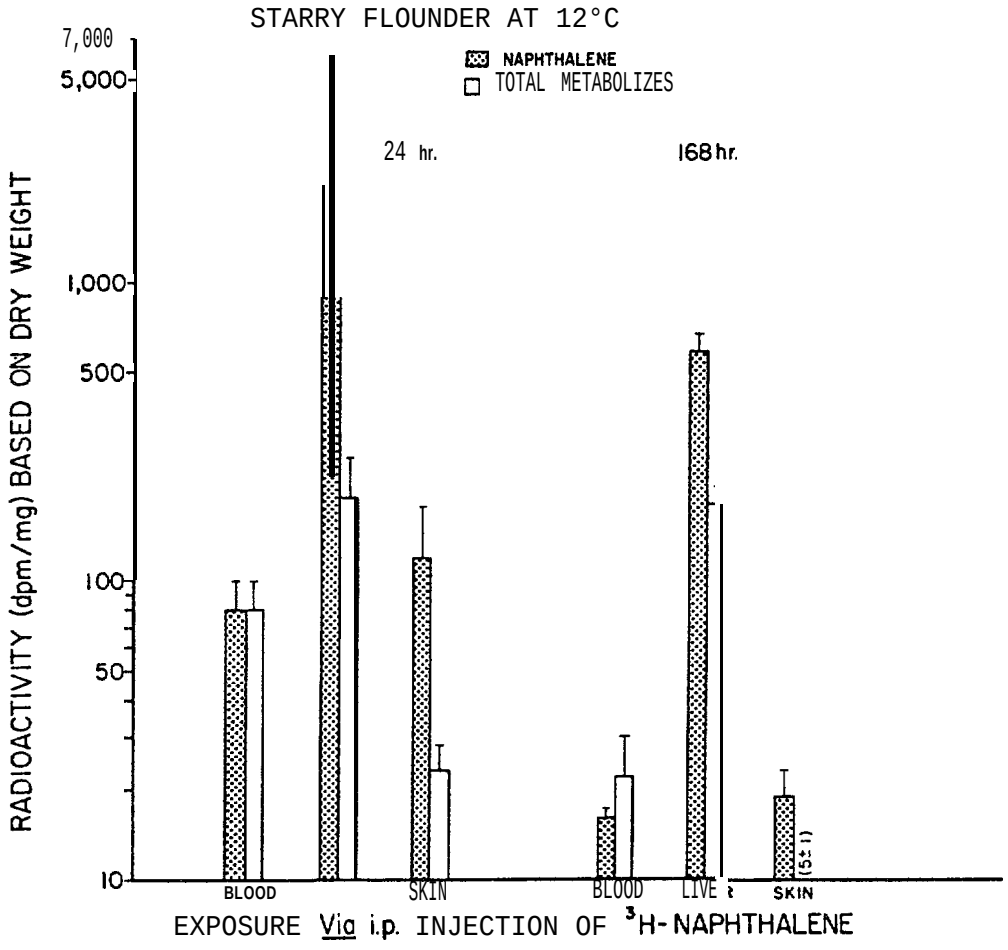


FIGURE 16. Concentrations of naphthalene and its metabolizes in tissues of starry flounder 24 and 168 hr after intraperitoneal injection of 56 µCi of 3H-naphthalene. Other pertinent details are given in Figure 14 and Table 13. (From Varanasi et al. 1979)

Concentrations of metabolizes in tissues of fish 24 hr after exposure were not substantially larger at the lower temperature (Table 15). Ratios of values for NPH concentrations in most tissues of fish at 4° vs. 12°C increased markedly from 24 to 168 hr; such a marked increase was not observed" for the metabolite concentrations. After one week, metabolize concentrations in most tissues of fish at 4°C were not much greater than those at 12°C (Table 15); exceptions were the blood and muscle of fish at 4°C which contained substantially higher concentrations of metabolizes than blood and muscle of fish at 12°C.

Table 14 Distribution of naphthalene in organs of coho salmon after force-feeding of 1-^{14}C-naphthalene

Organ	8 hr 4°C* ng/g Dry wt	8 hr 4°C* % Admin. dose	8 hr 10°C† ng/g Dry wt	8 hr 10°C† % Admin. dose	16 hr 4°C* ng/g Dry wt	16 hr 4°C* % Admin. dose	16 hr 10°C* ng/g Dry wt	16 hr 10°C* % Admin. dose
Brain	880 ± 290	0.022 ± 0.0087	630 ± 160	0.017± 0.0042	1400 ± 95	0.0.39 ± 0.001 7	640 ± 120	0.017 * 0.0035
Liver	3000 ± 1200	0.64 * 0.37	1000 ± 300	0.2 I ± 0.049	2300 ± 250	0.46 ± 0.035	400 ± 200	0.10 ± 0.052
Kidney	510*99	0.042 * 0.0012	540 ± 190	0.060 ± 0.018	930 ± I 20	0.092 ± 0.019	230 ± 110	0.046 ± 0.022
Gall bladder	950 ± 410	0.025 ± 0.010	420 *40	0.014 ± 0.0021	2200 ± 990	0.061 ± 0.044	1100 ± 580	0.035 ± 0.017
Dark muscle	1300 * 520	0.52 * 0.23	860 ± 200	0.39 * 0.1 I	2600 ± 1200	1.2 * 0.57	1700 ± 790	0.90 ± (').43
Light muscle	120 * 48	0.65 ± 0.33	85 ± 11	0.49 ± 0.035	230 ± 57	I .3 ± 0.39	77 ± 40	0.72 ± 0.35
Blood	140± 33	0.036 ± 0.0098	97 ± 8.0	0.067 ± 0.0021	200 ± 39	0.13 * 0.035	48 ± 21	0.041 ± 0.016
Gut + contents	NA‡	67 ± 9.9	NA	74 ± 17	NA	56 ± 9.6	NA	57 ± 28

*Average values for 3 fish ± S.E.M.
† Average values for 2 fish ± S.E.M.
‡ NA = not applicable—dry weight not determined.

(From Collier et al. 1978)

Table 15 Naphthalene and its metabolizes in naphthalene-fed starry flounder at 4°C

Tissue	Naphthalene pmoles/mg dry wt.	[C_4/C_{12}][a]	% admin. dose[b]	Metabolites pmoles/mg dry wt.	[C_4/C_{12}][c]	% admin. dose
	24 hr after feeding ^{3}H-naphthalene[d]					
Liver	35.7 ± 10.8[e]	[7.8][f]	6.7 ± 1.6	1.2 ± 0.4	[1.4]	0.22 ± 0.04
Muscle	1.9 ± 0.6	[5.6][f]	5.1 ± 1.3	0.4 ± 0.1	[2.1]	1.05 ± 0.02
Bile	6.2 ± 2.0	[3.3]	0.09 ± 0.01	24.7 ± 6.8	[2.2]	0.39 ± 0.05
Stomach	17.7 ± 8.9	[9.6][f]	1.30 ± 0.60	2.9 ± 1.4	[2.9]	0.22 ± 0.09
Intestine	86.1 ± 31.2	[15][f]	30.80 ± 12.7	9.5 ± 1.4	[2.7]	3.01 ± 0.48
Skin	1.2 ± 0.4	[3.6][f]	1.02 ± 0.3	0.2 * 0.1	[1.6]	0.16 ± 0.03
Brain	6.5 ± 2.0	[2.2]	0.08 ± 0.03	0.4 ± 0.1	[1.1]	0.005 ± 0.001
Blood	1.1 ± 0.4	[1.9]		0.9 ± 0.2	[2.5]	
Kidney	2.5 ± 0.6	[1.6]		1.7 ± 0.5	[1.3]	
Gills	2.5 ± 0.6	[3.7][f]		0.7 ± 0.2	[3.0][f]	
Mucus	0.4 ± 0.1	[5.1]		0.3 ± 0.1	[2.1]	
	168 hr after feeding ^{3}H-naphthalene					
Liver	2.75 ± 0.82	[34][f]	0.57 ± 0.16	0.52 ± 0.18	[1.6]	0.09 ± 0.03
Muscle	0.34 ± 0.11	[26][f]	1.30 ± 0.58	0.12 ± 0.04	[3.6][f]	0.33 * 0.10
Bile	2.42 z 0.97	[1.3]	0.04 ± 0.01	74.5 ± 35.3	[0.?]	1.21 ± 0.47
Stomach	0.42 ± 0.11	[4.2][f]	0.03 ± 0.004	0.28 ± 0.08	[0.1]	0.02 ± 0.003
Intestine	0.57 ± 0.16	[3.4][f]	0.14 ± 0.03	0.53 ± 0.17	[0.3]	0.14 ± 0.04
Skin	0.12 ± 0.02	[10][f]	1.30 ± 0.03	0.06 ± 0.02	[2.0]	0.06 ± 0.01
Brain	0.77 ± 0.29	[5.6][f]	0.010 ± 0.004	0.11 ± 0.04	[1.1]	0.0014 ± 0.0006
Blood	0.10 ± 0.03	[9.6][f]	—	0.36 ± 0.09	[5.6][f]	
Kidney	0.29 ± 0.07	[4.8][f]	—	0.55 ± 0.18	[1.6]	
Gills	0.28 ± 0.09	[7.0][f]	—	0.21 ± 0.06	[1.9]	

[a] Ratio of concentration of naphthalene in tissues of fish exposed at 4° and 12°C

[b] % administered dose was calculated using individual concentration value and total weight of each organ: each value for % admin. dose is mean ± S.E. (6 fish)

[c] Ratio of concentration of metabolites in tissues of fish exposed at 4° and 12°C

[d] Fish were fed 56 μCi (198 mCi/mMole) of ^{3}H-1-naphthalene

[e] Average value for six fish ± S.E.

[f] Concentrations at 4° and 12°C were significantly ($P < 0.05$) different from each other

(From Varanasi et al. 1981a)

Profiles of NPH Metabolites

The chromatograms in Figure 17 show profiles of metabolizes in liver, skin, and bile from a rock sole at 12°C. In livers of both rock sole (Table 16; Fig. 17) and flounder (Table 17), l,2-dihydro-l,2-dihydroxy NPH (dihydrodiol) was the major metabolize (40%) at 24 hr after feeding of the NPH at 12°C. Considerable proportions of conjugates (e.g., glucuronides, mercapturic acids and sulfate/glucosides) were also present in liver of rock sole and starry flounder (Fig. 17; Table 18). Profiles of metabolizes in the skin of starry flounder were similar to that in the skin of rock sole (Table 16) and were characterized by the presence of large concentrations of the dihydrodiol.

Analyses of the metabolizes in the liver of starry flounder exposed to the NPH via an i.p. injection at 12°C show that at 24 hr, liver contained primarily non-conjugated metabolizes of NPti (76.7%) of which the dihydrodiol (41.5%) and naphthols (21.3%) were the major components (Table 18). Comparison of data from Tables 17 and 18 reveals that,

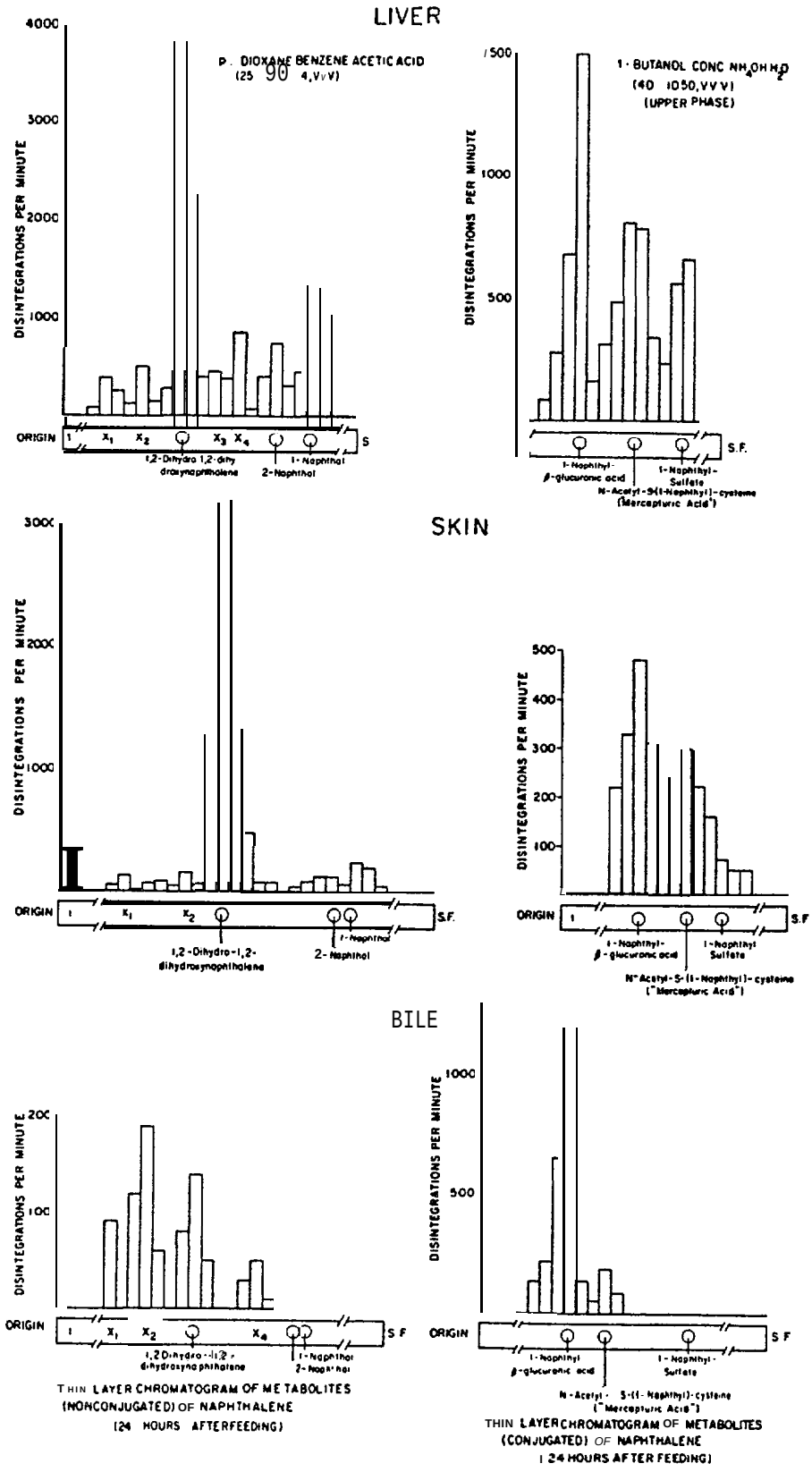


FIGURE 17. (A) Profiles of metabolizes in liver of a rock sole. (B) Profiles of metabolizes in skin of a rock sole. (C) Profiles of metabolizes in bile of a rock sole. (From Varanasi et al. 1979)

Table 16 Metabolites in rock sole

Metabolites	Liver[a] % of total[b] metabolizes	Liver[a] pmoles/mg dry wt.	Bile % of total[b] metabolites	Bile pmoles/mg dry wt.	Skin % of total[b] metabolites	Skin pmoles/mg dry wt.
	24 hr after feeding of ^{3}H-naphthalene					
Total conjugates	31.9 ± 2.7	0.8820.21	92.8 ± 0.4	172.6 ± 0.8	18.2 ± 9.4	0.08 ± 0.06
Total non-conjugates	68.1 ± 2.7[c]	2.08 * 0.01	7.2 ± 0.4	13.4 ± 0.8	81.829.4	0.30 ± 0.10
Glucuronides	12.8 ± 0.6	0.37 ± 0.06	80.7 ± 0.9	150.1 ± 1.6	8.6 ± 4.1	0.04 ± 0.03
Mercapturic acids	14.2 ± 3.1	0.41 ± 0.16	11.0 ± 0.7	19.7 ± 0.2	5.6 ± 1.6	0.02 ± 0.01
Sulfate/glucosides	4.1 ± 1.5[c]	0.11 ± 0.01	1.1 ± 0.2	2.0 ± 0.3	4.1 ± 4.0	0.02 ± 0.03
Dihydrodiol (1,2-isomer)	38.7 ± 5.(Y	1.19 ± 0.15	3.5 ± 0.2	6.5 ± 0.4	59.7 ± 2.9	0.23 ± 0.09
Naphthols (1- & 2-)	16.1 ± 4.4	0.51 ± 0.13	0.8 ± 0.1	1.550.2	10,7 ± 2.7	0.04 ± 0.01
Uncharacterized (X_1-X_4)	13.4 ± 3.0	0.34 * 0.03	2.9 ± 0.5	5.4 ± 1.0	11.5 ± 5.9[d]	0.04 ± 0.01
	168 hr after feeding of ^{3}H-naphthalene					
Total conjugates	54.5 ± 2.6	0.65 ± 0.06	96.2 ± 0.1	714.3 ± 1.1		
Total non-conjugates	45.5 ± 2.6	0.56 ± 0.10	3.8± 0.1	28.2 ± 1.1		
Glucuronides	27.2 ± 6.9	0.32 ± 0.06	85.9 ± 0.9	637.8 ± 6.4		
Mercapturic acids	13.3 ± 2.4	0.16 ± 0.05	8.5 ± 0.8	63.2 ± 5.9		
Sulfate/glucosides	14.0 ± 2.9	0.18 ± 0.07	1.8 ± 0.1	13.4 ± 0.5		
Dihydrodiol	6.8 ± 5.4	0.09 ± 0.08	1.4 ± 0.2	10.0 ±1.1		
Naphthols	29.3 ± 9.3	0.34 ± 0.09	0.720.1	5.6 ± 0.5		
Uncharacterized (X_1-X_4)	9.5 ± 6.3	0.13 ± 0.10	1.7 ± 0.1	12.6 * 0.4		

[a] Each value is mean ± s.d. of four samples

[b] Based on total extracted metabolizes, an average of 8, 10, and <0.5% of total radioactivity respectively, remained in liver, skin. and bile

[c] significantly (P c 0.001) different from corresponding value at 168 hr

[d] Only X_1 and X_2 were present in skin

(From Varanasi et al. 1979)

whereas profiles of metabolizes i n the 1 i vers of starry flounder from the feeding and injection studies were quite different, biliary metabolizes were similar for fish in both studies, and did not change significantly with time. Regardless of species or mode of exposure, there was an increase in the proportion of conjugated metabolizes in liver and muscle from 24 to 168 hr after NPH-exposure.

At both 4° and 12°C the dihydrodiol was the major metabolize (39.7-80.9% of total extractable metabolizes) present in liver and muscle of starry flounder at 24 hr after feeding of NPH (Table 19). At 24 hr, naphthols represented from 1.5 to 10.7% of the total extracted metabolizes in liver, muscle or bile of fish at 4° and 12°C (Table 19). The peak X_4 in chromatograms (Figs. 17 and 18) was tentatively identified as 1,2-naphthoquinone. Glucuronides, sulfates/glucosides and mercapturic acids were also present in liver, muscle, and bile from the NPH-exposed starry flounder (Table 19) at both 4° and 12°C. At 24 hr, no marked differences were observed in the proportions of most metabolize classes in liver of fish at 4° and 12°C.

Table 17 Metabolizes in starry flounder'

Metabolizes	Liver		Bile		Skin	
	% of total metabolites	pmoles/mg dry wt.	% of total metabolizes	pmoles/mg dry wt.	% of total metabolizes	pmoles/mg dry wt.
24 hr after feeding of ^{3}H-naphthalene						
Total conjugates	43.6 ±9.9[b]	0.5420.39	91.8± 0.3	4.83 ± 0.01	24.0 ± 1.6	0.04 ± 0.01
Total non-conjugates	56.4 ± 9.9[b]	0.62 ± 0.21	8.2 ± 0.3	0.4450.01	76.0 ±1.6	0.12 ± 0.01
Glucuronides	32.2 ± 7.2	0.4020.30	81.6 ± 0.1	4.29 ± 0.01	12.5 ± 0.4	0.0220.01
Mercapturic acids	9.5 ± 3.6	0.1220.10	8.9 ± 0.1	0.47± 0.01	9.7 ± 2.1	0.01 ± 0.01
Sulfate/glucosides	1.93 0.1[b]	0.01 ± 0.03	1.4 ± 0.1	0.07 ± 0.01	1.7 ± 2.5	0.01 ± 0.01
Dihydrodiol	39.72 13.9[b]	0.41 ± 0.04	4.0 ± 0.1	0.21 * 0.01	44.1 ± 1.3	0.07 ± 0.01
Naphthols (1- & 2-)	10.7 ± 3.9	0.14 ± 0.12	1.5 ± 0.1	0.08 ± 0.01	11.7± 0.4	0.02" ±0.01
Uncharacterized (X_1-X_4)	6.0 ± 0.7	0.07 ± 0.05	2.6 ± 0.3	0.1420.02	20.3 ± 3.3	0.03 ± 0.01
168 hr after feeding of ^{3}H-naphthalene						
Total conjugates	62.3 ± 8.8	0. M ± 0.03	91.5 ± 0.3	42.6 ± 0.1.		
Total non-conjugates	37.7 ± 8.8	0.10 ± 0.05	8.5 ± 0.3	4.0 * 0.1		
Glucuronides	16.6 ± 1.6	0.04 ± 0.01	81.7 ± 0.3	38.2 ± 0.1		
Mercapturic acids	10.9 ± 6.6	0.03 ± 0.03	8.8 * 0.1	4.1 ± 0.1		
Sulfate/glucosidcs	34.8 ± 14.8	0.08 ± 0.02	0.9 ± 0.1	0.4 ± 0.1		
Dihydrodiol	12.4 ± 11.7	0.03 ± 0.05	3.050.1	1.4 ± 0.1		
Naphthols	11.5 ± 3.9	0.03 ± 0.02	2.9 ± 0.2	1.4 ± 0.1		
Uncharacterized (X_1-X_4)	14.0 * 7.1	0.03 ± 0.01	2.6 ± 0.1	0.1 ± 0.1		

[a]See Figure 14 and Table 16 for details
[b]Significantly ($P < 0.05$) different from corresponding values at 168 hr

(From Varanasi et al. 1979)

From 24 to 168 hr, there was a significant ($p<0.05$) decrease in the proportion of the dihydrodiol fraction and an increase in sulfate/glucoside fraction in both liver and muscle of fish at 4° and 12°C (Table 19). Moreover, the proportion of the glucuronide fraction also increased with time in liver, muscle, and bile of fish at 4°C. Individual metabolize classes in liver and muscle of fish at 4° and 12°C did not vary directly with the concentrations of total metabolizes; ratios of concentrations of total metabolites (Table 15) at 4° and 12°C for the liver and muscle at 168 hr were 1.6 and 3.6, respectively, whereas ratios for the dihydrodiol at this time were 4.5 and 8.3, respectively (Table 19).

Metabolizes in bile from both groups of fish (4° or 12°C) at 24 and 168 hr were characterized by high percentages (>85%) of the conjugates, of which glucuronides were the major components (Table 19). Analyses of bile from fish at 8 hr after exposure at 12°C also showed the glucuronides to be the major components (Table 19, Footnote e). Very small proportions (<10%) of the nonconjugates (dihydrodiol and naphthols) were present in bile of starry flounder from 8 to 168 hr.

Table 18 Metabolites in starry flounder exposed to ^{3}H-naphthalene via ip injection'

Metabolites	24 hr after injection: Liver, % of total metabolizes	Liver, pmoles/mg dry wt.	Bile, % of total metabolites	Bile, pmoles/mg dry wt.
Total conjugates	23.2 ± 1.7[b]	0.099 ± 0.007	%.9 ± 0.7	21.70 ± 0.16
Total non-conjugates	76.7 ± L7[b]	0.328 ± 0.007	3.1 ± 0.7	0.70 ± 0.16
Glucuronides	8.3 ± 0.7	0.036 ± 0.003	88.7 ± 0.1	19.87 ± 0.03
Mercapturic acids	N.D.	N.D.	7.0 * 0.1	1.57 ± 0.01
Sulfate-glucosides	15.0 ± 0.7	0.064 ± 0.003	1.2 ± 0.1	0.26 ± 0.02
Dihydrodiol	41.5 ± 1.4[b]	0.178 ± 0.006	1.8 ± 0.3	0.42 ± 0.07
Naphthols (1- & 2-)	21.3 ± 0.8	0.091 ± 0.003	0.1 ± 0.2	0.01 ± 0.01
Uncharacterized (X_1-X_4)	13.8 ± 0.5	0.059 ± 0.002	1.2 ± 0.4	0.28 ± 0.10
	168 hr after injection			
Total conjugates	33.8 ± 1.5	0.13 ± 0.01	97.5 ± 0.4	108.9 ± 0.5
Total non-conjugates	66.2 ± 1.5	0.25 ± 0.01	2.520.4	2.8 ± 0.5
Glucuronides	9.720.3	0.04 ± 0.01	89.5 ± 0.5	100.0 ± 0.6
Mercapturic acids	N.D.	N.D.	6.7 ± 0.4	7.5 ± 0.5
Sulfate/glucosides	24.1 ± 0.3	0.09 ± 0.01	1.3 ± 0.1	1.4 ± 0.1
Dihydrodiol	29.1 ± 8.3	0.11 ± 0.03	0.8 ± 0.1	0.9 ± 0.1
Naphthols	20.7 ± 5.2	0.08 ± 0.02	0.6 ± 0.2	0.7 ± 0.2
Uncharacterized (X_1-X_4)	16.5 ± 4.6	0.06 ± 0.02	Lo ± 0.3	1.2 ± 0.3

[a] See footnotes under Figure 16 and Table 16 for details
[b] Significantly ($P < 0.05$) different than corresponding values at 168 hr

(From Varanasi et al. 1979)

Enzymatic hydrolysis of glucuronide and sulfate fractions isolated from bile reveal ed that these derivatives were formed primarily by conjugation with the dihydrodiol of NPH (Table 20; Fig. 19). Smaller amounts of naphthols (1- and 2-isomers) were also present in the hydrolysis products from the glucuronide fraction. Patterns of hydrolysis products of biliary glucuronides from fish at 4° and 12°C were similar at 24 hr after exposure (Table 20).

6.1.7 Uptake and Metabolism of Sediment-Associated NPH and BaP by Flatfish

The concentrations of BaP in sediment did not change significantly over 10 days (Table 21); however, a trend of declining NPH concentrations was evident. Chromatographic analyses of BaP-derived radioactivity (^{3}H) from sediment and SAW revealed that BaP remained largely (>93%) in the form of parent hydrocarbon throughout the experiment.

Table 19 Comparison of metabolize profiles in starry flounder exposed to dietary naphthalene at 4* and 12°C

Metabolites[a]	Liver			Muscle			Bile	
	c/c of total metabolites[b]			% of total metabolites[b]			% of total metabolites[b]	
	4°C	12°C[d]	[c,./c,*.]'	4°C	12°C	$[C_4/C_{12}]^c$	4°C	12°C[d,e]
	24 hr after feeding of ^{3}H-naphthalene							
Total conjugates	27.1 ± 1.8	43.6 ± 9.9	[0,5]	11.5 ± 0.3	9.4 ± 0.04	[2.7]	86.5 ± 1.6	91.8 ± 0.3
Total non-conjugates	72.9 ± 1.8[f]	56.4 ± 9.9'	[1.1]	88.5 ± 0.3'	90.6 ± 0.04[f]	[2.1]	13.5 ± 1.6	8.2 ± 0.3
Glucuronides	15.9 ± 0.4[f]	32.2 ± 7.2	[0,4]	4.6 ± 0.4[f]	5.4 ± 0.9[f]	[1.9]	73.9 ± 0.1[f]	81.6 ± 0.1
Mercapturic acids	11.2 ± 0.4	9.5 ± 3.6	[0.9]	3.2 ± 0.5	2.6 ± 1.1	[2,8]	10.9 ± 0.2	8.9 ± 0.1
Sulfate/glucosides	N.D.	1.9 ± 0. 1[f]	—	3.6 ± 0.9[f]	1.5 ± 0.2[f]	[5.4]	1.8 ± 0.3	1.4 ± 0.1
Dihydrodiol (1,2-isomer)	48.4 ± 0.2'	39.7 ± 13.9'	[1.1]	77.3 ± 0.7'	80.9 ± 0.5[f]	[2.1]	6.5 ± 1.4	4.0 ± (')1
Naphthols (1- & 2-)	8.8 ± 0.9	10.7 ± 3.9	[0.6]	1.8 ± 0.1	2.4 ± 0.01 [f]	[1.6]	2.8 ± 0.6	1.5 ± 0.1
Uncharacterized (X, - X_4)	1S.7 ± 0,6	6.0 ± 0.7	[2.1]	9.4 * 0.2	7.2 ± 0.9	[2.8]	4.2 ± 0.5	2,6 ± 0.3
	168 hr after feeding of ^{3}H-naphthalene							
Total conjugates	54.1 ± 1.3	62.3 ± 8.8	[i .7]	28.9 ± 0,5	46.1 ± 0.3	[2.6]	94.6 ± 0.2	91.5 ± 0.3
Total non-conjugates	45.9 ± 1.3	37.7 ± 8.8	[2.1]	71.1 ± 0.5	53.9 ± 0.3	[5.5]	5.5 ± 0.2	8.5 ± 0.3
Glucuronides	32.3 ± 0.3	16.6 ± 1.6	[3.8]	13.1 ± 1.3	23.7 ± 1.4	[2.3]	85.1 ± 0.2	81.7 ± 0.3
Mercapturic acids	11.7 ± 0.2	10.9 ± 6.6	[1.8]	7.2 ± 0.2	8.0 ± 0.9	[3.8]	7.7 ± 0.2	8.8 ± 0.1
Sulfate/glucosides	10.1 2 0.1	34.8 ± 14.8	[0.6]	8.7 ± 2.1	14:4 ± 0.5	[2.5]	1.8 ± 0.1	0.9 ± 0.1
Dihydrodiol (1,2-isomer)	29.7 ± 0.S	12.4 ± 11.7	[4.5]	47.4 ± 0.4	23.9 ± 0.9	[8.3]	2.5 ± 0.1	3.0 ± 0.1
Naphthols (1- & 2-)	6.2 ± 0.2	11.5 ± 3.9	[0.9]	6.9 ± 1.2	21.6 ± 0.6	[1.3]	0.6 ± 0.1	"2.9 ± 0.2
Uncharacterized (X, - X_4)	10.0 ± 1.1	14.0 ± 7.1	[5.1]	16.7 ± 1.1	8.4 ± 0.4	[8.2]	2.3 ± 0.3	2.6 ± 0.1

[a] Characterized by R_f values of TLC standards. Each value is mean ± S.D. of three samples of pooled homogenates prepared from 4 to 6 fish

[b] Based on total extracted metabolites; an average of 8, 13, and <0.570 of total radioactivity respectively, remained in liver, muscle, and bile from fish taken al 24 hr

[c] Ratio of concentration (pmole/mg dry wt) of each class of metabolize at 4°C to that at 12°C.

[d] Taken from Varanasi *et al.* (1979)

[e] Biliary metabolites were also analyzed at 8 hr after the exposure; major component (>75%) was glucuronide fraction. Free dihydrodiol and naphthols constituted less than 10% of the total extracted metabolizes

[f] Significantly ($p < 0.05$) different from the corresponding value at 168 hr

(From Varanasi et al. 1981a)

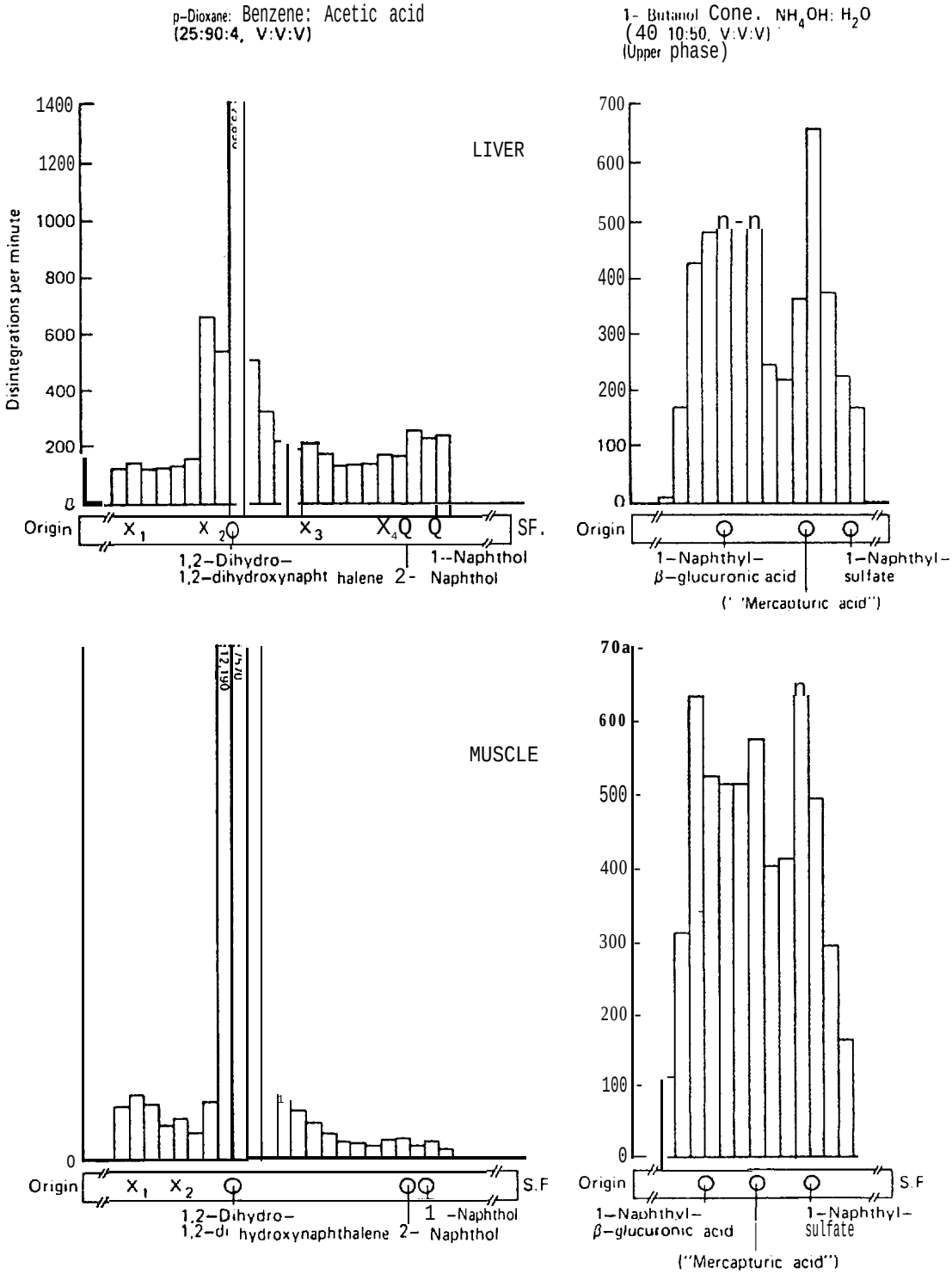


FIGURE 18. Metabolize profiles in liver and muscle of naphthalene-fed starry flounder at 4"C. (From Varanasi et al. 1981a)

Table 20 Ethyl acetate-soluble hydrolysis products of conjugated metabolizes [a] isolated from bile of naphthalene-fed starry flounder

Temp (°C)	Time after feeding (hr)	Conjugates	Hydrolysis products: dihydrodiol [b] (%)	1-naphthol [b] (%)	2-naphthol [b] (%)	% of conjugate fraction unhydrolyzed
12	8	glucuronides	6 2	8	30	45
12	24	glucuronides	7 1	7	22	28
12	168	glucuronides	8 9	4	7	28
4	24	glucuronides	7 4	6	20	56
12	168	sulfates/ glucosides	78	5	17	4s

[a] Metabolite fractions were incubated with β-glucuronidase from *Helix pomatia* which contained significant amounts of aryl sulfatase for 48 hr at 37°C. (pH = 5.0)
[b] characterized by R_f values of TLC standards

(From Varanasi et al. 1981a)

Analyses of NPH-derived (^{14}C) radioactivity in sediment also showed that no more than 7% of the total radioactivity was present as total metabolizes in sediment throughout the exposure (Table 21). For the first 3 days, the radioactivity (^{14}C) in the SAW was primarily due to NPH. However, samples of the water from days 4 to 10 contained on the average 16% of the total radioactivity (^{14}C) in the form of NPH metabolizes (Table 21).

Radioactivity derived from both BaP and NPH was detected in tissues and body fluids of English sole within 24 h after the fish were placed on the sediment containing ^{3}H-BaP, ^{14}C-NPH and 1% PBCO (Table 22). From 24 to 168 hr of exposure, BaP-derived radioactivity increased significantly ($p<0.05$) in liver (5 fold), and bile (16 fold), whereas NPH-derived radioactivity decreased for all tissues and fluids except for gill and bile. An increase of NPH-derived radioactivity occurred in the bile (Table 22).

Following 24 hr of exposure to oil-contaminated sediment, fish placed (for 24 hr) on "clean" sediment showed a significant decrease ($p<0.05$) in NPH-derived radioactivity from most tissues (Table 22). Except for a small decline in muscle, no significant change occurred in the levels of BaP-derived radioactivity.

Chromatographic analyses of BaP and its metabolizes in liver (Fig. 20) and bile of fish at 24 hr revealed that 2% or less of the total radioactivity in these samples was due to unconverted BaP (Table 23). TLC analyses of bile, both before and after enzymatic hydrolysis, showed the presence of nonconjugated BaP metabolizes (quinones, phenols, diols, and the more polar compounds as well as glucuronide (51%) and sulfate (15%) conjugates (Table 23).

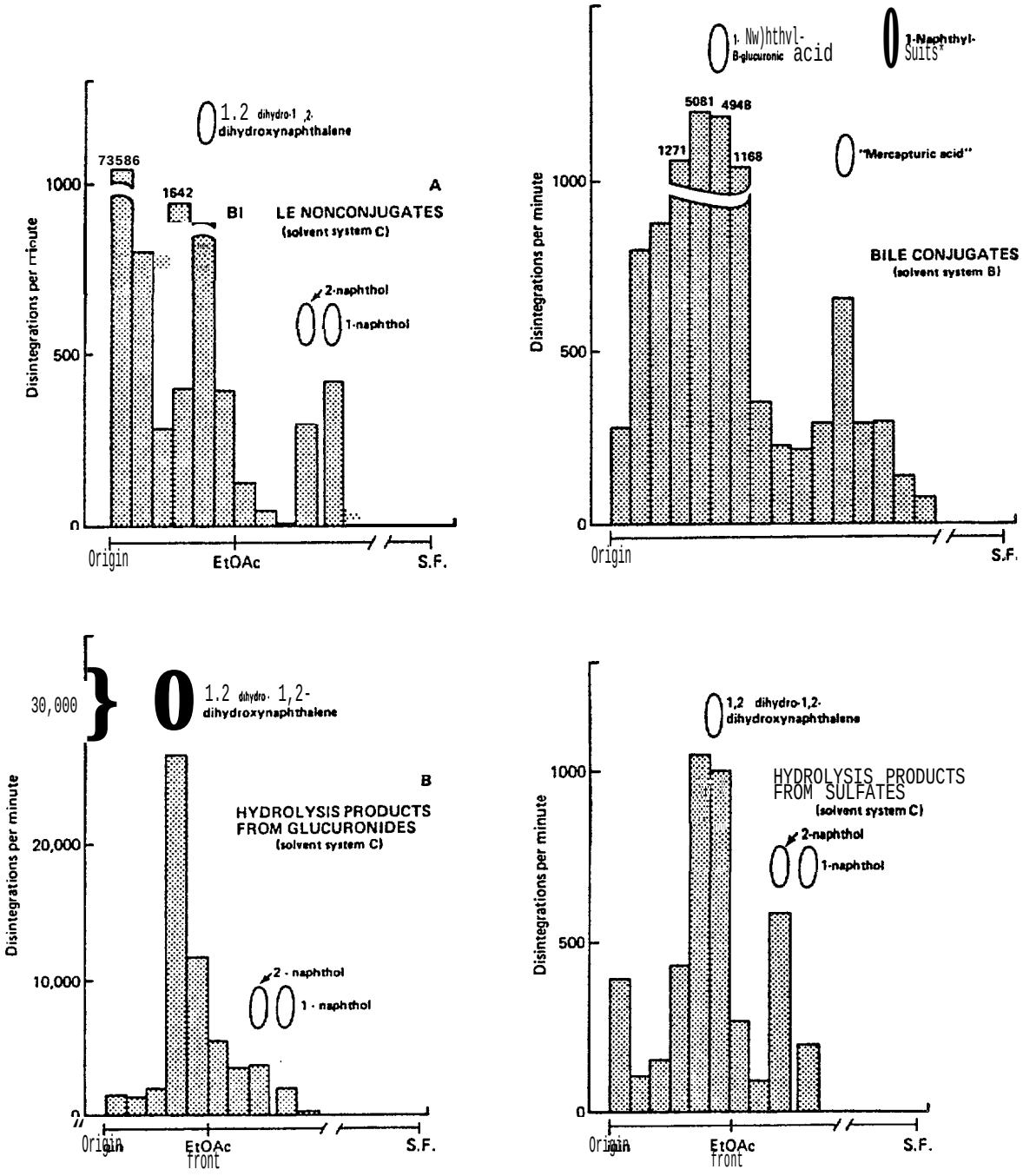


FIGURE 19. (A) Thin-layer chromatograms of biliary naphthalene metabolizes from naphthalene-fed starry flounder at 12°C (168 hr after feeding). (B) Thin-layer chromatograms of hydrolysis products of glucuronide and sulfate/glucoside fractions isolated from thin-layer chromatograms of (A). (From Varanasi et al. 1981)

TABLE 21

Concentrations of B[a]P- and NPH-derived radioactivity in sediment, sediment-associated water (SAW) and the water column."

		Sediment		Sediment-associated water (SAW)				Water column			
		B[a]P	NPH	B[a]P		NPH		B[a]P		NPH	
				unfiltered	filtered	unfiltered	filtered	unfiltered	filtered	unfiltered	filtered
Day	Event	pmol hydrocarbon equivalents/g of sample									
1	sediment was placed in aquarium	2200*	400(97)[b]	14000*	3000(94)	-[c]	-	-	-	-	-
2	Begin 24 h exposure	1700 ± 300(96)	13000* 2000(93)	190	10.6	960	238(93)	15	0.7	110	20
3	Remove 24 h exposed fish for sampling and deputation experiment; place 168 h exposure fish on oil-contaminated sediment	1300 ± 100	7200 ± 600	250	7.4 (97)	420	124(94)	4	0.3	72	38
4	Continue 168 h exposure of fish to oil-contaminated sediment	2500 ± 600(96)	3200 ± 700(94)	190	7.0(96)	-	212(84)	3	-	130	-
7		990 ± 150(93)	960 ± 140(94)	-	6.0 (96)	470	361(86)	-	-	-	-
9		1400 ± 200	600 ± 100(96)	130	6.1	-	-	2	0.1	-	36
10	Remove fish for sampling	2300 ± 340(96)	2500 ± 400(97)	-	14.2	-	317(81)	-	-	-	-

[a]Sediment and SAW samples were taken from 2 cm below the sediment/water interface as described in the text. SAW was decanted off from the sediment and analyzed before and after filtration. Three portions of sediment were analyzed to get average amount oft ritium and carbon-l 4 radioactivity in each sample. Concentration values (mean* SD) for sediment are based on dry weight. Samples of water at the air/water interface (water column) were also analyzed after filtration.

[b]Values in parentheses arc percentages of total radioactivity present as the parent hydrocarbon.

[c]Not done.

(From Varanasi and Gmur 1981a)

TABLE 22

Hydrocarbon-derived radioactivity[a] in tissues of English sole exposed to [^{3}H] B[a]P and [^{14}C]NPH in sediment containing 1% Prudhoe Bay crude oil.

	Gill	Blood	Liver	Skin	Muscle	Bile	Stomach	Intestine
24-h Exposure								
B[a]P	0.390	0.39	0.35	0.140	0.044	8.3	0.22	0.38
	* 0.049	*0.11	± 0.13	* 0.067	* 0.001	± 3.1	* 0.14	* 0.04
NPH	14.4	7,8	66	3.3	8.9	330	19.2	20.4
	* 4.5	± 1.8	*1 I	* 1.5	± 2.3	* 123	± 4.9	± 5.8
168-h Exposure								
B[a]P	0.55[b]	0.67	1.60[b]	'0.140	0.036[b]	130[b]	0.30	0.45
	* 0.10	± 0.38	± 0.06	± 0.016	* 0.003	± 15	± 0.18	± 0.17
NPH	55	3.3[b]	23[b]	1.5	1.2[b]	980[b]	5.2[b]	6.1[b]
	± 42	± 0.9	± 8	± 1.0	* 0.4	± 300	* 0.9	± 2.8
24-h Depuration after 24-h exposure								
B[a]P	0.5 I	0.460	0.66	0.100	0.032[b]	58	0.138	0.28
	± 0.10	± 0.052	± 0.21	± 0.011	* 0.004	± 32	± 0.015	* 0.12
NPH	5.3	2.5[b]	19[b]	2.9	2.3[b]	1600[b]	3.4[b]	6.3[b]
	* 1.0	± 0.6	* 9	± 2.3	± 0.9	± 600	* 1.0	± 0.6

● Values are expressed as nmoles of hydrocarbon equivalents per g of dry wt (mean ± SD).
[b]Significantly different ($P < 0.05$) from corresponding values at 24 h after the exposure.

(From Varanasi and Gmur 1981a)

Examination of the aqueous phase remaining after ethyl acetate extraction of liver showed that greater than one-third of the radioactivity in the aqueous phase was bound to cellular macromolecules in fish liver at both 24 and 168 hr after the BaP exposure (Table 23).

Analyses of NPH-derived radioactivity in the liver of fish at 24 and 168 hr revealed that 85% and 21%, respectively, of the total radioactivity was due to unconverted NPH (Table 24). Radioactivity in bile was due primarily to NPH-metabolizes (>99%) of which glucuronides were the major component. Hydrolysis of the glucuronide fraction revealed the presence of a large proportion (70%) of the 1,2-dihydro l,2-dihydroxynapthal ene and a much smaller proportion (11%) of 1-naphthol (Table 24).

Chromatographic analyses revealed that a large proportion of polar metabolizes having Rf values lower than BaP-9,10-dihydrodiol (Table 23) were conjugated with glucuronic acid. Smaller proportions of BaP-7,8-dihydrodiol, BaP-9,10-dihydrodiol and BaP-4,5-dihydrodiol were also conjugated with glucuronic acid. Analysis of this sample by HPLC (Fig. 21) confirmed the presence of the dihydrodiols and revealed several isomers of 7,8,9,10-tetrahydro-7,8,9, 10-tetrahydroxy-BaP in the hydrolysis products after the glucuronidase treatment of the bile sample. Moreover, the HPLC demonstrated that a number of isomeric phenols were

present in bile (Fig. 21). Hydrolysis products after the treatment of the aqueous phase of bile with aryl sulfatase were qualitatively similar to those produced after the glucuronidase treatment (Table 23).

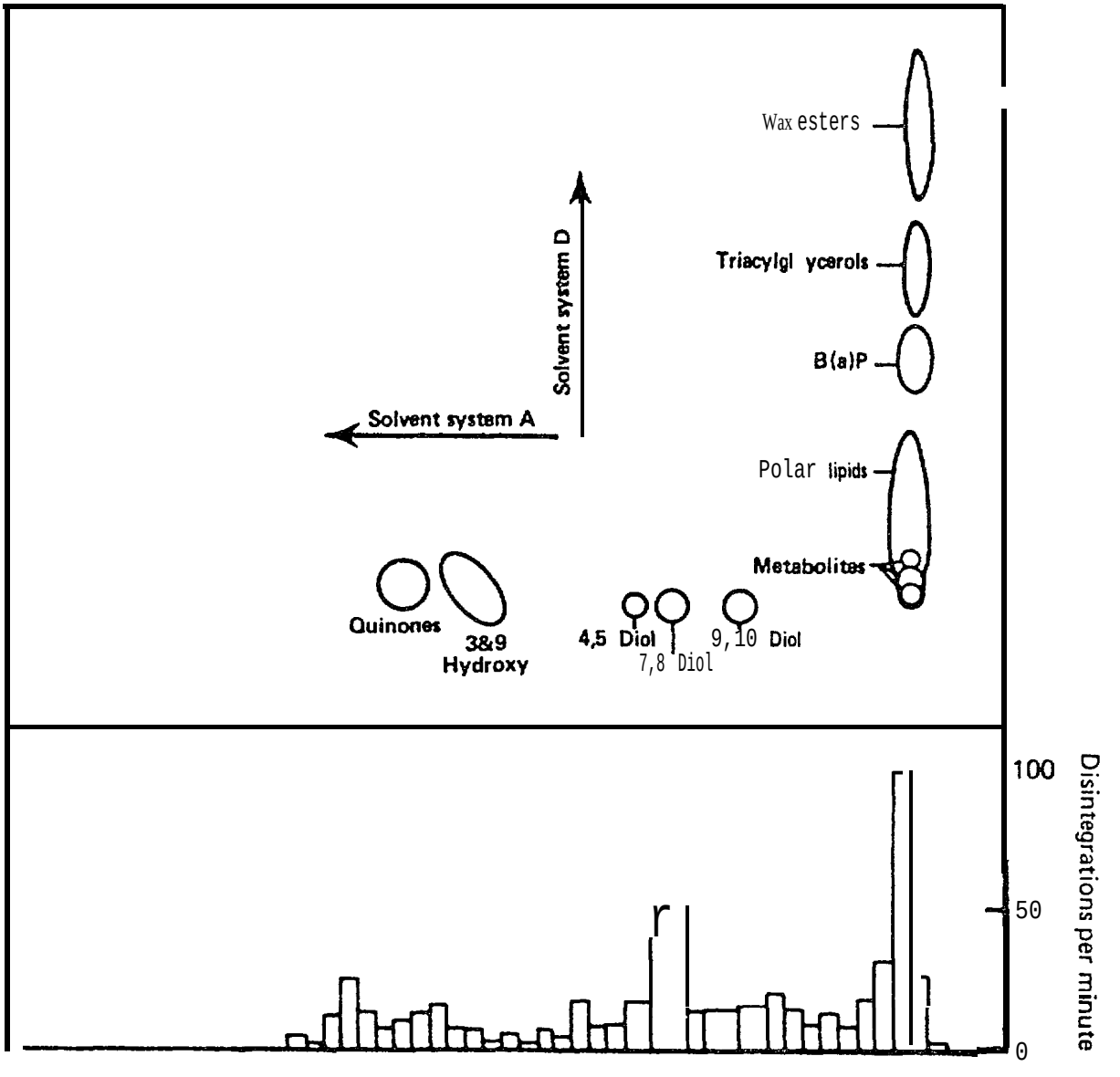


FIGURE 20. Two-dimensional TLC of liver extracts. Liver lipids and [^{3}H] B(a)P were separated from polar lipids and B(a)P metabolizes by developing the plate in one direction with the solvent system D (hexane:diethyl ether, 95:5;v/v) with appropriate standards. The plate was then turned 90° and redeveloped in solvent system A (toluene:ethanol, 9:1,v/v). The resulting profile of B(a)P-derived radioactivity is from one fish from the 168-h exposure group. (From Varanasi and Gmur 1981a)

TABLE 23

Proportions of B[*a*]P and its metabolites in liver and bile of English sole exposed simultaneously to [^{3}H]B[*a*]P and [^{14}C]NPH in sediment.

	Liver		Bile		
	Before enzymatic hydrolysis		Before enzymatic hydrolysis	After treatment of aqueous phase with β-glucuronidase	After treatment of aqueous phase with aryl sulfatase
	24 h[a]	168 h[b]		24 h[b]	
% of total radioactivity					
Unconverted B[*a*]P	2	0.8 ± 0.1	2 ± 3	0.3 ± 0.3	1.1 ± 0.5
Ethyl acetate extractable metabolizes	16	19 ± 2.6	14* 10	51 ± 3.6	15* 3.4
Radioactivity in aqueous phase	82[c]	80[c] ± 2.6	84 ± 12[d]	49 ± 3.6	84 ± 2.8
% of total metabolites in ethyl acetate					
Origin		40.8 ± 2.0	42.7 ± 2.9	12.5 ± 1.9	16.9 ± 4.6
Fraction P		20.6 ± 3.8	13.2 ± 1.6	27.9 ± 9.4	12.7 * 5.8
9,10-diol		2.4 ± 0.8	2.9 ± 1.9	6.8 ± 4.8	5.9 ± 2.4
x		2.3 ± 0.6	9.7 ± 4.0	12.4 * 2.3	5.7 * 6.1
7,8-diol		5.9 ± 0.4	4.5 * 0.3	12.2 ± 4.2	8.3 ± 1.6
4,5-diol		2.5 ± 0.4	3.6 ± 2.2	1.4 ± 2.4	6.5 ± 2.6
X'		n.d.	n.d.	5.6 ± 1.4	n.d.
Phenols		5.8 ± 1.5	1.9 ± 1.6	8.6 ± 1.0	10.2 * 1.7
Quinones		11.6 ± 1.6	3.6 ± 1.8	2.1 ± 1.3	9.0 ± 2.5
Unclassified		31.0* 6.3	18.0 ± 4.6	10.3 ± 5.7	25.0 ± 6.9

[a]Three livers were pooled and one set of values obtained.
[b]Each value is the mean of three individual values ± SD.
[c]41 and 35 ± 2%, respectively, of the radioactivity in the aqueous phase at 24 and 168 h was unextractable.
[d]Treatment with glucuronidase and sulfatase released a total of 66% of the radioactivity into ethyl acetate. The remaining radioactivity is assumed to be due to glutathione conjugates.

(From Varanasi and Gmur 1981a)

6.1.'8 BaP Metabolism by English Sole

Concentrations of BaP-derived radioactivity in muscle (1.4 ± 0.4 pmole/mg dry tissue) was substantially lower than that in liver (40 ± 1.5 pmole/mg) at 24 hr; however, the percent administered dose in the muscle (1.1 ± 0.5) was similar to that in the liver (1.4 ± 0.5); bile contained 2.6 ± 0.5% of the administered dose (Fig. 22). From 24 to 168 hr, concentrations of BaP derived radioactivity increased significantly in bile, decreased significantly in muscle, and did not change significantly in liver. (Fig. 22).

TABLE 24

Proportions of NPH and its metabolites in liver and bile of English sole exposed simultaneously to [^{14}C]NPH and [^{3}H]B[*a*]P in sediment.

	Liver		Bile[a]		
			Before enzymatic hydrolysis	After hydrolysis of glucuronide fraction[c]	After hydrolysis of sulfate/glucoside fractional
	24 h[b]	168 h		168 h	
% of total radioactivity					
Unconverted NPH	85	21 * 11	n.d.	n.d.	n.d.
Total metabolites	15[e]	79*11'	100	100	100
% of total metabolizes					
Glucuronides			88	n.d.	n.a.
Mercapturic acids			2	n.a.	n.a.
Sulfate/glucosides			3	ma.	4.0
1,2 dihydrodiol			0.3	70.0	73.0
1-naphthol			n.d.	11.0	3.0
2-naphthol			n.d.	8.0	2.0
Unclassified			7	11.0	18.0

[a]Three samples of bile from fish exposed for 168 h were pooled to get sufficient radioactivity for quantitation of metabolize classes.
[b]There was not sufficient radioactivity in metabolize fraction to allow quantitation of individual classes.
[c]Glucuronide fraction was isolated by TLC of the bile extract and then hydrolyzed with β-glucuronidase.
[d]Sulfate/glucoside fraction was isolated and then treated with arylsulfatase.
[e]This value includes one-third of the total radioactivity which was not extractable by solvents.
n.d. = not detected: n.a. = not applicable. .

(From Varanasi and Gmur 1981a)

As with fish exposed to BaP in sediment, radioactivity in liver and muscle of these fish was primarily in the form of metabolizes such as dihydrodiols, phenols and their sulfate and glucuronide conjugates (Table 25; Fig. 23). Less than 2% of the total radioactivity in these tissues (e.g. liver and muscle) was in the form of the parent hydrocarbon.

6.1.9 Binding of BaP Intermediates to DNA Catalyzed by Liver Enzymes of Fish

The results in Table 26 showed that the binding value for BaP to DNA was about 3 times greater for the untreated starry flounder than the corresponding value for English sole and rat. Moreover, pretreatment of English sole with PBCO resulted in an 18-fold increase in the binding value compared to the value for untreated fish; the increase in the binding in the case of PBCO-pretreated starry flounder was only 5-fold. The value for binding obtained with liver extracts from MC-pretreated starry flounder was about 10 times greater than that obtained

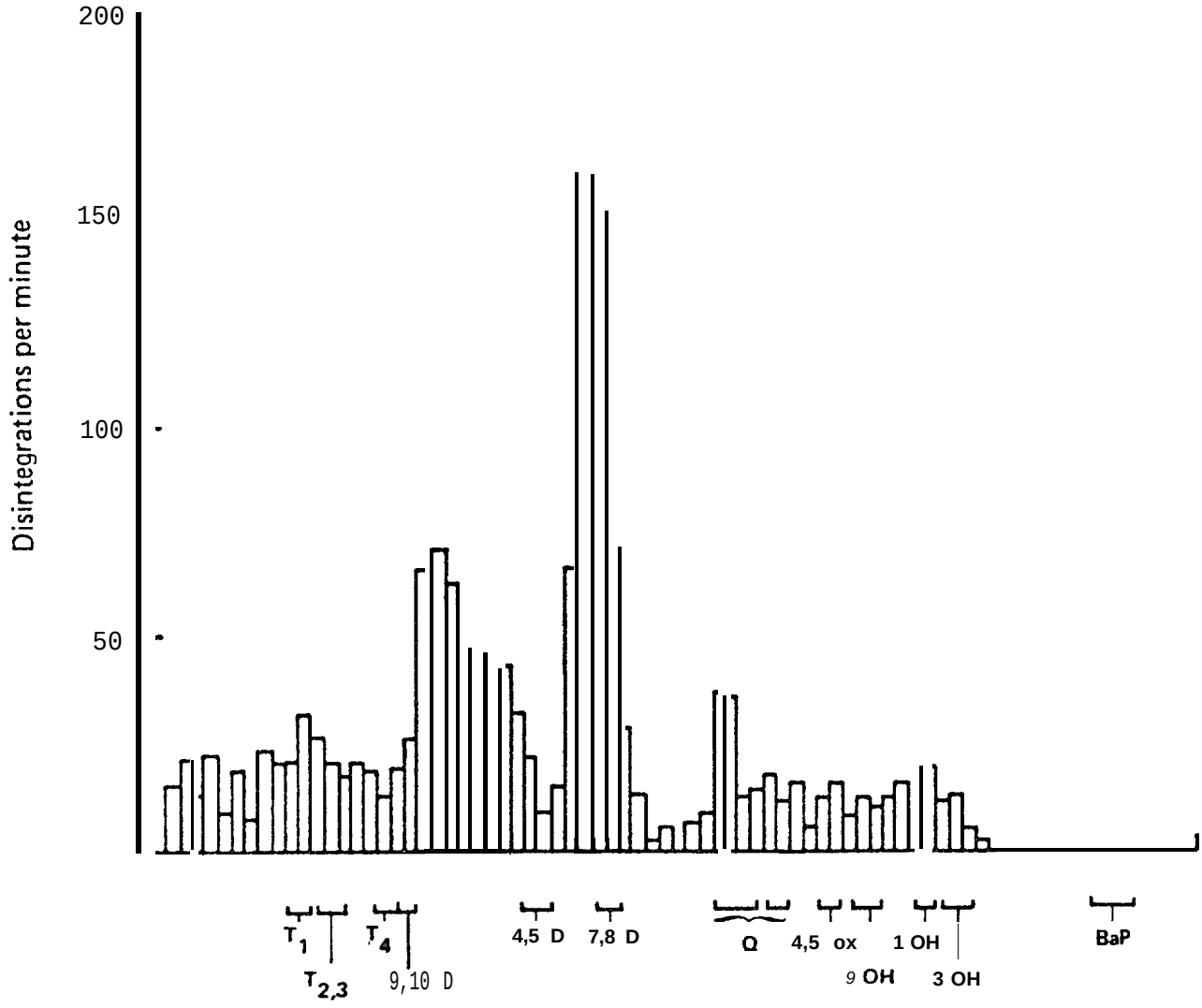


FIGURE 21. High performance liquid chromatography of ethyl acetate soluble metabolizes released after the treatment of aqueous phase of bile from English sole with β-glucuronidase. Metabolizes were separated by fractions collected at 15 sec. intervals. Abbreviations: T_1, T2,3 and T4 - tetrahydro tetrahydroxy BaP; 9,10 D-BP 9, 10- dihydrodiol; 4,5 D - BP 4,5-dihydrodiol; 7,8 D - BP 7,8 dihydrodiol; Q - quinones; 4,50X - BP 4,5 oxide; 1 OH, 3 OH and 9 OH-1-, 3- and 9-hydroxy BP. (From Varanasi and Gmur 1981b)

Amounts of Benzo(a)pyrene in tissue of English sole after force feeding.

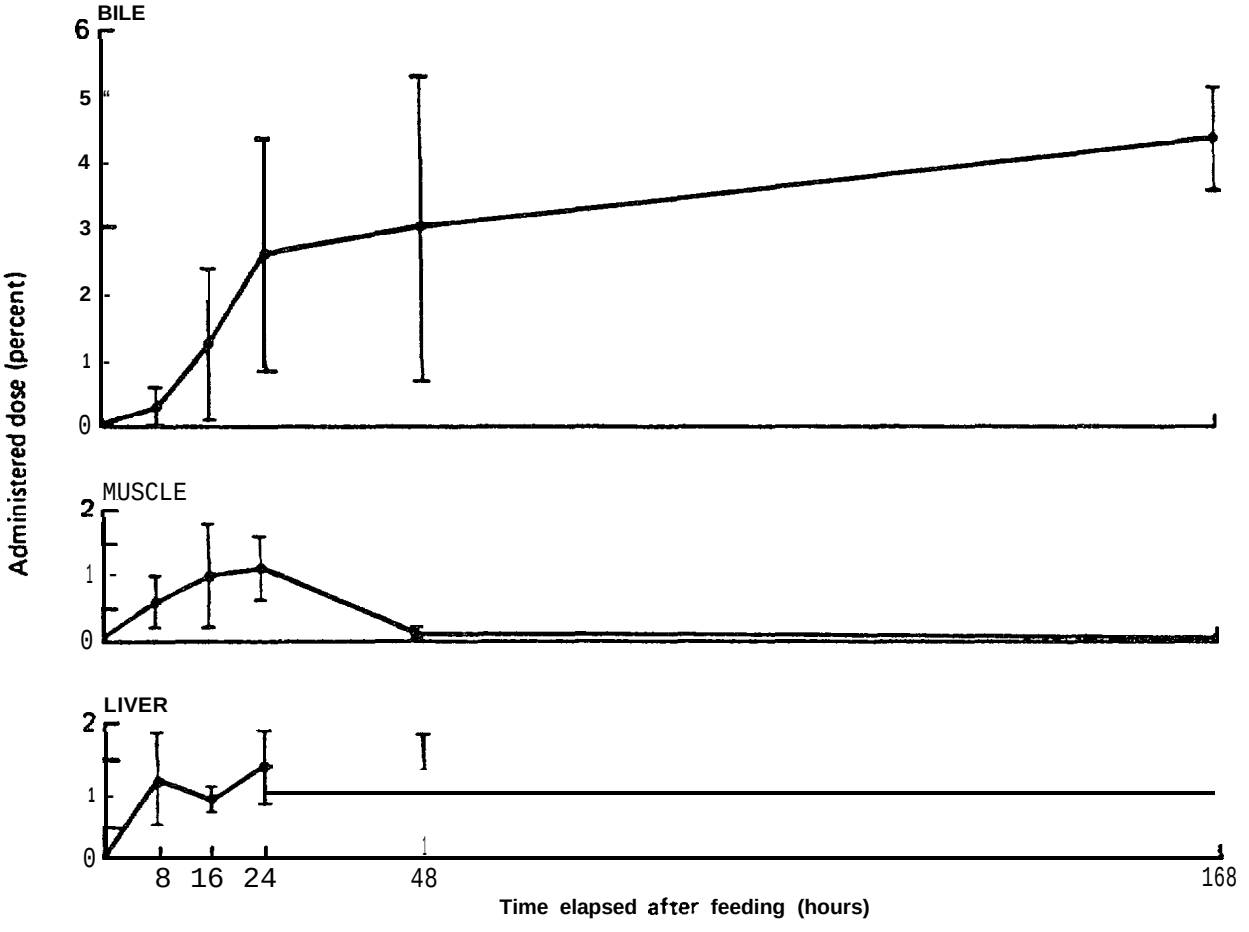


FIGURE 22. Benzo[a]pyrene-derived radioactivity in liver, muscle, and bile of English sole force-fed ^{3}H-BaP (2 mg/kg body weight). $\bar{X}$+S.D.

with the untreated fish; the binding of BaP to DNA was slightly greater when fish were pretreated with BaP than when they were pretreated with MC .

Figure 24 depicts HPLCs of ethyl acetate extractable metabolizes formed by liver enzymes of MC-pretreated fish species and rat. The data revealed that for all three fish species 9,10-dihydro-9,10-dihydroxy-benzo(a)pyrene (BaP 9,10-dihydrodiol) and 7,8-dihydro-7,8-dihydroxy-benzo(a)pyrene (BaP 7,8-dihydrodiol) were the major metabolizes; 3-hydroxy BaP was also present in considerable amounts. The metabolize profile for the NC-pretreated rat revealed the presence of a high proportion of phenols and quinones together with lower, but significant amounts of the non K-region dihydrodiols (BaP 7,8-dihydrodiol and BaP 9,10-dihydrodiol).

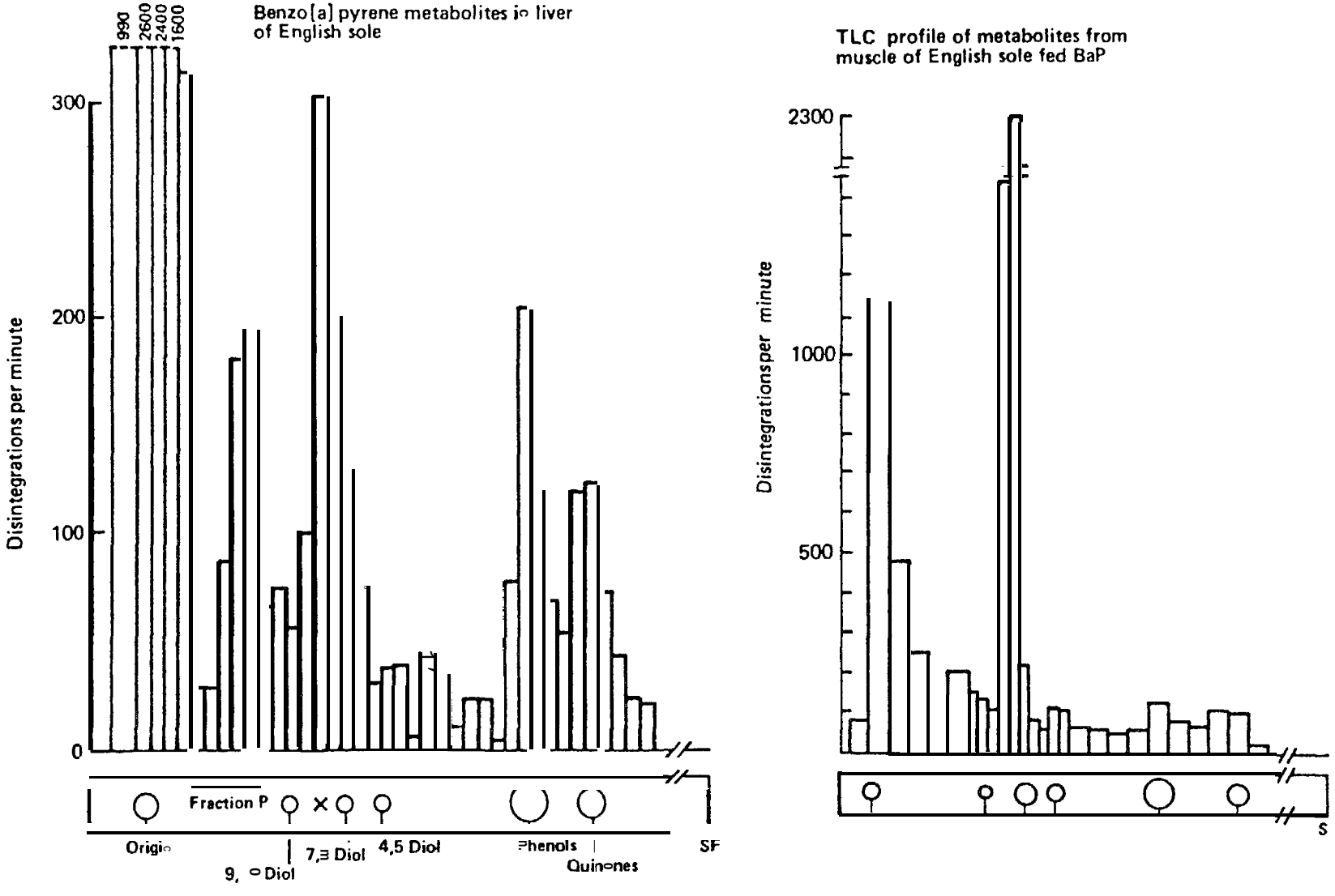


FIGURE 23. Thin-layer chromatograms of organic-solvent soluble BaP metabolites from liver and muscle of English sole force-fed ^{3}H-BaP (2 mg/kg body weight). Abbreviations are Fraction P, metabolites with R_f values between the origin and BaP 9,10-dihydrodiol; 9,10-diol, BaP 9,10-dihydrodiol; 7,8-diol, BaP 7,8-dihydrodiol; 4,5-diol, BaP 4,5-dihydrodiol.

TABLE 25. Distribution of BaP-derived radioactivity in liver and bile of English sole exposed to ^{3}H-BaP.[a]

	Liver	Bile
Unmetabolized BaP	1±0.1	<<1
Ethyl acetate-soluble metabolizes	16±1	17±3
Glucuronides		46±4
Sulfates	83±1	1124
Other aqueous-soluble metabolizes		26±5

[a] Fish were force-fed ^{3}H-BaP (2mg/kg)dissolved in corn oil; liver and bile were analyzed 24 hr after the feeding. Each entry, expressed as percent of total radioactivity, is the mean ± S.D. of three values. (From Varanasi and Gmur 1981b)

6.1.10 Activities of Aryl Hydrocarbon Monooxygenases in Different Species

The activities of aryl hydrocarbon monooxygenases (AHM) are reported for marine species from Alaska waters, as follows: 15 samples of flathead sole (Hippoglossoides elassodon), 14 of arrowtooth flounder (Atharestes stomias), 24 of rock sole, 15 of pollock (Theragra chalcogramma), 4 of butter sole (Lepidopsetta isolepis), 19 of Pacific cod (Gadus macrocephalus), 4 of sea snail (Fusitriton sp.), and 8 of tanner crab (Chionoecetes sp.). The snails were found to have no detectable activity of AHM in whole body samples. The AHM activities of the species examined are presented in Table 27.

The specific activities of hepatic AHM for the fish ranged from 0.006 to 0.927 nmoles of BaP products per 20 min per mg of protein; values for the crab viscera AHM ranged from 0.005 to 1.03 nmoles/ 20 min/mg protein.

The activities of hepatic AHM for Pacific cod which had pseudobranchial tumors were indistinguishable from the activities found for the ordinary Pacific cod. The data for the tumor-bearing cod revealed AHM activity of 0.31 ± 0.16, while the normal cod provided AHM activity of 0.33 ± 0.26 Units/mg protein. There was no correlation between sex of fish and AHM activity.

TABLE 26. In Vitro Binding of Activated BaP to DNA Catalyzed by Liver Supernatants from Fish and Rat*

Species	System[b] (Supernatant)	pmole of BaP Equivalent/mg DNA/mg Protein[c]	% of Control Value
Starry flounder	Control	0.15	100
	MC	1.62	1,100
	BaP	1.70	1,100
	PBCO	0.74	500
	MC[d]	0.53	[d]
English sole	Control	0.06	100
	PBCO	1.05	1,800
	MC	0.16	—[e]
Coho salmon	Control	0.02	100
	MC	0.97	4,900
	BaP	1.06	5,300
	MC[d]	0.30	—[e]
Rat	Control	Q.06	100
	M C	0.69	1,200

"Maximum standard deviation between values from two sepa rate experiments was 14 percent.

[b]Liver supernatants (10,000 x g) were obtained from either untreated (control) animals or those injected with 10 mg/kg of 3-met hylcholanthrene (MC), benzo(a)pyrene BaP, or Prudhoe Bay crude oil (PBCO) when the water temperature for fish was 13°C.

[c]Liver supernatants (=5 mg protein) from different animals were incubated in the dark with 5 nmole of BaP, 2 mg of salmon sperm DNA, and cofactors for 15 minutes at 25°C (for fish) and 37° C (for rat). Each value is an average of two experiments and three replicate measurements using pooled liver extracts from five animals. Binding values for incubation without NADPH were less than 0.001 and are subtracted from the values reported.

[d]Liver supernatants were obtained from MC-pretreated fish when the water temperature was 8°C.

[e]No control fish were sampled at 8° C.

(From Varanasi et al. 1980)

6.1.11 Uptake, Metabol ism and Toxicity of Hydrocarbons in Invertebrates

(a) The changes that are induced by NPH were studied using the gametes from artificially spawned adults. Mussel gametes were combined i n separate solutions containing 1. 10. and 100 ppb NPH. The survival of the resulting larvae, after 24 hr, was 27, 32", and 2%, respectively> for the three exposures compared to 69% survi val for controls. In the 100 ppb solution, 24% of the exposed eggs failed to fertilize

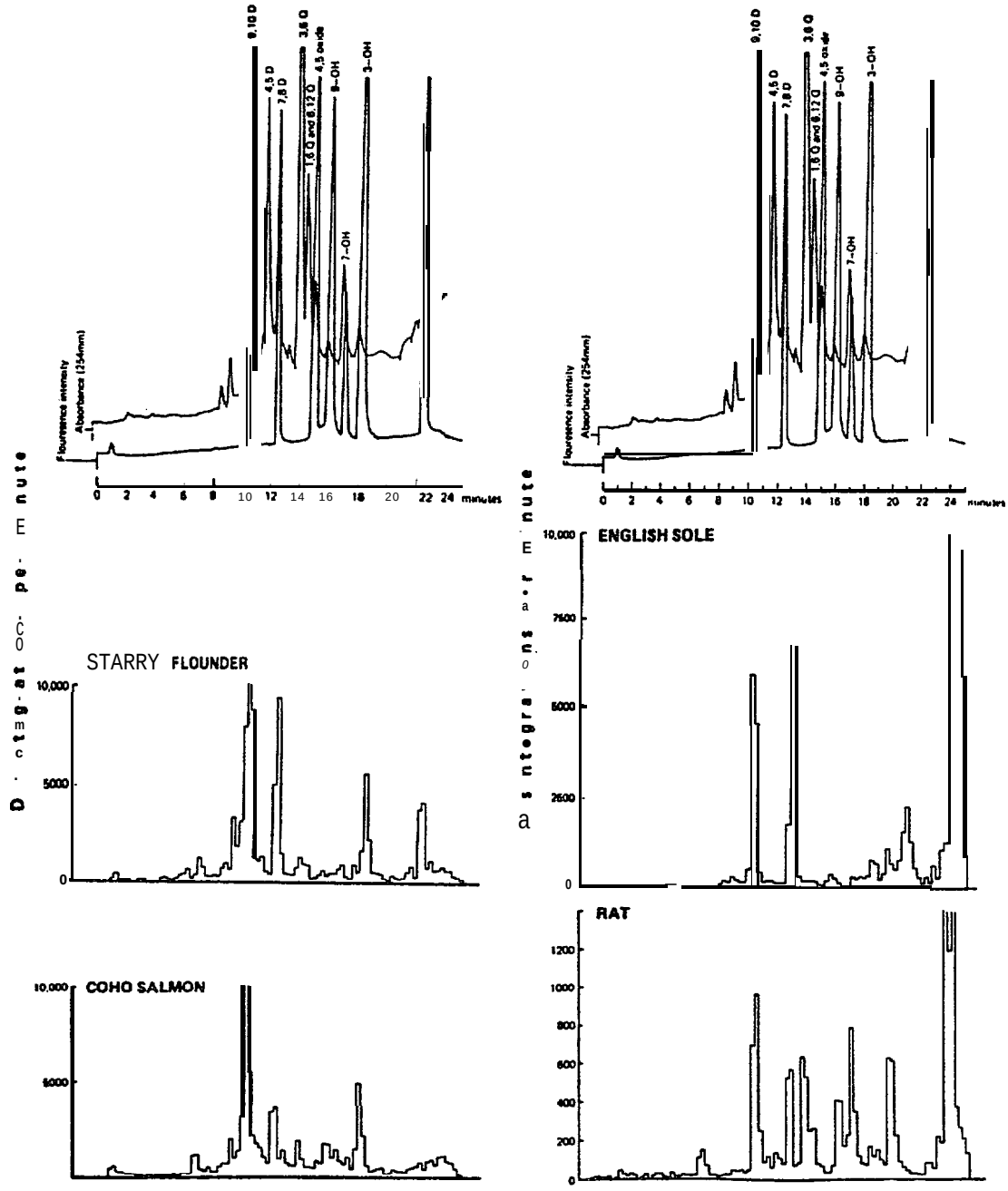


FIGURE 24. HPLC of reference BaP metabolizes and ethyl acetate-extractable metabolizes of ^{3}H-BaP produced by liver supernatants (10,OO(I x g) of MC pretreated starry flounder, coho salmon, English sole, and rat. Reference compounds and metabolizes were detected by both UV and fluorescence spectrometry. Metabolites formed by incubating 5 nmole ^{3}H-BaP with liver supernatants (5 mg protein) of fish (at 25°) and rat at (37°) for 15 minutes were separated by fractions collected at 15-second intervals. Abbreviations are 9,10-L), BaP 9,10-dihydrodiol; 4,5-D, BaP 4,5-dihydrodiol; 7,8-D, BaP 7,8-dihydrodiol; 3,6-Q, BaP 3,6 quinone; 1,6-Q, and 6,12-Q, 1,6-BaP quinone and 6,12-BaP quinone; 9-OH, 9-OH-hydroxy BaP; 7-OH, 7-OH-hydroxy BaP; 3-OH, 3-hydroxy BaP; BaP, benzo(a)pyrene. (From Varanasi et al. 1980)

TABLE 27. Hepatic aryl hydrocarbon monooxygenases (AHM) activities for marine species from Alaska waters.[a]

Species	Sex	No.	Body Weight (g)	Body Length (cm)	Liver Weight (g)	AHM (Units per mg of protein)[b]
Flathead sole	M	7	136± 26	26.5±2.5	1.15±0.42	0.30±0.31
	F	8	232± 62	29.7±2.5	2.62±0.60	0.15±0.072
	M&F	15	187± 69	27.7±2.9	1.94±0.91	0.22±0.22
Arrowtooth flounder	M	9	185±129	27.2±4.5	1.50±0.85	0.063±0.037
	F	5	514±305	36.0±8.5	2.84±1.68	0.063±0.041
	M&F	14	303±256	30.3±7.3	1.98±1.33	0.063±0.038
Rock sole	M	7	240± 91	28.1±3.7	1.64±0.74	0.22±0.19
	F	17	399±237	31.375.3	2.88±1.80	0.2170.20
	M&F	24	352±216	27.9±8.6	2.52±1.65	0.21±0.19
Pol lock	M	8	324±227	33.0±7.6	3.38±1.27	0.19±0.20
	F	7	497~281	39.2±8.0	5.21±2.61	0.22±0.17
	M&F	15	405±260	35.9±8.1	4.24±2.15	0.21±0.18
Butter sole	F	4	244± 45	29.7±1.8	1.75±0.38	0.13±0.080
Pacific cod	M	9	980±404	44.4±5.4	3.31±1.05	0.43±0.26
	F	10	887~269	43.8±4.5	3.88±0.93	0.23±0.22
	M&F	19	9315333	44.1±4.8	3.61±1.00	0.33±0.26
(pseudobranchial tumors)	?	5	449±110	34.9±2.7	---	0.31±0.16[c]
Tanner crab (viscera)	F	8	---	---	---	0.25±0.35

[a] Each value represents $\bar{X}$±S.D.

[b] Using benzo(a)pyrene as-substrate, one unit of AHM will convert 1.0 nanomole of benzo(a)pyrene to 1.0 nmole of oxidation products per 20 minutes at pH 7.5 at 25°C. Source of protein was the 9,000x g (or 10,000 x g) - 20 min. supernatant fraction of a 20% (wt/vol.) homogenate in cold 0.25M sucrose solution.

[c] Hepatic AHM from tumor-bearing Pacific cod.

compared to 3% failure in the controls. The highest concentration also produced abnormal embryological development in 25% of the animals after 1.5 hr of exposure, compared to 2% of the controls. After 24 hr all of the larvae exposed to 10 ppb water-borne NPH had reached the "straight hinge" stage. No animals at the other concentrations or the controls developed to straight hinge larvae within 24 hr.

Table 28 Hydrocarbon content of thoracic and abdominal segments of adult spot shrimp (*P. platyceros*) exposed to a water-soluble fraction of Prudhoe Bay crude oil.

Hydrocarbons	Water-soluble fraction† (rig/g)	Thorax§ (rig/g)	Bio-concn.	Abdomen§ (ng/g)	Bio-concn.
Cyclohexane	29	—‡		—‡	
Benzene	5·5	—‡		—‡	
Toluene	55	—‡		—‡	
Ethylbenzene	0·7	—‡		–%	
Xylenes	37·1	320	9	350	9
C_3-substituted benzene	4"1	220	54	150	37
C_4/C_5-substituted benzenes	t"4	540	386	180	129
Subtotal		1080		680	
Naphthalene	0·4	10	25	20	so
1-Methylnaphthalene	0·4	100	250	40	100
2-Methylnaphthalene	0·4	110	275	60	150
C_2-substituted naphthalene	1·4	240	171	100	71
C,-substituted naphthalene	0·7	190	271	20	29
Subtotal: Cl-C, -substituted naphthalenes		640		220	
Total hydrocarbons	110	1730		920	

The shrimp were exposed for 7 days to a concn. of 100 p.p.b. of the water-soluble fraction of the oil.

† Calc. from representative analysis before dilution.
‡ Not quantifiable due to analytical procedure.
§ Concn. was determined as ng/g wet wt., detectable limit was 10 ng/g.

(From Sanborn and Malins 1980)

(b) The decrease in fertilization demonstrated in the mussel experiments and the effect of NPH on sperm and eggs were further explored using oyster gametes. The oyster sperm exposed to 10 ppb and 1 ppb of NPH and then combined with uncontaminated eggs resulted in 14 and 9% unfertilized eggs, respectively, while no effect was seen at 0.1 ppb NPH. However, only 5% of the eggs exposed to 10 ppb and 1 ppb NPH and then combined with uncontaminated sperm failed to fertilize. This compares to a 4% failure to fertilize when uncontaminated eggs or eggs exposed to 0.1 ppb NPH and uncontaminated sperm were combined.

(c) The data on accumulation of SWSF hydrocarbons in one-year-old spot shrimp show that detectable levels of low molecular weight aromatic hydrocarbons were readily accumulated in thoracic segments (Table 28) (probably associated with the hepatopancreas). The abdominal segments were found to contain significant concentrations of identified aromatic hydrocarbons. The data given in Table 28 represent hydrocarbon accumulations in experimental animals with respect to data obtained from a control group.

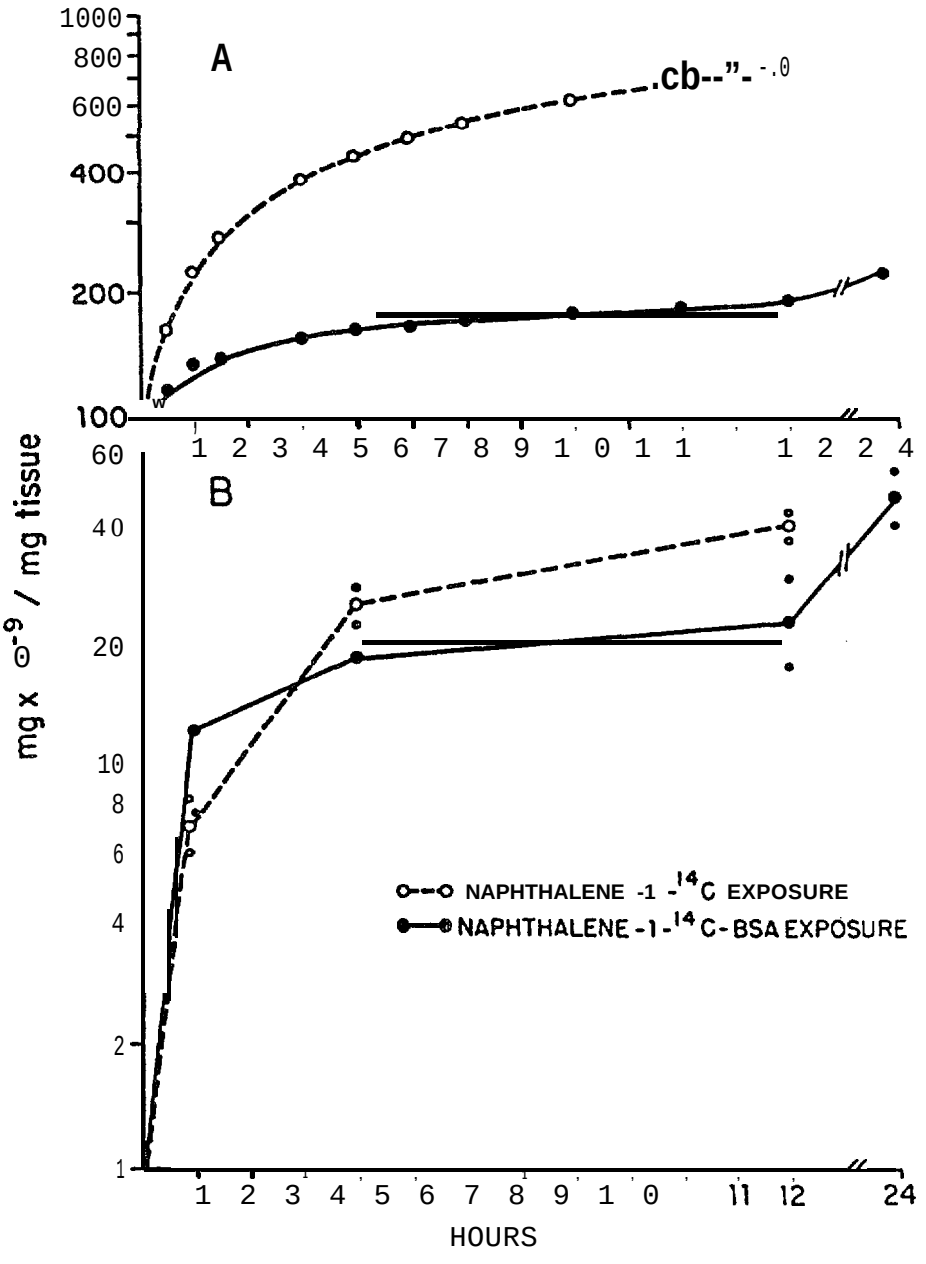


FIGURE 25. Accumulation of naphthalene and metabolic products (expressed as naphthol) on exposure of stage V spot shrimp (Pandalus platcyeros) to 8-12 ppb of water-borne [1-^{14}C]naphthalene and [1-^{14}C]naphthalene complexed with BSA. (A) Regression lines of concentrations of [1-^{14}C]naphthalene with sampling points indicated. (B) Median values of metabolic products with data ranges. (From Sanborn and Malins 1977)

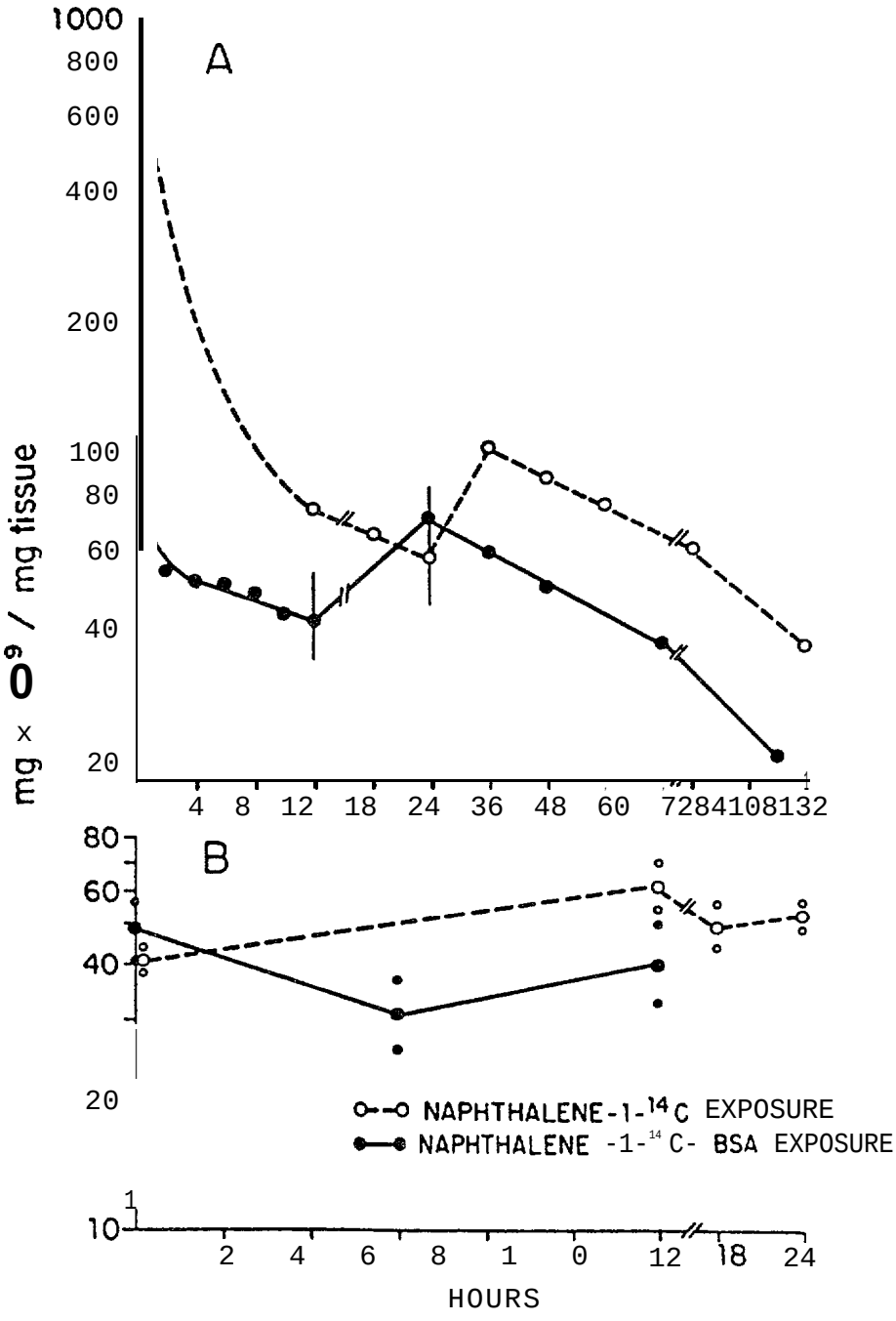


FIGURE 26. Deputation of naphthalene and metabolic products (expressed as naphthol) after exposure of stage V spot shrimp (Pandalus platyceros) to 8-12 ppb of water-borne [1-^{14}C]naphthalene (12 hr) and [1-^{14}C]naphthalene complexed to BSA (24 hr). (A) Regression lines of concentrations of [1-^{14}C]napthalene up to sampling points indicated by vertical lines; thereafter, not a regression line and points represent primarily metabolic products containing only small amounts of [1-^{14}C]naphthalene. (B) Median values of only metabolic products with data ranges. (From Sanborn and Malins 1977)

(d) The results of studies on shrimp and crab larvae indicated that 8 to 12 ppb of NPH-1-^{14}C and NPH-1-^{14}C bovine serum albumin (BSA) complex in flowing seawater caused 100% mortality in 24 to 36 hr in Dungeness crab zoea and in Stage 1 and Stage V spot shrimp larvae. Maximum accumulation of NPH in Stage V spot shrimp was nearly 4 times greater than in shrimp larvae exposed to the NPH-BSA complex (820 ppb vs 220 ppb) (Fig. 25). The percent of total radioactivity attributable to metabolic products (calculated as 1-naphthol) in larvae after 24 hr exposure to NPH was 9%. However, after 24 hr exposure to NPH complexed with BSA, 21% was present as metabolizes. NPH was almost entirely released from tissues in 24 to 36 hr, whereas metabolic products were resistant to deputation (Fig. 26).

(e) The adult and Stage I larval spot shrimp exposed to 80 ppb and 18 ppb NPH, respectively, formed a number of conjugated and nonconjugated metabolizes (Table 29). In adults, nonconjugated forms (quinones, naphthol, and dihydrodiol) represented 69% of the total metabolizes. In the larval shrimp 39% of the metabolizes were present as sulfate conjugates while 44% were present as naphthol.

Table 29 Conversion products formed by adult and larval spot shrimp (*P. platyceros*) exposed for 10 h to [^{3}H]- and [^{14}C]naphthalene.

Conversion product	% total conversion products		
	Adult		Larvae
	[^{3}H]Naphthalene	[^{14}C]Naphthalene	[^{3}H]Naphthalene
Naphthyl glucuronide	2	2	6(?)
Naphthyl sulphate	7	1	39
Unknown No. 1†	5	4	—
Unknown No. 2†	16	17	—
Naphthalene-1,2-dihydrodiol	17	20	4
Naphthyl glycoside	1	>1	—
Naphthoquinone†	36	32	7
α-Naphthol	16	24	44

† Not corrected for extraction efficiency.

(From Sanborn and Malins 1980)

6.1.12 Food Chain Transfer of 2,6-DMN to Sea Urchins via Algae

The maximum accumulation of 2,6-DMN in algae (Fucus distichus) occurred in 20-25 hr (Fig. 27). It was shown that greater than 99% of the tritium accumulated in Fucus after 25 hr was organic-solvent soluble; TLC of a Fucus extract showed no evidence of 2,6-DMN metabolism. Thus it was concluded that all of the tritium was associated with unmetabolized 2,6-WIN.

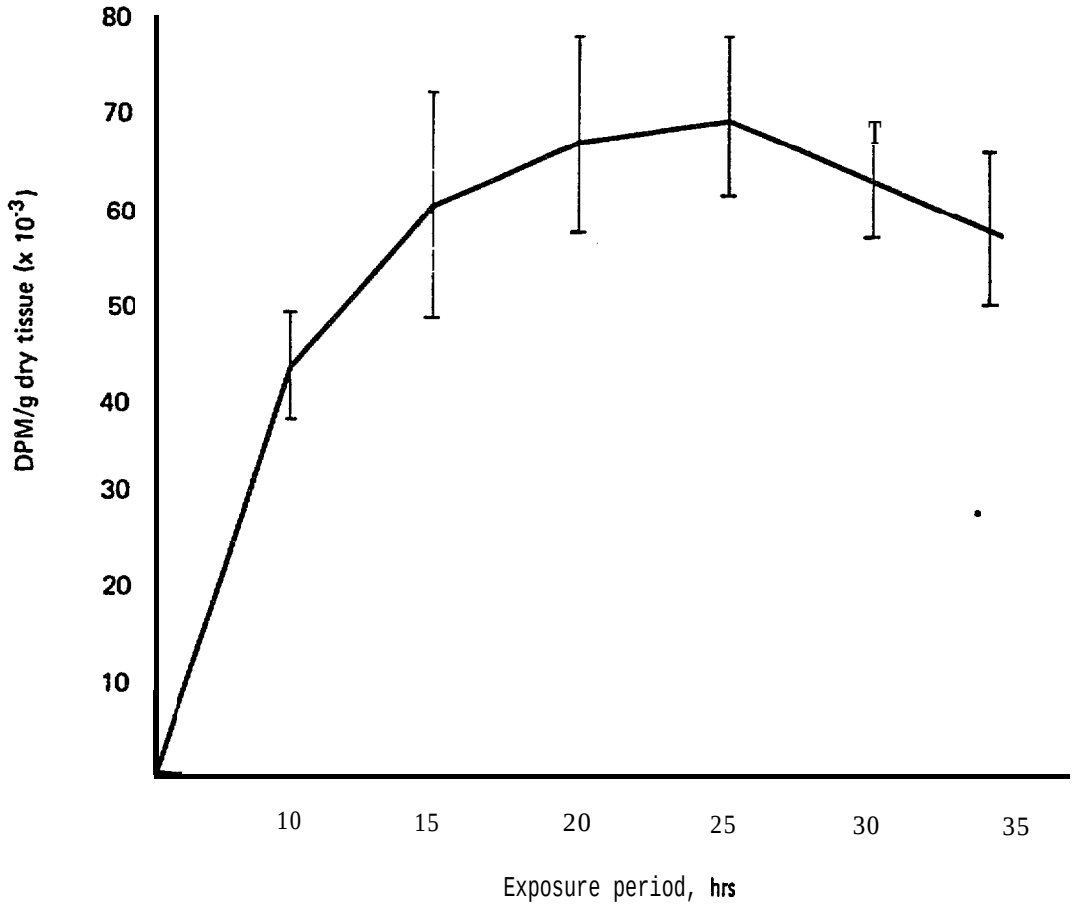


FIGURE 27. Accumulation of 3H-2,6-dimethylnaphthalene in seaweed (F. distichus) exposed through seawater. Values are expressed as mean + SD of three aliquots from the same exposure experiment. (From Mattins and Koubal 1982)

The distribution of tritium in sea urchins (S. droebachiensis) after feeding on Fucus for 3 days was investigated (Fig. 28). Digestion of the methanol-extracted exoskeleton with tissue solubilizer, a process potentially destructive to metabolizes, released 64% of the total radioactivity accumulated by the sea urchins. Other methods to extract radioactive compounds from the exoskeleton (e.g., treatment with organic solvents, dilute acid, EDTA, and a proteolytic enzyme) were not effective. The remaining extractable radioactivity was distributed among the soft tissues as shown in Figure 28.

A substantial amount of the 2,6-DMN-derived radioactivity isolated from the digestive tract and gonadal tissue from the 3 and 14 day experiments was in the form of conjugated metabolizes (Fig. 29), primarily the sulfate. Hydrolysis of the sulfate fraction from the 3 day experiment with aryl sulfatase, followed by TLC showed fractions corresponding to the 3- (80%) and 4-hydroxy-2,6-DMN (20%).

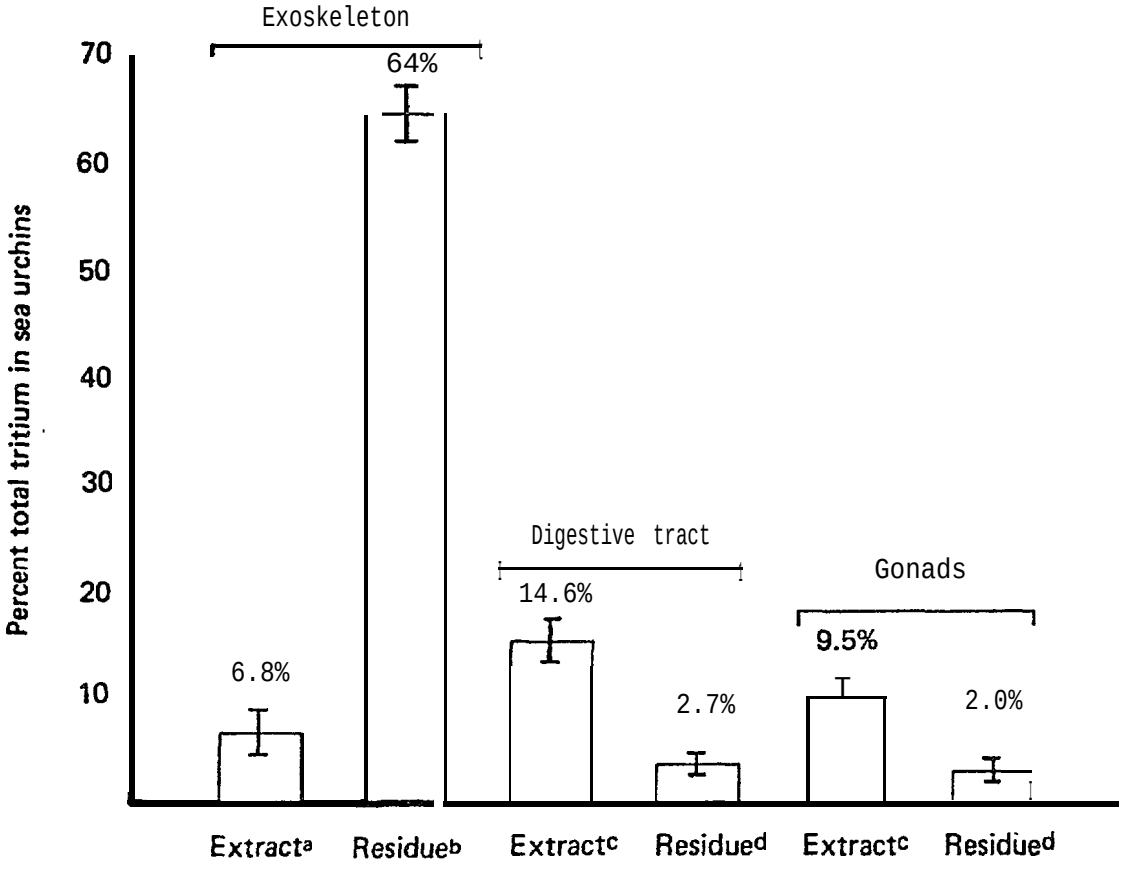


FIGURE 28. Tritium in sea urchins (S. droebachiensis) that consumed ^{3}H-2,6-dimethyl naphthalene for three days. Values are expressed as mean ± SD. [a]Extracted with hot methanol. [b]Residue from hot methanol extract. Solubilized in Soluene Tissue Solubilizer and then analyzed by scintillation spectrometry. (From Malins and Roubal 1982)

TLC of soft tissue extracts after 3 and 14 days exposure gave no evidence for conjugated or nonconjugated methanol derivatives, and only minor amounts (<0.06 ng total/g dry tissue) of nonconjugated metabolizes were found. Both 3,4-dihydro-3,4-dihydroxy-2,6-DMN and 3-hydroxy-2,6-DMN were detected in digestive and gonadal tissues after 14 days; however, after 3 days, only the former compound was found in the digestive tract. An unknown polar compound was detected in both digestive tract and gonadal tissues from animals exposed for 3 and 14 days.

6.1.13 Biological Fate of Metals

(a) Tissue Concentrations of Lead and Cadmium.

Both coho salmon and starry flounder exposed to ppb concentrations of lead and cadmium in seawater attained ppm concentrations of these

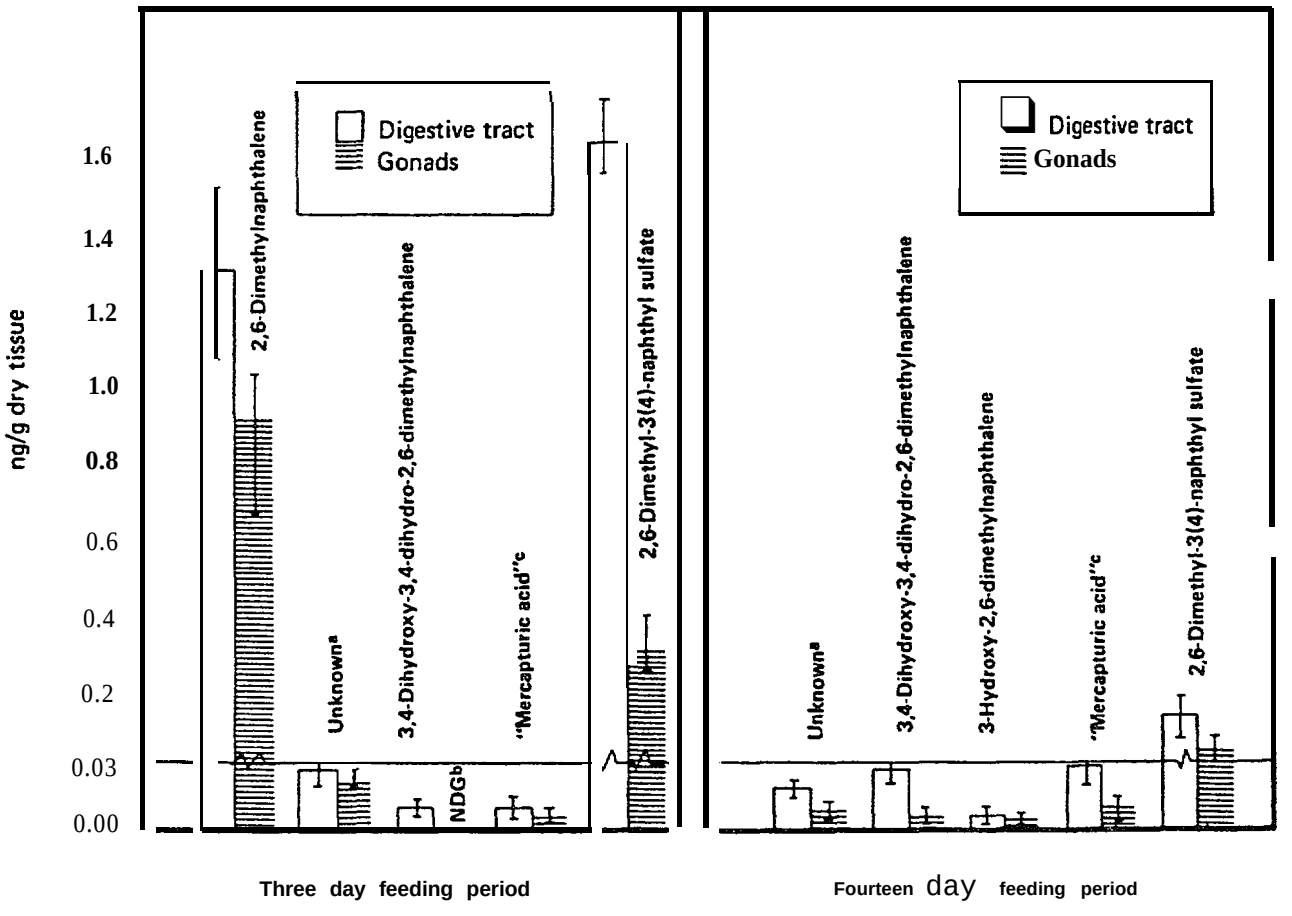


FIGURE 29. Aromatic compounds isolated from sea urchins (S. droebachiensis) that fed on Fucus distichus containing 2,6-dimethyl naphthalene. Values are expressed as mean ± SD. [a]Considered to be as 3-hydroxy-2,6-DMN for purposes of quantitation. [b]NDG; not detected in gonads. [c]R_f value corresponds to 1-naphthyl mercapturic acid. (From Malins and Roubal 1982)

metals in various tissues (bioconcentration values given in Tables 30 and 31 are based on wet weight of tissues). Data in Tables 30 and 31 show that high concentrations of lead accumulated in the brain of starry flounder, and that both species accumulated substantial concentrations of lead and cadmium in kidney, especially in the posterior section. Concentrations of metal in epidermal mucus of coho salmon were slightly greater than the levels in the surrounding water; however, metals (especially lead) were bioconcentrated to a much greater extent in starry flounder mucus.

The results in Table 30 and 31 show that the temperature of the surrounding water had a definite influence on the uptake and accumulation of metals in both fish species under investigation. Skin, liver, and kidney, of fish held at 10"C had considerably higher metal concentrations than the tissues of fish held at 4"C.

TABLE 30. Effect of temperature on bioconcentration[a] of lead and cadmium in tissues of saltwater-adapted coho salmon (Oncorhynchus kisutch) exposed to water-borne metals.[b]

Metal	Temp. °C	Mucus	Scales[c]	Liver	Brain	Kidney[d] a	Kidney[d] p
Pb	4	1.5	10.0	1.8	ND[e]	1.8	7.3
	10	1.8	24.9	3.0	ND	3.1	12.0
Cd	4	1.0	0.3	1.1	ND	0.7	0.9
	10	1.2	0.7	2.0	ND	1.2	2.5

[a] Bioconcentration=metal concentration in tissue (rig/g, wet wt)/metal concentration in seawater (rig/ml).
b Fish were exposed to 150 ppb of either lead or cadmium for two weeks.
[c] Scales with dermal and epidermal cells attached.
d a=anterior kidney; p=posterior kidney.
[e] ND=not detected.

(From Varanasi and Markey 1978, Reichert et al. 1979).

TABLE 31. Effect of temperature on bioconcentration[a] of lead and cadmium in tissues of starry flounder (Platichthys stellatus) exposed to water-borne metals.[b]

Metal	Temp. 'c	Mucus	Ski nc	Liver	Brain	Posterior kidney
P b	4	4.2	1.2	3.9	1.3	5.7
	10	2.1	2.4	5.0	3.5	8.6
Cd	4	1.3	O*3	3.0	NDd	0.5
	10	1.3	1.1	10.5	ND	1.9

a Bioconcentration=Pb concentration in tissue (wet wt)/Pb concentration in water.
b Fish were exposed to 150 ppb of either lead or cadmium for two weeks.
c Skin with scales.
d ND=not detected.

(From Varanasi and Markey 1978, Reichert et al. 1979).

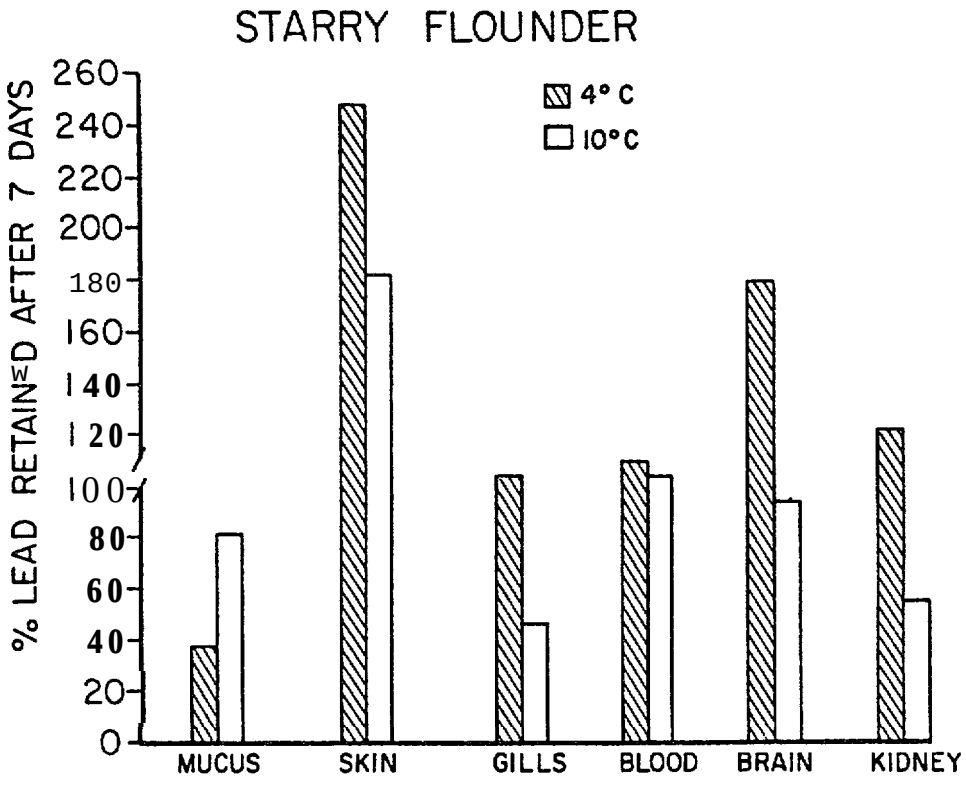


FIGURE 30. Effect of temperature on lead concentrations retained in the tissues of lead-exposed starry flounder after one week of deputation. (From Varanasi 1978)

Temperature of the surrounding water appears to have a distinct effect on retention of metals in tissues of starry flounder. For example, after 7 days of deputation, fish maintained at 10"C retained much lower concentrations of lead in tissues such as skin, gills, brain, and kidney compared to the lead concentrations in tissues of fish at 4°C (Fig. 30). No such temperature effect was observed for coho salmon.

When the metal-exposed coho salmon were placed for 7 days in seawater free of radioactive metals, lead concentrations increased in most tissues but decreased in blood, indicating transfer of lead from the blood to other tissues. For example, liver, scales, posterior kidney, and blood of lead-exposed coho salmon contained 109, 114, 110, and 74%, respectively, of the lead concentrations accumulated in these tissues at the end of the two-week exposure at 10°C. Conversely, cadmium concentrations decreased significantly in all tissues except kidney of cadmium-exposed coho salmon after 7 days of deputation. Liver, scales, posterior kidney, and blood of these fish contained 87, 53, 156, and 79%, respectively, of the original cadmium concentrations. In starry flounder, after a 7-day deputation, lead was also retained to a much greater extent than cadmium in these tissues.

After 37 days of deputation, substantial (>50%) concentrations of both lead and cadmium still persisted in most tissues of coho salmon. A notable finding was that metal concentrations in the posterior kidney of coho salmon continued to increase even after the fish were placed in seawater for a period of 5 wk. Also, more than 50% of the lead and cadmium accumulated in the scales of coho salmon was still present after five weeks of deputation. Deputation of starry flounder tissues was not studied for periods longer than 7 days, because after 7 days of deputation the mortality rate increased in both control and exposed fish.

(b) Distribution of Cadmium and Lead in Subcellular Fractions.

Samples of liver and kidney removed from the above coho salmon and starry flounder exposed to water-borne lead and cadmium were also used to investigate subcellular distribution of these metals. Cytosol fractions of liver and posterior kidney of cadmium-exposed starry flounder contained 72 and 80% of total tissue cadmium (^{109}Cd), respectively, and cytosol fractions of these tissues from coho salmon contained 62 and 48% of total tissue cadmium (^{109}Cd), respectively. Microsomal fractions of liver and kidney each contained 18% of the cadmium present in the tissue. Distribution of lead was somewhat different: liver of lead-exposed coho salmon contained almost equal proportions of lead in mitochondria, microsomes, and cytosol.

Intravenous injections of ^{109}Cd were employed to assess if cadmium in gills, liver, and kidney of cadmium-exposed fish was bound to metalloproteins (M.W. 10,000 daltons). Results with coho salmon show that similar to mammalian systems, a major fraction of the ^{109}Cd was bound to cadmium-binding proteins, CdBP (<8,900), in liver and kidney of coho salmon. Moreover, the percentage of the total ^{109}Cd in cytosol bound to CdBP was greater in those fish which had been exposed to 200 ppb Cd in seawater for two weeks, compared to controls in seawater *only* (Table 32). The studies with unexposed coho salmon indicate that CdBP concentrations ingills are low. Yet, within 24 hr after metal challenge there is an appreciable increase in the accumulation of CdBP-bound cadmium indicating induction of CdBP.

(c) Effect of Metal Exposure on Synthesis of Epidermal Mucus

To assess the effect of water-borne metals on mucus production, weight of mucus was determined for each test and control coho salmon. The results show that at 10*C, 150 ppb of either lead or cadmium in surrounding water induced significantly higher ($p<0.05$) mucus production in coho salmon (Table 33).

Our studies also show that when the fish were injected with either lead (^{210}Pb) or cadmium (^{109}Cd) salts substantial concentrations of lead (164 ppb, dry wt) and cadmium (744 ppb, dry wt) were present in the mucus for at least 2 days following the injection.

Table 32 Distribution of injected ^{109}Cd in coho salmon gill, liver, and kidney cytosol at 10°C

Organ	Time after injection (hr)	Percent of total Cd^{+2} of soluble fraction in: Coho[a] High M.W.[c] fraction	CdBP	Cd in cytosol[d]	Coho-M[b] High M.W. fraction	CdBP	Cd in cytosol
Liver	3	21[e,f]	79	85.3	11	89	82.5
	24	55	45	103.0	26	74	81.2
	48	16	84	200.2	7	93	97.7
Gills	3	91	9	21.7	45	55	29.6
	24	77	23	22.8	56	44	39.5
	48	76	24	57.3	51	49	18.8
Kidney	3	66	34	63.0	23	77	38.5
	24	71	29	83.4	39	61	70.0
	48	--[f]	--	--	22	78	88.9

[a] Fish not exposed to water-borne cadmium prior to injection.
[b] Fish exposed to 150ppb water-borne cadmium for 2 weeks prior to injection.
[c] Molecular weight greater than 13,000 mol.wt.
[d] ng ^{109}Cd/mg cytosolic protein.
[e] Three fish were sampled for each time period and the organs were pooled.
[f] All values were normalized to 100% (more than 95% of the radioactivity was recovered).
[g] Fractions were lost.

(From Reichert et al. 1979)

Table 33 Average weight of epidermal mucus obtained from control and metal-exposed fish*

Sample	Metal	Mg mucus/g fish
Control†	—	7.2 ± 1.6 (10)‡
Test	Pb	12.7 ± 2.8 (10)§
Test	Cd	13.1 ± 2.9 (10)§

* Fish were exposed to 150 ppb of either lead or cadmium for a period of 2 weeks at 10°C.

† Control seawater contained < 5 ppb of lead and 2 ppb of cadmium.

‡ Values represent mean value ± S.D. and numbers in parentheses indicate number of individual fish.

§ Student r-test was performed on the data and values for the test were significantly ($P < 0.05$) different from the control value.

(From Varanasi and Markey 1978)

6.2 Pathology

6.2.1 Effects of Petroleum on Disease Resistance

Salmonids. In initial tests with coho salmon no alteration in disease resistance was observed from peroral exposure to PBCO. The LD_{50} dose of *V. anguillarum* for coho maintained on a diet containing 1,000 ppm PBCO for 34 days was not significantly different (P=0.05) from that of control coho fed a normal diet; LD_{50} values and their 95% C.I. were 1.1 x 10^3 (2.5 x 102 - 4.9 x 104) and 8.6 x 10^2 (1.3 x 10^2 - 5.7 x 103) bacteria for tests and controls, respectively. Similarly, no difference in mortality following bacterial challenge could be demonstrated between fish exposed to a SWSF of PBCO (ca 0.8 ppm) for 14 d and the controls. In this second experiment, however, the LD_{50} doses were less than 20 bacteria in both the oil-exposed and non-oil-exposed groups. The actual percent mortalities at the 20 bacteria dose level were 60% and 80%, respectively; these levels of mortality are not significantly different (P=0.05).

The results of additional tests with salmonids showed that rainbow trout perorally exposed to 10 or 1,000 ppm PBCO for 10 mo were not demonstrably immunosuppressed. As shown in Table 34 and Figure 31, trout exposed to PBCO for 10 mo and vaccinated against *V. anguillarum* survived bacterial challenge to the same degree as vaccinated, non-exposed controls; and in all cases the immunized fish survived at a higher rate than the nonimmunized controls.

The results of a preliminary assay with dispersant suggested, however, that simultaneous exposure of juvenile coho salmon to the oil-dispersant Corexit 9527 and *V. anguillarum* induced a greater rate of infection and subsequent mortality than that which occurred in fish similarly exposed to bacteria only (Table 35).

TABLE 34. LD_{50} values for oil-exposed and control rainbow trout immunized against *Vibrio anguillarum* and challenged with live bacteria.

Group	Concentration of PBCO in diet[a]	LD_{50} dose of *V. anguillarum*[b]
Immunized	1,000 ppm	9.2 X 10^4
	10 ppm	9.2 X 104
	None (control)	9.2 X 104
Nonimmunized	None (control)	2.3 X 10

a Fish were fed a PBCO-contaminated diet for 10 mo.
b LD_{50} values were calculated by the method of Reed and Muench (1938) using 5 bacterial concentrations with 10 fish/concentration.

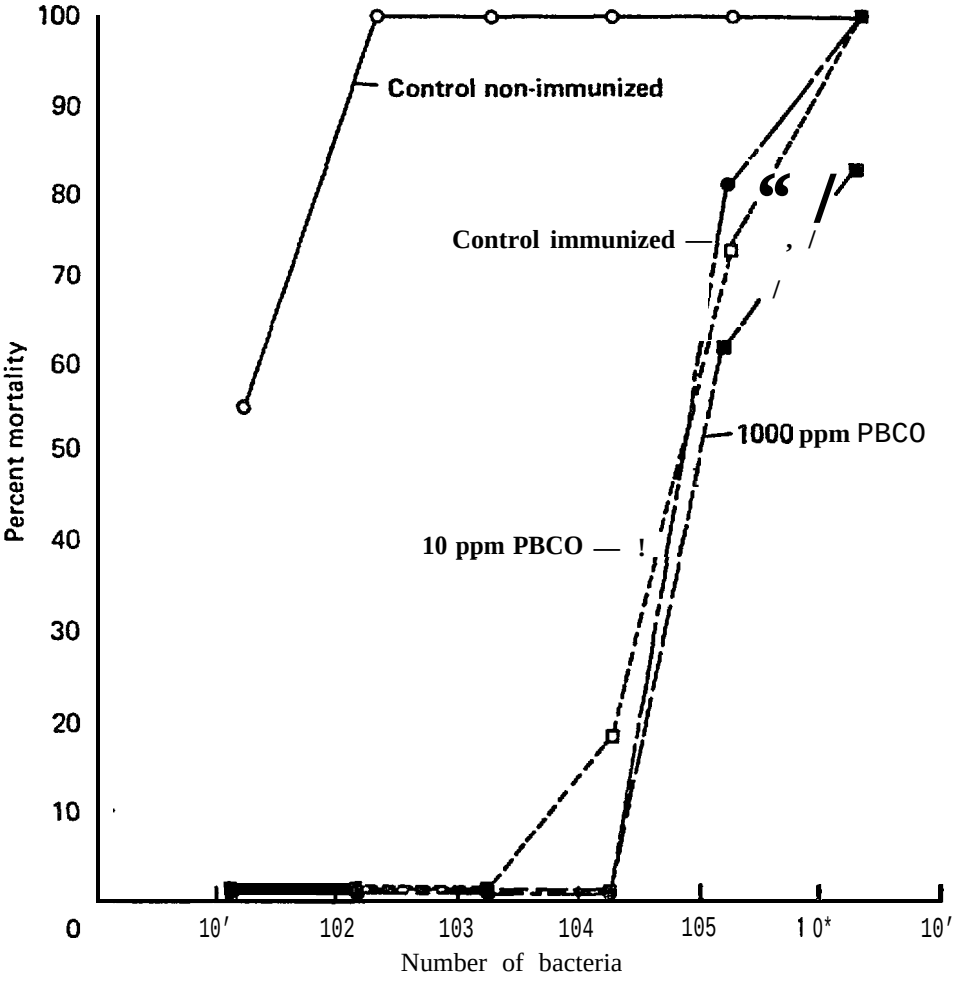


FIGURE 31. Effects of peroral exposure to PBCO for 10 mo on the immunocompetence of rainbow trout. Fish were vaccinated against Vibrio anguillarum by injection of heat-killed bacterial cells and challenged 21 days later with various concentrations of live bacteria. Each point represents percent mortality in a 10 fish group.

It was further demonstrated that exposure to PBCO for 10 mo did not markedly affect antibody levels or antibody formation. As shown in Table 36, the ability to synthesize antibodies, as reflected by agglutinin titer, appeared to decline slightly as a result of oil exposure; however, the difference was not statistically significant ($\alpha = 0.05$). Moreover, the number of antibody-forming cells demonstrable in the spleens and anterior kidneys of trout exposed to 1,000 ppm PBCO did not differ substantially from those of controls. Numbers of plaque-forming cells per 106 lymphoid cells ($PFC/10^6$ lymphoid cells) in a 5-fish pool of kidney tissue from oil-exposed and control fish were 275 and 107, respectively. Numbers of $PFC/10^6$ lymphoid cells in splenic tissue were 193 and 218, respectively.

TABLE 35. Percent mortality among juvenile coho salmon exposed for 30 min to various concentrations of *V. anguillarum* and 30 ppm Corexit 9527, alone and in combination. Mortality was monitored for 10 days after exposure.

Treatment	Mortality % and (# dead/total #)
10^5 bacteria + Corexit	27 (8/30)[a]
10^5 bacteria only	3 (1/30)
10^4 bacteria + Corexit	17 (5/30)
10^4 bacteria only	3 (1/30)
Corexit only	0 (0/30)

a Significantly different than control of bacteria only (P=0.05)

Results of mitogenesis and polyclonal activation tests also suggested that long-term peroral exposure to PBCO did little to alter immunocompetence. Mitogenic stimulation indices (defined as the ratio of ^{3}H-thymidine incorporated by 106 Con A treated leukocytes/^{3}H-thymidine incorporated by 106 nontreated leukocytes) of splenic leukocytes from rainbow trout perorally exposed to 1,000 ppm PBCO for 10 mo and non-exposed controls were 15.0 + 2.8 ($\bar{x}$ + SE) and 24.5 + 5.0, respectively. Polyclonal lymphoid cell activition indices (Expressed as the number of PFC/10^6 lymphoid cells) of peripheral blood leukocytes from rainbow trout similarly exposed to PBCO and controls were 17.3 $\pm$ 7.3 ($\bar{x} \pm$ SE) and 14.6 $\pm$ 5.7, respectively.

One noteworthy finding of the rainbow trout studies was the observation of a significantly changed (reduced, P = 0.05) spleen/body weight in those fish maintained on a diet containing 1,000 ppm PBCO for 15 mo. The average ratio of spleen weight (in mg) to body weight (in g) for control fish was 0.69 while the ratio was 0.49 for the PBCO-exposed group. Subsequent testing indicated this reduction did not correlate with either a reduced number of erythrocytes or leukocytes-thrombocytes in the spleen or head kidney (Table 37), or in the peripheral blood (Table 38).

Flatfish. The results of disease resistance screening tests with adult starry flounder and adult rock sole exposed to PBCO are shown in Table 39. No significant differences (α=0.05) in mortality were observed in either starry flounder exposed for 2 or 6 wk to 1800 ppm PBCO in sediment or rock sole exposed for 2 wk to 2500 ppm PBCO in sediment and non-exposed controls when challenged with the same concentration of pathogenic bacteria.

TABLE 36. Effect of peroral exposure to PBCO for 10mo on antibody formation in rainbow trout. Antibody formation was assessed by measuring agglutinating antibody titer to *V. anguillarum* bacterin 21 days after immunization. Results represent the geometric mean titers (log_2) of 10 fish from each exposure regime.

Concentration of PBCO added in diet	Mean antibody titer against *V. anguillarum* bacterin
1,000 ppm	4.3
10 ppm	5.2
None, immunized	5.7
None, non-immunized	<1

TABLE 37. Comparison of mean erythrocyte (rbc) and leukocyte-thrombocyte (wbc-t) counts from homogenates of spleen and anterior kidney of PBCO-exposed (10 ppm in diet for 15 mo) and control rainbow trout. Values are expressed as cells/mg of homogenate. ($\bar{X} \pm$ SD, N=10).

Group	Anterior kidney		Spleen	
	rbc	wbc-t	rbc	wbc-t
Oil-exposed	400:134	600 ± 316	1400 ± 346	750:209
Control	260 ± 100	530 ± 83	1100 ± 409	1000 ± 259

TABLE 38. Comparison of mean erythrocyte (rbc) and leukocyte-thrombocyte counts in the peripheral blood of rainbow-trout perorally exposed to 1,000 ppm PBCO for 15 mo, and controls.

	Exposure	
	1,000 ppm PBCO	Control
No. of rbc's[a]	1.2 x 10^6	1.4 x 10^6
No. of leukocytes-thrombocytes[a]	4.1 x 10^5	4.2 x 105

a Values expressed are cells per cm^3 and represent the average of 10 fish.

TABLE 39. Result of screening of flatfish for changes in disease resistance after exposure to PBCO-contaminated sediment.

Test species and exposure conditions	Number of bacteria	Percent Mortality oil-exposed	control
Starry flounder exposed 2 wk on sediment contaminated with 1800 ppm PBCO[a]	1.1X108	80	60
	1.1X107	60	40
	1.1X106	0	0
	1. 1x10^5	0	0
Starry flounder exposed 6 wk on sediment contaminated with 1800 PBCO	1.3X108	80	60
	1.3X107	60	40
	1.3x10^6	0	0
	1.3x10^5	0	0
Rock sole exposed 2 wk on sediment contaminated with 2500 ppm PBCO	2.2X108	80	80
	2.2x10^7	60	40
	2.2X106	0	0
	2.2X105	0	0

[a] Concentration of PBCO was determined at the start of exposure period.
b 5 fish per group

The results of testing of the effects of CICO on disease resistance of juvenile flatfish are shown in Table 40. English sole exposed to oil-contaminated sediment for up to 2 wk showed no significant difference (α=0.05) from controls in their ability to survive a laboratory bacterial challenge. The accompanying analyses of sediment, water, and liver tissue documented both the availability and uptake of petroleum hydrocarbons by the exposed fish.

Spot Shrimp. The results of tests in which adult spot shrimp were exposed for up to 4 wk to sediment contaminated with CICO are summarized in Table 41. LD_{50} values computed from mortality data following challenge with V. anguillarum indicated no significant differences (α=0.05) in disease resistance. Results of the analyses of sediment, water, and soft tissue for TEPH demonstrated that the-petroleum hydrocarbons were both bioavailable and taken up by the exposed shrimp.

6.2.2 Pathological Changes in Flatfish from Exposure to Oil-Contaminated Sediment

Five experiments were conducted in 1977 through 1979; parameters of the individual experiments are listed in Table 42.

TAME 40. Results of disease resistance tests *on* CICO-exposed and non-exposed juvenile English sole and related petroleum hydrocarbon analyses.

Sediment Condition	Duration of Exposure *(h)*	LD_{50}[a] (95% confidence interval)	Total Petroleum Hydrocarbons (ppm)		
			Sediment	Water[b]	Liver Tissue[c]
Oil-contaminated	o	NDd	461.0	2,900	NO
Control	o	ND	0.3	0.002	ND
Oil-contaminated	24	6.2x105 (3.3x105 -1.2x1o6)	295.0	0.180	4.2
Control	24	1.4x106 (7.3 X105 -2.6 X106)	0.3	0.004	1.7
Oil-contaminated	168	2.9x106 (1.3x106 -4.9x1o6)	282.0	0.037	8.0
Control	168	4.ox1o6 (1.5x10[6]-6 .8x106)	0.3	0.009	1.0

[a] LD_{50} values and their 95% confidence intervals were calculated by logit analysis using 5 bacterial concentrations.
b Water was collected 2 cm above the sediment-water interface.
[c] Values represent composite sample from 3 fish.
d ND=not determined.

TABLE 41. Results of disease resistance tests on CICO-exposed and non-exposed adult spot shrimp and related petroleum hydrocarbon analyses.

Sediment Condition	Duration of Exposure (h)	LD_{50}[a] (95% confidence interval)	Total petroleum hydrocarbons (ppm)		
			Sediment	Water[b]	soft Tissue[c]
Oil-contaminated	0	ND[d]	1,015,0	0.690	ND
Control	0	NO	0.4	0.019	6.1
Oil-contaminated	24	2.4x10[6] (1.2 X106 -4.9 X106)	714.0	0.077	40.4
Control	24	2.5x10[6] (1. IX106-5.4X1O6)	0.4	0.016	11.0
Oil-contaminated	168	2.5x107 (1.2x10[7]-5.1x10[7])	475.0	0.002	16.3
Control	168	1.9 X10"7 (1. OX1O7-6.4X1O7)	0.5	0.002	10.1
Oil-contaminated	672	2.8x10[7] (1.3x107 -6.4x1o7)	234.0	0.023	11.8
Control	672	1.4x10[7] (7.8xIo6-2.4x1o7)	0.3	0.018	5.0

[a] LD_{50} values and their 95% confidence intervals were calculated by logit analysis using 5 bacterial contractions with 20 shrimp/concentration.
b Water was collected 2 cm above the sediment-water interface.
C Values represent a composite sample of abominal and thoracic tissues from 3 shrimp.
d No, not determined.

TABLE 42. Experimental conditions employed in evaluating pathological changes in flatfish resulting from exposure to oil contaminated sediment.

Experiment Number	Month/ Year	Initial Oil Cone. % (vol/vol)	Flatfish Species	Age (years)	Number of fish	Sediment type	Duration (days)
1	2/77	0.2	English sole	1-3	35	high-sand	120
2	2/78	0.5	English sole	1-2	41	high-silt	29
			Rock sol e	1	41		
3	7/78	0.5	Starry flounder	O-1	50	high-sand	62
4	12/78	1.0	English sole	o-1	44	high-silt	126
5	9/79	1.0	English sole	o-1	50	high-sand	42

Concentrations of Sediment-Associated Petroleum Hydrocarbons

Sediment characteristics greatly influenced the retention of petroleum hydrocarbons. For example, in experiments 2 and 3 in which two different types of sediments received 0.5% PBCO, the high-sand type lost 77% of the TEPH during the first men' h; the high-silt type lost only 16% during the same period (Fig. 32). This difference between the rates of release of petroleum hydrocarbons was also reflected in the concentrations of TEPH in the interstitial water of the sediments (215 ppm in high-sand and 14 ppm in high-silt) 24 hr after they were mixed with oil and placed in aquaria with flow" ng seawater.

FIGURE 32. Concentrations of TEPH in PBCO-contaminated sediments from Experiment 2 (high-silt content sediment) and Experiment 3 (high-sand content sediment). *Indicates remixing of sediment. (From McCain and Malins 1982)

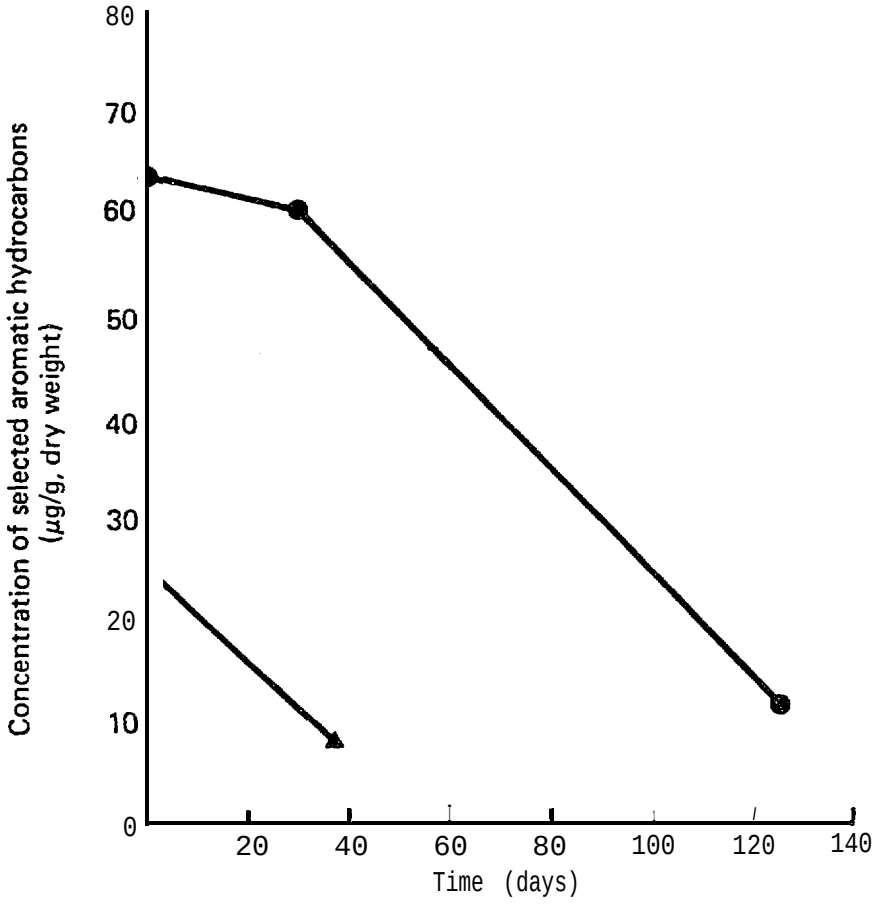


FIGURE 33. Concentrations of major aromatic hydrocarbons (see Figs. 34, 35) in PBCO contaminated sediments from Experiment 4 (high-silt content sediment = ●) and Experiment 5 (high-sand content sediment =A). Both experiments had initial PBCO concentrations of 1.0% (v/v).

Similar results were obtained in Experiments 4 and 5, in which the sediments received 1% PBCO (Fig. 33). The high-sand sediment lost 70% of the petroleum hydrocarbons during the first 38 days; the high-silt, only 4% after 30 days. By 126 days the high-silt sediment had lost an additional 77%. (The high-sand experiment was terminated after 42 days.)

The composition of the aromatic hydrocarbons (AHs) in the two sediment types from Experiments 4 and 5 differed slightly. Initially and at 30 days the relative concentration of the low molecular weight AHs in the high-silt sediment (Fig. 34) was higher than in the high-sand sediment at 38 days (Fig. 35). After 126 days all hydrocarbons measured in high-silt content sediment were greatly reduced and approximated the hydrocarbon concentrations found in high-sand content sediment after 38 days.

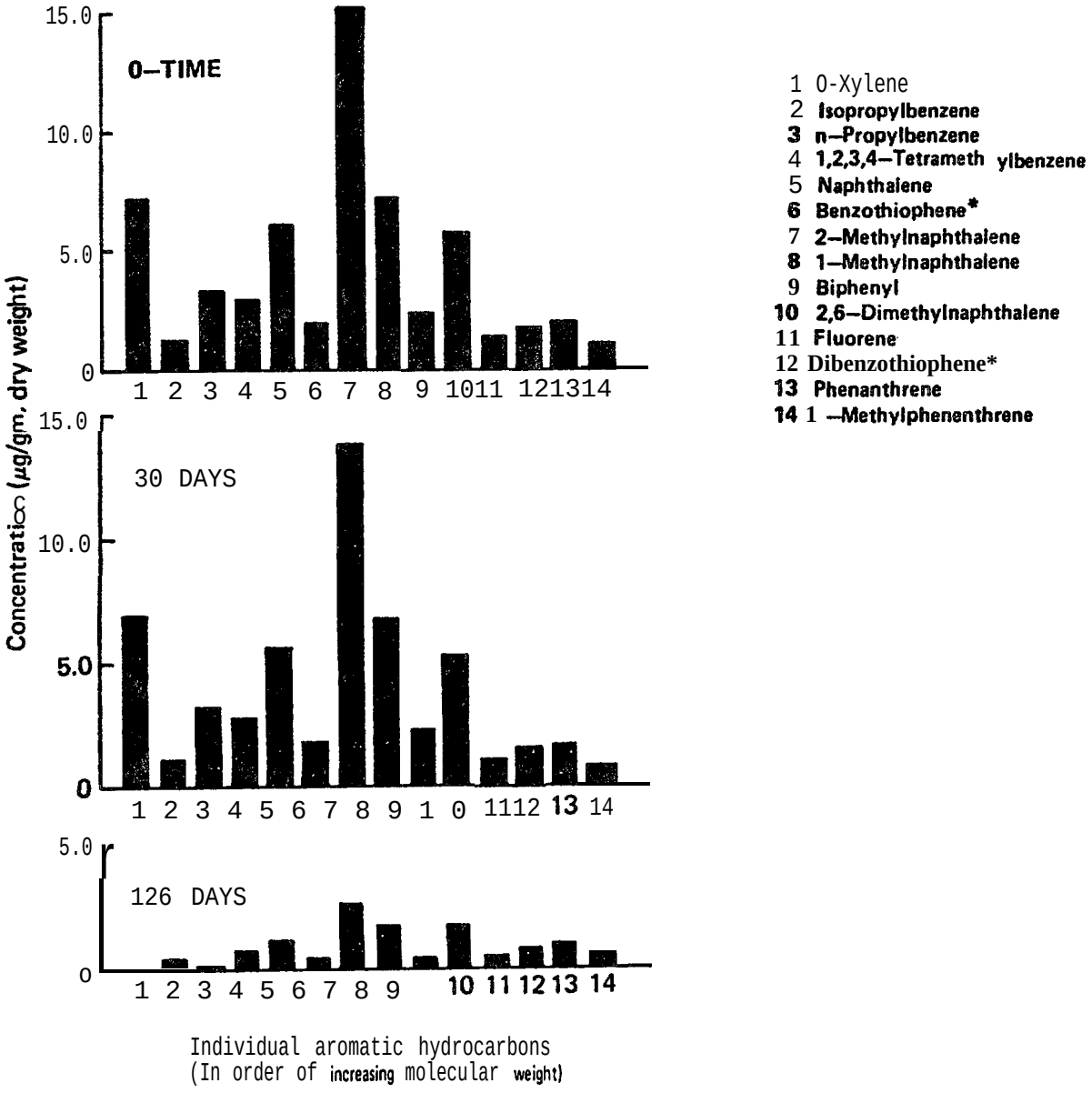


FIGURE 34. Concentrations of major aromatic hydrocarbons in PBCO-contaminated high-silt content sediment (Experiment 4) initially (O-time], and at 30 and 126 days. *Sulfur containing compound.

Tissue Uptake of Sediment-Associated Petroleum Hydrocarbons

In Experiment 1 (Table 42), using high-sand sediment and 0.2% PBCO, several aromatic hydrocarbons were detected in liver, skin and muscle of fish analysed after 11 days of exposure (Fig. 36). No AHs were found in the tissues of control fish. Test fish analyzed at 27 and 60 days had detectable levels of AHs only in liver tissue. 1-Methylnaphthalene, 2-methylnaphthalene, and l,2,3,4-tetramethylbenzene were

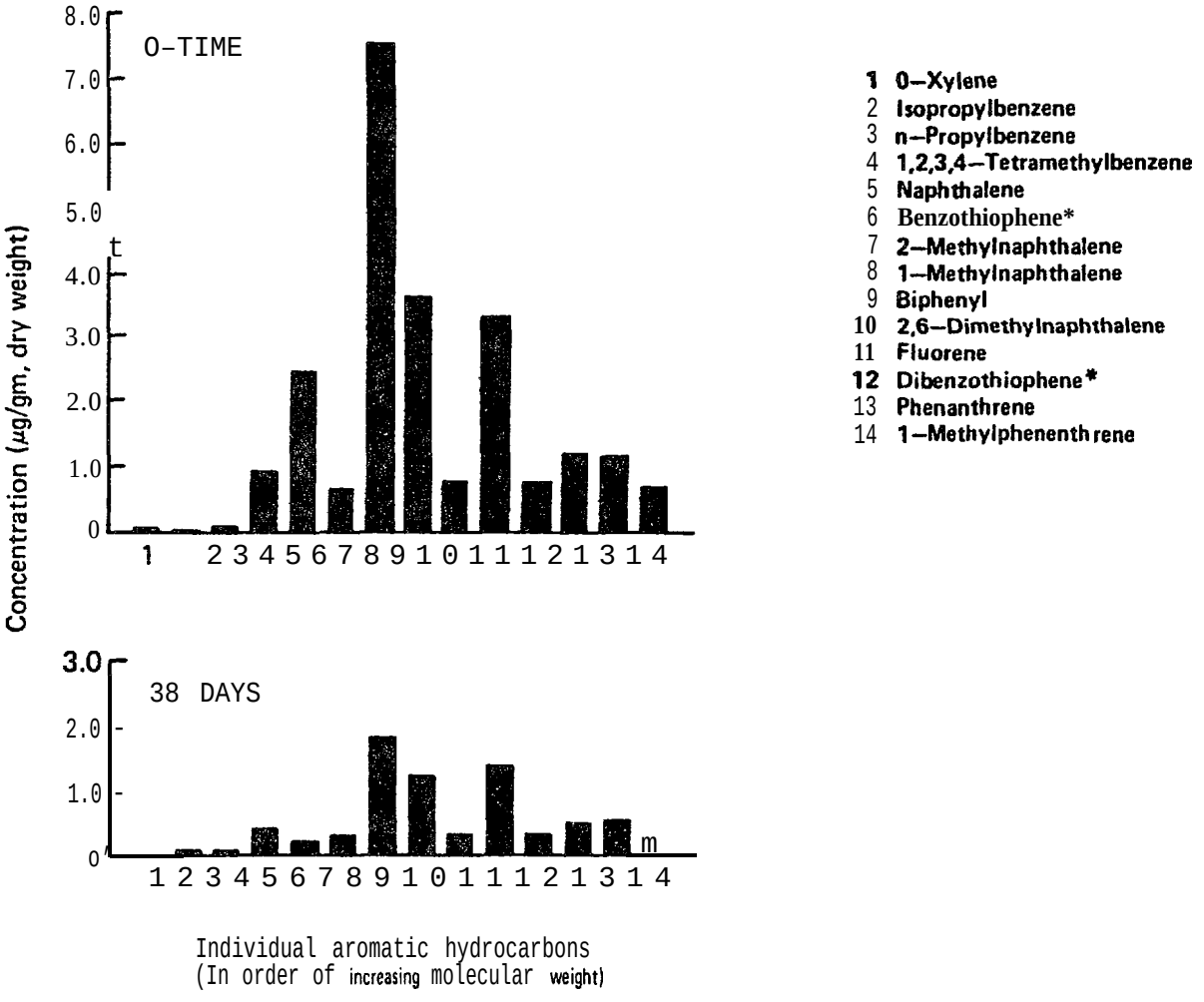


FIGURE 35. Content rations of major aromatic hydrocarbons i n PBCO-contami ned high-sand content sediment (Experiment 5) initially (O-time), and at 38 days. *Sulfur containing compound.

the most abundant AHs detected in the livers during the first 4 wk of exposure. Detectable amounts of only the 2 latter hydrocarbons were found after 60 days.

Tissues of test fish from Experiment 1 also had substantial levels of several alkanes (C-11, 13, 17, 26, 28, and 31); concentrations of some of these compounds were as much as five-fold higher than tissues from control fish. At 27 and 60 days, however, the alkane concentrations were the same in both exposed and control groups.

The uptake of AHs by English sole and rock sole in Experiment 2 with high-silt sediment and 0.5% PBCO (Fig. 37) was less than the AH uptake from high-sand sediment with 0.5% PBCO (Fig. 36). The AHs were noticeably higher in tissues of rock sole than in tissues of English sole (Fig. 37).

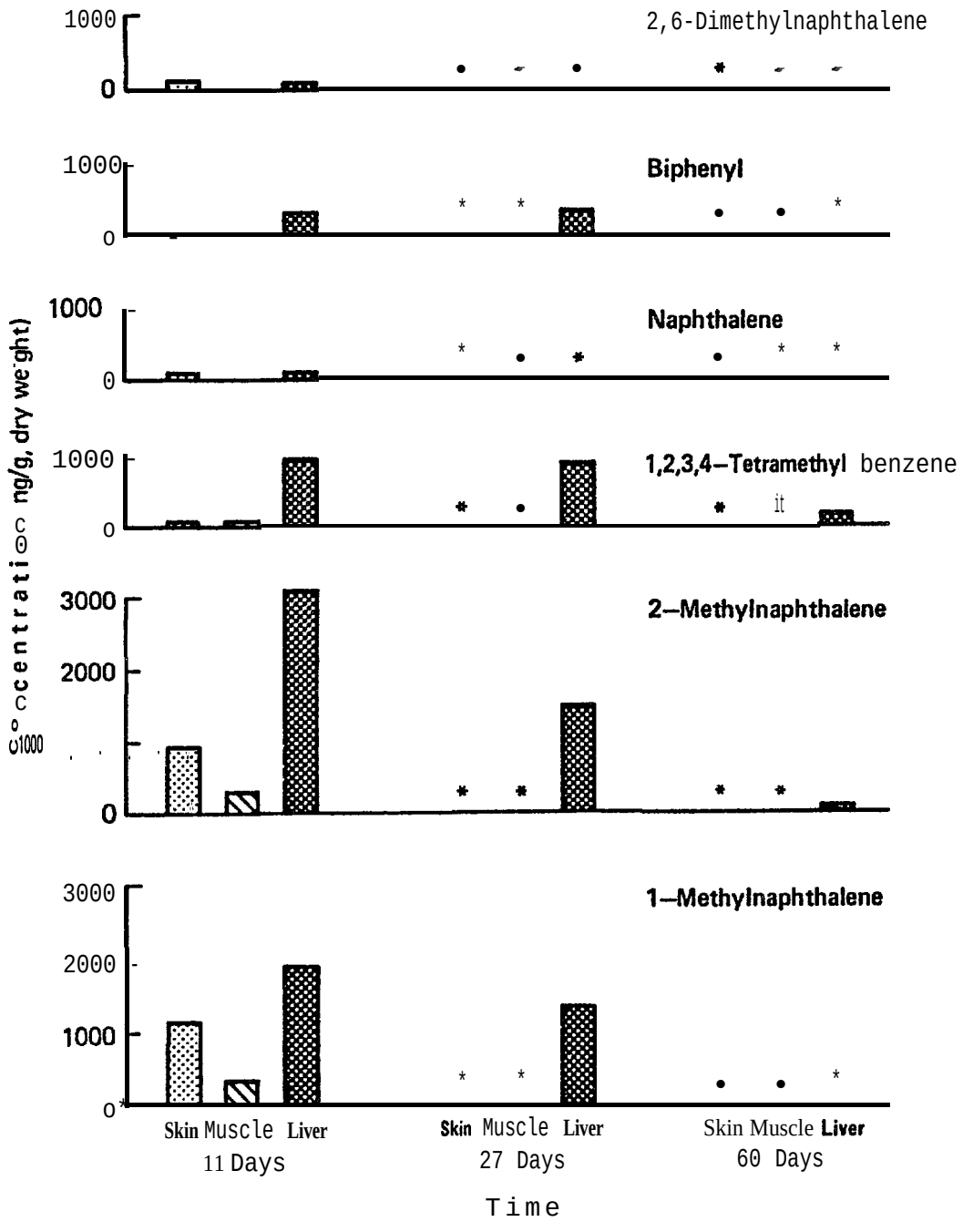


FIGURE '36. Concentrations of aromatic hydrocarbons in tissues of English sole exposed to PBCO-contaminated sediment for 11, 27, and 60 days. *Indicates not detected. (Derived from McCain et al. 1978)

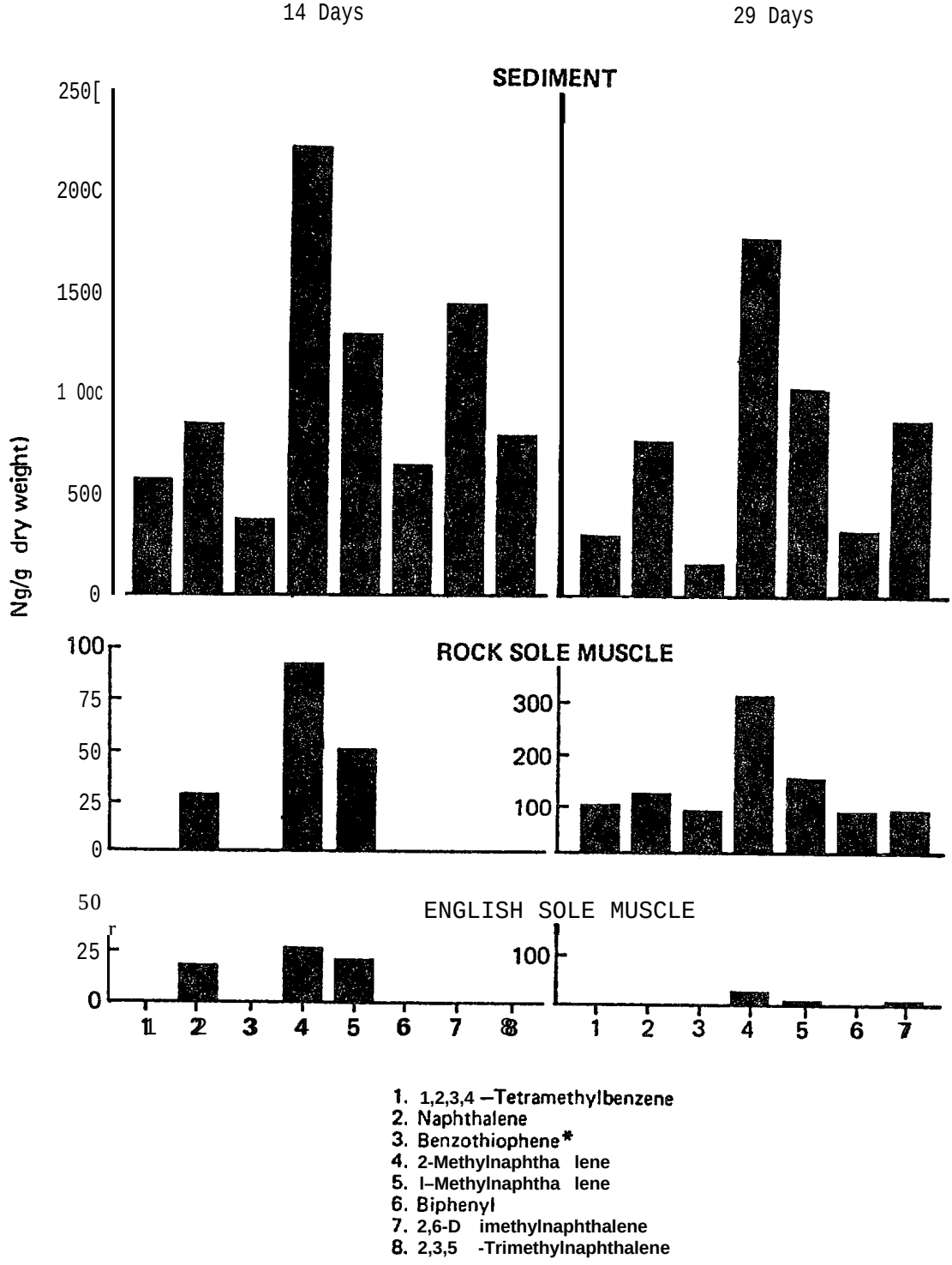


FIGURE 37. Concentrations of major aromatic hydrocarbons in PBCO-contaminated high-silt sediment and in the English sole and rock sole associated with the sediment at 14 and 29 days. Tissue levels are an average of 10 g muscle samples from 2 fish (Experiment 2). *Sulfur containing compound. (From McCain and Malins 1982)

Muscle tissue of English sole exposed for 91 and 126 days in Experiment 4 (high-silt, 1.0% PBCO) had no detectable AHs. However, the muscle tissue of the English sole exposed to 1.0% PBCO in high-sand sediment (Experiment 5) had detectable concentrations (26 and 41 ppb dry wt) of 1- and 2-methyl naphthalene after 38 days.

Biological Effects of Exposure to Oiled Sediments

Rock sole and starry flounder sustained high mortalities (30-50%) in both control and exposed groups after 30-60 days, whereas English sole had low mortalities in both of the 120 day tests (Experiments 1 and 4). As a result English sole were used to assess the biological effects of long-term exposure to oil-contaminated sediments.

In Experiment 1 both test and control fish lost weight during the first 2 mo, with the oil-exposed group losing more than the controls (Fig. 38). After this period, the control fish began to regain weight and at 4 mo, only 27% of the animals weighed less than at 2 wk. The oil-exposed fish were slower in their recovery from the initial weight loss, and at 4 mo 69% weighed less than they did initially. The numbers of fish gaining and not gaining weight at 4 mo were significantly different between the experimental and control groups (P=0.05). These results are apparently related to reduced feeding in the oil-exposed group, as indicated by consistently more uneaten food removed from the test aquarium than from the control aquarium.

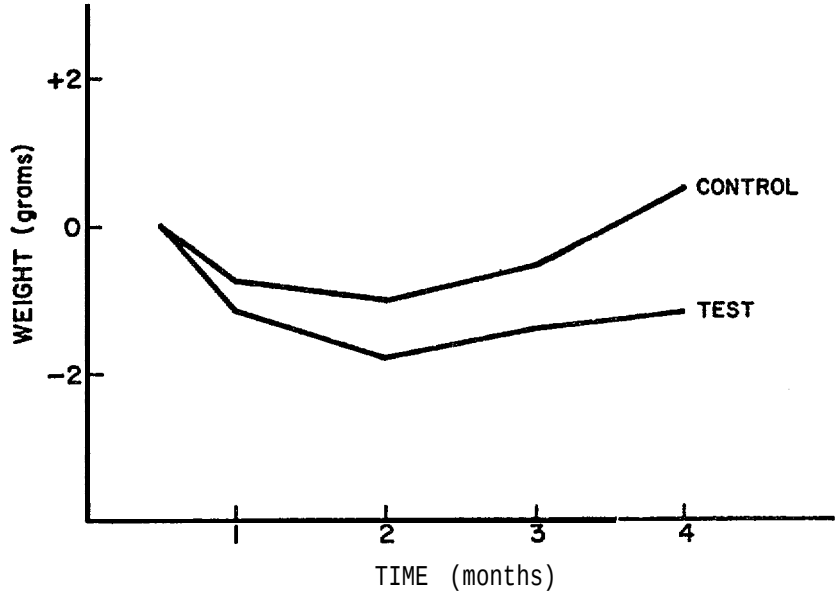


FIGURE 38. Weight changes in oil-exposed and control English sole over a period of 4 months (Experiment 1). Each observation at monthly intervals represents the average change (g) from weights at 2 wk for 16 test and 15 control fish. (From McCain et al. 1978)

During the first month of Experiment 4, there were slight but comparable weight losses in both test and control groups. Both groups recovered the lost weight, however, by the second month. Feeding responses were similar in both groups.

There was no consistent differences in any of the hematological parameters measured (hematocrit, hemoglobin, and total RBC; total and differential leukocyte counts) that could be related to oil exposure. Examination of spleen, kidney, intestine, fins, gills, or skin of English sole for histopathological changes revealed no differences between exposed and unexposed animals in either Experiment 1 or 4. In Experiment 1, however, more of the livers of oil-exposed fish had hepatocellular lipid vacuolization (HLV). HLV is manifested as a replacement of the cytoplasm by lipid vacuoles and was considered severe when 95% replacement occurred. This condition was found in its most severe form in half of the exposed animals during the first month of exposure; none of the controls of developed HLV. During the first month of Experiment 4 most test and control fish had severe HLV. After the first month this condition was rarely identified in either group.

6.2.3 Cytopathology

Effects of PBCO on Adult Flatfish and Salmonids.

Coho salmon and starry flounder exposed to 100 ± 90 ppb ($\bar{x}$, + range) of the SWSF of PBCO for 5 days in a flow-through saltwater system (Roubal et al. 1977b) developed gill lesions resulting from the loss of the surface cells (Fig. 39). Immature mucous glands below the surface were exposed when the surface sloughed and their contents, in some instances, were exuded. The area of sloughing varied among gill filaments: 10 to 30 cells were lost in the smaller lesions and, in a few cases, the surface of the entire filament lost its outermost layer of cells.

Rainbow trout were fed PBCO in Oregon moist pellet (OMP) for five days per week (average of 120 mg PBCO/kg body wt/day). After 2 weeks of feeding, the hepatocytes of control fish contained large amounts of glycogen, whereas those of the oil-exposed fish had virtually none. Proliferation of the endoplasmic reticulum was evident in hepatocytes from oil-exposed fish only, and cochlear ribosomes, a common feature of cells rapidly synthesizing proteins (i.e., in embryos), were apparent in oil-exposed fish hepatocytes (Fig. 40).

In a 75 day feeding experiment with the same exposure regime, all fish gained weight and no mortalities were observed. At the termination of the experiment, the control fish had gained an average of 95.5% in body weight; the oil-fed fish, 70.5%. Glycogen deposits in the hepatocytes of test fish showed the same striking decrease as observed in the previous 14 day experiment. In addition, liver lipid reserves were reduced in the oil-fed fish.

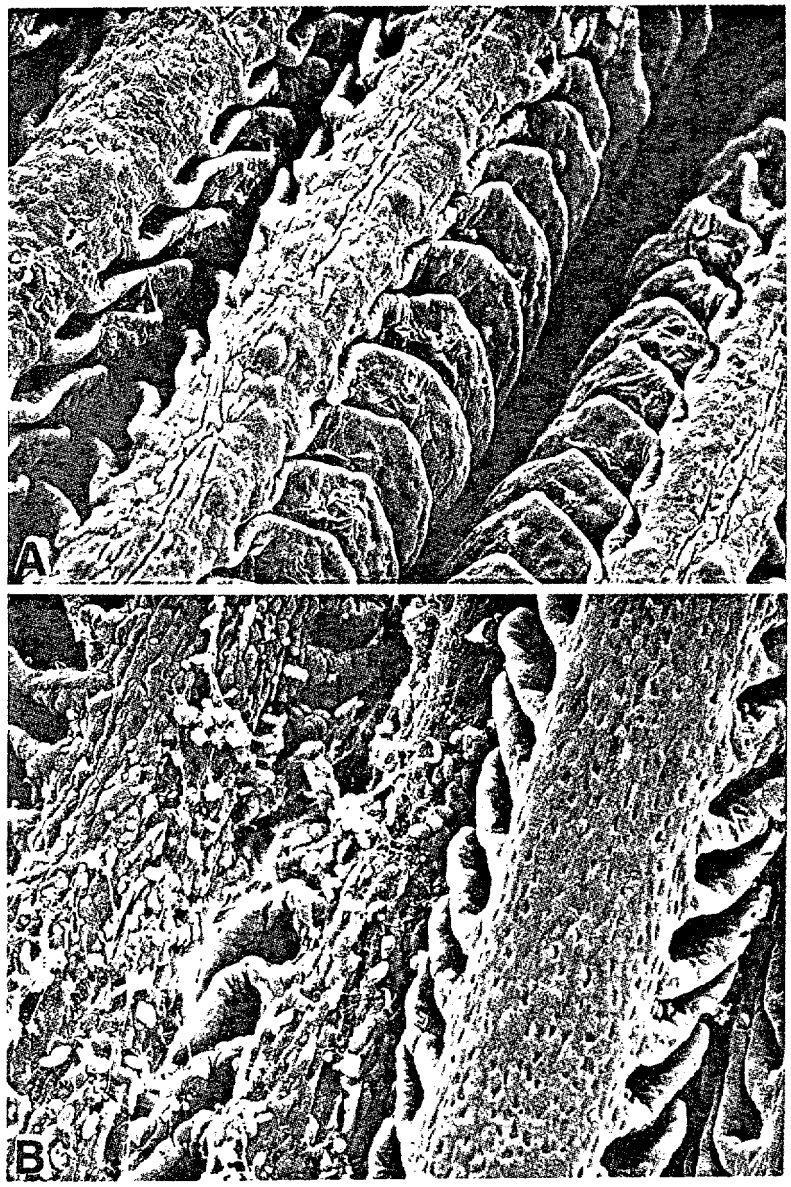


Figure 39A. SEM of gill from an untreated adult coho salmon. X 370. (From Hawkes 1977.)

Figure 39B. SEM of gill from an adult coho salmon exposed to 100 ppb of the SWSF of PDCO. Sloughed surface cells and an abundance of exuded mucus are present on two of the three gill filaments. X 370. (From Hawkes 1977.)

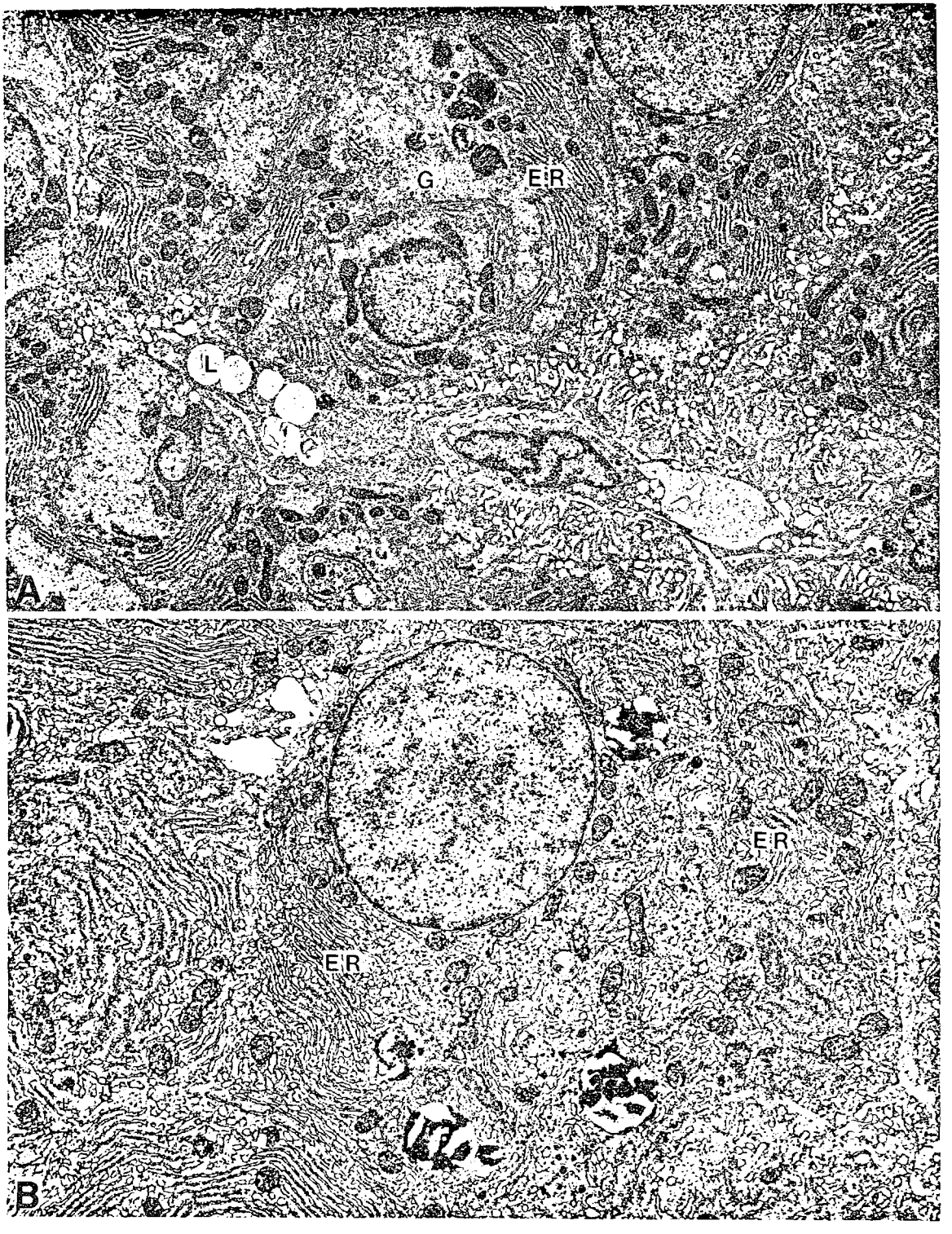


Figure 40A. TEM of untreated adult rainbow trout liver. There are abundant glycogen (G), lipid droplets (L), and several layers of endoplasmic reticulum (ER) at the periphery of the hepatocytes. X 6,400 (From Hawkes 1977.)

Figure 40B. TEM of liver section from an adult rainbow trout fed petroleum for 75 days. In addition to a lack of glycogen in the hepatocytes, the endoplasmic reticulum (ER) had proliferated, nearly filling the cytoplasm. x 7,500. (From Hawkes 1977.)

Rainbow trout fed a PBCO-contaminated diet (1000 ppm added to food) for 8 mo were sampled for microscopy at the time of spawning. Both TEM and LM analysis indicated an abnormal increase in collagen around the liver sinusoids. Trout from this same study continued to be fed the oil-contaminated diet for another 4 to 5 mo after spawning; these fish exhibited abnormally soft and enlarged eye lenses. Of trout fed PBCO for 12-13 mo the eye lens volume was double that of controls; 226 ± 81 mm^3 ($\bar{x} \pm SD$) and 111 ± 19 mm^3, respectively.

The lens is composed of ribbon-like filaments which interdigitate and form a sphere. The filaments have simple projections on their broad surfaces which plug into pits on the adjacent fiber; in addition, there are complex interlocking series of protuberances on their thin side (Fig. 41A). In trout exposed to a crude oil contaminated diet for either 75 days or one year, the fiber structure changed: the surface became distorted and the interdigitating projections were irregular. In a longer experiment, rainbow trout were fed PBCO (120 mg/kg body wt/day) for 3 years; three of five petroleum-exposed fish developed cataracts, whereas all three control fish had normal lenses. In lenses with cataracts, the fiber surface was irregular and the lateral projections were severely deformed (Fig. 41B).

Effects of weathered PBCO on early development of sand sole.

SEM examination of 5 sand sole larvae exposed to 164 ppb of the SWSF of weathered PBCO during embryogenesis revealed 4 of 5 with severely reduced (both numbers and length) olfactory cilia (Fig. 42); the fifth appeared normal (comparable to controls). In fish with reduced olfactory cilia, the epidermal microridges, a feature of normal keratinocytes, were absent from the keratinocytes surrounding the olfactory epitheliums. In addition, the keratinocytes of these four larvae were rounded and protruded from the skin surface, an indication of cellular hypertrophy.

TEM examination of sections from 8 sand sole larvae (4 exposed to 164 ppb of the SWSF as embryos, and 4 controls) revealed ultrastructural changes in the mitochondria of epidermal cells of oil-exposed larvae. Although the sample size was too small to definitively correlate observed changes to oil exposure, the abnormal mitochondria showed classic hydropic changes, a reduction in cristae and a reduction in the electron density of the matrix. Separation between the outer and inner mitochondrial membranes was quite pronounced and breaks in both membranes were observed. Other organelles such as the nuclei, Golgi assemblies, and microtubules were normal.

Effects of weathered CICO on early development of surf smelt.

Morphology of unexposed embryos. Sections of entire 21 or 27-day-old control embryos were examined with light microscopy and electron microscopy and all tissues appeared normal. In neurons of the brain the heterochromatin was evenly distributed in small clusters throughout the nuclei. The

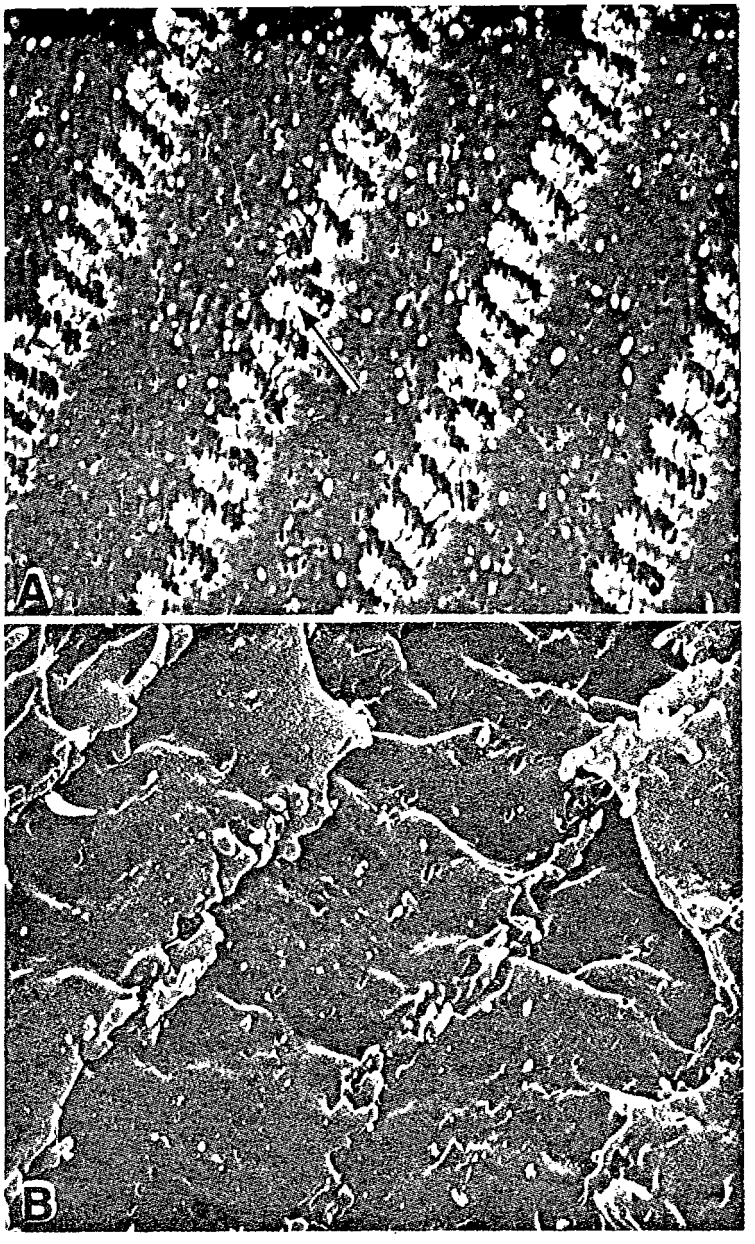


FIGURE 41A. SEM of normal lens fiber cells from adult rainbow trout. The lateral projections (arrow) suture the lens fibers together. X 2,900. (From Hawkes 1980)

FIGURE 41B. SEM of lens fiber cells from an adult trout perorally exposed to PBCO for three years. This fish had a cataract in both lenses. The lens fiber lateral projections are absent or grossly misshapen and the fiber surface is irregular. X 2,900. (From Hawkes 1980)

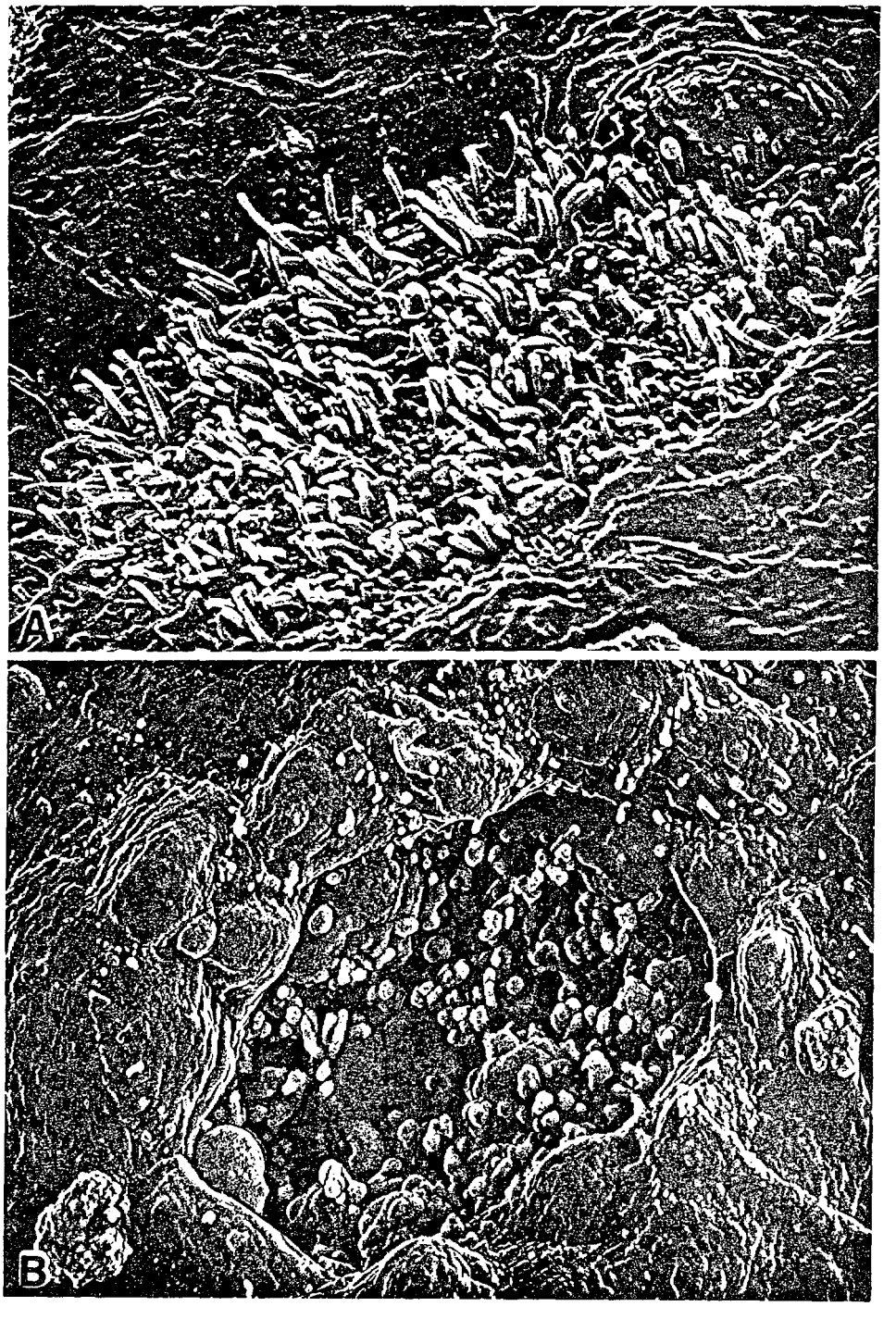


FIGURE 42A. SEM of normal olfactory epitheliums from a sand sole larva. Chemosensory cilia about 2.5 um long protrude from the surface of the olfactory organ. X 3,000. (From Hawkes 1980)

FIGURE 42B. SEM of olfactory epitheliums from a sand sole larva exposed to 164 ppb of the SWAF of CICO. The chemosensory cilia are reduced in size and numbers. x 3,000. (From Hawkes 1980)

cytoplasm of the neurons stained pale blue with Richardson's stain, and and the mitochondria, Golgi complexes, and polyribosomes appeared normal.

The retinas of both 21- and 27-day-old embryos had well-developed, melanized pigment epithelia with processes that extended between portions of the receptor cells. Only one type of receptor cell was observed in the embryos. This is typical of embryonic teleost cone cells (Ali 1959, Blaxter 1974), having a broad outer segment of tightly stacked membranes (saccules) containing the visual pigment, a connecting cilium, an inner segment with both ellipsoid and myoid regions, and a nuclear region at the innermost portion of the cell (Figs. 43A and 44A). At the outer boundary of the receptor cell, the processes of the pigment epitheliums surrounded the tip of the cell, thus enclosing the area where the tightly stacked receptor membranes were located. The ellipsoid region was tightly packed with mitochondria and the myoid region was distinguished by numerous Golgi complexes, free ribosomes, and short profiles of rough endoplasmic reticulum. In addition to the cell nucleus, the inner portion of the receptor cells had synaptic complexes. Adjacent to the receptor cells were horizontal cells (Yamada and Ishikawa 1965, Kaneko 1970) of the neural zone of the retina. A region of embryonic neurons, 5-10 cells deep, lay between the horizontal cells and the internal plexiform layer. Adjacent to the internal plexiform layer was the ganglion cell layer followed by an optic nerve fiber layer which completed the inner retinal zones.

Morphology of CICO-exposed embryos. Neuronal damage was evident in the brain and eye in both age groups of embryos after exposure to 54 and 113 ppb CICO (Table 43); however, all other tissues appeared normal. In both the brain and eye, necrotic neurons appeared intensely blue-colored after application of Richardson's stain. These neurons also stained intensely with PAS reagents; no PAS reaction was evident in neuronal tissues of control embryos. Ultrastructurally, necrotic neurons were easily identified by their electron-dense nuclei and cytoplasm. In some nuclei, the heterochromatin was clumped in a central mass; in others, the heterochromatin was also condensed but distributed along the inside of the nuclear membrane. The cytoplasm of many of the necrotic cells was uniformly granular. However, some had clusters of autolysosomes which contained fragments of organelles such as mitochondria.

The retinal receptor cells of smelt embryos sampled 21 days after fertilization were normal, but the retinal receptors in 60 to 80% of the embryos sampled 6 days later had lesions (Table 43) localized in the ellipsoid and myoid regions of the inner segment. The most severe damage was in the myoid region (Figs. 43Band 44B). In contrast, the tightly stacked membranes of the outer segment were normal or only slightly disorganized, and the basal portion of the cell, which contains the nucleus, also appeared normal. In some embryos, the cytoplasm of the inner segment was filled with clear vacuoles. In damaged receptor cells, the mitochondria were observed in various stages of lysis. Commonly, the outer mitochondrial membrane was intact but the cristae were disrupted and vacuous areas were evident in the matrix.

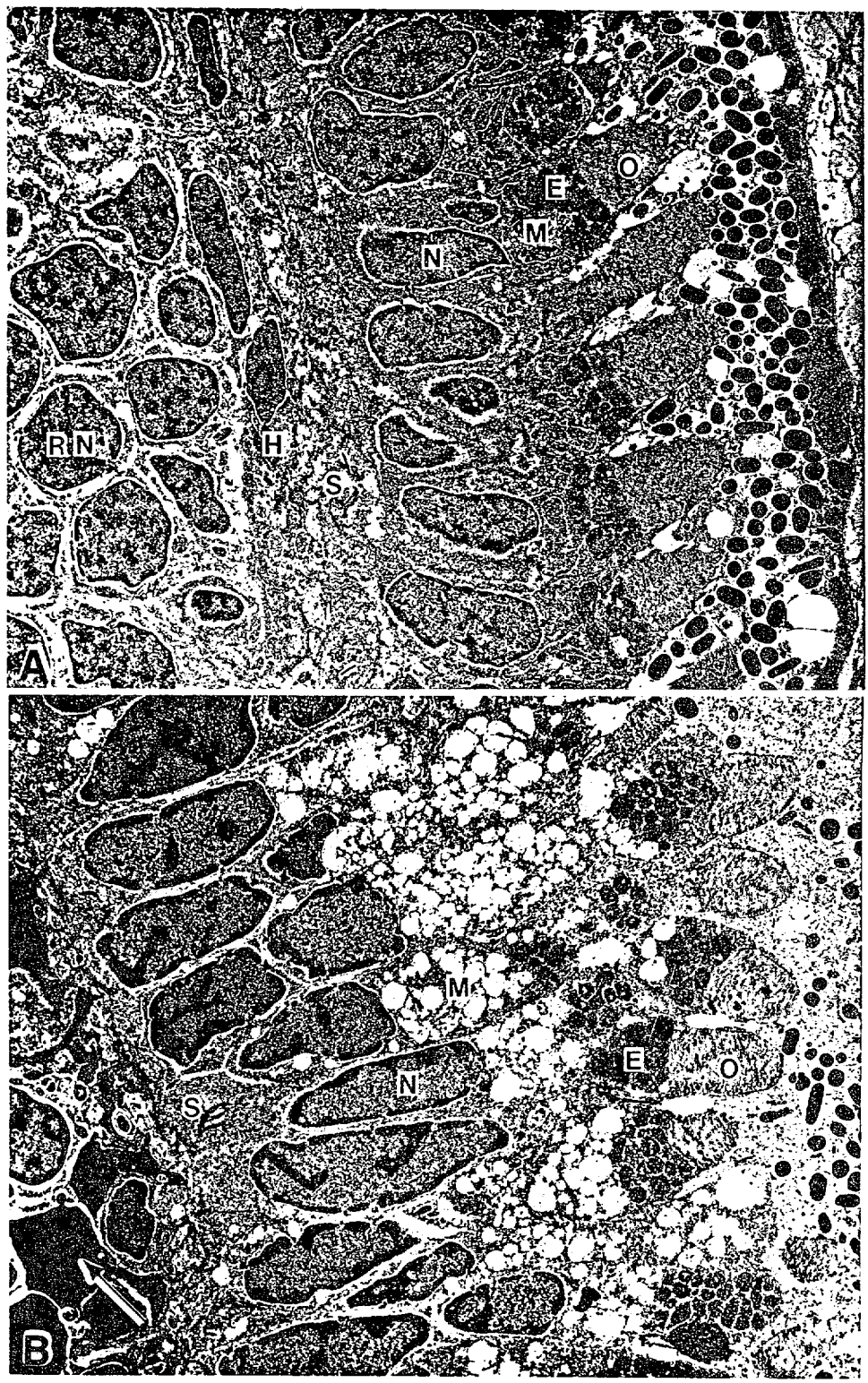
TABLE 43. Percent surf smelt embryos with cellular abnormalities of the eye and brain sampled 21 and 27 days after fertilization (45 and 63 hr oil exposure)

Average hydrocarbon concentration (ppb)	Percent with Cellular abnormalities					
	21 days post-fertilization			27 days post-fertilization		
	Vacuolated retinal receptor cells	Necrotic retinal neurons	Necrotic forebrain neurons	Vacuolated retinal receptor cells	Necrotic retinal neurons	Necrotic forebrain neurons
control	0 (4)[a]	0 (4)	0 (5)	0 (4)	0 (4)	0 (5)
54	0 (6)	83 (6)	72 (7)	80 (5)	80 (5)	80 (5)
113	0 (4)	75 (4)	80 (5)	75 (4)	80 (5)	66 (6)

a Number of embryos examined.
(From Hawkes and Stehr 1982)

FIGURE 43A. TEM of a portion of the retina from a norms? 28-day-old smelt embryo. Regions of the receptor cells include the outer segment (O), ellipsoid region (E), myoid region (M), nucleus (N), and synaptic junctional complex (S). Neural cells of the retina shown in the micrograph include horizontal cells (H) and retinal neurons (RN). X 5,000. (From Hawkes and Stehr 1982)

FIGURE 43B. TEM of a portion of the retina of a 28-day-old exposed (113 ppb CICO) surf smelt embryo. Vesiculation is evident in the myoid regions (M) of the receptor cells and necrotic neurons are present (arrow). X 5,000. (From Hawkes and Stehr 1982)


E
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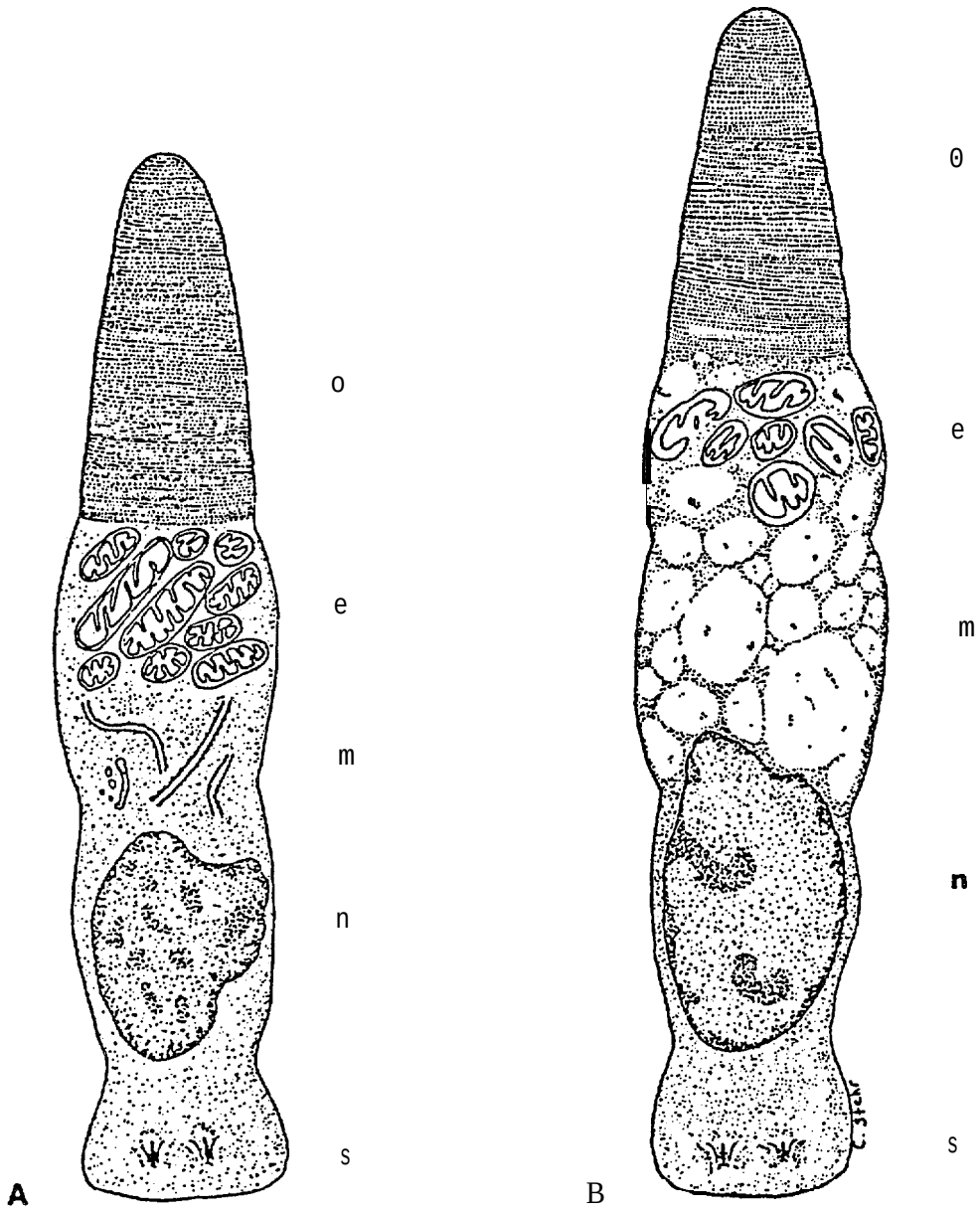

FIGURE 44. Schematic drawing of receptor cells from 28-day-old surf smelt embryos. (A) Control. (B) Exposed to 113 ppb of the SWSF of CICO (hypertrophy, vacuolization and chromatin marination are depicted). Regions of the cells are labelled: outer segment (0), ellipsoid region (e), myoid region (m), nucleus (n), and synaptic complex (s). (From Hawkes and Stehr 1982).

6.3 Behavior and Physiology

6.3.1 Vertebrate Studies

Behavior of Pacific Salmon Exposed to Petroleum Hydrocarbons

(a) Olfactory Disruption in Juvenile Coho Salmon. The first series of electrophysiologi cal tests to evaluate the effect of petroleum hydrocarbons on the olfaction of seawater-adapted juvenile salmon were of an exploratory nature, and the data were not evaluated statistically. Exposure of the olfactory epitheliums to both benzene (0.2 to 17.0 ppm) and NPH (0.2 to 2.0 ppm) for 25 seconds induced a rapidly diminishing oscillatory pattern from the olfactory bulb indicating stimulation, but no immediate injury to, or irritation of, the olfactory epitheliums. Following perfusion of the nares with either 2 ppm NPH or benzene for 10 minutes, there was no reduction or inhibition of the immediate subsequent response to 103 L-serine, L-methionine, or L-alanine. When one part of 1.5 ppm NPH was mixed with an equal part of 10^{3} L-serine the EEG response approximated that of the L-serine solution alone.

There was no evidence of the salmon detecting 2,6-dimethylnaphthalene, or 2,3,6-trimethyl naphthalene at concentrations of 0.2 to 2.0 ppm. The EEG response to these aromatics was similar to that of rinsing the nares with filtered saltwater; that is, there was a slight temporary decrease in baseline activity. In contrast, the SWAF of PBCO (1.8 to 20.0 ppm) was detected by the fish; however, there was no decrease in subsequent responses to amino acids. Threshold concentrations for discrimination were not determined.

In the second series of electrophysiological tests, activity of the olfactory bulb (EEG responses) of coho salmon following exposure of the nares to a standard stimulant (10^{-3} M L-serine) were compared with EEG responses induced by different concentrations of an aromatic hydrocarbon mixture representative of the SWAF of PBCO (Table 44). A significant increase in the amplitude of the EEG response to the aromatic hydrocarbon mixture occurred at concentrations between 1.9 and 2.8 ppm; this is less than the 2.8 to 3.7 ppm concentrations which resulted in statistically significant avoidance of the hydrocarbons by juvenile coho salmon tested at the same time of year and at the same temperatures (Maynard and Weber 1981).

Coho salmon exposed to a 4.(I ppm aromatic hydrocarbon mixture for up to 20 min showed no significant change in EEG response to 10^{-3} M L-serine given before and after exposure (Table 45).

(b) Migratory and Homing Behavior in Adult Salmon

Avoidance reaction. Control observations (i.e., percentage of salmon migrating up the east or west fish ladders when aromatic hydrocarbons were not added) and tests with hydrocarbons were made during the peak of salmon migration in Chambers Creek. More than 1,400 salmon ascended the darn during control periods; 56% of these

TABLE 44. Effect of a mixture of monocyclic aromatic hydrocarbons (see Table 1) on the olfactory bulb EEG as measured by amplitude of response to test mixture compared response to control water, and as percent of 10^{-3} M L-serine response.

Hyd rota rbon Mixture (ppm)	Number of testsa	Average amplitude of EEG response (SD)[b] Control	Test	Significance	% of 10^{-3} M L-serine EEG response (average)
1.9	10	37 (27)	23 (24)	NS	7
2.8	20	35 (24)	87 (52)	***	25
3.7	15	31 (19)	117 (49)	***	32

[a] Equal numbers in each of control and test groups.
b Relative values calculated from area (in cm^2) of integrated EEG response between 2 and 6 Sec after introduction of stimulant to naris.
c NS, not significant ($p>0.05$); ***$P<0.001$
(From Maynard and Weber 1981)

TABLE 45. Comparison of olfactory bulb EEG response to 10^{-3} M L-serine before and after rinsing nares with a 4.0 ppm mixture of monocyclic *aromatic* hydrocarbons.

	Duration of hydrocarbon mixture rinse (rein) <1	10	20
Difference ($\bar{x}\pm SE$)	6.4±16.7	42.7±17.5	-36.2334.1
Number of fish tested	6	6	5
Significance	NS[a]	NS	NS

a Relative values calculated from area (in CM^2) of integrated EEG response between 2 and 6 sec after introduction of L-serine.
b NS, not significant (= 0.05).
(From Maynard and Weber 1981)

chose the west ladder (Table 46). Another 1,149 salmon migrated over the dam during test periods when hydrocarbons were added to the water in the west fish ladder.

Of the total number of salmon ascending the two fish ladders during the study period, 99% were coho salmon and the remaining 1% were a mixture of pink, chum, and chinook.

Data was analyzed using hydrocarbon concentrations based on GC analysis rather than the approximately 40% greater concentrations calculated from the amount of hydrocarbon mixture used and water flow. The Francis formula used for calculating water flow in the fish ladder is a rough approximation of weir discharge, and tends to underestimate water volume at high velocities representative of the field study site. Because of the consistency of hydrocarbon concentrations in replicate

TABLE 46. Numbers of salmon ascending fish ladders of Chambers Creek dam when aromatic hydrocarbons were, and were not (control), present in water of west ladder.

Date	Observation and Test Period(s) (h)	Salmon Ascending Ladders Total	% East	% West	Hydrocarbon Concentration in West Ladder (ppm) Calculated	GC Analysis Top of 1 adder x + range	GC Analysis Middle of ladder x + range
Cent rol							
29 Oct. to 15 Nov.	6 to 24	1431	44 (±7.8 sd, N=9)	56	0		
Test							
2 Nov.	22.0	236	56	45	1,900	1330:280	b
3 Nov.	6.0	107	69	31	4,900	2690 ± 280	2900 ± 360
8 Nov .	18.5	56	75	25	5,300	2130 ± 30	2130 ± 160
11 Nov.	7.0	59	64	36	2,700	1490:140	950[c]
12 Nov.	16.0	139	55	45	1,900	1160 ± 70	970[c]
13 Nov.	4.5	35	71	29	4,400	2500 ± 10	2250:30
13 Nov.	6.0	61	93	7	6,100	37503110	3720[c]
14 Nov.	5.0	126	56	44	1,000	530 ± 50	b
16 Nov.	5.0	100	69	31	3,800	2070 ± 110	2080 ± 20
17 Nov.	19.5	149	45	55	300	180 + 1	180[c]
18 Nov.	20.0	60	57	43	2,600	1530 ± 70	1410 ± 60
19 Nov.	6.0	21	95	5	7,000	4590 ± 150	3960 ± 30

[a] Based on ml/min of hydrocarbon mixture used and water flow in fish ladder
b No water sample collected
[c] No replicate water sample collected.
(From Weber et al. 1981)

water samples taken at the top and in the middle of the fish ladder we elected to use the results of chemical analysis as being the more appropriate for representing the hydrocarbon concentration. The hydrocarbon concentration at which 50% of the fish expected to ascend the west ladder would avoid it (EC_{50}) was 3.2 ppm; the EC_{50} value was calculated from a regression equation based on results of individual avoidance tests (Fig. 45). Water samples for hydrocarbon analysis by GC were also collected downstream from the dam at the confluence of flows from the fish ladders and spillway. When hydrocarbons were present in the west fish ladder the concentration in midstream was 0.7 ppm or less. Thus, all fish approaching the dam during a test period may have encountered the aromatic hydrocarbons downstream.

Disruption of homing capability. Results of capture-oil exposure-transport-release experiments indicate that exposure of up to 40 ppm of the FWAF of PBCO for 14-18 hr, or up to 2 ppm of an aromatic hydrocarbon mixture for 8-22 hr, does not impair homing capability.

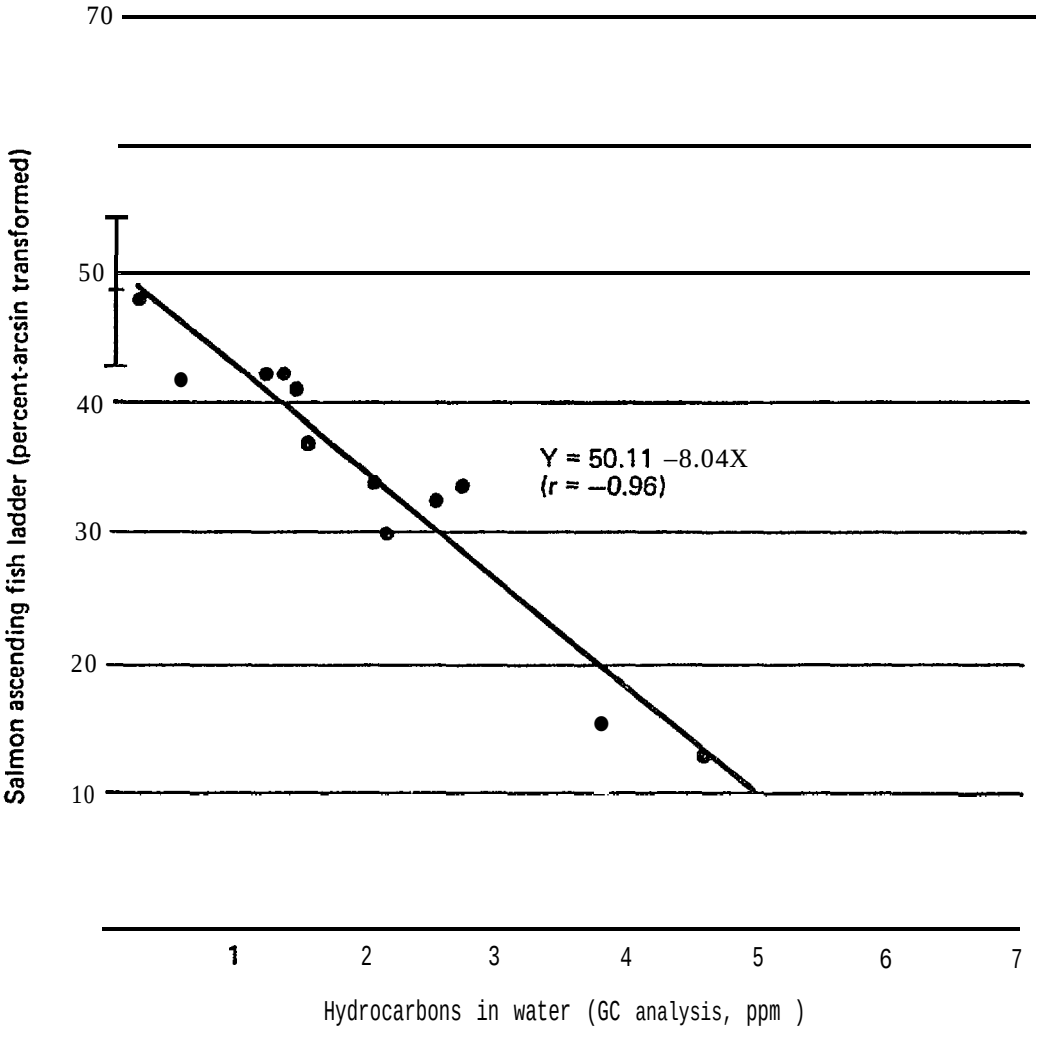

FIGURE 45. Percentage of adult salmon migrating up (west) fish ladder and concentration of aromatic hydrocarbons in the water as determined by GC analysis. Mean and standard deviation for control observations are given at left. (From Weber et al. 1981)

The percentage of chinook salmon returning to the University of Washington homing pond after exposure to 0.5 ppm PBCO was 10% greater than the percentage of returning controls (Table 47). Fish exposed to 5 ppm returned 11% less frequently than their controls, and fish exposed to 40 ppm returned 2% less frequently. These percentages are in marked contrast to the olfactory occluded salmon which returned at a frequency which was 44% less than their controls (6% vs 50%), suggesting that the concentrations of PBCO used for exposure did not adversely affect olfaction. Analysis of data yielded no significant difference ($P>0.05$) between the numbers of returning control fish and fish exposed to either 5 or 40 ppm of PBCO, or in time of return between control and oil exposed fish.

TABLE 47. Release and recovery data for mature chinook salmon at University of Washington following exposure to the FWAF of PBCO.

Release date	Exposure (hr)	concentration ppm Calc.[a]	concentration ppm Analyzed[b] x̄+range	Number released Control	Number released Exposed	Number recovered (%) Control	Number recovered (%) Exposed	Average days to return Exposed-Control
Oct. 30	15	0.5	0.005±.004	7	6	4 (57)	4 (67)	+8.5
Nov. 3-8[c]	14-18	5.3	0.045±.02	19	.22	9 (47)	8 (36)	+1.1
Nov. 10-16[d]	14-18	40.6	0.100±.05	30	32	10 (33)	10 (31)	+0.4
	Total			56	60	23 (41)	22 (37)	+1.9

[a] Determined from volume of PBCO injected and water flow.
[b] Determined by GC (sum of aromatic hydrocarbons).
[c] Four separate release groups.
[d] Five separate release groups.

TABLE 48. Release and recovery data for jack coho salmon at Tulalip following exposure to a mixture of monocyclic aromatic hydrocarbons (Table 1).

Release date	Number released Control	Number released Test	Exposure (hr)	Transported (km)	Hydrocarbon concentration ppm[a]	Number recovered (%) Control	Number recovered (%) Test	Average days to return Exposed-Control
Ott 21	23	23	22	4.7	1.0	14 (61)	16 (70)	-0.4
Ott 28	55	44	22	1.6	1.0	24 (44)	18 (41)	-1. B
Ott 31	62	66	8	1.6	1.0	17 (27)	25 (3B)	-5.1
Nov 3	26	25	B	1.6	1.0	15 (58)	9 (36)	-1.9
Nov 4	64	65	8	1.6	2.(-I	28 (44)	29 (45)	-4.5
Nov 6	52	52	8	1.6	2.0	23 (44)	19 (37)	-2.3
Tot al	282	275				121 (46)	116 (45)	-2.7

[a] Determined from volume of hydrocarbons injected and water flow.

In the first 4 tests at Tulalip Creek, jack coho salmon were exposed for 8-22 hr at calculated concentrations of aromatic hydrocarbons of 1 ppm, and for the last 2 tests the fish were exposed for 8 hr to 2 ppm. Analysis of individual tests indicated no significant difference ($P>0.05$) between the number of returning control fish versus number of hydrocarbon exposed fish (Table 48). However, the exposed salmon were delayed an average of 3 days in their return to Tulalip Creek ($P<0.05$).

TABLE 49. Comparison of calculated vs. measured concentrations of aromatic hydrocarbons in the Tulalip Creek exposure tank and the relation to number of fish exposed.

Number of fish	Hydrocarbon concentration (ppb) Calculated	GC analysis ($\bar{x}$ ± range) (N=2)
3	1500	1597 ± 76
12	1020	378 ± 25
25	960	154 ± 43
52	2200	2143106
65	2100	24 ± 11

In the Tulalip study, a marked discrepancy (ranging from 0.6 to 2.0 ppm) was found between the calculated concentration of aromatic hydrocarbons present in the flow-through exposure tank and concentrations determined by GC analysis (Table 49). Since the eductor system used to introduce monocyclic aromatic hydrocarbons into the water was shown to be accurate (see Fig. 3, Section 5.3.2[c]), an explanation for this discrepancy was sought. The difference between the calculated concentrations of aromatic hydrocarbons present and the concentrations determined by GC analysis are apparently related to the number of fish present in the exposure tank. Figure 46 shows that the more fish present in the exposure tank the larger the discrepancy between calculated and GC determined concentrations. A test of this relationship with linear regression demonstrates a highly significant ($P<0.01$) correlation between the two variables. It appears that the coho salmon were rapidly taking up the aromatic hydrocarbons from the water, and either the aromatics were retained in the tissues or they were being excreted as metabolic products not detectable by the analytical methods employed.

(c) Predator-Prey Behavior. The SWSF concentrations to which the fry were exposed averaged 350 ppb and ranged from 130 to 620 ppb. Mortality of oil-exposed fish was approximately 5% during exposure compared to <1% for controls. Of 280 oil-exposed fry only 85 survived predation, whereas of 280 control fry, 161 survived predation. The hypothesis that the survival of prey chum fry exposed to the predators is independent of exposure to the SWAF of CICO was tested by chi-square analysis and rejected in every case: at 24 hr, $P<0.01$; 48 hr, $P<0.01$; 72 hr, $P<0.001$; 96 hr, $P<0.05$; entire experiment, $P<0.001$. At 96 hr, however, significantly fewer oil-exposed fish were consumed in comparison to controls.

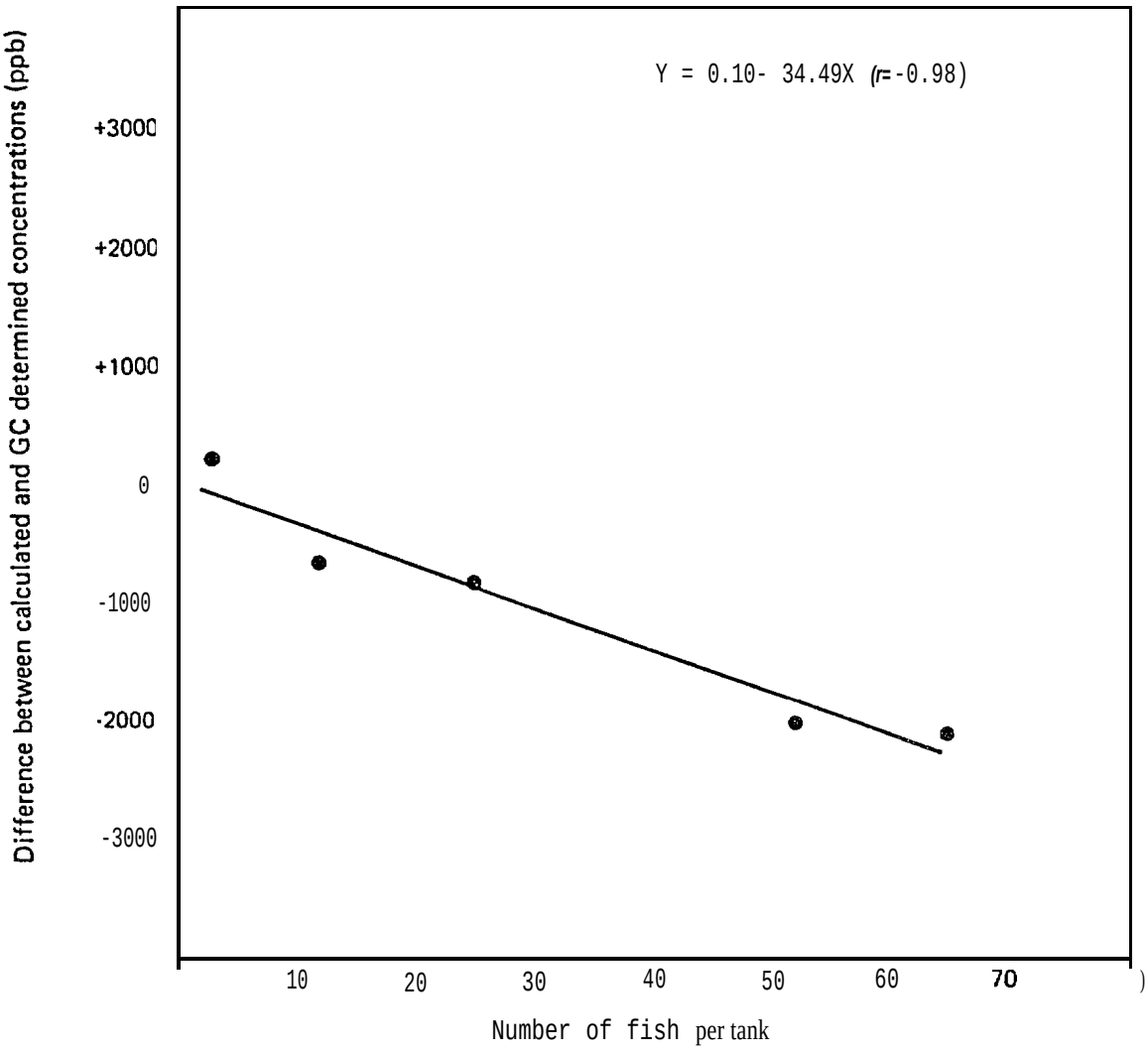

FIGURE 46. Difference between calculated concentrations of aromatic hydrocarbons present in the water and concentrations determined by GC analysis plotted against number of fish exposed.

Many of the coho predators exposed to 343 + 93 ppb ($\bar{x}$ + SD) of the SWSF of CICO began to show behavioral modifications by the tenth day of exposure. In general, the oil-exposed predators appeared lethargic and showed little or no interest in the prey presented to them (these predators were designated as noneaters). However, one of the oil-exposed subgroups (number 6, designated as eaters) demonstrated none of these behavioral modifications and continued to feed at rates comparable with those of the unexposed predators. Similar behavioral responses with eater and noneater groups in coho predators exposed to No. 2 fuel oil were also observed in another experiment (L. Folmar, NWAFC, personal communication).

Figure 47 depicts the numbers of rainbow trout fry consumed during 10 min of exposure to the control or oil-exposed predators at three time intervals. An initial Yates X2 evaluation showed a significant difference in prey consumption between the control and oil-exposed predator

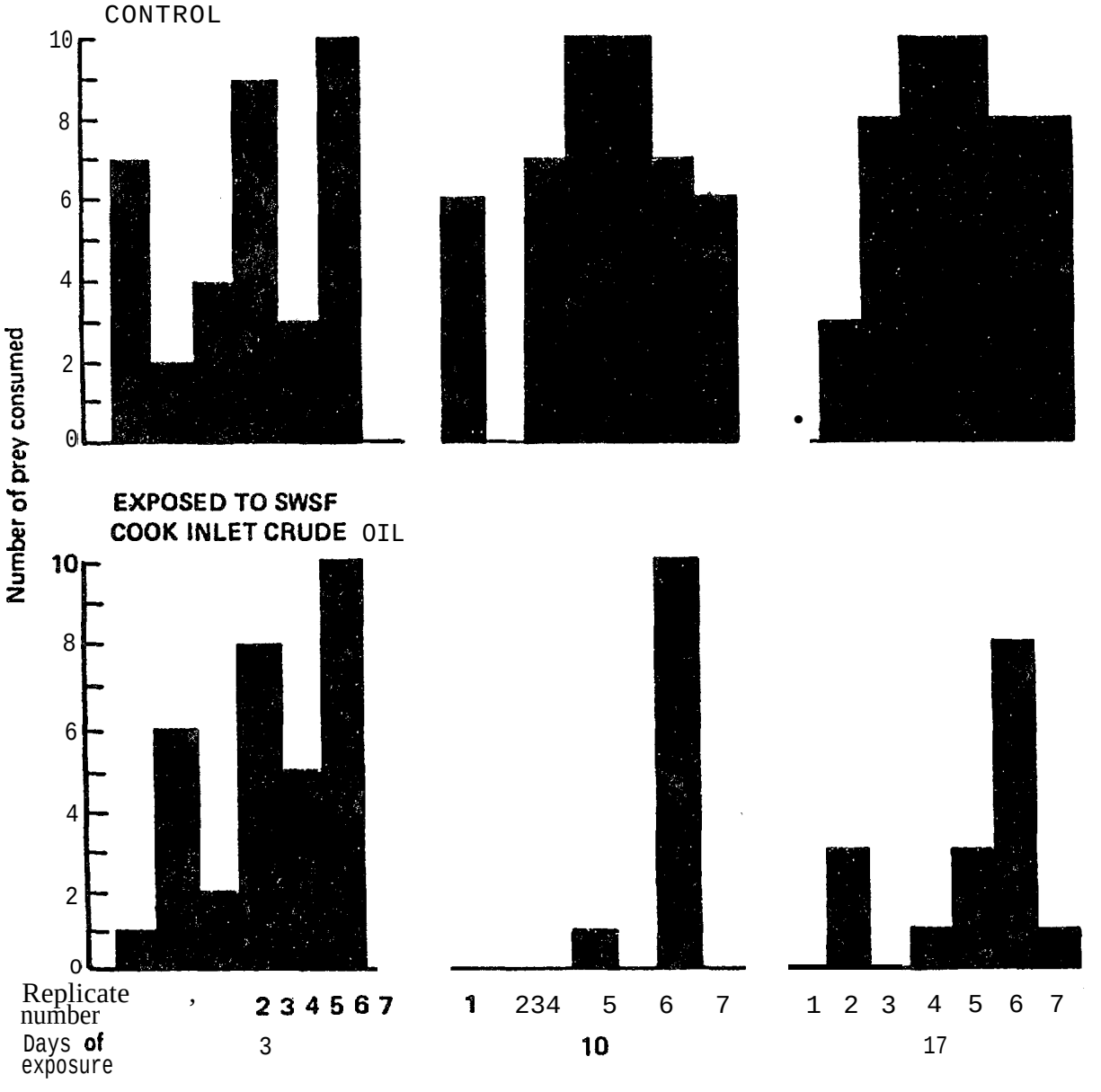

FIGURE 47. Ten rainbow trout fry were offered to each of the seven (3 fish) replicate control and oil-exposed coho salmon predator subgroups after 3, 10, and 17 days of oil exposure. The histograms represent the number of prey consumed by each of the control and oil-exposed subgroups at the three sampling periods. Each numbered subgroup represents the same three predators throughout the experiment. * = no data due to mortalities. Other absences of a bar represent no prey consumed. (From Folmar et al. 1981)

subgroups ($P<0.005$). To determine within- and between-group differences with the small sample sizes, the data were subjected to predictive sample reuse analysis (Geisser and Eddy 1979). With a sample size of seven, this test is designed to select the correct model >95% of the time. The data were used to select one of two models: Model 1 (test populations were equivalent) or Model 2 (test populations were unequal). Model 1 (=) was selected for the control observations at 3, 10, and 17 days, as it was for the comparison tests of control and oil-exposed fish after 3 days of exposure. However, Model 2 (#) was selected for the control and oil-exposed fish comparison tests after 10 and 17 days of exposure. Model 1 (=) was also selected for the comparison of the 10 and 17 days oil-exposed predators. Even with the bias of Subgroup 6 toward the control values, our results clearly indicate that there was reduced predation by the adult coho salmon exposed to the SWAF of CICO for periods of 10 and 17 days.

(d) Avoidance Behavior of Flatfish Exposed to Oil-Contaminated Sediment

Control tests with uncontaminated sediment on both sides of the choice chamber (Fig. 6, Section 5.3.3[d]) indicated that after 22-24 hr an average of 54% of the fish were found on the side of introduction (Table 50).

Tables 51 and 52 present the results of 3 series of tests with oil-contaminated sediment on one side of the test apparatus and uncontaminated sediment on the other. The first two series were conducted with an initial oil-to-sediment concentration of 2,5%; the third, with 5% oil. At the start of Test 1 (2.5% oil) a sediment sample taken immediately after mixing contained over 23,000 ppm TEPH which decreased by 62% after 2 hr of rinsing. Subsequent sediment samples did not show an appreciable decline in TEPH over an 8 day period. At the

TABLE 50. Percent juvenile English sole found in each side of the testing apparatus when no oil was present in the sediment.

Test dates	No. fish introduced	Hours in test apparatus	% fish	
			left	right
8/9	20	24	45[a]	55
8/23	20	22	55	45a
8/24	20	24	70[a]	30
9/6-1 1	20	24	45	55a
		48	50	50
		76	50	50
		117	55	45

[a] Side on which fish were introduced.

TABLE 51. Percent juvenile English sole on each side of the test apparatus when oil-contaminated sediment was present on one side at an initial oil concentration of 2.5% (v/v). Hydrocarbon analysis of sediment was by the gravimetric method; above-sediment water analysis was by GC.

Test dates	No. fish	Hours in test apparatus	% fish		Hours oiled sediment in apparatus	Hydrocarbon concentration (ppm)			
						sediment (dry wt.)		above-sediment water	
			left	right		left	right	left	right
Test 1									
8/14	0				0[a]	23315.9	17.3		
8/14	0				2	8974.8	17,5	.001	<.001
8/16	20	23	25	75[b]	46	8404.0	17.5	.003	.026
8/17	20[c]	21	60	40[b]	71	7140.0	34.1	.078	.040
8/18	20[c]	22	45	55b	94	8056.9	43.2	NAd	NA
Test 2									
8/29	0				22	12.2	8194.3	.025	.026
8/30	20	22	75b	25	46	12.2	8496.0	.032	.037
8/31	20[c]	22	70[b]	30	69	13.3	5258.1	.050	.062
9/1-5	20[c]	23	65b	35	93	14.7	6350.2	NA	NA
		98	35	65	192	15.9	6821.8	NA	NA

a Sediment sample taken before filling test apparatus with seawater.
b Side on which fish were introduced. In each instance fish were initially placed on the non-oil-contaminate side of the apparatus.
c Previous 20 fish removed and a new group of 20 fish placed in chamber on same non-oil-contaminated sediment.
d NA, not analyzed.

start of Test 3 (5% oil) the TEPH concentration after 4 hr rinsing was 18,600 ppm (Table 52); however, after 26 hr rinsing, with fish in the test apparatus, the oil concentration in sediment was reduced to 8,300 ppm and remained fairly constant thereafter. Thus, regardless of the initial oil-sediment concentration, the carrying capacity of the sediment appeared to be about 8,000 to 10,000 ppm.

At the start of each test series the total extractable organic material from the non-oiled sediment was 12 to 17 ppm. During avoidance testing there was a gradual increase to a maximum of 43 ppm in the uncontaminated tray, indicating a probable transfer of petroleum hydrocarbons from the oil-contaminated side.

In Test 3, after the introduction of fish into the choice apparatus, the average TEPH in the oil-contaminated sediment between 26 and 361 hr was 8,860 ± 1,240 (SD) ppm. The TEPH found in the sediment-associated water (SAW) of the same oil-contaminated sediment averaged 6,980 ± 762 (SD) ppm, indicating that only about 20% of the crude oil was bound to the sediment. Of the total extractable organic material found in uncontaminated sediment, less than 20% was found in the SAM. SAW samples from the oil-contaminated sediment were analyzed by GC; no change in the relative composition of aromatic and alkane constituents from the beginning to the end of each test was observed.

TABLE 52. Percent juvenile English sole on each side of the test apparatus when oil-contaminated sediment was present on one side at an Initial oil concentration of 5% (v/v). Hydrocarbon analysis of sediment was by the gravimetric method; above-sediment water analysis was by GC.

Test dates	No. fish	Hours in test apparatus	% fish, left	% fish, right	Hours oiled sediment in apparatus	Hydrocarbon concentrate on (ppm) sediment (dry wt.) left	sediment (dry wt.) right	above-sediment water left	above-sediment water right
Test 3									
9/18	0				4	18594.5	17.1	.016	.010
9/19	20	21	30	70[a]	26	8282.8	23.8	.081	.079
9/20	20[c]	21	50	50[a]	46	9323.9	26.0	.266	.062
to		91	40	60	96	8469.5	26.6	.074	.014
10/3	(19)[b]	166	58	42	171	6936.1	28.2	.136	.045
	(18)[b]	214	50	50	219	9668.7	31.8	.109	.014
	(17)[b]	356	41	59	361	10498.3	33.9	.024	.017

[a] Side on which fish were introduced. In each instance fish were initially placed on the non-oil contaminated side of the apparatus.
b Reduced number of fish due to mortality in initial group of 20 fish.
c Previous 20 fish removed and a new group of 20 fish placed in chamber on same non-oil-contaminated sediment.

Since analysis of the TEPH in the sediment indicated that the sediment was essentially saturated with oil at concentrations of less than 2.5%, all tests were comparable with respect to petroleum hydrocarbon exposure. There was no significant avoidance of oil-contaminated sediment by juvenile flatfish when all 21 to 23 hr tests were combined (P=0.265). However, there was a significant difference (P=0.017) between numbers of fish on oil-contaminated sediment and numbers of fish on uncontaminated sediment for the first groups of 20 fish introduced into the avoidance apparatus at the beginning of each of the three test series. Prior to initiation of each test series, when the oil-contaminated sediment had not been disturbed, the average percentage of flatfish found on the oil-contaminated side after the first 21 to 23 hr was 27%, compared to 46% for controls. That is, within this experiment a high percentage of fish were found at each observation period on oiled sediment. For those fish repeatedly tested, there was no predictable pattern for preference of the right or left side, regardless of the presence of oil-contaminated sediment. Also, no differences were detected in feeding responses of fish on either oil-contaminated or uncontaminated sediment, and visual assessment of stomach fullness at the end of each test indicated that all fish were feeding except in the test which extended 15 days. During this extended test (Table 52) there was a 15% (3 of 20) mortality, and at termination of the test 24% (4 of 17 of the survivors) were not feeding.

(e) Effect of Petroleum Hydrocarbons on Development of Embryos and Larvae

(1) Hydrocarbon Exposure. In experiments assessing the effects of petroleum hydrocarbons on embryos and larvae, salmon and flatfish were exposed to PBCO and surf smelt were exposed to CICO. A list of the major components in PBCO and CICO as determined gravimetrically is

TABLE 53. Comparison of major petroleum fractions of fresh PBCO and CICO. Analysis determined gravimetrically, and each fraction expressed as percent of total.

Fraction	CICO[b]	PBCO[c]
Naphtha[a]	27.9	18.6
Saturates	39.5	48.9
Aromatics	19.7	19.4
Polars	11.5	13.8
Insoluble	2.3	1.4
	100.9	102.1

a Naphtha fraction includes saturates and aromatics boiling at less than 210° c.
b Personal communication, Paul Robisch, NWAFC.
[c] Malins et al. (1 978)

given in Table 53. A more detailed composition of aliphatic and aromatic fractions of PBCO and CICO, as determined by glass-capillary GC, is presented by MacLeod et al. (1980). The major differences between these two oils are in the naphtha and saturate fractions. In our experiments the low molecular weight saturates through $n\text{-}C_{11}$ and most of the naphtha fraction of both PBCO and CICO disappeared from the SWAF in the first 30-48 hr of weathering. The result of weathering on the hydrocarbon content of water-accommodated CICO is shown in Figure 48. Generally, as weathering of CICO progresses, there is a rapid initial loss of both akyl-substituted benzenes and low molecular weight alkanes. With increasing time the naphthalenes disappeared from the SWAF, and after over 500 hr of weathering the dominant hydrocarbons were compounds less volatile than $n\text{-}C_{14}$. PBCO has the same general pattern of weathering as CICO with the more volatile benzenes and naphthalene compounds disappearing over time (Fig. 49).

Chum salmon. Chum salmon embryos and alevins were exposed to the SWAF of weathered PBCO for an average of 3 hr/day, 4 days a week, for 16 consecutive weeks. The concentration of petroleum hydrocarbons remained relatively constant for the first 3 days of exposure and then dropped on day 4 (Table 54). The average hydrocarbon concentration in the water during exposure was 470 ppb.

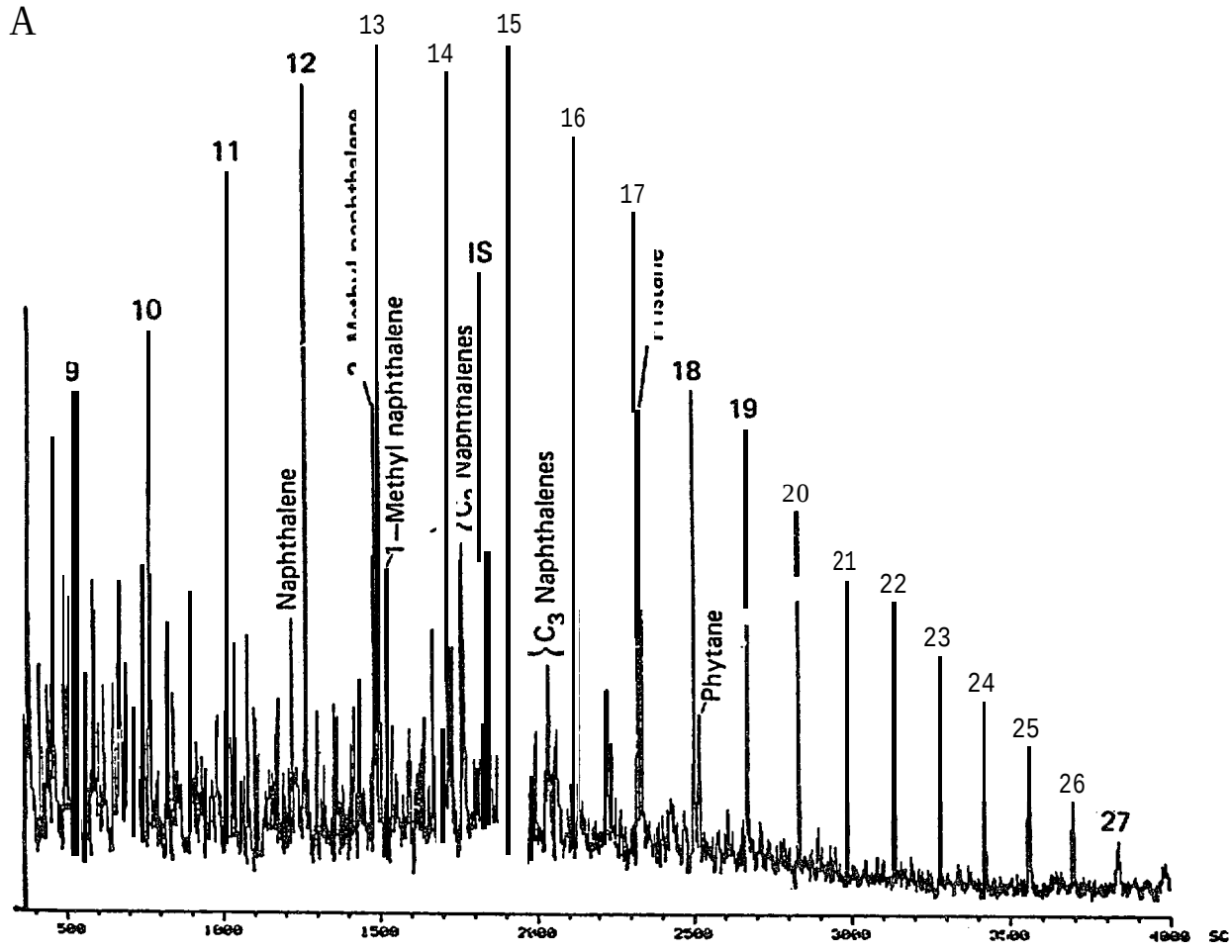

FIGURE 48. Gas chromatograms of fresh CICO and seawater-accommodated CICO, all normalized to n-pentadecane (n-C_{15}). A. Fresh CICO. B. CICO weathered 48 hr. C. Weathered 192 hr. D. leathered 528 hr.

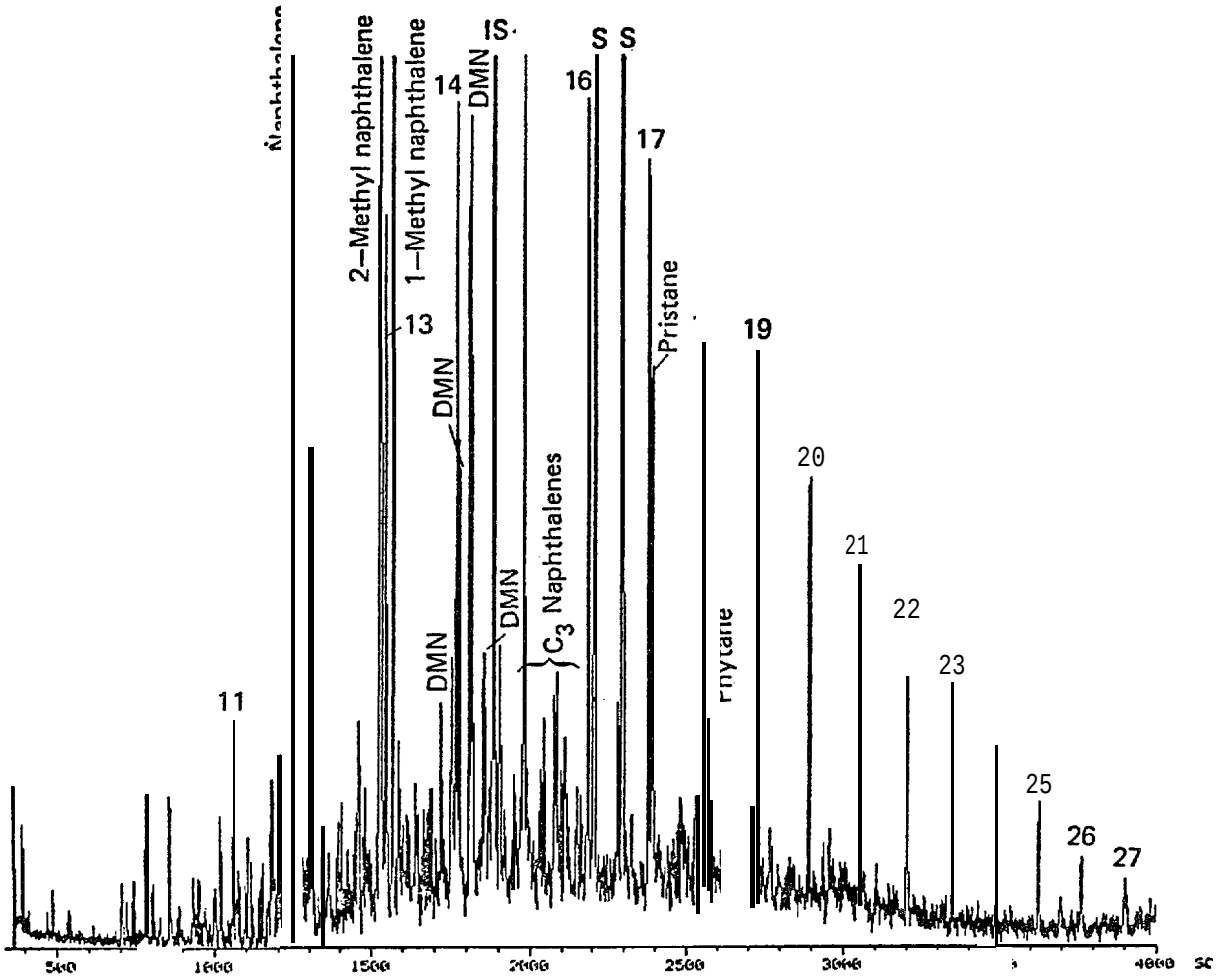

FIGURE 48. B. **CICO** weathered 48 hr.

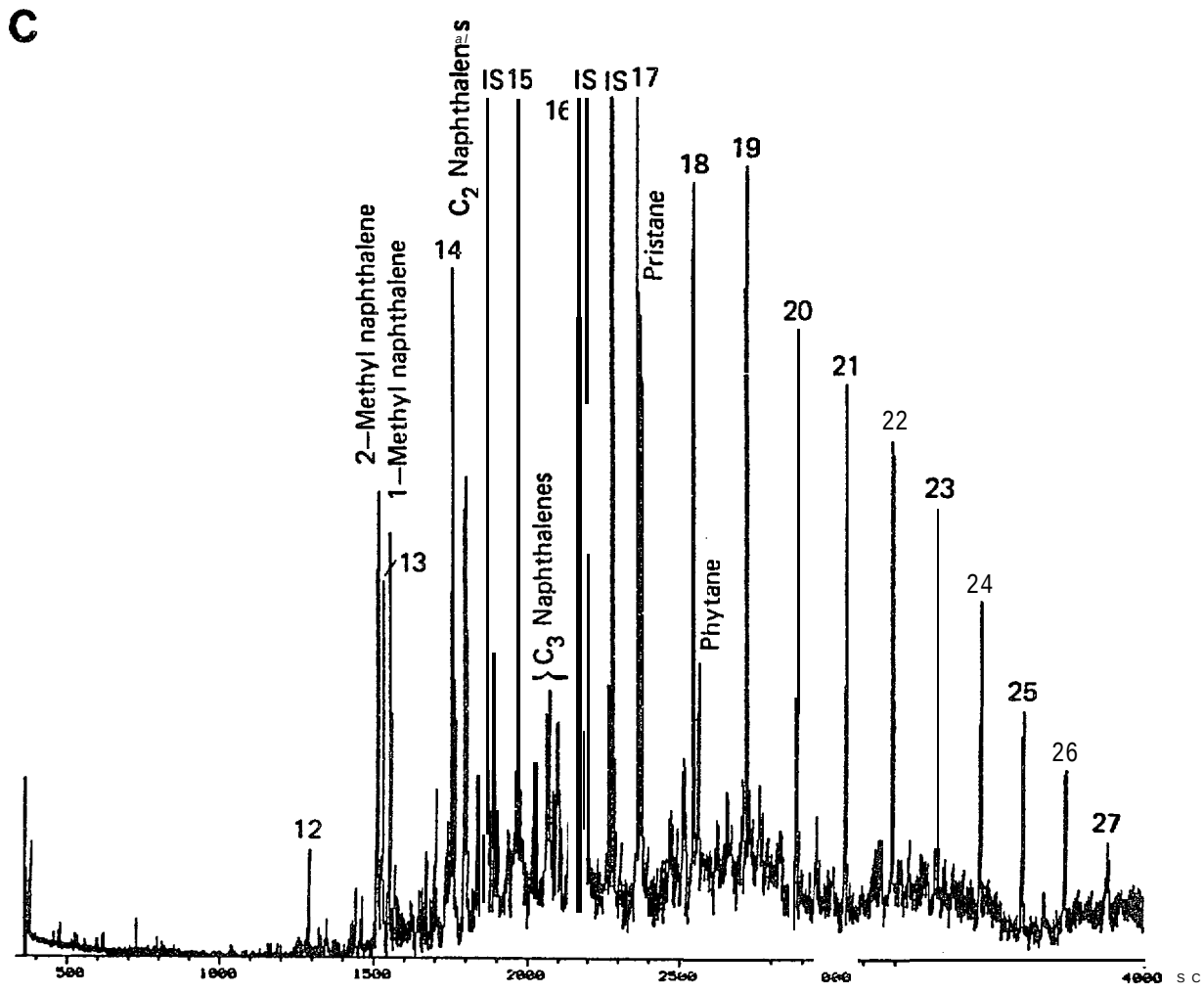

FIGURE 48. C. CICO weathered 192 hr.

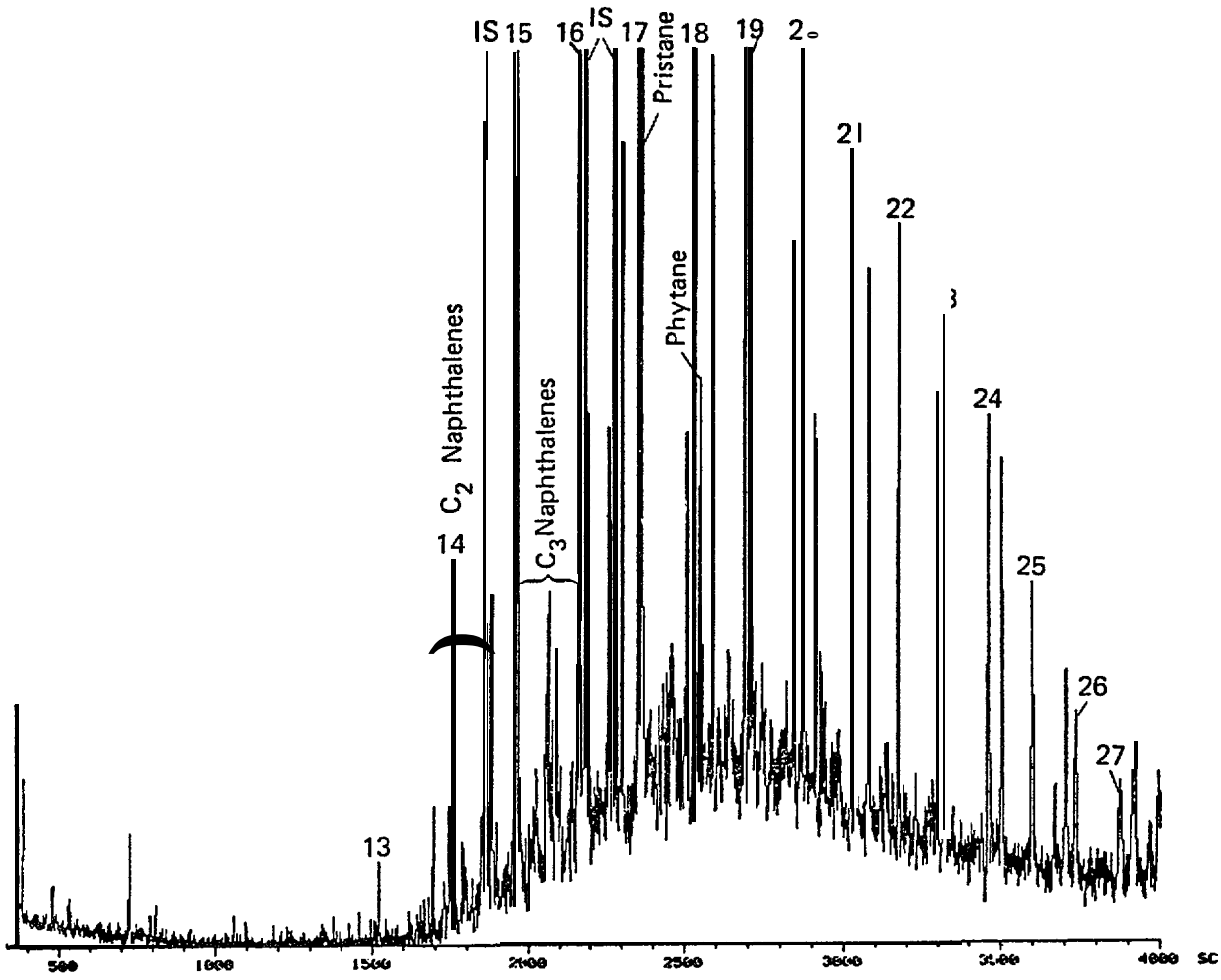

FIGURE 48. D. CICO weathered 528 hr.

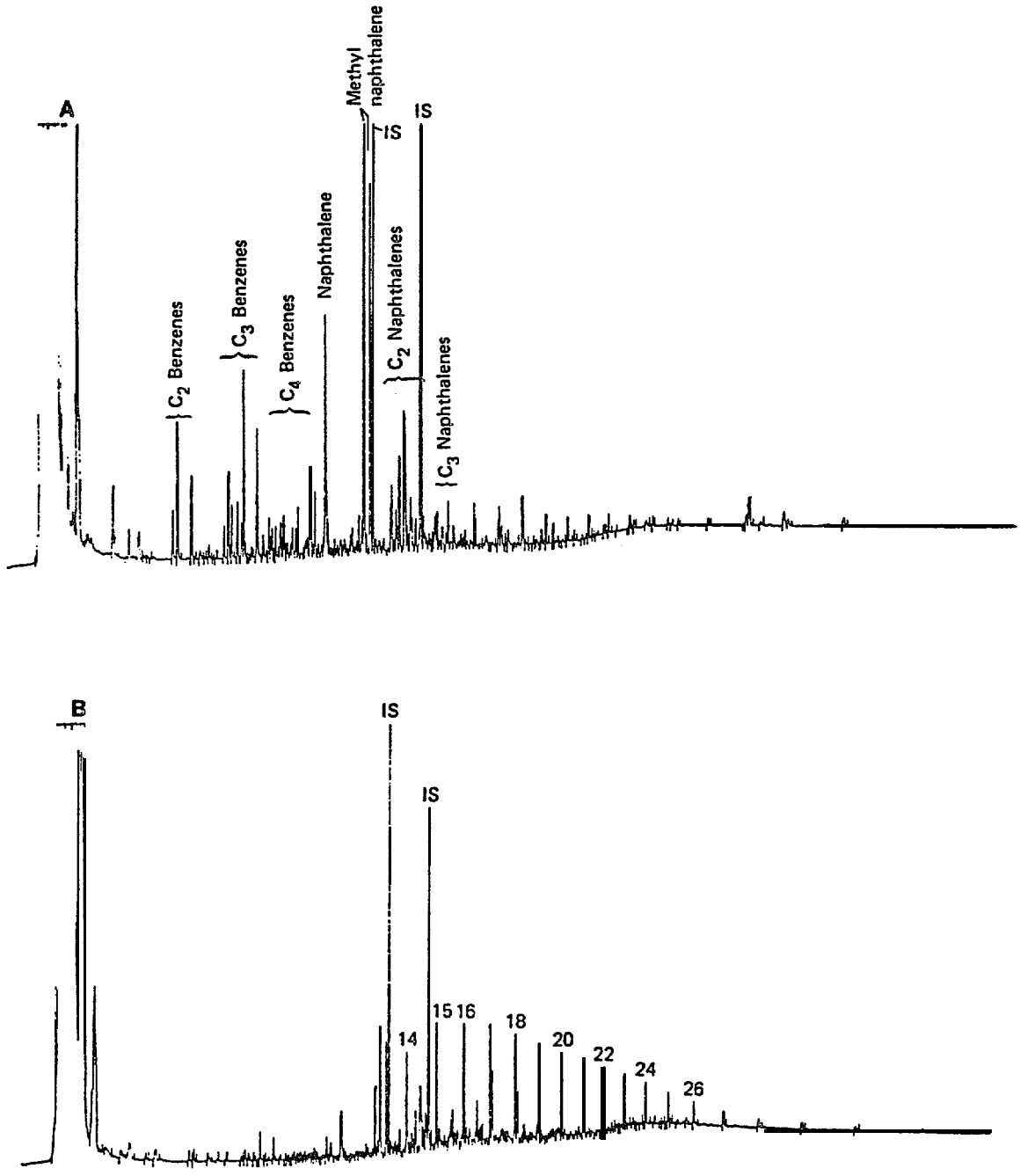

FIGURE 49. Gas chromatograms of the SWAF of PBCO after weathering for: A. 30 hr and B. 54 hr.

TABLE 54. Concentration of petroleum hydrocarbons in the SWAF of PBCO during exposure of chum salmon embryos and alevins.

Day of exposure	Hours oil weathered	Number of samples	Total hydrocarbon concentration in ppb ($\bar{x}$±SD)
1	30	10	730±540
2	54	10	420±480
3	78	9	450±51 o
4	102	5	70±30

Flatfish. Exposure of flatfish embryos to the SWAF of PBCO was initiated one day after fertilization at which time the oil had weathered 48 hr. A water sample taken from one rearing vessel at 72 hr (mid-incubation) indicated almost total loss of hydrocarbons (Fig. 50). At mid-incubation the SWAF was replaced with an aliquot of the original SWAF. The hydrocarbon concentrations found in the water at the start of oil exposure, half-way through incubation, and at time of hatching, are given in Table 55.

TABLE 55. Concentration of petroleum hydrocarbons in the SWAF of weathered PBCO to which English and sand sole embryos were exposed.

Species and treatment	Total hydrocarbon Concentration (ppb) Days post-fertilization 1	4	8	$\bar{X}$	Number of samples
Sand sole					
Control	2	4	13		3
Test 1	430	25(193)[a]	11	1:4	4
Test 2	200	9(91)[a]	15	79	4
English sole					
Control	4	10(2)[a]	4	5	4
Test	278	60(184)[a]	8	133	4

[a] Hydrocarbon concentrations of replacement aliquots of original SWAF, or control water, are in parentheses.

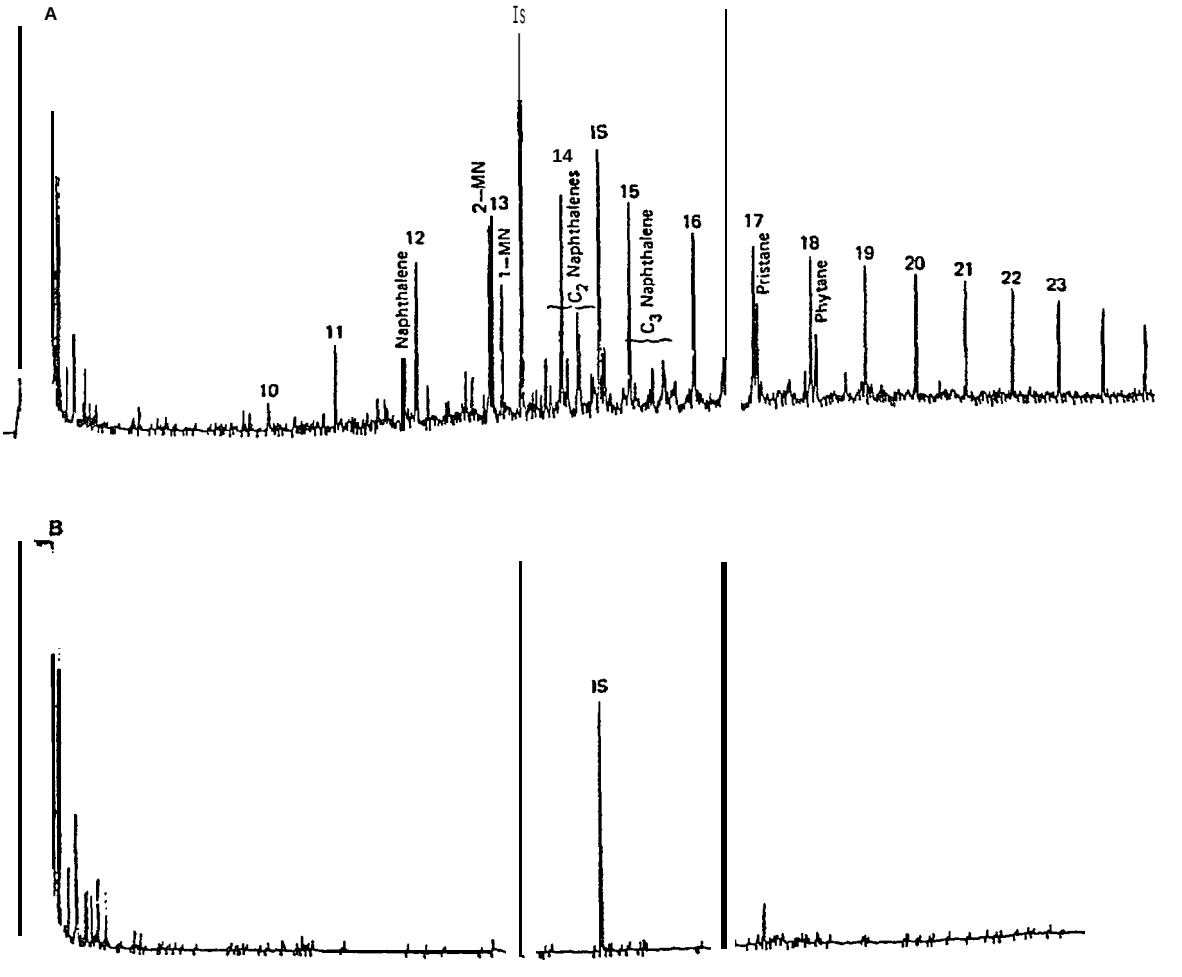

FIGURE 50. Gas chromatograms of the SWAF of PBCO used in exposure of flatfish embryos. A. 48 hr weathered PBCO sampled at initiation of experiment. B. Same SWAF as above sampled half-way through incubation 72 hr later.

Surf smelt. Experiments in which surf smelt embryos were exposed to the SWAF of weathered CICO were replicated, and the hydrocarbon concentrations found in water samples taken throughout each of the two tests are shown in Figure 51. Eggs collected in November were first exposed to the SWAF 4 days after fertilization; the oil having been weathered previously for 48 hr. The undiluted hydrocarbon concentration, as measured by GC analysis, averaged 324 $\pm$ 125 ($\bar{x} \pm$ SD) ppb for the first 7 days of exposure and then dropped to an average concentration of 77 $\pm$ 64 ppb for the remaining two weeks of the test. The overall undiluted hydrocarbon concentration throughout the 23 days of embryo exposure averaged 173 $\pm$ 152 ppb. The SWAF was also mixed directly with uncontaminated seawater to give diluted hydrocarbon concentrations, calculated from water flow as 53% and 25% of the original SWAF. This results in calculated average hydrocarbon concentrations for these dilutions of 92 and 43 ppb, respectively.

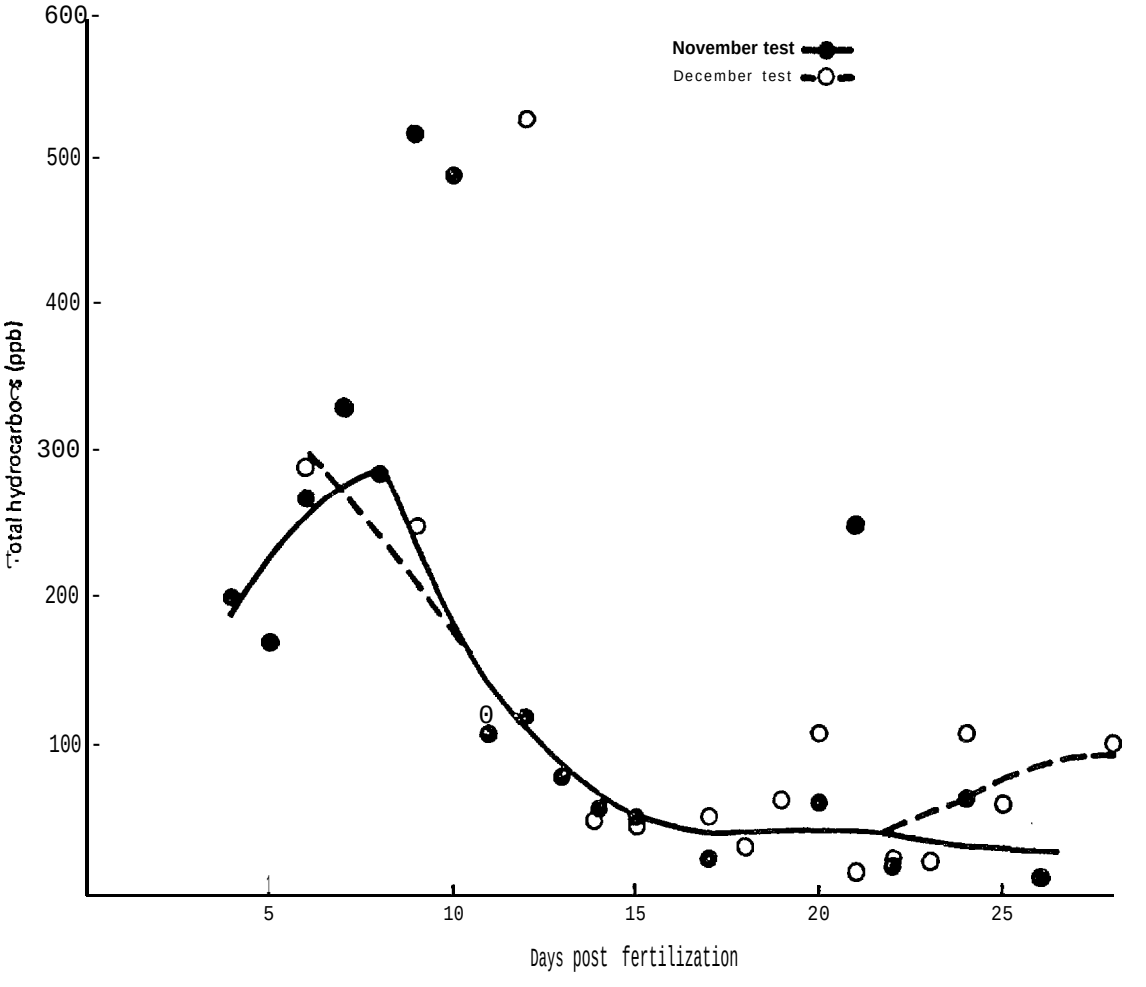

FIGURE 51. Total petroleum hydrocarbons in the SWAF of weathered CICO as related to time of surf smelt embryo exposure following fertilization. Circles represnt hydrocarbon concentration in water samples collected from the undiluted SWAF during each test (Nov. and Dec.). Corresponding solid and dashed lines represent smoothed data calculated by method of Cleveland (1979).

For surf smelt exposed to weathered CICO in December, the general trend of hydrocarbon concentration with time is similar to the November test (Fig. 51). After the oil had weathered for 8 days (12 days post-fertilization) the SWAF concentration in December dropped from an average of 298 $\pm$ 148 ($\bar{x}$ $\pm$ SD) ppb to an average concentration of 56 $\pm$ 32 ppb. The overall hydrocarbon concentration for the December test was 113 + 128. The calculated dilutions of the original SWAF for the December-test were 48% and 23% (54 and 26 ppb, respectively).

(2) Chum salmon. Mortality of embryos from oil-treated and control groups was evaluated at time of hatching (Table 56). Embryos treated for the full 75 day development period (122 hr exposure) showed a significant increase in mortality over controls (P=0.01).

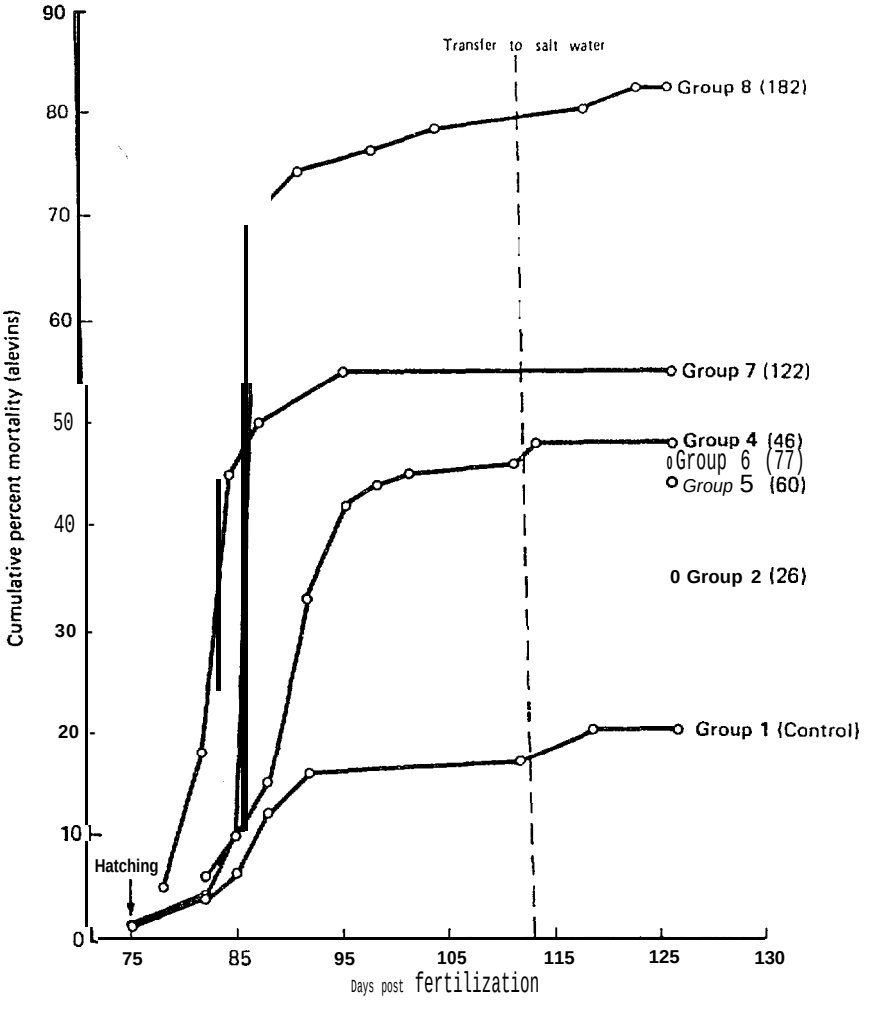

FIGURE 52. Cumulative percent mortality for alevin chum salmon exposed to the SWAF of" weathered PBCO as embryos and/or alevins. Refer to Figure 8 for exposure at stage of development (groups). Total hr of exposure for each group are given in parentheses,

All groups of chum salmon embryos began hatching 75 days after fertilization, and on day 76 in the oil-exposed groups, 50% to 95% of the viable embryos had hatched as compared to only 15% of the controls. Six days later the percent of viable embryos hatching in the controls still lagged behind the oil-exposed groups.

After hatching, 5 groups of chum salmon were held for 50 days to observe delayed mortalities. Exposure to the SWAF of weathered PBCO was continued in group 8, which had been exposed continuously since fertilization, and group 5, alevins which had not been exposed to the SWAF as embryos. In group 3, late alevin exposure (day 92 after hatching to day 113), there were no mortalities; the cumulative mortality for all other groups of alevins is shown in Figure 52. Generally, the

TABLE 56. Percent mortality of chum salmon embryos at time of hatching (75 days post-fertilization), and percent of viable eggs hatched on days 76 and 82.

Group(s)[a]	Exposure period (days post-fert.)	Hours exposed	Average percent mortality	Cumulative viable eggs 76	% hatch of on days 82
1,3,5	(control)	0	24	15	70
2	(14-26)	26	28	60	95
4	(1-26)	46	25	95	99
6	(26-75)	77	19	80	98
7,8	(1-75)	122	42	50	96

a See Figure 8, Section 5.3.3.(e)
b Data adjusted for 2.6% of eggs which were unfertilized.

longer the duration of exposure to the SWAF the lower the survival. Highest mortality occurred in group 8, which was exposed continuously from fertilization; in this group 70% of the alevins were dead 12 days after hatching as compared to a 12% mortality in controls. The second highest mortalities occurred in group 7. These were exposed continuously as embryos, but with no oil exposure as alevins; 50% of this group died within 12 days after hatching.

Alevins were removed from each trough 12 days after hatching . and examined for gross abnormalities. Three types of abnormalities were evident: malformed yolk sac, "dome" head, and kyphosis (angular curvature of the notochord).

The normal yolk sac is elliptical compared with the foreshortened and bulbous sac observed in some oil-exposed individuals. This abnormality occurred in the majority of oil-exposed groups, but was most frequent (61%) in those exposed continuously from fertilization (Table 57, group 8). Normally developing chum salmon alevins have heads with slight "dome" shape above the optic lobes of the brain (Distler 1954). Oil-exposed individuals had single- or double-"dome" heads which were much more pronounced than in Distler's illustrations or in our controls, and resembled lake charr (Salvelinus namaycush) alevins reared in water with low dissolved oxygen content (Balon 1980). Dissection of alevins with cephalic abnormalities revealed no evidence that the "dome" shape was a result of hydrocephaly. Kyphotic notochords occurred only in alevins exposed to oil continuously (143 hr); other groups did not exhibit any obvious notochord deformities.

Sixteen days after hatching, samples of 10 to 15 alevins from each group were fixed and stained to examine development of fin rays. Fin ray development in alevins exposed to the SWAF of weathered PBCO only during embryonic development was similar to controls; however, for those exposed to oil after hatching (groups 5 and 8) there appears to be an inhibition of ray development, particularly of the dorsal fin (Table 58).

TABLE 57. Gross morphological abnormalities observed in chum salmon alevins 12 days after hatching (87 days post-fertilization).

Group	Exposure period (days post-fert.)	Hours exposed	Sample Size	% abnormalities		
				Yolk sac	head	kyphosis
1	(Control)	0	204	0	0	0
2	(14-26)	26	15	0	9	0
3	(1 -26)	46	19	16	16	0
4	**(75-87)**	**21**	**10**	**10**	**33**	**0**
5	(26-75)	77	15	0	33	0
6	(l-75)	122	6	33	33	0
8	(1-87)	143	16	61	44	11

a Pictorial description of yolk sac and cephalic abnormalities are shown in Malins et al. (1980).

TABLE 58. Effect of weathered PBCO on fin ray counts of chum salmon alevins 16 days after hatching (91 days post-fertilization).

Group	Exposure period (days post -fert.)	Hours exposed	$\bar{X}$ Number of fin rays with calcification		
			Caudal	Anal	Dorsal
1	(Control)	0	17	7.8	8.3
2	(14-26)	26	18.7	11.2	9.2
4	(l-26)	46	12.5	8.7	6.3
5	(75-91)	29	10.0	1.2	0
6	(26-75)	71	20.1	12.7	10.9
7	(l-75)	122	15.0	11.7	6.0
8	(1-91)	151	5.0	3.5	0

[a] Following staining with alizarin red S.

A minimum of 10 alevins from each group were measured at termination of the experiment (127 days after fertilization). There were no differences in growth, as measured by total length, between control and oil-exposed alevins.

(3) Flatfish. Effects of the SWAF of weathered PBCO on English and sand sole embryos and larvae are given in Table 59 and summarized in Figure 53. Flatfish eggs and larvae were categorized using the following nomenclature:

Nondeveloped eggs: Consisted of two types of eggs which were indistinguishable as to the cause of nondevelopment--(l) nonviable eggs which were not successfully fertilized; (2) embryos which died in early cell division as a result of oil exposure or natural failure (embryo not formed).

TA8LE 59. Effects of the SWAF of weathered PBCO on egg, embryo, and larval development of English sole and sand sol e. Data were collected at time of hatching (end of 8 day exposure) and are reported in percent of total eggs introduced.

Species and hydrocarbon concentration (ppb)	Eggs		Embryo		Larvae			
	Number of eggs introduced	Non-developed (%)	Normal (%)	Abnormal or dead (%)	Normal (%)	Abnormal (%)	Grossly abnormal (%)	Dead (%)
English sole								
Control	348	13.5	0.9	0	74.7	5.2	0	5.7
133	372	22.0	0	3.0	0	0	64.5	10.5
Sand sole								
Controls	1469	7.4	0.1	0	89.7	1.8	0	1.0
164	365	15.6	0	4.9	0	43.6	22.2	13.7
79[a]	968	5.5	0.4	0.2	78.9	8.9	1.6	4.5

a Tests repeated and data pooled.

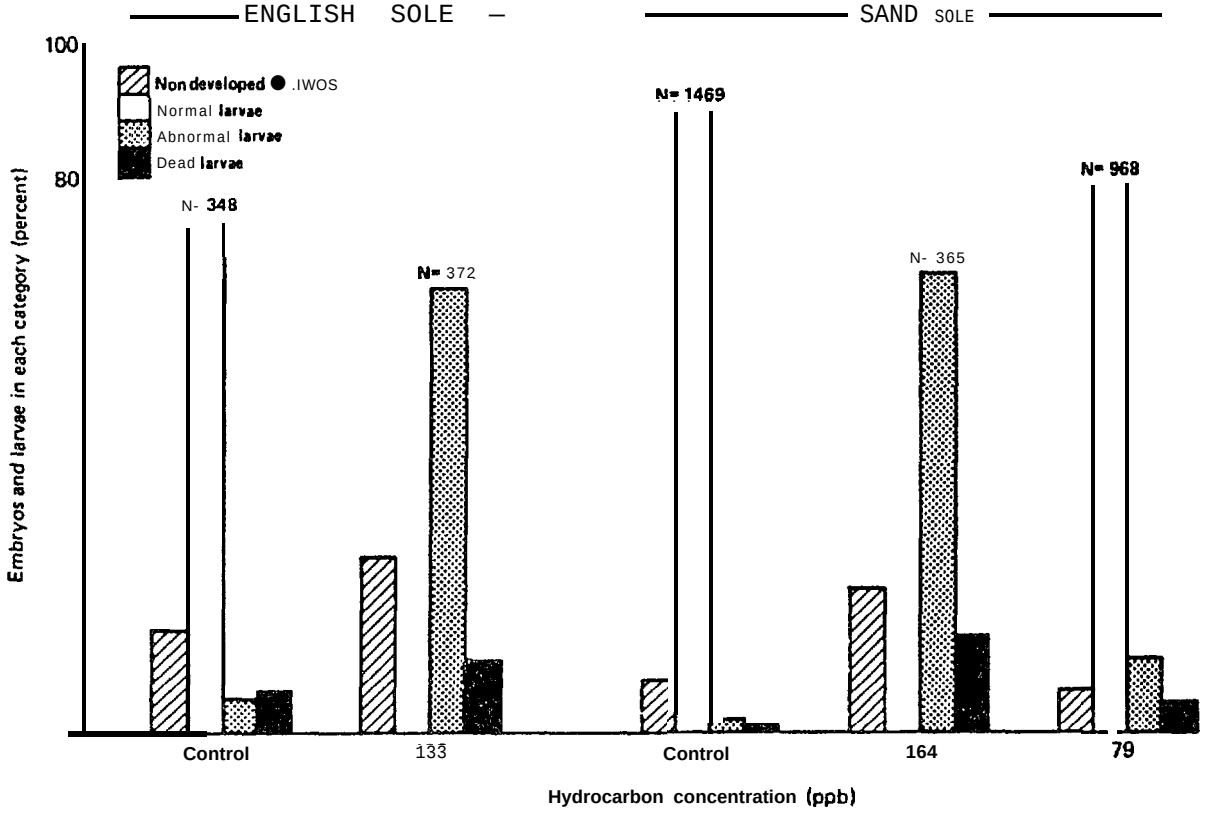

FIGURE 53. Hydrocarbon concentration of SWAF of PBCO and percent English and sand sole in each category at termination of tests. Data from Table 59 (% eggs and embryos, and abnormal larvae categories combined).

Normal embryo: Embryo transparent, with regular heart contractions.

Abnormal or dead embryo: Opaque, often scoliosis evident, and intermittent or no heart contractions.

Normal larvae: Regular heart contractions, notochord straight, finfold continuous, digestive tract complete, and pigmentation complete.

Abnormal larvae: Regular heart contractions, slight curvature of the notochord and entire body, generally a lateral curvature of up to 45°.

Grossly abnormal larvae: Regular heart contractions, but with body curvature exceeding 45° (some with notochord curvature of 180° and double 180° curvatures). Finfold deformed, digestive tract incomplete pigmentation not in patches but scattered, lying motionless on bottom, opaque.

Dead larvae: No heart contraction, generally opaque, and usually contorted.

A single test with English sole eggs indicated that exposure to an average hydrocarbon concentration of 133 ppb resulted in a high percentage of hatching, but all larvae were either abnormal or died shortly after hatching.

In control tests with sand sole an average of 90 ± 2% ($\bar{x}$ ± range) of the eggs hatched into normal larvae. Exposure to the SWAF at a concentration of 79 ppb resulted in an average of 79 + 2% normal larvae and only 10 ± 1% deformed larvae. At a SWAF concentration of 164 ppb embryos developed and hatched, but two-thirds of the larvae were deformed; the most common abnormality was scoliosis.

(4) Surf smelt. An accounting of all surf smelt embryos and larvae in the two replicate experiments was conducted when hatching of live control embryos appeared complete. These data are presented in Tables 60 and 61 along with the average hydrocarbon exposure concentrations. A majority of the eggs were unaccounted for at the end of both experiments, and as no intact embryos could be lost from the incubation baskets, this loss is attributed to early embryo death, and subsequent embryo disintegration; for controls this averaged 49 ± 3% ($\bar{x}$ ± range).

In both experiments the control embryos and those exposed to the lower hydrocarbon concentrations produced an equal number of live larvae (controls, 44 ± 4%; the 26 and 43 ppb exposure groups, 43 ± 1%). However, eggs exposed to the two higher hydrocarbon concentrations produced few live larvae with most dying in the embryonic stage (89 ± 9% as dead embryos or disintegrated eggs).

Cumulative hatching rates for both experiments are shown in Figures 54 and 55. Control larvae in the December experiment hatched approximately 6 days later than those in the November experiment, but the total percent hatching was similar. At an average hydrocarbon

TABLE 60. Effects of a 57 hr exposure to the SWAF of weathered CICO on hatching success of surf smelt embryos (November 1979 experiment). Oata compiled at completion of hatching in controls, and reported in percent of total embryos introduced.

Hydrocarbon concentration (ppb)	Embryos				Larvae	
	Initial no. (N)	Alive (%)	Dead (%)	Disintegrated (%)	Alive[a] (%)	Dead (%)
Control	440	3.2	3.6	51.9	40.6	0.7
43	425	0	3.0	52.1	42.2	2.7
92	420	9.6	13.8	66.1	9.1	1.4
173	425	1.7	47.1	50.5	0.7	0

[a] Live larvae were collected daily immediately after hatching, and the percent presented is cumulative.

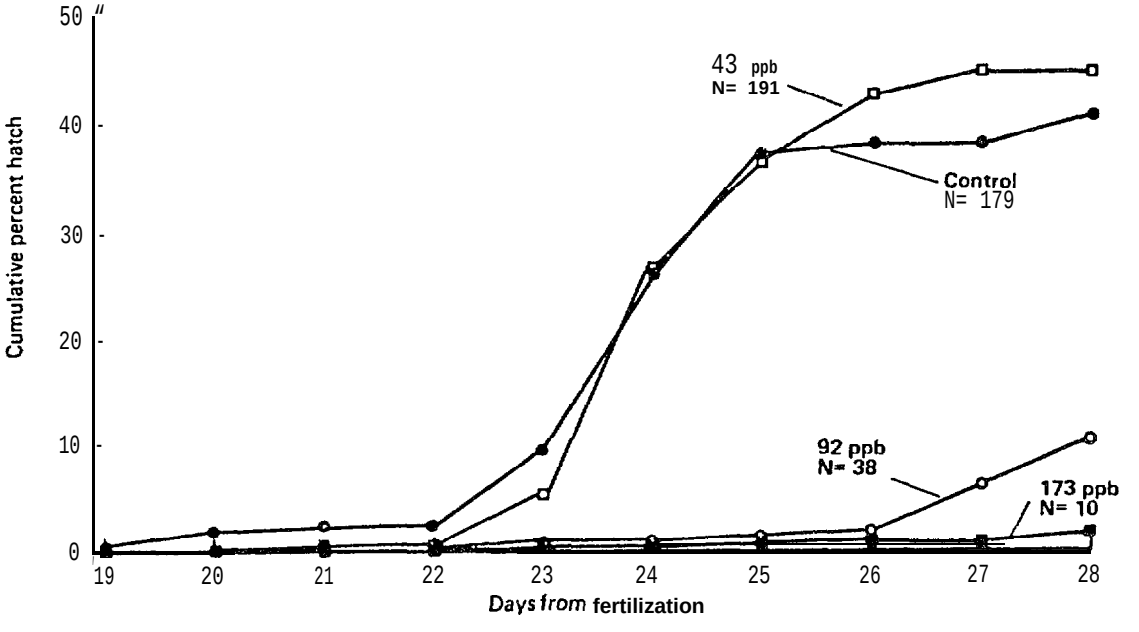

FIGURE 54. Cumulative percent hatching of surf smelt embryos exposed to 3 different concentrations of the SWAF of weathered CICO for a total of 57 hr (November 1979 experiment). Average hydrocarbon concentration for undiluted SWAF (173 ppb) was determined by GC analysis. The two lower SWAF concentrations (92 and 43 ppb) were calculated from proportional dilutions of the undiluted SWAF.

TABLE 61. Effects of a 63 hr exposure to the SWAF of weathered CICO on hatching success of surf smelt embryos (December 1979 experiment). Data compiled at completion of hatching in controls, and reported in percent of total embryos introduced.

Hydrocarbon concentration (ppb)	Embryos				Larvae	
	Initial no. (N)	Alive (%)	Dead (%)	Disintegrated (%)	Alive[a] (%)	Dead (%)
Control	461	0	2.2	46.2	47.7	3.9
26	458	0.7	12.9	28.6	43.2	14.6
54	455	4.0	12.8	71.1	5.9	6.2
113	445	0.7	4.9	86.8	4.0	3.6

a Live larvae were collected daily and held in uncontaminated seawater to evaluate post-exposure survival; the percent represented is cumulative.

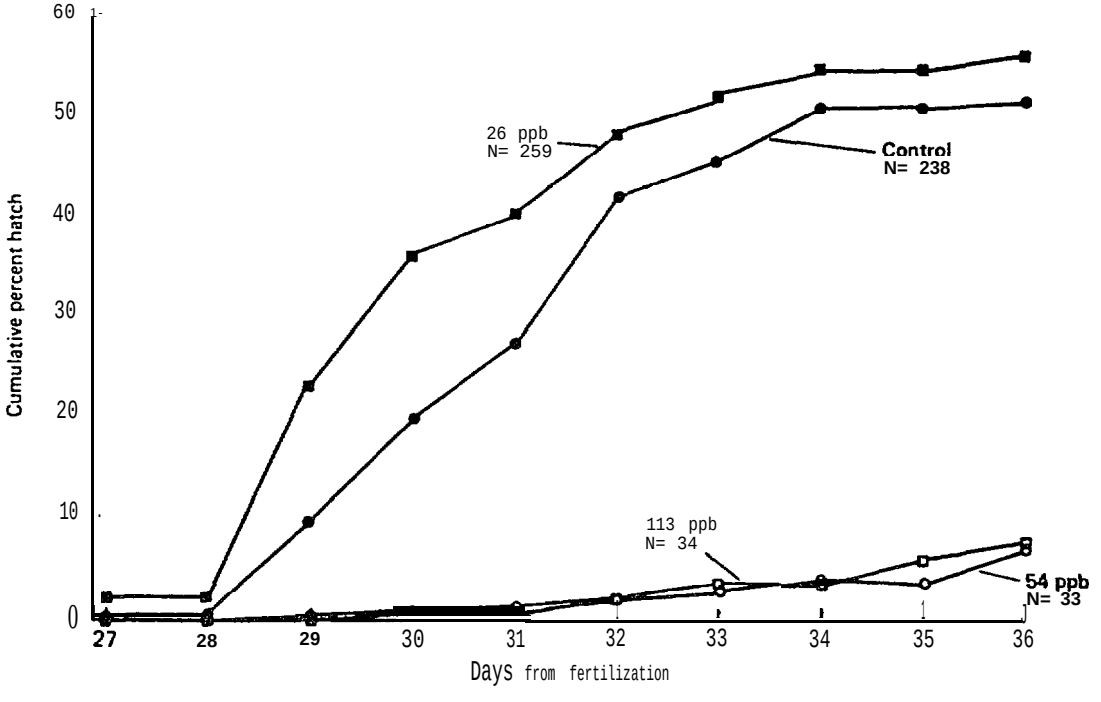

FIGURE 55, Cumulative percent hatching of surf smelt embryos exposed to 3 different concentrations of the SWAF of weathered CICO for a total of 65 hr (December 1979 experiment). Average hydrocarbon concentration for undiluted SWAF (113 ppb) was determined by GC analysis. The two lower SWAF concentrations (54 and 26 ppb) were calculated from proportional dilutions of the undiluted SWAF.

concentration of 26 ppb (December experiment) there was an apparent acceleration in hatching similar to that observed for chum salmon embryos exposed to the SWAF of weathered PBCO.

Samples of developing embryos were collected at two points during the December experiment (Table 62). On day 14 post-fertilization, mortality in control embryos was greater than in oil-exposed embryos; however, in embryos sampled one week later (approximately two-thirds of the way through incubation), this was reversed. The most appropriate statistical model (Geisser and Eddy 1979) for the 22 day post-fertilization data indicated that the percent abnormal and dead embryos in the control group and those exposed to 26 ppb petroleum hydrocarbons were similar, but differed from embryos exposed to 54 and 113 ppb. These latter two in turn differed from each other.

In the December 1979 experiment, three consistent types of gross abnormalities were observed in the oil-exposed embryos: reduced coiling (an index of growth by length); absence of eye rotation and closure of the choroid fissure; and diffused pigment. The most frequent abnormality was reduced coiling. As embryos develop, the body coils inside the chorion attaining a maximum of 2.5 coils just prior to hatching. All abnormal embryos, exposed to a hydrocarbon concentration of 113 ppb exhibited reduced growth, with only 1.5 coils after 22 days of development; the same number of coils was observed one week earlier in controls. Approximately 50% and 20% of abnormal embryos exposed to hydrocarbon concentrations of 54 and 26 ppb, respectively, also exhibited reduced growth after 22 days incubation. The eyes of affected embryos did not develop beyond the 15-day post-fertilization stage. Arrested eye development was the predominate abnormality in embryos incubated in 26 ppb total hydrocarbons.

Surf smelt embryos have a single row of contracted, dark, melanophores along the ventral midline, and smaller, stellate-shaped melanophores scattered over the ventral surface of the yolk. Fifty percent of the abnormal embryos exposed to 54 and 113 ppb had diffused pigment in these melanophores.

In order to observe any latent effects of oil exposure, newly hatched larvae from the December 1979 experiment were held in uncontaminated water, and fed rotifers (Brachionus plicatilus). After holding an average of 10 days (depending on time of hatching) survivors were counted (Table 63). Although the percent of normal appearing larvae hatching in the 26 ppb concentration of the SWAF was nearly identical to the control group, their survival as larvae was significantly less ($P<0.001$); only 8.7% survived compared with 42.5% in controls.

Surf smelt embryos from the December 1979 experiment were sampled 14 days after fertilization and whole eggs (embryo and egg envelope) were analyzed for petroleum hydrocarbons by GS/MS. Aromatic hydrocarbons found in control eggs and eggs exposed to the SWAF of weathered CICO are given in Table 64. Alkanes are commonly found in marine organisms, and prominent differences were not observed in concentrations from either oil-exposed or control eggs.

TABLE 62. Percent normal, abnormal, and dead surf smelt embryos sampled from the December 1979 experiment 14 and 22 days after fertilization (28 and 53 hr exposure).

Hydrocarbon concentration (ppb)	Sampling date (post-fertilization) 14 days (N)	Normal (%)	Dead (%)	22 days (N)	Normal (%)	Abnormal (%)	Dead (%)
Control	99	83	17	27	85	0	15
26	103	97	3	26	69	19	12
54	100	99	1	26	23	19	58
113	100	100	0	28	4	32	64

TABLE 63. Percent survival of surf smelt larvae hatched from embryos exposed to three concentrations of the SWAF of weathered CICO. Percent survival was determined 10 days after hatching.

	Control	Average hydrocarbon concentration (ppb) during embryo incubation 26	54	113
Original number of larvae introduced	219	196	23	18
Percent survival	42.5	8.7	8.7	5.6

TABLE 64. Concentrations of aromatic hydrocarbons in control surf smelt eggs and eggs exposed for 26 hr to the SWAF of weathered CICO.

Hydrocarbon	Hydrocarbon concentration in Nanograms/g(Wet Wt.) Control	Oil -exposed[a]
Naphthalene	33	22
2-Methylnaphthalene	<30	72
1-Methyl naphthalene	<15	46
Biphenyl	<35	37
2,6-Dimethyl naphthalene	<15	110
2,3,5 -Trimethyl naphthalene	<15	125
Fluorene	<15	66
Phenanthrene	<15	63

a Sample size of 99 eggs from control group and 100 eggs from 113 ppb oil-exposed group.

(f) Effect of Crude Oil Ingestion on Salmonid Reproductive Success

Fifteen of the 48 test fish died 1 to 3 months after spawning; all of these animals were heavily infected with fungus. None of the 12 controls were similarly affected.

Maturation and Reproductive Success. The first males were in spawning condition by mid-December 1975; the first females were ripe 2-3 weeks later (Fig. 56). Although the first ripe fish were from the test group, there appeared to be no pronounced acceleration or retardation of maturity related to petroleum exposure. Eggs were collected from ripe females starting in early January 1976, and collections continued weekly through mid-February.

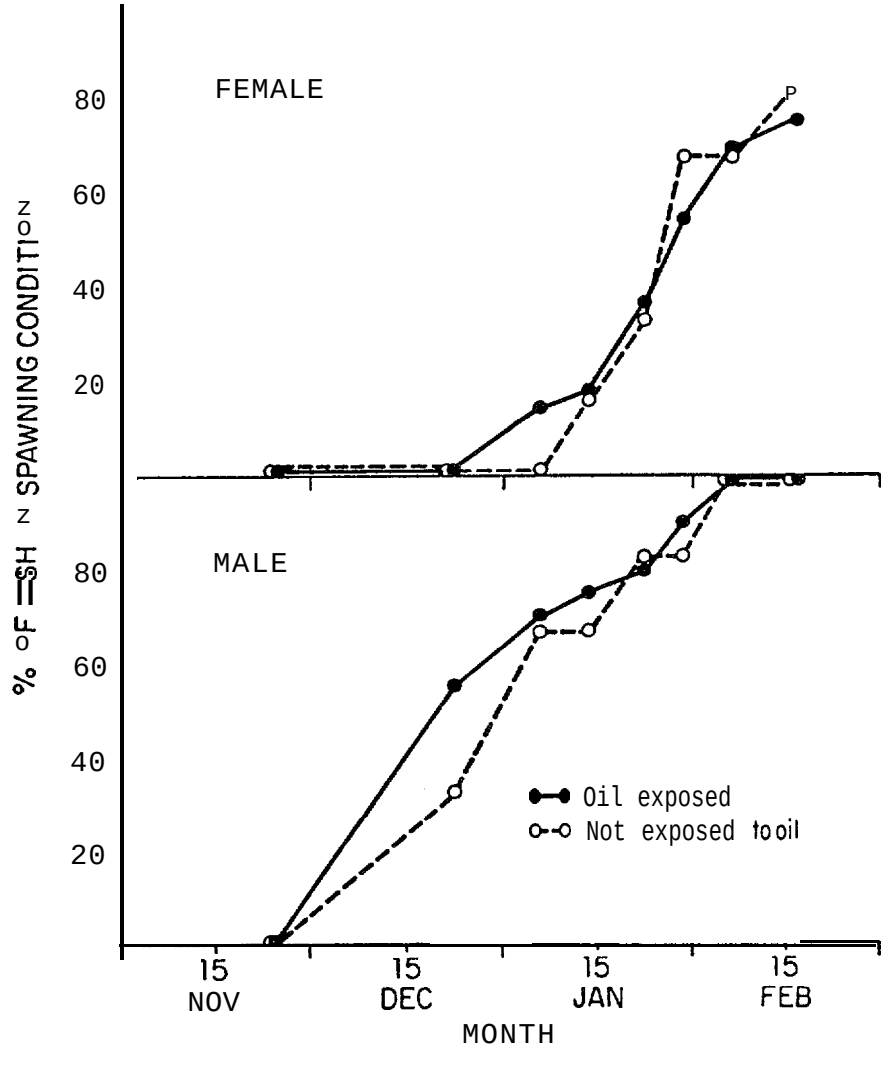

FIGURE 56. Timing of maturation for petroleum-exposed and non-petroleum exposed rainbow trout. (From Hodgins et al. 1977)

No significant difference (P=0.10) in hatching success (percent survival) among crosses in which sperm was used from petroleum-fed and control males was observed (Table 65). One particular cross did, however, result in very low survival (5.1%) and slightly lowered the average percent hatching success of eggs fertilized with sperm from control males.

Hatching success ranged from 32.4% to 99.5% for eggs from petroleum-exposed females and from 79.2% to 96.8% for control eggs (Table 66), but the respective means of 86.4% and 90.3% were not significantly different (P=0.10). Lowered survival of eggs from 2 test females reduced the average survival of the test group.

Average survival of alevins was higher, although not significantly (P=0.10), for control than for test fish (Table 67). Again, low survival occurred in one petroleum-exposed group.

Chemical Analyses. In using the spectrofluorometric method of analysis, interference from non-hydrocarbon fluorescing compounds prevented precise quantitation of PBCO in adult trout muscle and eggs; only qualitative and semi-quantitative results were possible. An emission maximum (364 nm) superimposed on the background of fluorescing compounds was observed for all samples from fish fed PBCO; this maximum was not observed for any of the samples from fish fed the control diet (Fig. 57). The ratios of the average relative intensities at an excitation wavelength of 262 nm and an emission wavelength of 364 nm of petroleum-fed fish to

TABLE 65. Survival of eggs fertilized with sperm from petroleum-exposed and non-petroleum exposed male rainbow trout.

Female	% Survival through hatching	
	Crossed with petroleum-exposed males	Crossed with non-petroleum-exposed males
Non-petroleum exposed	96.8	96.7
	81.3	77.3
	95.7	96.3
	89.9	87.7
	88.5	94.4
Petroleum exposed	99.4	99.6
	98.2	98.1
	98.6	95.2
	95.9	95.2
	36,9	37.3
	58.9	5.1
	95.8	98.3
	94.7	94.5
	78. 5[a]	72.5[b]
	$\bar{x}$ = 86.4	$\bar{x}$ = 82.0
	SD = 18.0	SD = 27.7

[a] Pool of eggs from two females
b Pool of eggs from three females.
(From Hodgins et al. 1977)

control fish for muscle tissue and eggs were 2.8:1 and 3.8:1, respectively. A total of 14 analyses of muscle and 7 analyses of eggs from petroleum-fed fish and 4 analyses of muscle and 2 of eggs from control fish were performed. The background of fluorescing compounds was sufficiently high for the control and petroleum-impregnated food so that no definitive results could be obtained via spectrofluorometry.

TABLE 66. Survival of eggs through hatching from petroleum-exposed and non-petroleum exposed female rainbow trout.

% Survival for eggs from non-petroleum-exposed trout	% Survival of eggs from petroleum-exposed trout
96.8	96.8
79.2	98.4
96.0	98.9
88.4	9B.5
91.3	99.5
	9B. 1
$\bar{x}$ = 90.3	97.0
SD= 7.1	95.6
	37.2
	32.4
	97.0
	94.6
	75.6[a]
	89.8[b]
	86.5[c]
	$\bar{x}$ = 86.4
	SD = 21.9

a Pool of eggs from two females.
b Pool of eggs from three females.
c Pool of eggs from four females.
(From Hodgins et al. 1977)

TA8LE 67. Survival of alevins from petroleum-exposed and non-petroleum-exposed female rainbow trout.

% of offspring surviving from hatching to swim-up	
Non-petroleum exposed	Petroleum exposed
99.0	97.4a
99.4[a]	96.2[a]
89. 1	90.3a
76.3	87.3[c]
	81.1[a]
$\bar{x}$ = 91.0	79.8[b]
SD 10.9	74.1
	68.0
	61.7[a]
	25.0[a]
	$\bar{x}$ = 76.1
	SD = 21.4

a Pool of offspring from two females.
b Pool of offspring from three females.
c Pool of offspring from four females.
(From Hodgins et al. 1977)

6.3.2 Invertebrate Studies

Defense Behavior of Sea Urchins Exposed to Petroleum Hydrocarbons.

Aromatic hydrocarbons were found to markedly inhibit the chemosensory-mediated defense response of green sea urchins at concentrations in the low ppb range. The effect of the SWSF of PBCO and its major identified monocyclic aromatic hydrocarbon components are shown in Figures 58-60.

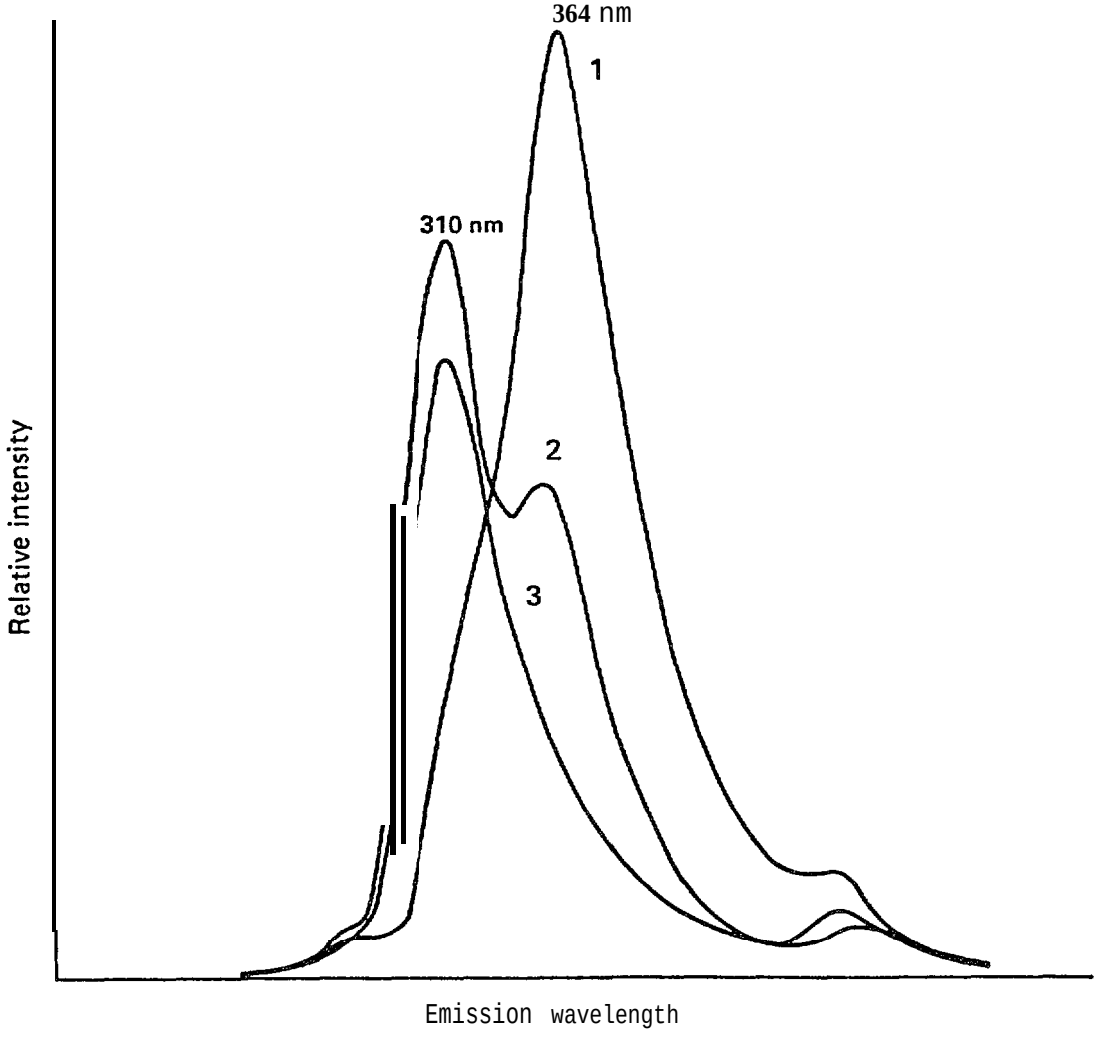

FIGURE 57. Spectrophotofluorometric curves of PBCO and extracts of trout eggs.
No. 1 - Prudhoe Bay crude oil
No. 2 - Oil-fed fish egg extract
No. 3 - Control fish egg extract
(From Hodgins et al. 1977)

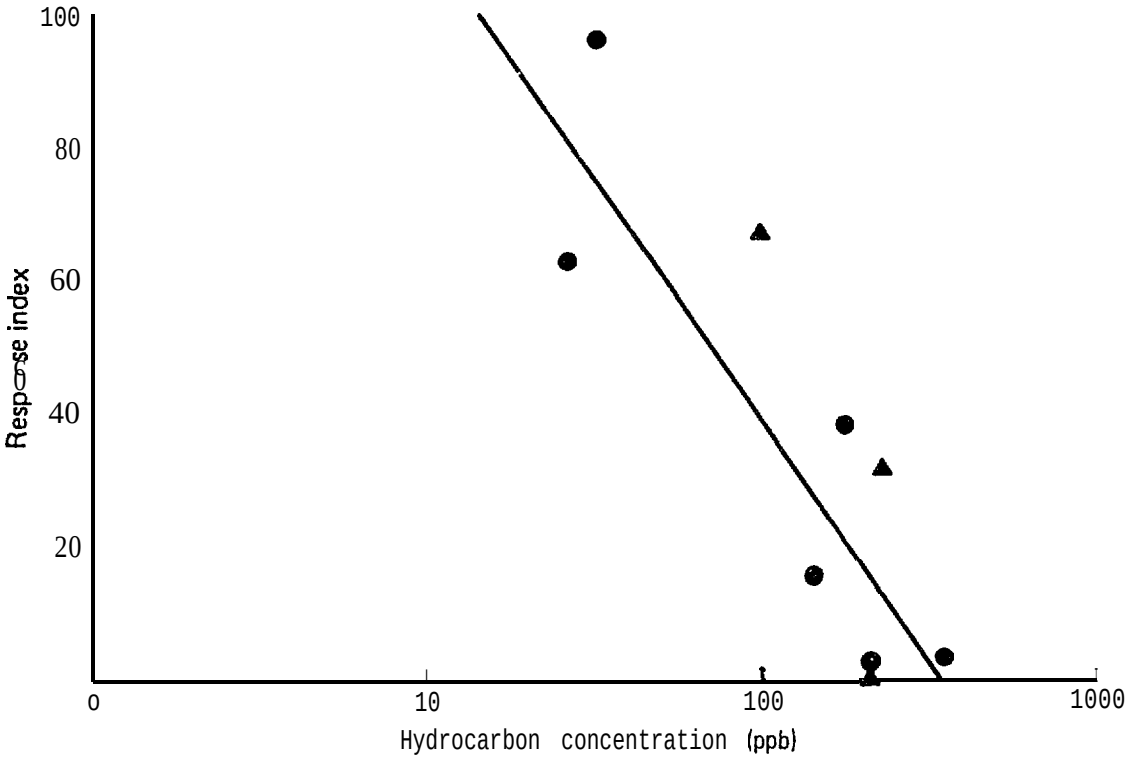

FIGURE 58. Effect of the SWSF of PBCU on the pedicellarial defense response of green sea urchins. ● = 24 hr exposure; ▲ = 10 min exposure. The 10 min exposures are not included in calculation of regression line: Y = 182 - 72 $\log_{10}$ (ppb SWSF); r = -0.88.

The relationship between the logarithm of the hydrocarbon concentration and the resulting response index was apparently unaffected by the duration of exposure to the hydrocarbons. In general, 10 min exposure periods produced inhibitions of the defense response equal to those following 24 hr exposures. Thus, it can be assumed that the urchins, in terms of their assayed defense behavior, equilibrate with the hydrocarbons very quickly.

Based on the calculated regression given in Figure 58, the PBCO SWSF concentration at which the pedicellarial response is inhibited by 50% (EC_{50}) is 60 ppb. The EC_{50} for toluene, the major constituent of the SWSF, is 200ppb. For o-xylene, ethylbenzene, and trimethylbenzene, the EC_{50}'s are 350, 400, and 350 ppb, respectively. [1]

[1] Analysis of dissolved benzene concentrations were insufficient to Permit a regression analysis. Benzene analyses that were completed averaged 194 ± 62 ppb ($\bar{x}$ + range) and the corresponding average pedicellarial response indices-were 32% for 24 hr exposures and 32% for 10 minute' exposures.

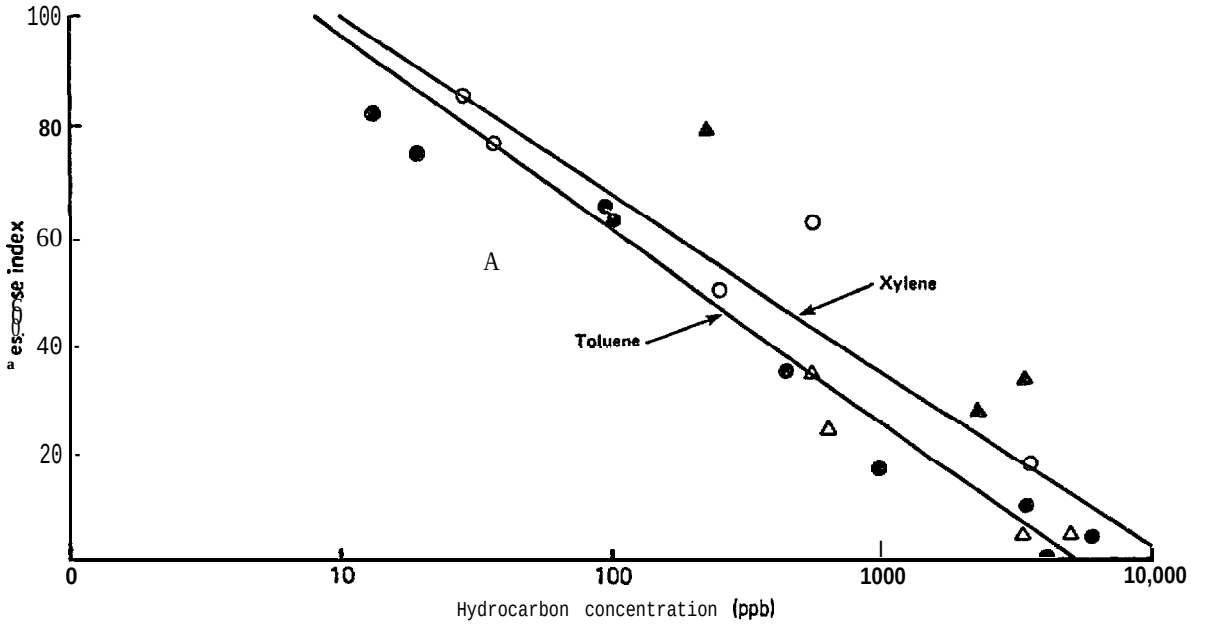

FIGURE 59. Effect of toluene and o-xylene on the pedicellarial defense response of green sea urchins. Closed circles and triangles are for toluene exposure, and 10 min exposures are not included in calculation of regression lines. e = 24 hr toluene exposure; ▲ = 10 min exposure; Y = 133 - 36 $\log_{10}$ (ppb toluene); r = -0.98; o = 24 hr xylene exposure; Δ = 10 min exposure; Y = 131 - 32 $\log_{10}$ (ppb xylene); r = -0.89.

Four groups of 5 sea urchins each were also exposed to 1-methylnaphthalene for either 1 or 24 hr. Although naphthalenes were usually detected in the SWSF of PBCO, their concentrations normally amounted to only 3% or less of the total SWSF. The effect of l-methylnaphthalene on the pedicellarial response is shown in Figure 61. The EC_{50} (60 ppb) is similar to that found for the SWSF of PBCO. It is doubtful that naphthalenes are responsible for the effects noted in the SWSF tests since their concentrations in the SWSF were generally less than 2 ppb; such a concentration would probably not inhibit pedicellarial response.

In order to determine the competence of the pedicellariae to respond, 0.5 ml isotonic KC1 was injected into the body cavity of 3 groups of 5 sea urchins each. Injections of KC1 into the control group, which previously showed an average 64% response to starfish extract, caused 100% opening of the pedicellariae. In the two parallel groups of urchins which had been exposed for either 10 min or 24 hr to 210 ppb of the SWSF an average of 93% responded. These tests indicate that pedicellariae which demonstrated reduced behavioral sensitivity as a result of hydrocarbon exposure are fully competent to respond if artificially provoked.

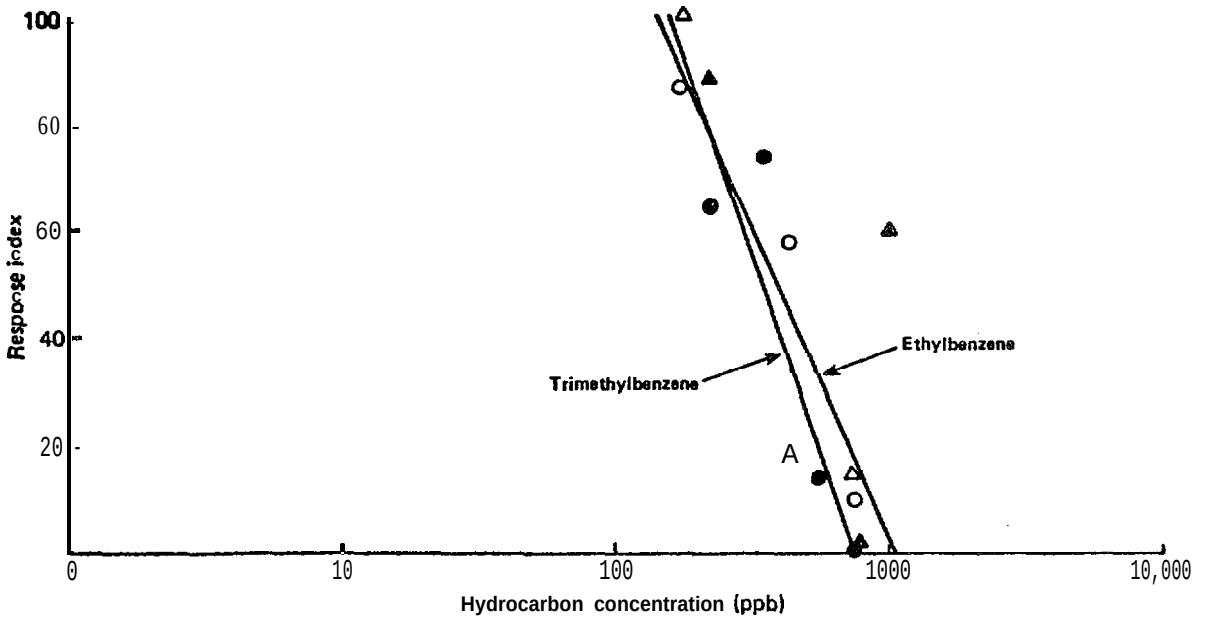

FIGURE 60. Effect of 1,2,4-trimethylbenzene and ethylbenzene on the pedicellarial defense response of green sea urchins. Closed circles and triangles are for trimethylbenzene exposure, and 10 min exposures are not included in calculation of regression lines. ● = 24 hr toluene exposure; ▲ = 10 min exposure; Y = 429 - 148 $\log_{10}$ (ppb trimethylbenzene); r = -0.88; o = 24 hr ethylbenzene exposure; △ = 10 min exposure; Y = 363 - 120 $\log_{10}$ (ppb ethylbenzene); r = -0.96.

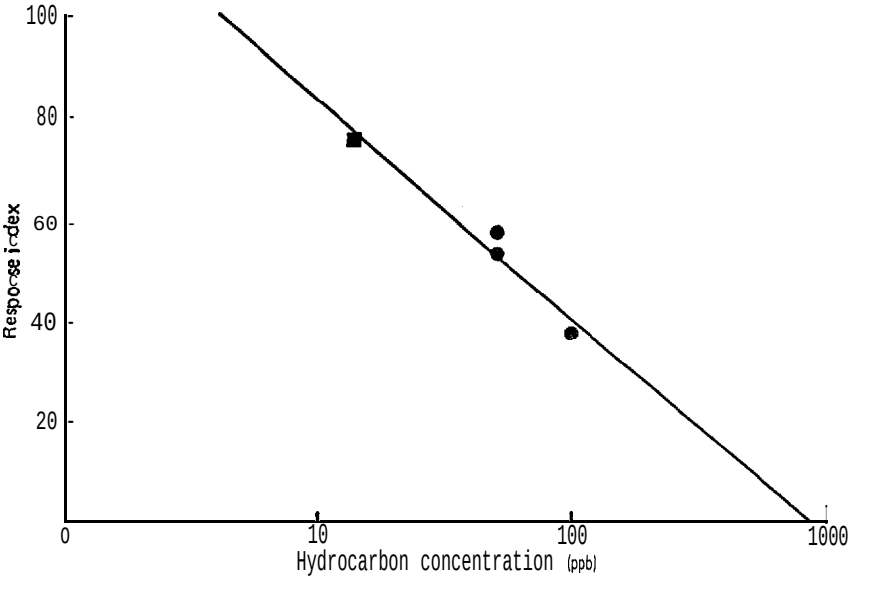

FIGURE 61. Effect of l-methyl naphthalene on the pedicellarial defense response of green sea urchins. ● = 24 hr exposure; ■ = 1 hr exposure: Y = 128 - 44 $\log_{10}$ (ppb methyl naphthalene); r = -0.98.

Hydrocarbon-induced inhibition of the pedicellarial response was found to be reversible if the urchins were allowed to depurate in clean, flowing seawater. The results of 3 assays involving various deputation periods and hydrocarbon treatments are shown in Figure 62. These data show that the hydrocarbon-induced pedicellarial inhibitions diminished much more slowly than they ensued.

In addition to the assays of defense behavior considered above, numerous observations of hydrocarbon-treated urchins were made concerning other types of activity. In general, urchins exposed to less than 1 ppm hydrocarbon were able to feed, respond to touch, adhere to the glass substrate, right themselves, and move about in an apparently normal fashion. No urchins were killed by the hydrocarbon treatments employed in this research, and narcosis was generally evident only at hydrocarbon concentrations approaching or exceeding 1 ppm.

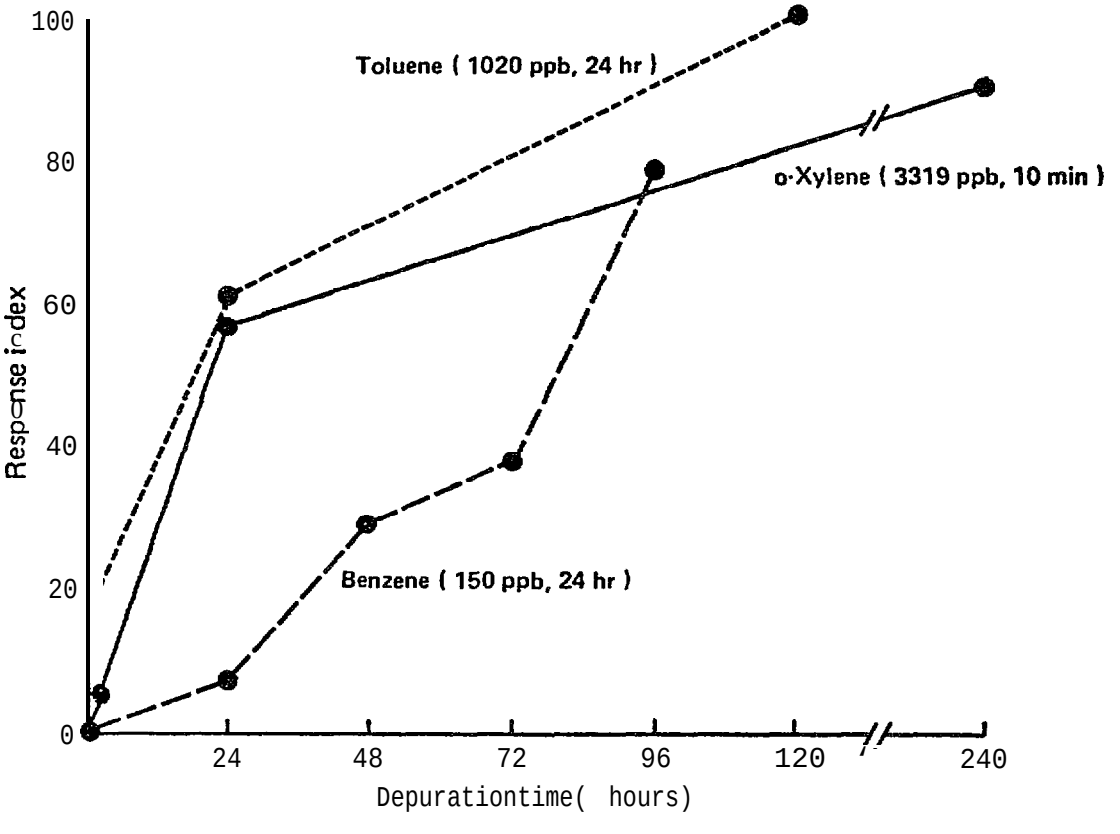

FIGURE 62. Recovery of the pedicellarial defense response as a function of deputation time in clean flowing seawater. Points represent average response index for each group of five green sea urchins. Lines are labeled with hydrocarbon treatments administered to each group.

In predation experiments equal numbers of control urchins and urchins exposed to the SWSF of PBCO were placed into a tank with three Pycnopodia. The results showed that more hydrocarbon-treated urchins were eaten than untreated controls in each of the four trials (Table 68). The combined data for each of these trials indicates that predation of PBCO-treated urchins was significantly greater than on control urchins (P=0.01).

Reproductive Behavior of Dorid Nudibranchs Exposed to Petroleum Hydrocarbons

(a) Chemosensory disruption. Prior to testing of individual dorid nudibranchs for their behavioral response to an aggregate of conspecifics, the individuals were exposed" for 24 hr to-3 different concentrations of the SWSF of PBCO; 15 ± 3 ppb ($\bar{x}$ ± range), 60 ± 20 ppb, and 420 ± 254) ppb. There was no direct relation between hydrocarbon concentration and chemotatic response; thus, the data are combined (Table 69).

TABLE 68. Effect of 24 hr exposure to a SWAF of PBCO on predation of sea urchins by Pycnopodia.

Exposure concentration (ppb)	Trial duration (hr)	Treated		Control	
		N	% eaten	N	% eaten
470	2	7	100	7	43
359	2	5	100	5	40
233	24	5	60	5	40
145	2	5	100	5	60
$\bar{x}$ = 302			88		49

TABLE 69. Chemotatic response of control and SWSF exposed (15 to 420 ppb) dorid nudibranchs to an aggregate of reproductive conspecifics in "stimulus" chamber.

Treatment	Number tested	Percent movement		
		Toward "stimulus" chamber	Toward "blank" chamber	No choice
Control	19	84	5	11
24 hr exposure	24	62	33	4

The percentage of nudibranchs moving into the "stimulus" chamber (toward the nonspecific aggregate) after exposure to the SWSF of PBCO was significantly different from that of controls (P=0.05). Their mobility (denoted by the number that did not make a choice) was not affected by exposure to petroleum hydrocarbons. For these tests a 50-50 distribution is interpreted as random movement.

(b) Embryological development. Exposure of mature dorid nudibranchs to increasing concentrations of the SWSF of PBCO resulted in two microscopically measurable effects: a delay in egg laying in the highest exposure group, and an immediate and long-term decrease in the total weight of eggs laid (Table 70). The egg laying delay was not a factor in total weight of eggs deposited after 12 days since all groups had sharply declined oviposition by the end of the 18 day experiment.

Microscopically, exposure of mature dorids and their subsequent spawn to the SWSF of PBCO resulted in a direct relationship between the number of abnormal embryos and SWSF concentration (Fig. 63). There was no evidence of an increase in embryo abnormalities with duration of exposure to the SWSF at any concentration tested. Nearly half (44%) of the abnormalities observed in eggs spawned by adults exposed to 225 ppb did not have capsules. This type of abnormality was never observed in control samples, and occurred at a frequency of only 0.7 and 1.1% in the 7 and 22 ppb exposure groups, respectively.

Hatching commenced 9 to 10 days after egg deposition and was completed by the llth day in all groups except the one exposed to the highest SWSF concentration. Embryos exposed to 225 ppb of the SWSF exhibited a marked delay in development, with only 25% of those which appeared normal reaching the shelled stage; none were observed to hatch after 11 days of development.

TABLE 70. Effect of the SWSF of PBCO on egg deposition of dorid nudibranches. Each exposure group contained 20 mature nudibranches with a total wet weight of 2.1 g per group.

Hydrocarbon concentration (ppb)	First egg masses laid			Total egg mass weight over 12 days (mg)
	Time after start of adult exposure (days)	Number of egg masses	Wet weight of masses (mg)	
Control	1	16	370	938
7[a]	1	14	320	832
22a	1	10	345	821
225	6	5	185	516

a Concentrations based on dilutions of highest concentration which was 225±80 ($\bar{x}$±SD, N=4).

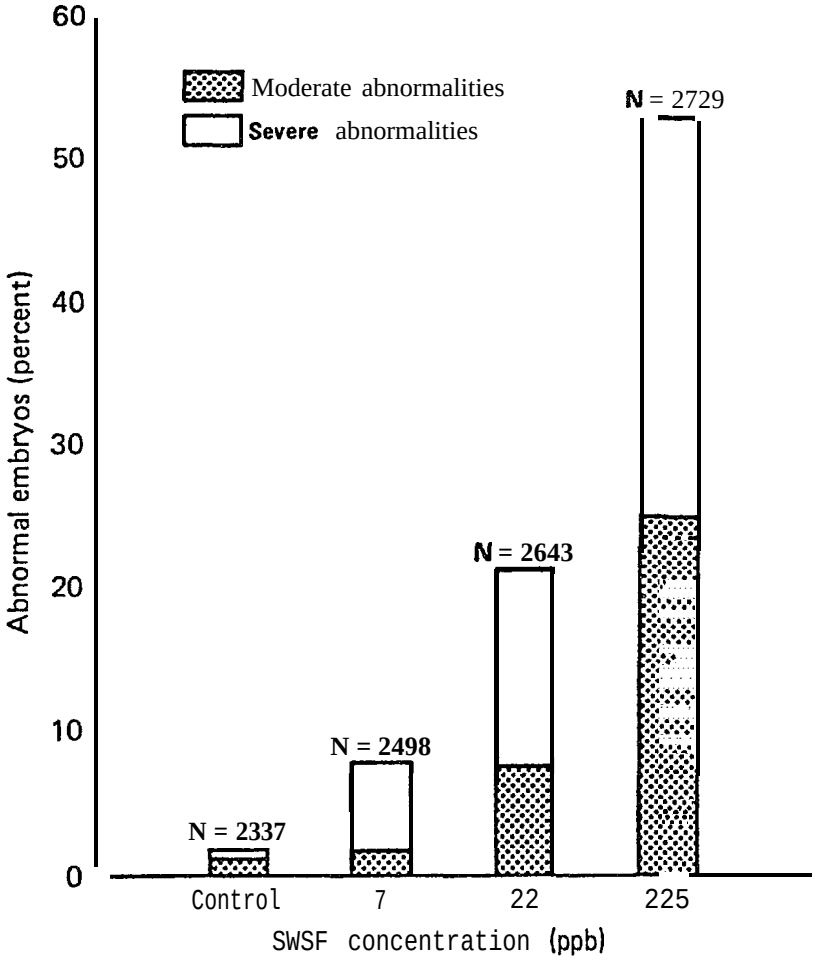

FIGURE 63. Effect of the SWSF of PBCO on embryonic development of the dorid nudibranch. Percent abnormal embryos averaged from all samples taken in first 10 days of exposure.

Feeding Behavior of Shrimp Exposed to Petroleum Hydrocarbons

Results of experiments on the feeding response of spot shrimp indicate that exposure to the SWSF of PBCO causes a decrease in feeding activity, particularly those activities involving searching and contact with the stimulus source (Fig. 64). Both of these higher order behaviors show an EC_{50} of about 25 ppb.

These results represent a composite of observations taken over a 6-day exposure period at each SWSF concentration. Generally there was not a progressive increase or decrease in searching or feeding behavior with time. At the highest concentration several of the shrimp exhibited a loss of equilibrium following 3 days' exposure; observations on these shrimp are not included.

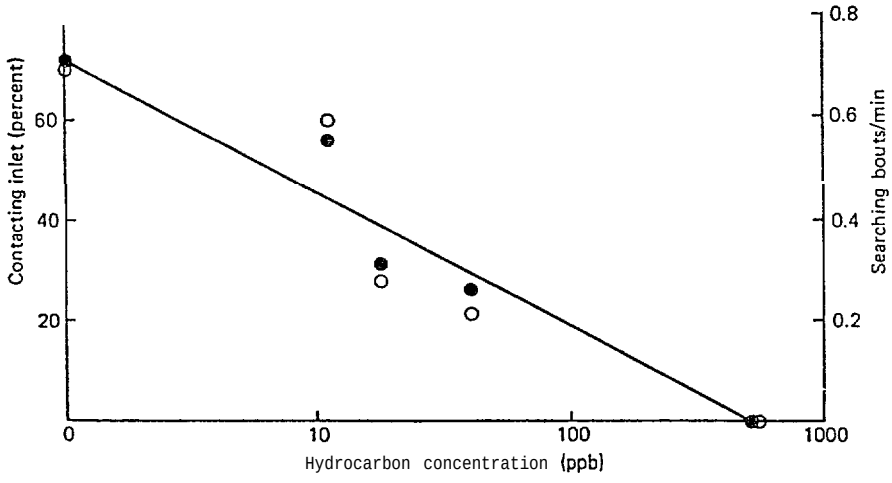

FIGURE 64. Effect of the SWSF of PBCO on pereopod searching behavior (bouts/rein) and percent of spot shrimp contacting stimulus inlet in response to squid extract. Each point reflects response to stimulus minus background activity. Regression line based on data of percent shrimp contacting stimulus inlet (closed circles): $Y = 73 - 27 \log_{10}$ (ppb SWSF); $r = -0.97$. For searching behavior (open circles): $Y = 0.72 - 27 \log_{10}$ (ppb SWSF); $r = -0.93$.

7. DISCUSSION

7.1 Chemistry

7.1.1 Accumulation and Biotransformation of Specific Aromatic Hydrocarbons in Salmonids

Results (Roubal et al. 1977a) indicated that benzene, NPH, and anthracene are readily deposited in coho salmon tissues. Concentrations of these compounds increased in tissues, such as liver and brain, in the order of benzene < NPH < anthracene. This finding suggests that within certain molecular weight ranges, the accumulation of aromatic hydrocarbons in salmonid tissues may be directly related to the number of benzenoid rings in the molecule; however, as discussed below, the degree of alkyl-group substitution on the parent hydrocarbon also appears to influence hydrocarbon concentrations in tissues of fish (Roubal et al. 1978).

The hydrocarbon metabolizes appeared in considerable amounts in all tissues examined, including the brain, of coho salmon. A substantial decline in hydrocarbon concentrations over time was accompanied by a steady increase in the proportions of metabolic products in all tissues examined.

This study showed that metabolizes of NPH formed by salmonids are similar to those formed by mammals (Boyland and Solomon 1955, 1956, Terriere et al. 1961, Sims 1964). Moreover, the study showed for the first time, that conjugated metabolizes of NPH were formed in fish.

7.1.2 Accumulation of Petroleum Hydrocarbons by Fish Exposed to SWSF of PBCO

Roubal et al. 1978 reported that substituted benzenes, NPH, and substituted NPH accumulated both in coho salmon and starry flounder when the fish were exposed to the SWSF of PBCO. Moreover, as the number of alkyl substituents increased on benzene and naphthalene, so did the accumulation of these compounds.

The C_4- and C_5-substituted benzene fraction of PBCO was the most prominent fraction accumulated in muscle of both coho salmon and starry flounder. However, substantially greater concentrations of this fraction accumulated in the muscle of starry flounder than in muscle of coho salmon. Hydrocarbons of the SWSF were not detected in the liver and gills of the test coho salmon. However, substantial amounts of the substituted benzenes, NPH, and substituted NPHs were found in the liver and gills of starry flounder. The ability of starry flounder to bioconcentrate aromatic hydrocarbons suggests that starry flounder residing on sediments contaminated with petroleum hydrocarbons may accumulate substantial concentrations of petroleum hydrocarbons in various tissues.

7.1.3 Metabolism of NPH by Coho Salmon

This study (Collier et al. 1978) represents the first use of HPLC for characterizing NPH metabolizes in fish. The presence of both non-conjugated and conjugated derivatives in the liver and gall bladder indicates that coho salmon have a significant ability to metabolize NPH and excrete the metabolizes. However, the question still remains to what extent low levels of these metabolizes are retained for extended periods in tissues of salmonid fish.

7.1.4 NPH and Its Metabolizes in Fish Skin and Mucus

Skin, The results (Varanasi et al. 1978) show that within a few hwrs of NPH exposure, appreciable concentrations of both the hydrocarbon and its metabolizes were present in the skin of coho salmon, starry flounder, and rainbow trout, regardless of the mode of exposure or the salinity of the medium. Moreover, comparison of these results with those obtained for liver of rainbow trout (Collier et al. 1980) and starry flounder (Varanasi et al. 1979) reveals that the pattern of uptake and release of NPH and its metabolizes in skin was similar to that observed for the liver. NPH concentrations in skin declined more rapidly than the concentrations of NPH metabolizes, demonstrating a tendency of skin to preferentially retain metabolizes. This finding is in agreement with results of Lee and coworkers (1976), Sanborn and Malins (1977), and Roubal et al. (1977a), which showed that the parent hydrocarbon was more rapidly discharged either directly or via biotransformation and that small but detectable concentrations of metabolizes persisted in tissues of crustaceans and fish over a long period. Whether metabolic products detected in the skin of our test fish arise from the direct transport of these compounds by blood to the skin and/or from biotransformation of NPH by AHM that may be present in the skin

remains to be seen. It should be noted that maxima in concentrations of both NPH and its metabolites in the skin were preceded by the corresponding maxima in the livers (Varanasi et al. 1978, Collier et al. 1980). It appears therefore that injected or ingested NPH was first transported to the liver and subsequently transported to the skin via the blood stream.

Mucus. Epidermal mucus of fish serves as a physical and perhaps an immunological barrier to pathogenic organisms (Harris and Hunt 1973). It is also believed to be associated with osmoregulation (Jakowska 1963) and is an important factor in controlling the swimming speed of the fish (Rosen and Cornford 1971). The results (Varanasi et al. 1978) from the force-feeding and injection studies, which showed that NPH and its metabolic products were present in epidermal mucus of rainbow trout for several days after the initial exposure to NPH, strongly suggest that epidermal mucus of salmonids is involved in excretion of hydrocarbons and their metabolizes. Epidermal mucus is in constant flux; its relative importance in the excretory mechanisms of fish would depend on the rate of discharge of these compounds from the mucus into the surrounding water as well as on the turnover or sloughing rate of the mucus itself. Varanasi and Markey (1978) reported that the presence of certain metals in water induces increased mucus production in fish, thereby accelerating the turnover of the mucus layer. Whether hydrocarbons exert such an influence on mucus production in fish is unknown; however, copious amounts of mucus were produced by soft-shell clams exposed to No. 2 fuel oil in water (Stainken 1975).

7.1.5 Uptake and Biotransformation of NPH by Flatfish

The results (Varanasi et al. 1979) show that pleuronectids, like salmonids (Roubal et al. 1977a, Varanasi et al. 1978, Collier et al. 1978), are able to absorb and metabolize dietary NPH, and that NPH and its metabolic products are broadly distributed in tissues and body fluids of the exposed fish. Rock sole contained statistically significant larger concentrations of NPH and its metabolizes than did starry flounder and almost 4 times as much radioactivity remained in the digestive tract of rock sole compared to that in the starry flounder at 24 hr after feeding of ^{3}H-NPH. The digestive tract of rock sole has pyloric caeca, whereas that of starry flounder does not; in addition to other species-specific differences, perhaps structural and functional variations in the digestive tracts (Barrington 1957) of these fish are responsible for the observed differences in the levels of accumulated radioactivity. In fact, a major fraction of the administered radioactivity may have been directly discharged from the gastrointestinal tract of starry flounder.

Support for this observation comes from results showing that when the same dose of NPH was administered to starry flounder via i.p. injection, subsequent concentrations of NPH in tissues {e.g., liver) were comparable to those obtained for NPH exposed rock sole and were much higher than those for starry flounder force-fed NPH. It may be that absorption of NPH over a longer period, from the gastrointestinal (GI) tract in the case of rock sole in the feeding study and from the abdominal cavity

in the case of starry flounder in the injection study, was responsible for the results showing that the decline in the concentrations of NPH from the tissues (e.g., liver, skin, and blood) of these fish was much slower than that for starry flounder force-fed NPH.

In terrestrial mammals, the route of administration can alter the pharmacokinetics of a given compound (Gibaldi and Perrier 1974). The mode of hydrocarbon-exposure did influence concentrations and relative proportions of NPH and its metabolic products as well as the types of metabolizes accumulated in pleuronectid fish. Metabolism of NPH in the GI tract may be responsible for the observed differences in the pattern of metabolizes accumulated in livers of starry flounder in the injection and feeding experiments at 12°C. Thus, it can be speculated that differences in patterns of metabolizes may occur when the major route of uptake of PAH is other than the GI tract (e.g., gills and skin when PAH is present in water or sediment).

Dietary aromatic hydrocarbons are cleared from terrestrial animals primarily via biliary and to a lesser extent, via renal excretion (Daniel et al. 1967, Guarino et al . 1972). As with salmonids (Roubal et al. 1977a, Melancon and Lech 1978, Statham et al. 1978, Collier et al. 1978), bile of flatfish in both dietary and i.p. exposures accumulated large amounts of NPH metabolic products. This suggests that biliary excretion was one of the major routes by which the hydrocarbon was cleared from these pleuronectids. Metabolizes in the bile of the NPH-exposed flatfish were characterized by the preponderance of glucuronides (>80%). Radioactivity excreted in urine was not measured, but considerable radioactivity was present in the kidney of the test fish over a long period (up to 6 wk). Excretion of both NPH and metabolic products via epidermal mucus (Varanasi et al. 1978) and gills (Thomas and Rice 1981) seems to occur in salmonids. Epidermal mucus and gills of pleuronectids exposed to NPH contained detectable concentrations of NPH and, more importantly, its metabolic products, which suggests that in addition to biliary and renal excretion, clearance via epidermal mucus and gills may also take place in these fish. The relative importance of each of these pathways in clearance of xenobiotics in fish remains to be assessed.

The concentrations of NPH and its metabolic products in muscle were one-tenth of those for the liver of the individual species, indicating that no marked tendency was shown by muscle to accumulate the hydrocarbon or its metabolizes. Liver and various extra-hepatic tissues of marine organisms are shown to possess AHM (Varanasi and Malins 1977, Bend and James 1978), but the presence of this enzyme system in the muscle of fish has not yet been demonstrated. Our results show that the pattern of metabolizes accumulated in muscle were qualitatively similar to that in the liver of the same fish. However, in both species, the proportion of non-conjugates, specifically 1,2-dihydro-1,2-di hydroxy-NPH (dihydrodiol), was higher in the muscle than in the liver. If the presence of metabolic products in muscle was primarily due to their transport from the liver, then selection seems to take place in the types of metabolizes that are deposited in muscle.

In agreement with the results with salmonids (Roubal et al. 1977a), proportions of metabolizes relative to NPH increased with time after exposure in tissues (e.g., liver) of flatfish. Moreover, the findings show that the types of metabolizes accumulated in flatfish liver were dependent on the time elapsed after the administration of NPH. With time, the pattern of metabolizes changed in the liver of test fish, regardless of species or mode of administration of NPH; there was an increase in the proportion of conjugates--specifically sulfate/glucoside fraction--and a decrease in the proportion of the dihydrodiol. Because certain dihydrodiols of PAH are known to be mutagenic and carcinogenic (Swaisland et al. 1974, Levin et al. 1976), a decreased proportion of the dihydrodiol may imply increased detoxification of the hydrocarbon.

7.1.6 Effect of Temperature on Disposition of NPH and Its Metabolizes in Fish

Water temperature had a pronounced effect on NPH accumulation and retention in the major organs of coho salmon exposed to NPH via force feeding (Collier et al. 1978). An inverse relationship between environmental temperature and NPH retention was observed for brain, liver, kidney and blood. The work of Fucik and Neff (1977) with clams, Rangia cuneata, and Harris et al. (1977) with copepods, Calanus helgolandicus, indicated a similar relationship between temperature and NPH retention in marine invertebrates. Thus, it seems that the nature and severity of any toxic effects of NPH in marine organisms may be different in colder environments; cold-adapted organisms may retain NPH longer than those in warmer waters.

In another study, Varanasi et al (1981a) reported that a decrease in water temperature from 12° to 4°C resulted in the retention of substantially higher concentrations of NPH in tissues of NPH-exposed starry flounder. The increase in NPH concentrations could be due to several factors: Differences in rates of absorption of the ingested dose, differences in rates of excretion of NPH from tissues, and differential rates of biotransformation of NPH at these two temperatures.

Environmental temperature is known to have a marked effect on the activity of the alimentary canal of fish; both the rate of passage of food and the rate of its absorption are much slower in fish at lower temperatures (Barrington 1957). The results of Varanasi et al (1981a) indicate that both absorption and elimination of the ingested dose from the alimentary canal were slower for fish at 4°C than at 12°C. This may explain the slower decline in NPH concentrations from 24 to 168 hr in tissues of starry flounder at the lower temperature. Lowering the water temperature may also influence the rate of elimination of NPH from tissues of fish. For example, Collier et al. (1978) did not observe any effect of lowered water temperature on the retention of ingested NPH in the gut of coho salmon, but did report statistically significant higher concentrations of NPH in tissues of fish at 4°C compared to those at 10°C.

The effect of decreased temperature on metabolize concentrations in starry flounder tissues was not as marked as that observed for the NPH concentrations, which suggests that a much smaller proportion of NPH was biotransformed by liver of starry flounder held at the lower temperature. However, such a result may also be due to altered rates of excretion of NPH and its metabolizes at the lower temperature. Nevertheless, when considering consequences (e.g., toxicity) of increased concentrations and increased residence times of NPH in cold-acclimated fish, the decreased bioconversion of NPH should be taken into account.

A change in environmental temperature is also known to alter the activities of certain enzymes in fish (Hochachka and Somero 1971). Stegeman (1979) reported that in vitro activities of hepatic BaP hydroxylase measured at 25°C were significantly greater for Fundulus heteroclitus maintained at 6.5°C than those at 16.5°C. Egaas and Varanasi *(1982)* reported that lowering the environmental temperature increased both the initial hydrocarbon metabolism and the time needed for the effect of the chemical inducer to be evident. A change in the water temperature of a fish brings about changes in proportions of polyunsaturated fatty acids (PUFA) in phospholipids associated with membranes (Hazel 1979a, 1979 b), which may result in altered rates of absorption and excretion of lipophilic PAHs and their metabolizes. Wills (1980) reported a linear relation between the rate of BaP oxidation and PUFA content in endoplasmic reticulum in rat. Thus, environmental temperature can alter concentrations of individual metabolizes formed and retained in tissues of fish exposed to PAHs. A decrease in water temperature brought about marked changes in individual metabolize classes in flatfish, which would have gone undetected if only total metabolize concentrations were determined.

Virtually no information is available on the toxicity of different NPH metabolizes on fish. However, in mammals phenols and dihydrodiols of certain PAHs [e.g., benzo(a)pyrene] are more toxic than the parent compound, and some of these metabolizes also interact with cellular macromolecules (Burke et al. 1977, Kapitulnik et al. 1977, Varanasi et al. 1981 b). Certain hydroxylated metabolizes of NPH also irreversibly bind to cellular protein (Hesse and Mezger 1979). Accordingly, the results showing a statistically significant increase in the concentrations of the dihydrodiol derivative of NPH in liver and muscle of cold-maintained starry flounder is noteworthy. Moreover, it should be noted that 1 wk after NPH-exposure, fish held at 12°C contained very low concentrations of NPH, whereas tissues (e.g., liver and muscle) of fish at 4°C still retained substantial concentrations of NPH, a potential source of metabolic products.

7.1.7 Uptake and Metabolism of Sediment-Associated NPH and BaP by Flatfish

Several studies report on the uptake and metabolism of NPH by fish (Lee et *al.* 1972, Roubal et al. 1977a, 1978, Melancon and Lech 1978, Collier et al. 1978, 1980, Varanasi et al. 1979, 1981a). Studies on uptake of BaP are fewer (Lee et al. 1972, Lu et al. 1977, Gerhart and Carlson 1978, Martin 1980) and to our knowledge, until the work of Varanasi and Gmur (1981a), no information was available on the extent

of in vivo metabolism of BaP by fish liver. In this study, when English sole were exposed simultaneously to ^{14}C-NPH and ^{3}H-BaP in sediment, a substantial percentage of NPH-derived radioactivity in the liver of fish was present in the form of the parent hydrocarbon, whereas most of the BaP in the liver of the same fish was biotransformed. Moreover, the results show that the ratio of radioactivity in bile vs liver was substantially higher for BaP than that for NPH, demonstrating that BaP was more extensively metabolized in liver and transferred to bile. A large proportion of NPH may have been excreted, prior to metabolism, via gill (Thomas and Rice 1981) and skin/mucus (Varanasi et al. 1978).

These results (Varanasi and Gmur 1981a), together with our previous results (Varanasi et al. 1979), show that whether the fish were exposed to NPH or BaP, singly or in the presence of other hydrocarbons, the liver metabolized BaP more extensively than NPH. This probably explains why BaP is detected in very low concentrations or not detected at all in liver of fish (Veldre et al. 1979, Malins et al. 1980) sampled from areas containing considerable concentrations of BaP, since the currently available techniques can detect only the parent hydrocarbon. It is obvious from the results that if fish tissues are examined for hydrocarbons alone to assess whether the organisms have been exposed to these compounds, grossly misleading results may be obtained; the magnitude of error will be dependent on a number of factors (e.g., tissue type, duration of exposure, type of hydrocarbon). Therefore, metabolize analyses together with hydrocarbon analyses should be included in order to assess properly the levels of xenobiotics in marine organisms.

One of the major difficulties in tissue analyses of PAHs, such as BaP, has been the presence of lipids. A new method using two-dimensional TLC was developed to separate BaP and its metabolizes from each other and from lipids present in liver (Varanasi and Gmur 1981a). This method may be adapted to separate PAHs and their metabolizes from liver and muscle of fish sampled in field studies.

Metabolizes of NPH in the bile of English sole in the present study were similar to those reported for starry flounder and rock sole exposed to dietary NPH (Varanasi et al. 1979, 1981a). The glucuronide of 1,2-dihydro-l,2-dihydroxynaphthalene was the major metabolize. The tendency of pleuronectid fish to produce a greater proportion of glucuronide conjugates than sulfate conjugates was also evident when bile samples were analyzed for both NPH and BaP metabolizes. Glucuronidation is the major detoxification pathway in hamster embryo cells (Baird et al. 1977) and rat hepatocytes (Burke et al. 1977); however, marine invertebrates tend to produce large proportions of sulfate conjugates (Corner et al. 1960, Malins and Roubal 1982). Moreover, a preliminary report (Von Hofe et al. 1979) has shown that a higher proportion of sulfate conjugates than glucuronide conjugates was present in bile of Fundulus heteroclitus injected (i.p.) with BaP. Species specific differences as well as differences in mode of exposure to hydrocarbons may be responsible for the observed differences in types of conjugates formed by pleuronectids and Fundulus.

A number of metabolizes known to be toxic to mammals were detected in liver and bile of English sole exposed to sediment-asociated BaP. Studies with mammals have demonstrated that BaP-7,8-dihydrodiol is further metabolized to BaP-7,8-dihydroxy-9,10-epoxy-7,8,9, 10-tetrahydrobenzo(a)pyrene, which is known to bind covalently with DNA (Sims et al. 1974). The diolepoxide is hydrolyzed to 7,8,9,10-tetrahydroxy-7,8,9, 10-tetrahydrobenzo (a)pyrene. HPLC analyses of polar metabolizes in the present study revealed the presence of tetrols in bile of English sole, indicating that the diolepoxide, which is implicated as the ultimate carcinogen of BaP, was produced in liver of these fish. Recently Varanasi et al. (1981b) have demonstrated that English sole metabolizes BaP into reactive metabolizes that bind to liver DNA. Such interaction of BaP with DNA is believed to be an important step in chemical-induced neoplasia.

In contrast to the extensive conversion of BaP by English sole liver, BaP in sediment and sediment-associated water remained primarily as the parent compound over a period of several days. The results also show that while BaP concentrations in sediment did not change markedly, NPH concentrations declined steadily. Greater susceptibility to microbial degradation (Herbes and Schwall 1979, Lee 1977) and higher water volubility (Brown and Weiss 1978) of NPH compared to BaP are two likely causes for the observed decline in NPH concentrations in sediment during the experiment.

The statistically significant decrease in concentrations of NPH-derived radioactivity in most tissues of English sole from 24 to 168 hr was attributed to the net release of NPH and/or its metabolizes from tissues and not to decreased availability of NPH because NPH concentrations in SAW did not decline markedly. In contrast to NPH, BaP-derived radioactivity was continually accumulated and retained by English sole. This tendency to retain and bioconcentrate BaP-derived radioactivity is not unexpected. Roesijadi et al. (1978) reported that clams bioconcentrated radioactivity due to PHN, chrysene and BaP in the order of increasing molecular weight of PAH. Roubal et al. (1977a) with coho salmon fed benzene, NPH, or anthracene found that the degree of retention of hydrocarbon-derived radioactivity in fish tissues was in direct correlation with increasing molecular weight of the parent hydrocarbon.

7.1.8 BaP Metabolism by English Sole

English sole force-fed BaP converted it into a number of metabolizes that have been characterized as mutagenic and carcinogenic in a variety of biological assays. Both liver and muscle contained toxic metabolizes such as BaP 7,8-dihydrodiol, BaP 9,10-dihydrodiol and phenols. The two findings showing that concentrations of BaP metabolizes decreased significantly with time in muscle and that a number of toxic metabolizes were present in edible tissue of flatfish are important from a human health point of view. Further studies on the characterization of, mutagenic potency of, and rate of release of PAH metabolizes from muscle of fish are needed to gain a better understanding of the fate and effects of these hydrocarbons in edible tissue of fish.

7.1.9 Binding of BaP Intermediates to DNA Catalyzed by Liver Enzymes

The results show that liver enzymes of coho salmon, starry flounder and English sole converted BaP into reactive intermediates that bind to DNA (Varanasi and Gmur 1980, Varanasi et al. 1980). The metabolizes formed by fish liver extracts were characterized by a preponderance of non K-region dihydrodiols (e.g., BaP 7,8-dihydrodiol and BaP 9,10-dihydrodiol). It should be noted that mainly non K-region metabolizes of PAH covalently bind to DNA of PAH-exposed mammals.

Many environmental and species-specific differences may influence the xenobiotic metabolizing capabilities of aquatic animals; it would be imprudent to attribute the marked difference observed in the binding of values for the three fish species to any particular factor. Moreover, wide variations in AHM activity have been reported to occur among different strains of a single species of fish or mice. However, it is possible that starry flounder and English sole, being demersal fish, are habitually exposed to a multitude of xenobiotics (some of which are inducers of AHM) present in bottom sediments, which could result in an initially high value for the in vitro covalent binding of BaP to DNA. This would also explain why pre-exposure of flatfish to 3-methyl-cholanthrene (MC) or BaP did not result in an increase in binding as great as that for coho salmon. The results (Varanasi et al. 1980) showing that the increase in the formation of reactive DNA binding intermediates was much greater for English sole after treatment with PBCO than that for starry flounder suggest that English sole was more sensitive to petroleum exposure than starry flounder. This can be compared with results described in the Pathology Section 6.2.2 showing that English sole were sensitive to PBCO when exposed to PBCO contaminated sediment.

Our results (Varanasi and Gmur 1980, Varanasi et al. 1980) and those of others (Ahoka et al. 1979) show that the magnitude of increase in in vitro binding of BaP to DNA on pre-exposure of fish or rat to PAH (MC or BaP) was relatively greater than the increase in AHM activity. Covalent binding of BaP to DNA may prove to be more sensitive than AHM activity as an index of pre-exposure of fish to certain toxic chemicals. Moreover, the binding value serves as a useful index for correlating metabolism with the carcinogenicity of a PAH. The carcinogenic potential of a compound to mammals is roughly correlated with the extent of covalent binding to DNA (Buty et al. 1976, Pelkonen et al. 1978). Accordingly, studies to assess the extent of covalent binding of a variety of xenobiotics to cellular DNA in various target tissues of marine organisms may give useful information. In a recent study (Varanasi et al. 1981a) binding of BaP intermediates to DNA in English sole liver was reported to be as high as that for mammalian tissues susceptible to BaP carcinogenesis.

7.1.10 Activities of Aryl Hydrocarbon Monooxygenases (AHM) in Different Species

Investigations of the biotransformation of aromatic hydrocarbons and the fate of PAH metabolizes in marine organisms should also include

analysis for the presence or absence of aryl hydrocarbon monooxygenase enzyme systems, e.g., benzo(a)pyrene monooxygenase (BPMO). In the last decade (Philpot et al. 1976, Payne 1977, Gruger et al. 1977, Kurelec et al. 1977, Bend et al. 1977, Bend and James 1978) it has been demonstrated that many freshwater and marine fish species possess hepatic and extrahepatic AHM activity. The results of Sanborn and Malins (1977, 1980) and Malins and Roubal (1982) discussed elsewhere in this report show the ability of several marine invertebrates to metabolize NPH and 2,6-DMN *in vivo*. Bend and James (1978) pointed out that those marine invertebrates that do metabolize xenobiotics *in vivo* by cytochrome P-450-dependent mixed-function oxidases do so at a slower rate than do fish. Microsomal mixed-function oxidation and aryl monooxygenation *in vitro* are also very slow in crustacean hepatopancreas, relative to fish hepatic microsomes. The present research adds eight more species to the growing list of aquatic organisms which possess aryl hydrocarbon monooxygenase activity.

7.1.11 Uptake, Metabolism and Toxicity of Hydrocarbons in Invertebrates

Exposure of Mollusc Larvae and Gametes to NPH. Mussel and oyster gametes and the resultant larvae were sensitive to NPH at concentrations as low as 1 ppb. The major effect at low concentrations was a reduction in survival of the larvae. At higher concentrations, a decrease in fertilization of eggs resulted when NPH exposed sperm was used. Embryological abnormalities also were more prevalent at concentrations greater than 1 ppb. All of these factors indicate a reduction in the ecological fitness of petroleum-contaminated mollusc gametes and their resulting larvae.

Exposure of Crustaceans to Hydrocarbons. Larval forms of spot shrimp and Dungeness crabs were found to be extremely sensitive to water-borne NPH and NPH bound to protein (Sanborn and Malins 1978). The compounds were lethal in the low ppb range to Stage I and Stage V spot shrimp and newly hatched Dungeness crab zoea. These results are consonant with the generally accepted belief that early developmental stages are more sensitive to petroleum than adult marine organisms (Malins and Hodgins 1981). The findings also suggest that Stage I and Stage V spot shrimp are capable of accumulating from 25 to 100 times the environmental concentrations of NPH, depending upon whether the NPH is free or protein-bound (Sanborn and Malins 1978). The findings showing that NPH is readily released from Stage I and Stage V spot shrimp and the metabolites retained for several days (Sanborn and Malins 1978) are similar to those observed with fish (Roubal et al. 1977a, Varanasi et al. 1979, Collier et al. 1980).

The findings with *P. platyceros* suggest that the mature animals readily accumulate hydrocarbon components of the SWSF of crude oil (Sanborn and Malins 1980). The thoracic segments contained both low molecular weight benzenes and NPHs. It is likely that the hepatopancreas located in the thorax is a major site of hydrocarbon accumulation and metabolism. Abdominal segments also contained low molecular weight benzenes and NPHs indicating that shrimp sequester water-soluble hydrocarbons in edible tissue. Bioconcentration values are low in comparison to

values observed with fish (Roubal et al. 1978). The bioconcentration of substituted benzenes was in direct relationship to the degree of alkylation, a finding that corresponds to results obtained with fish (Roubal et al. 1978).

Both adult and larval spot shrimp are capable of forming a wide variety of metabolic compounds (Sanborn and Malins 1980). The profile of metabolizes in adult shrimp was not markedly dissimilar from that observed in NPH-exposed fish (Varanasi et al. 1979, Collier et al. 1980). The fact that larvae formed metabolizes indicates that early developmental stages have well developed enzyme systems capable of converting NPH to both conjugated and nonconjugated metabolizes.

7.1.12 Food Chain Transfer of 2,6-DMN to Sea Urchins via Algae

The present work (Malins and Roubal 1982) shows that ^{3}H-2,6-DMN readily accumulates in Fucus exposed to this hydrocarbon through the water column. Moreover, the 2,6-DMN is not metabolized in the algae and thus was transferred to the echinoderms as the parent compound (Malins and Roubal 1982).

It was not expected that the sea urchins would accumulate a large proportion of the ingested tritium in the exoskeleton, particularly because the 2,6-DMN was administered through the diet and no evidence was found for radioactive hydrocarbons and metabolizes in surrounding sea water. However, this finding raised the question of whether tritium exchange occurred with the result that tritiated polar compounds (e.g., water) accumulated in the exoskeleton. This possibility was dismissed because the $^{3}H/^{14}C$ ratios in exoskeleton and soft tissues in the dual-label experiment indicated that such an exchange did not occur to a significant degree (Malins and Roubal 1982). The question of whether the tightly bound aromatic fraction in the exoskeleton is available to interfere with normal cellular functions of soft tissues remains to be answered.

The preferential formation in echinoderms of 2,6-DMN metabolizes arising from oxidation of the aromatic ring is an interesting finding. In rat liver, 7,12-dimethylbenzanthracene is converted primarily to methanol derivatives, although a shift to ring oxidation occurs after pretreatment with other aromatic hydrocarbons (Jellinck and Goudy 1967). Moreover, Kaubisch et al. (1972) demonstrated that in rat liver microsomes oxidation of 1- and 2-methyl naphthalene and 1,2-DMN leads principally to naphthoic acids and methylnaphthoic acids, respectively. Little comparable information is available on marine organisms; however, Gruger et al. (1981) showed that starry flounder accumulate primarily methanol derivatives in bile after oral administration of 2,6-DMN. Thus, the metabolism of methyl-substituted NPHs in this species of echinoderm appears to be similar to that by aromatic hydrocarbon-pretreated rat.

Sulfotransferases are responsible for the conjugation of a variety of compounds (Pasternak et al. 1963, Wortman 1961). Little information exists on these metabolic transformations in marine invertebrates;

however, the lobster (Homarus americanus) is reported to have a poor ability to convert aromatic hydrocarbons to oxygen-containing derivatives" (Elmamlouk and Gessner 1978). Yet, if hydroxy compounds (e.g., p-nitrophenol) are administered, they are readily conjugated through the sulfotransferases (Elmamlouk and Gesser 1978). The present work indicates that echinoderms are different from lobsters in having active enzyme systems for biosynthesizing the hydroxy compounds, as well as for converting these oxidation products to aryl sulfates.

7.1.13 Biological Fate of Metals in Fish

The results (Varanasi and Markey 1978, Varanasi 1978, Reichert et al. 1979) demonstrated that fish held at lower temperatures accumulated relatively smaller concentrations of metals. These findings suggest that the effect of temperature on the turnover and retention of metals should be taken into account when considering the overall effect of trace metals on arctic biota. These studies also show that fish exposed to low levels (ppb) of water-borne metals readily accumulate substantial concentrations (ppm) in tissues. High levels of lead, a neurotoxin, were found in the brain of starry flounder, which suggests possible neurological damage that could have behavioral consequences (Varanasi 1978). Moreover, the results indicate that coho salmon exposed to either water-borne lead or cadmium accumulate about twice as much metal in the posterior kidney as in the anterior kidney (Reichert et al. 1979). These results cannot be adequately explained at present; however, it is suggested that this tendency may be associated with the excretory function of the posterior kidney in salmonids (Smith and Bell 1976).

Termination of exposures to metals does not automatically mean that metal concentrations will necessarily remain constant or decline in the kidney and possibly other tissues. Even when a decline in cadmium or lead concentrations was observed, e.g., in gills, considerable levels still remained in gills after 5 wk deputation in control seawater (Reichert et al. 1979). The strong tendency to sequester cadmium and lead in gills (Reichert et al. 1979) during uptake and deputation periods raises questions about possible interference with osmoregulation and oxygen consumption (Thurberg et al. 1973).

More than 50% of lead and cadmium accumulated in the scales of coho salmon was still present after 5 wk of deputation (Varanasi and Markey 1978). It is known that in salmonids, skin and scales play an important role in calcium transport and regulation (Podoliak and Holden 1965). The persistence of high concentrations of lead and smaller concentrations of cadmium may interfere with transport and perhaps regulation of calcium in fish. Moreover, scales of salmonids are known to be resorbed during maturation and the mineral components of the scales are utilized for general metabolism and production of gametes (Wallin 1957). In the metal-exposed fish, resorption of scales may result in the release of toxic metals in the bloodstream at the time of stress. The consequences of release of such toxic metals on eggs and sperm remains to be assessed. It should be noted that increased levels of environmental or dietary calcium (Varanasi and Gmur 1978)

significantly reduces uptake and retention, and presumably toxicity, of lead in coho salmon.

The finding (Varanasi and Markey 1978) that coho salmon had lower epidermal mucus concentrations of metals than did starry flounder, along with the results on rainbow trout (Varanasi et al. 1975) indicated that the level of metals accumulated in epidermal mucus are dependent largely on a particular species of fish rather than the salinity of the medium. In addition, the results (Varanasi and Markey 1978) show that short-term exposure to pollutants triggers increased synthesis of mucus; however, the consequence of long-term exposure on mucus production remains to be assessed. Sherwood and Bendele (1975) reported that in Dover sole (Microstomus pacificus) collected from Pales Verdes, an area of known metal contamination, fin erosion was accompanied by reduction in epidermal mucus (visual observation). Whether or not there exists a relationship between altered rate of mucus production and pathological surface conditions is not known.

Epidermal mucus of salmonids exists in a state of continuous flux (Pickering 1976)--that is, small amounts of mucus are continuously sloughed off and renewed. Thus, our results showing that metals were present in the mucus of the test fish for several days after termination of exposure suggested that metals were discharged in epithelial mucin via the mucous cells and excreted in the mucus (Varanasi and Markey 1978, Varanasi 1978). The intriguing possibility exists that a certain critical concentration of a pollutant in blood at a given temperature may trigger increased synthesis of epithelial mucin, resulting in rapid turnover of mucus which would expedite discharge of the pollutant. With respect to this hypothesis, it should be noted that NPH and its metabolic products are also shown to be released in epidermal mucus of NPH-exposed salmonids (Varanasi et al. 1978) and flatfish.

Heavy metal-binding proteins in gills of marine species have been reported previously (Marafante 1976, Bouquegneau et al. 1975, Noel-Lambot et al. 1978). Noel-Lambot et al. (1978) found extremely low concentrations of cadmium bound to a cadmium binding protein (CdBP) in the gills of eels that had been exposed to 200 ppm of cadmium for 5 hr. Ours studies with unexposed coho salmon indicate that CdBP concentrations in gills are low. Yet within 24 hr after metal challenge there is an appreciable increase in the accumulation of CdBP-bound cadmium, indicating induction of CdBP. Accordingly, it is likely that CdBP may play an important role in binding cadmium in the salmonid gill; this reaction may serve to protect vital physiological processes (e.g., osmoregulation) from the potentially destructive influences of this metal.

In rat liver, the proportion of cytosolic cadmium bound to CdBP (identified as metal lothionein) increases steadily for at least 48 hr after injection (Frazier and Puglese 1978, Cempel and Webb 1976). Similar to studies with rat, induction of CdBP in the liver and kidney of salmonids was observed after the fish were exposed to cadmium (Reichert et al. 1979). Also, unexposed coho salmon apparently have appreciable concentrations of CdBP present in liver. However, there

appears to be no specific low molecular weight protein like metal lothionein in the cytosolic fraction of the kidney, liver, and gills of coho salmon that has a high affinity for lead. Similar observations have been made in mammals. Consequently, this metal binds with a variety of proteins in the cytosol and may alter protein structures.

In conclusion, the biochemical fate and biological effects of metals are probably influenced by temperature, calcium and other metal ions in the marine environment. Synergistic and antagonistic effects associated with multiple systems of metals on marine organisms are important subjects for future study.

7.2 Pathology

7.2.1 Effects of Petroleum on Disease Resistance

The results of a series of tests designed to assess the effects of petroleum hydrocarbons on disease resistance of salmonids, juvenile and adult fl atfish, and adult spot shrimp failed to identify a marked impairment. Whether or not a lowered host resistance could occur under other stress and exposure conditions and to different diseases cannot be predicted from the present data. These data do, however, strengthen an argument that exposure to environmentally realistic concentrations of petroleum does not markedly impair disease resistance in the tested and related species.

In contrast, a preliminary assay with a representative petroleum dispersant (Corexit 9527) suggested the potential for an adverse effect of oil-dispersant mixtures on disease resistance of salmon.

7.2.2 Pathological Changes in Flatfish from Exposure to Oil-Contaminated Sediment

Flatfish were shown in these series of experiments to take up petroleum hydrocarbons in muscle and liver tissue following contact with PBCO-contaminated sediments. Bioavailability and bioaccumulation appears to be dependent on a number of factors including aromatic hydrocarbon structure, sediment characteristics, and biotransformation mechanisms in the flatfish tissues.

The differential uptake and retention of 1- and 2-methyl naphthalenes and tetramethylbenzene suggests that aromatic structure influences bioavailability. Such selectivity has been reported for starry flounder by Roubal et al. (1978), and has also been inferred from other studies (see Section 6.1.2). The reduction of tissue levels of petroleum hydrocarbons over time, despite a persistently high sediment concentration of hydrocarbons, further suggests that increased clearance rates and/or increased metabolic transformation of petroleum hydrocarbons to oxidation products are occurring (see Section 6.1.7).

The silt content of the sediments clearly influence the dynamics of release of sediment-associated petroleum. There appears to be a slower and more continuous release of hydrocarbons from high-silt sediment than from high-sand sediment.

The significance of severe hepatocellular lipid vaculoization (HLV) is unknown. Although this condition has been observed to occur at a higher frequency in crude oil-exposed English sole than in controls, it was observed at approximately the same frequency in both oil-exposed and control rock sole and starry flounder. HLV can be induced by a variety of causes, including nutritional deficiencies (Snieszko 1972), exposure to pesticides (Couch 1975), and by contact with polluted environments (Pierce et al. 1978). Also, under the experimental conditions employed severe HLV appears to be reversible.

7.2.3 Cytopathology

(a) Adult Fish Exposures

Gill. Both salmon and flatfish exposed to 100 ppb concentrations of the SWSF of PBCO exhibited gill lesions resulting from loss of surface cells. Similar conditions have been observed in a number of fish species exposed to waterborne contaminants. Trace metals such as cadmium, nickel, and lead damage gills by causing sloughing of epithelial cells (Voyer et al. 1975, Schwiger 1957, Haider 1964). Excessive mucus production is also characteristic of exposure of fish to trace metals (Varanasi et al. 1975, and Section 6.1.13 of this report) and is thought to cause death by suffocation (Gardner 1975, Haider 1964). Similarly, epithelial sloughing and discharge of mucous glands was observed in marine fish captured near an oil slick off the Texas and Louisiana coast (Blanton and Robinson 1973). The secretory cells of the pseudobranch of the Atlantic silverside (Menidia menidia) degenerated after exposure to either 0.14 ppm Texas-Louisiana crude oil or 100 ppm waste motor oil (Gardner 1975, Gardner et al. 1975); light micrographs revealed severe vacuolization of the entire pseudobranch but no data on adjacent gill tissue was presented.

The effects of phenol on gill tissue have been documented in several freshwater species. Rainbow trout sloughed epithelial cells and developed a general inflammation of the gills after exposure to 6.5 to 9.6 ppm phenol (Mitrovic et al. 1968). Another freshwater fish, the bream (Abramis brama), was exposed to lower concentrations of phenol for a longer duration and suffered similar destruction of the gill epithelial cells; in addition test fish suffered damage to the blood vessels and extravasation of blood in the gill lamellae (Waluga 1966). Fourteen species of fish sampled from phenol-polluted portions of the Rhine and Elbe Rivers revealed discharged mucous glands and generalized gill inflammation (Reichenbach-Klinke 1965).

Liver. Increases in rough endoplasmic reticulum in the hepatocytes of rainbow trout were observed after exposing the fish to high peroral doses of PBCU for 8 months. In one of the few field studies

that included ultra structural data, a similar increase in rough endoplasmic reticulum was reported in the hepatocytes of Fundulus heteroclitus sampled from an area which had received an oil spil 1 8 years earlier (Sabo and Stegeman, 1977). Also, the hepatocytes of channel catfish (Ictalurus punctatus) showed proliferation of endoplasmic reticulum and bizarre" whorls of both the smooth and rough endoplasmic reticulum after chlorobiphenyl exposure via gastric incubation for 21 days (Hinton et al. 1978, Klaunig et al. 1979). Circular arrays of smooth endoplasmic reticulum in liver cells have been reported following dietary exposure of rainbow trout to aflatoxin B (Scarpelli 1976), and to phenylbutazone (Scarpelli 1977). Hawkes et al. (1980) showed that after 28 days exposure to a mixture of 5 ppm chlorobiphenyls in diet, the liver cells of chinook salmon had vesiculated rough endoplasmic reticulum and circular arrays of smooth surfaced membranes that closely resembled the arrays of smooth endoplasmic reticulum reported in catfish (Hinton et al. 1978, Klaunig et al. 1979). Such changes in the endoplasmic reticulum, and particularly the changes resulting in circular arrays of smooth endoplasmic reticulum, have been suggested to be a response to substances that induce the mixed-function oxidase system (Klaunig et al. 1979).

The process that results in changes in endoplasmic reticulum has been hypothesized to include the disruption of lipoprotein synthesis. Triglycerides norms'lly utilized for lipoprotein formation are thought to accumulate in the hepatocytes (Tanikawa 1979). An increase in hepatocyte lipid content has been reported in English sole exposed to oiled-sediment (McCain et al. 1978), in channel catfish (Hinton et al. 1978) and rats (Kasza et al. 1978) exposed to dietary chlorobiphenyls, and in humans and other mammals exposed to a variety of toxic substances (Lombardi 1966; Tanikawa 1979). In contrast, our studies showed exposure of rainbow trout to dietary crude oil resulted in depletion of lipid in the hepatocytes, and although there was an increase in rough endoplasmic reticulum, no vesiculation of the cisternae was found (Hawkes 1977).

Lens. Opacities in lenses of mammals are preceded by hydration of the lens fiber cells (Hollwich et al. 1975); lens changes observed in rainbow trout perorally exposed to petroleum also appear to include hydration. Generally, long-term peroral exposure of trout to PBCO caused progressive degeneration of lens tissue characteristic of early cataract formation. Payne et al. (1978) also reported tissue changes in the lens of cunner (Tautogolabrus adspersus) exposed to an oil slick for 6 mo. In mammals, lens abnormalities associated with exposure to naphthalene have been well documented (Hollwich et al. 1975). The sequence of metabolic events that precedes cataract formation in rats and rabbits after exposure to naphthalene appears to include enzymatic oxidation reactions that lead to the formation of 1,2-dihydroxynaphthalene, a compound which may be autooxidized to 1,2-naphthoquinone; this compound may react with lens components. The enzymes that perform some of the oxidation reactions have been found in the eye of rabbits (Van Heyningen 1979, Van Heyningen and Pirie 1967) and, recently, in the eyes of fish (Stegeman, personal communication).

(b) Embryonic Fish Exposures

Olfactory epitheliums. Scanning electron microscopy of sand sole larvae exposed to 164 ppb of the SWAF of weathered PBCO during embryogenesis revealed degenerative changes in the chemosensory cilia and a loss of the microridges that circumscribe the perimeter of the epithelial cells surrounding the olfactory organs. Whether olfaction was impaired in these larvae is not known; however, the degree of structural alteration observed indicated severe damage to the receptor organelles.

These findings are in accordance with those of Gardner (1978), who identified the olfactory epitheliums of fish as a site of damage from petroleum hydrocarbons. Gardner exposed the Atlantic silverside, Menidia menidia, to whole crude oil, the water-soluble fraction of crude oil, and the water-insoluble fraction of crude oil for 7 days. Pathological changes in the olfactory organ differed with exposure regime; whole crude oil induced a marked hyperplasia; epithelial metaplasia developed after exposure to the water-soluble faction; and submucosal blood vessels were dilated and congested after exposure to the water-insoluble fraction.

Brain and Eye. The late appearance of cytopathological changes in the retinal receptor cells of surf smelt exposed to the SWAF of CICO may be related to the differentiation times of these cells. Typically, teleost retinas are relatively undifferentiated throughout embryogensis and, in some species (Ali 1959, Blaxter 1974), do not begin to mature until late in larval development. In surf smelt, the receptor cells develop outer segment membranes (a necessary structure before the cells can function as photon receptors) between day-15 and day-21 of embryogenesis. The first eye damage was observed in 21-day old embryos; this suggests that receptor cell damage from exposure to petroleum hydrocarbons is not evident cytologically until these cells are fully differentiated.

In addition to morphological alterations of the receptor cells, the effects of exposure to the SWAF of CICO on surf smelt embryos were reflected by necrosis in the neurons of both the brain and eye. In some fish and mammals, neural tissue appears to be particularly vulnerable to certain petroleum hydrocarbons; brain tissue has been reported to bioaccumulate relatively large amounts of these compounds. For example, naphthalene was sequestered in the brain of salmonids in amounts comparable to that in the liver (Roubal et al. 1977a, Collier et al. 1980, and Section 6.1.6 of this report). Neurotoxic effects of low-molecular-weight hydrocarbons in mammals have been attributed to accumulation of either the parent hydrocarbons or their metabolizes in brain tissue (Savolainen 1977). Although the effect of changes in the forebrain of the surf smelt is difficult to assess, the observed neuronal damage may have been sufficient to account for the five-fold increase in mortality of larvae exposed to the SWAF of CICO as embryos as compared to unexposed controls (See Section 6.3.1 [e]).

7.3 Behavior and Physiology

7.3.1 Vertebrate Studies

Behavior of Pacific Salmon Exposed to Petroleum Hydrocarbons

(a) Olfactory disruption. The waterborne aromatic hydrocarbon components of petroleum are thought to be a likely cause of chemosensory disruption in aquatic organisms (Takahashi and Kittredge 1973). Although the mechanism of disruption is unknown, it has been suggested that such contaminants may mask the chemoreceptive sites, thus blocking incoming chemical signals at the receptor level (Sutterlin 1974). It is also possible that olfactory lesions, such as those observed by Gardner (1975) in Atlantic silversides exposed for 7 days to the SWSF of Texas-Louisiana crude oil, might account for disruption. Our data indicate that the olfactory receptor sites of salmon responsive to certain amino acids are not masked by aromatic hydrocarbons; further, comparison of our EEG data with results of similar studies using other pollutants indicates that short-term exposure (20 rein) to a 4 ppm aromatic hydrocarbon mixture does not result in disruption of the olfactory epitheliums. In contrast, when Hara (1972) infused the nares of juvenile coho and sockeye (O. nerka) salmon for 10 sec with either 2.7 ppm $HgCl_2$ or 1.6 ppm $CuSO_4$, the olfactory response to all stimulants was eliminated. Also, Hara et al. (1976) found a significant decrease in rainbow trout EEG response to stimulants after 30 min exposure to 0.3 ppm $HgCl_2$.

A statistically significant increase in olfactory bulb EEG response was observed at hydrocarbon concentrations of 2.8 ppm and greater. However, an olfactory EEG response does not preclude the fishes' use of other sensory modalities (such as taste) for identification and concomitant behavioral responses to hydrocarbons. Also, the olfactory bulb EEG response is the result of integrated neural activity and does not necessarily reflect the minimal olfactory detection of aromatic hydrocarbons.

(b) Migratory and Homing Behavior in Adult Salmon

Avoidance reaction. Fifty percent of the mature salmon migrating up Chambers Creek during the peak of the run avoided a mixture of hydrocarbons in the water at concentrations greater than 3.2 ppm. These tests represent avoidance of hydrocarbons under estuarine rather than strictly freshwater conditions since movement of adult salmon into Chambers Creek occurred predominantly at high tide when seawater intrusion covered 1/2 to 3/4 of the fish ladders. Whether or not similar relationships between hydrocarbon concentration and avoidance hold for higher salinity waters is not known.

Disruption of homing capability. The concentrations of hydrocarbons used in our homing tests are well below the acutely toxic levels reported for Pacific salmon (Morrow 1973, Bean et al. 1974, Moles et al. 1979). Sublethal concentrations of petroleum, however, have been shown to cause a number of behavioral, physiological, and

histological changes in salmonids (Bean et al. 1974, Rice et al. 1976, Cardwell 1973, Hawkes 1977). Thus, the 3 day delay of coho returning to Tulalip Creek following exposure to monocyclic aromatics may be attributed to a variety of causes. Several lines of evidence suggest that the most likely cause is central nervous system disruption in the form of narcosis. The three major components of the aromatic hydrocarbon mixture (toluene, xylene's, and benzene) have all been established as vertebrate narcotics. Other investigators have noted a similar "narcosis" in fish exposed to monocyclic aromatics (Strushaker 1977, Strushaker et al. 1974, Pickering and Henderson 1966), and there is strong evidence that central nervous system tissue concentrates monocyclic aromatics (Kern et al. 1976, Roubal et al. 1977b, and Section 6.1.2 of this report). Also, observations made during hydrocarbon exposure and at time of release in our studies indicated a consistently reduced level of activity of salmon exposed to petroleum hydrocarbons.

In the Tulalip study the difference between the calculated concentration of aromatic hydrocarbons in the exposure tank and the concentrations determined by GC analysis (Table 9, Section 6.3.1[b]) would indicate that the coho salmon were not exposed to 1-2 ppm hydrocarbons, but to a series of lower concentrations ranging from 25 to 375 ppb. Analysis of the data using the latter hydrocarbon exposure concentrations suggests that the percent returning to Tulalip Creek was inversely related to prior exposure concentration. (For details of this method of analysis see Malins et al. 1978.) However, the overall results of our studies indicate that, outside of a possible slight delay, short-term exposure of Pacific salmon to petroleum hydrocarbons does not impair homing ability.

(c) Predator-Prey Behavior

Exposure of chum salmon fry to low levels of SWAF of CICO for periods of 24 to 72 hr resulted in a statistically significant increase in their consumption by non-oil-exposed coho predators. However, the longest exposure period (96 hr) did not cause the greatest loss to predation. Predation by coho predators was impaired only after the predators were exposed to the SWAF of CICO for longer than 72 hr.

An interesting observation associated with predation by oil-exposed predators was that levels of the parent hydrocarbons were markedly higher in the tissues of predators actively eating compared to those that were not eating. Brain and liver hydrocarbon concentration differences between the eater and noneater subgroups suggest differential uptake, excretion and/or metabolism of these chemicals. These results are in apparent contrast with reported findings that acute neurotoxic effects (Savolainen 1977) and behavioral changes (Dixit and Anderson 1977) were related to accumulation of the parent hydrocarbon compounds.

Avoidance Behavior of Flatfish Exposed to Oil-Contaminated Sediment

Twenty-five to 60% of the juvenile English sole tested did not avoid sediment contaminated with 8,000-10,000 ppm PBCO. Although this oil concentration is high , it is environmentally attainable. In a review

of the literature on observed concentrations of petroleum in the marine environment, Clark and MacLeod (1977) presented data which show that hydrocarbon levels in sediment of polluted coastal areas are usually less than 1,000 ppm; however, they have been measured at concentrations exceeding 12,000 ppm.

The significance of avoidance or non-avoidance cannot be definitively resolved in a laboratory situation because of numerous factors in the natural environment that cannot be duplicated. For example, Rice (1973) has shown that pink salmon fingerlings will avoid the seawater-soluble fractions of PBCO and suggests that this may cause them to move into offshore waters where food supplies are less abundant and predation may occur -- a situation which may promote return to an oil-contaminated area. It is evident, in any event, that without consideration of behavioral parameters, predictions of effects of petroleum hydrocarbons on many marine organisms may be grossly exaggerated or, conversely, overlooked.

Effect of Petroleum Hydrocarbons on Development of Embryos and Larvae

(a) Chum salmon. Embryos and alevins were exposed 3 hr/day in a flow-through system to an average of 470 ppb of the SWAF of weathered PBCO. Survival of embryos exposed during either the first third or last two-thirds of development was similar to controls, but continuous exposure throughout development reduced embryo survival. The primary effect of oil exposure during embryonic development was observed in alevins in the first 10 to 15 days after hatching. Of embryos and alevins exposed continuously, only 20% of those actually hatching survived through yolk sac absorption; 85% of the control alevins survived during a comparable period. Oil exposure of newly hatched alevins, not previously subjected to oil contamination, resulted in mortality double that of controls.

A somewhat comparable study was conducted by Rice et al. (1975) assessing the effect of PBCO on pink salmon embryos and alevins. They found that, generally, embryos and alevins were equally resistant to the SWAF of PBCO. These findings are similar to ours in that chum salmon alevin mortality was 44-48% regardless of whether they were exposed to the SWAF of PBCO as embryos (46 to 77 hr exposure) or as alevins (60 hr exposure). In contrast to our studies, Rice et al. (1975) noted a difference in growth between control pink salmon alevins and those exposed for 10 days to sublethal hydrocarbon concentrations (average of 725 ppb measured by infrared spectrophotometry). Although we did not observe any differences in total length of oil-exposed and control chum alevins, the lack of calcification of fin rays in chum alevins exposed to the SWAF of PBCO may be a reflection of developmental retardation.

(b) Flatfish. Eggs were exposed to the SWAF of weathered PBCO using a static-relacement regime with the SWAF being renewed at mid-incubation. Incubation of embryos at a concentration of 80 ppb resulted in a percentage of normal larvae comparable to that of controls. At hydrocarbon concentrations of 130 to 165 ppb embryo survival and percent hatching

was high, but all hatched larvae were either abnormal or died shortly after hatching. Similar events were observed by Kuhnhold (1972) during exposure of cod embryos to slightly weathered Iranian crude oil. Even though the percent hatching of oil-exposed cod embryos was high, most of the larvae were deformed, unable to swim normally, and died within one day.

(c) Smelt. In duplicate experiments, surf smelt embryos were exposed in a flow-through system to the SWAF of weathered CICO for 3 hr/day during embryonic development. At the highest hydrocarbon concentrations (55 to 175 ppb) less than 10% of the embryos hatched into normal larvae. At the lowest hydrocarbon concentrations (25 to 45 ppb), 43% of the exposed embryos hatched into apparently normal larvae -- a percentage similar to that of controls. The hatched larvae were held in uncontaminated water and the survivors counted 10 days later. Over 40% of the control larvae survived, whereas survival was less than 10% for any group of larve exposed to the SWAF of weathered oil as embryos.

One day prior to hatching, normal appearing surf smelt embryos from the above experiment were sampled and examined microscopically. Ultrastructural analysis indicated that up to 80% of the oil-exposed embryos had cellular abnormalities of the eye and brain; no abnormalities were observed in the controls (see Section 6.2.3). In a similar study Cameron and Smith (1980) exposed Pacific herring embryos to the SWAF of slightly weathered PBCO at a concentration of 680 ppb and examined the newly hatched larvae for cytopathological changes. Although the oil-exposed larvae showed no gross abnormalities, there were ultrastructural disruptions which the investigators believed would have severely decreased larval survival.

(d) Mortality of Controls. In all our experiments there were varying levels of mortality in controls, ranging from a low of 10% for sand sole to a high of 50% for embryos of surf smelt. These mortalities are attributed, in part, to stresses induced by simulating natural environmental conditions. For example, chum salmon embryos and alevins were subjected daily to wide fluctuations in salinity, and surf smelt embryos were subject to desiccation and thermal stress. Thus, the effects we observed are most likely the result of stress from a combination of chemical, physical, and biological factors, and not from petroleum acting alone.

Effect of Crude Oil Ingestion on Salmonid Reproductive Success

The quantities of petroleum components consumed by experimental fish in the reproduction study clearly exceeded that which would be encountered in natural food supplies; however, it was our intention to examine an extreme case of exposure. The fish readily consumed the petroleum-impregnated food and continued to grow and develop. Although there were no mortalities of petroleum-fed fish prior to spawning, the post-spawning mortality of petroleum-exposed trout with fungus infections suggests some possible interaction betweeen petroleum exposure and recovery

from spawning. It is also possible, however, that the differential post-spawning mortality between the test and control groups may have been related to a greater density of fish in the test tank compared to that in the control tank.

There was no statistically significant impairment of hatching success related to the petroleum exposure. Survival percentages of 86 to 90% compare well with survivals of 90 to 95% for the hatchery program from which the fish were obtained (M. Albert, Hatchery Manager, personal communication, 1976), as well as with published values for other studies using rainbow trout (Anon., 1973). However, eggs from two of the test females had low survivals, and it may be that certain individual fish were adversely affected by the petroleum exposure. There is no indication that the dietary petroleum exposure had any effect on male fertility; in fact, the lowest survival was associated with a control male.

Many other behavioral and physiological aspects of natural reproduction were not examined in these studies. Clearly, activities such as homing, mate selection, redd-building behavior, and territoriality could be disrupted by petroleum consumption and contribute to poor reproductive success in the natural environment.

The fluorescence spectra associated with the trout muscle indicated that certain fluorescing compounds were mobilized from the food through the circulatory system in the fish, and localized in the tissues. Similarly, the evidence suggests that trout are capable of transporting certain hydrocarbons into eggs when the fish are exposed to petroleum in food.

There is no evidence from these studies to suggest that chronic dietary exposure to concentrations of the less volatile components of PBCO that are likely to occur in the environment would result in reproductive failure of rainbow or steelhead trout; however, the histological abnormalities of eye lenses and livers observed in some of these fish (see Section 6.2.3) exposed to petroleum are potentially deleterious.

7.3.2 Invertebrate Studies

In marine organisms the chemosensory system plays a prominent role in behavioral activities related to feeding, avoidance and escape responses, and to reproduction. These responses can be induced in laboratory-maintained animals by specific compounds at levels of parts-per-trillion (Kittredge et al. 1971) and have been abolished by water-soluble oil fractions at levels of 1 ppb (Jacobson and Boylan 1973). In our studies the SWSF of PBCO was found to disrupt, at low ppb concentrations, chemosensory mediated behavior in three diverse invertebrate organisms: the green sea urchin (an echinoderm); the dorid nudibranch (a mollusc); and the spot shrimp (an arthropod).

Defense Behavior of Sea Urchins Exposed to Petroleum Hydrocarbons

The pedicellarial defense behavior of the green sea urchin is mediated by chemical substances released by the starfish; following exposure of urchins to hydrocarbons, this pedicellarial response is moderated. At hydrocarbon concentrations of less than 1 ppm detected behavioral effects were limited to pedicellarial responses; urchin mobility was not markedly impaired, nor was the competence of the pedicellariae to respond eliminated (as shown by elicitation of pedicellarial response following the injection of isotonic KCl). The mode of action of hydrocarbons on the pedicellarial system is not known but the results are provocative in several respects. Inhibition of the defense response ensues within 10 min and thereafter the response index shows no marked change following continuous exposure of up to 48 hr, indicating that equilibrium is quickly established between the pedicellarial response system and the hydrocarbon concentration. In contrast to the rapid onset of inhibition, recovery of the defense reponse during deputation is relatively slow.

Of the five aromatic hydrocarbons tested individually for defense reponses in the sea urchin , only l-methyl naphthalene approximates the responses elicited by the total SWAF. The concentration of 1-methyl-naphthalene found in the SWSF, however, is not sufficient by itself to account for the inhibition of the pedicellarial response. The same lack of sufficient concentration in the SWSF applies to ethyl benzene and trimethylbenzene which, when tested individually, are ineffective at concentrations less than 100 ppb. The greatest concentration reached by either of these latter two aromatics in the SWSF was 21 ppb.

In experiments on interaction between sea urchins and Pycnopodia sp. there was an 80% difference in predation of oil-exposed urchins over controls. This is probably attributable to the inhibition of the pedicellarial defense response, but there is the possibility that Pycnopodia are attracted to, and prefer, oil-tainted urchins. (The attraction to low concentrations of hydrocarbons has been observed in some marine organisms, particularly crustacean.) It is also possible that oil-treated urchins are less successful at avoiding the starfish. Regardless of the causes, the results show selective predation for oil-exposed sea urchins.

Reproductive Behavior of Dorid Nudibranchs Exposed to Petroleum Hydrocarbons

The movement of a nudibranch toward an aggregate of other mating conspecifics is thought to be a chemotactic response mediated by a sex pheromone. As little as 1 day of exposure to the SWSF of PBCO at a concentration of less than 15 ppb significantly decreased the percentage of nudibranchs responding to the aggregate. This effect of SWSF exposure appears to involve the chemoreceptive system and is not a general narcosis, as evidenced by equal movement for both SWSF-exposed and control animals.

As a follow-up to the doridmating behavior experiment, the effect of the SWSF on egg laying and embryonic development was also studied. At the highest exposure level (225 ppb) egg deposition and development was retarded, and approximately half of the eggs laid were either not encapsulated or showed other abnormalities. At lower exposure concentrations the effect of the SWSF on egg development was reduced accordingly.

Feeding Behavior of Shrimp Exposed to Petroleum Hydrocarbons

Observations concerning the effect of the SWSF of PBCO on the feeding behavior of spot shrimp generally showed a decrease in responsiveness by the shrimp as hydrocarbon concentrations increased from 0 to 500 ppb. The actual physiological mechanisms involved in alteration of the observed behaviors is unknown and may involve either narcosis or simple masking effects at the chemoreceptor level.

In an attempt to differentiate between general narcosis and chemosensory disruption, we exposed 3 crayfish (Astacuspacifastacus) to 2-4 ppm of a model mixture of aromatic hydrocarbons (Tabee II, Section 5.3.2) and monitored reflex reactions of the heart and scaphognathite to physical and chemical stimuli using the methods of Larimer (1964) and Wilkins et al. (1974). The results indicated that both under control conditions, and during exposure to hydrocarbons, changes in heart and ventilation frequencies to sound and light stimuli were similar, occurring 68 ± 12% (range) of the time. The 75% control reflex response to chemical stimuli was reduced to 29% when hydrocarbons were present. The decrease in reflex response to chemical stimuli, and not light or sound stimuli, may indicate specific action of petroleum hydrocarbons on the chemosensory system.

8. CONCLUSIONS

8.1 Chemistry

8.1.1 Accumulation and Biotransformation of Aromatic Hydrocarbons by Marine Species

Coho salmon, starry flounder and adult shrimp exposed to <1.0 ppm of a SWSF of PBCO accumulated substantial concentrations of aromatic hydrocarbons representing a broad spectrum of individual compounds in all tissues examined (Roubal et al. 1978, Sanborn and Malins 1980). Starry flounder accumulated substantially greater concentrations of aromatic hydrocarbons than did either coho salmon or shrimp. For example, bioconcentration values for naphthalenes in the muscle of starry flounder and coho salmon where about 500 and 30 respectively, and the value for the abdomen of shrimp was about 80. In addition, the results indicate that fish (Gruger et al. 1977) and shrimp (Sanborn and Malins, 1978, 1980) have a capability for metabolizing aromatic hydrocarbons to potentially toxic products, as indicated by enzyme (AHM) studies and determination of total and individual metabolizes in tissues. Characterization of the individual NPH metabolizes in coho

salmon showed that hydroxylated and conjugated compounds were formed (Roubal et al. 1977a, Collier et al. 1978); these compounds are similar to metabolizes found in studies with mammals. These findings showing that fish and shellfish accumulate hydrocarbons and metabolic products in a variety of body tissues suggest that potentially deleterious effects on the organisms could arise, and raises questions about the suitability for human consumption of fish and shellfish exposed to petroleum.

8.1.2 Factors Influencing Uptake and Metabolism of Naphthalene by Fish

Starry flounder and rock sole readily accumulated and extensively metabolized dietary NPH. The extent of biotransformation of naphthalene and the types of metabolizes remaining in tissues of these flatfish were shown to be greatly influenced by both the mode of exposure and elapsed time after exposure (Varanasi et al. 1979). However, regardless of mode of exposure, species, or structure of hydrocarbon, it was demonstrated that hydrocarbon metabolizes remain in tissues of fish and crustaceans over a longer period than do the parent compounds (Roubal et al. 1977a, Collier et al. 1978, 1980, Sanborn and Malins 1978, 1980, Varanasi et al. 1979, 1981, Varanasi and Gmur 1981a,b,). Thus, when evaluating the overall consequences of petroleum exposure to marine fish, it is extremely important to include determinations of tissue metabolize concentrations together with the concentration of parent hydrocarbon.

Lowering the water temperature resulted in higher levels of NPH in several organs of NPH-fed coho salmon (Collier et al. 1978). In addition, starry flounder maintained at a lower temperature showed an increase in both concentrations and resident times of NPH and its metabolizes in tissues of fish exposed to dietary NPH; however, the increase in concentration was much greater for NPH than for its metabolizes, indicating that the bioconversion of NPH was considerably less at the lower temperature. Lowering of the temperature also influenced the relative concentrations of metabolize classes accumulated in tissues of starry flounder (Varanasi et al. 1981b). It is evident from these studies that environmental temperature may sharply influence both the nature and severity of toxic effects of NPH in fish.

8.1.3 Naphthalene and Its Metabolizes in Fish Skin and Mucus

The results demonstrate that the skin of salmonids and flatfish was actively involved in the retention and discharge of NPH and its metabolizes. A notable finding was that in starry flounder four times more metabolizes as parent hydrocarbon were present in the skin one week after exposure, indicating either preferential retention of metabolizes or extensive metabolism of naphthalene. In addition, data on the role of epidermal mucus in the excretion of naphthalene and its metabolizes in fish suggest that mucus must also be considered as one of the routes of excretion (Varanasi et al. 1978, Varanasi and Gmur 1978).

8.1.4 Uptake and Metabolism of Sediment-Associated Naphthalene and Benzo(a)pyrene by Flatfish

English sole exposed to NPH and BaP in oil-contaminated sediment took up and readily metabolized these hydrocarbons (Varanasi and Gmur 1981a,b). A number of mutagenic and carcinogenic metabolizes of BaP were identified in the liver. The metabolizes of BaP were retained in flatfish liver over a much longer period than NPH and its metabolizes. Furthermore, BaP tends to remain largely unconverted in sediment and thus can be available for continued uptake by benthic organisms. Continued uptake, greater retention, and more extensive metabolism of BaP than NPH by benthic fish indicate that although BaP is a minor component of petroleum, its derivatives can be bioconcentrated in tissues of demersal organisms. The substantial bioconversion of BaP in fish liver very probably explains why BaP is usually not detected in fish tissues even when a considerable concentration of BaP is detected in the environment. The ultimate consequences of the presence of BaP metabolizes in fish tissues are not known, but implications on toxicity can be drawn from biological activities of these metabolizes from various assays (e.g. , Ames Test, cytotoxicity, carcinogenicity).

8.1.5 BaP Metabolism by English Sole

When English sole were force-fed BaP, a number of toxic metabolizes were found in both the liver and edible muscle of the fish. The concentration of BaP-derived radioactivity in muscle was much lower than in liver and the decrease in concentration of BaP and its metabolizes in muscle with time was greater than in liver. These findings indicated that muscle of English sole may not be a major storage site of BaP and its metabolizes, which is important, since muscle of English sole is a consumer product.

8.1.6 Examination of Aryl Hydrocarbon Monooxygenase Activity in Different Species and Binding of BaP Intermediates to DNA Catalyzed by Liver Enzymes

Examination of tissue aryl hydrocarbon hydroxylase activities of marine species from Alaskan waters revealed that highly variable enzyme activities are common to hepatic tissues of vertebrate species tested.

In other studies (Section 6.1.9), liver enzymes from flatfish were able to convert benzo(a)pyrene (BaP) into reactive intermediates that bind to DNA in vitro, and the level of binding increased with pre-exposure of flatfish to PAH or PBCO. Binding of metabolizes of PAH to DNA is a presumed prerequisite step in the chain of events leading to chemical-induced genetic damage and neoplasia.

8.1.7 Uptake, Metabolism and Toxicity of Hydrocarbons in Invertebrates

The acute toxicity (mortality preceded by narcosis) of about 10 ppb of naphthalene to early life stages of crustacea suggests that these life forms are highly sensitive to this component of the SWSF of crude

oil. The tendency of early developmental stages of shrimp to metabolize and retain NPH metabolizes at unchanged concentrations while concentrations of the parent hydrocarbon decline is of concern because evidence links aromatic hydrocarbon metabolizes to toxicity in mammals, The high susceptibility of larval and other developmental stages to aromatic hydrocarbons must be considered an important factor in the environmental effect of arctic and subarctic petroleum operations.

8.1.8 Food Chain Transfer of 2,6-DMN from Algae to Sea Urchins

Sea urchins feeding on 2,6-dimethylnaphthalene-exposed algae accumulated and metabolized this hydrocarbon to hydroxy compounds and their conjugates, thus demonstrating a food chain transfer of an aromatic hydrocarbon (Malins and Roubal 1982).

8.1.9 Fate of Metals in Fish

Salmon and starry flounder readily accumulate lead and cadmium from seawater (Reichert et al. 1979). The metals in liver and kidney were stored to a substantial degree in cellular fluids where biochemical and physiological processes occur (e.g., cytosol). Cadmium was preferentially bound by low molecular weight proteins (9,000 daltons), but was also associated with high molecular weight proteins of the cytosol. Lead showed a strong preference for neural tissue (e.g., brain). The findings imply that low concentrations of metals entering marine waters are likely to increase the metal burden of key tissues of fish and thereby possibly alter normal physiological processes.

Epidermal mucus of coho salmon is involved to some extent in excretion of lead and cadmium (Varanasi and Markey 1978). The importance of this route in relation to other excretory tissues (e.g., kidney), however, remains to be assessed. Exposure to sublethal levels of lead and cadmium for periods of up to two weeks resulted in increased production of epidermal mucus in coho salmon. This increased rate of production may induce alterations in physiochemical and theological properties of mucus.

Our results also show that skin and scales act as storage and perhaps detoxification sites for metals in both salmonids and flatfish. Substantial amounts of metals persisted in the skin and scales several weeks after fish were returned to clean water. Whether persistence of high concentrations of toxic metals would have adverse effects on skin structure remains to be seen.

8.2 Pathology

8.2.1 Effects of Petroleum on Disease Resistance

Laboratory studies evaluating the effects of petroleum hydrocarbons on immunocompetence and disease resistance of selected fish and shellfish

failed to demonstrate a marked impairment. Preliminary assays did, however, suggest petroleum dispersants may enchance the incidence of infectious disease in salmon.

8.2.2 Pathological Changes in Flatfish from Exposure to Oil-Contaminated Sediment

Experimental exposures of 3 species of flatfish (English sole, rock sole, and starry flounder) to PBCO-contaminated sediments indicates that English sole are the most likely to be adversely affected. Although the pathological changes observed appeared to be reversible and not directly life-threatening, they do reflect alterations that may reduce fitness, and thereby survival, of flatfish in heavily oiled environments.

8.2.3 Cytopathology

Cytopathological changes occurred in flatfish and smelt embryos exposed throughout embryogenesis to low ppb concentrations of the SWAF of crude oil. The olfactory cilia of flatfish developed abnormally and extensive necrosis was evident in the eye and brain of smelt embryos. Structural changes were also observed in liver and lens tissues of adult rainbow trout perorally exposed to high levels of petroleum hydrocarbons for 2 to 12 mo.

8.3 Behavior and Physiology

8.3.1 Vertebrate Studies

Behavior of Pacific Salmon Exposed to Petroleum Hydrocarbons

Mature Pacific salmon migrating upstream during the peak of the run substantially avoided a mixture of monocyclic aromatic hydrocarbons in the water at concentrations of 3.2 ppm (EC_{50}) and higher. Also, electrophysiological recordings from the olfactory bulb of juvenile salmon indicated that short-term exposure to these high concentrations of aromatic hydrocarbons would not be likely to disrupt the olfactory system.

Mark-recapture experiments were conducted to assess the ability of adult chinook and coho salmon to repeat their spawning migration following exposure to petroleum hydrocarbons. Chinook salmon exposed for 14 to 18 hr to FWAF of PBCO showed no alteration of homing capability. Similarly, the spawning migration of coho salmon was not disrupted by 8 to 22 hr exposure to a mixture of monocyclic aromatic hydrocarbons. However, the return of the coho to their captive site was delayed by 3 days.

Effects of Petroleum Hydrocarbons on Predator-Prey Behavior of Salmon

Exposure of chum salmon fry to the SWSF of CICO at a hydrocarbon concentration of 350 ppb for periods of 24 to 72 hr resulted in a

statistically significant greater consumption of the oil-exposed prey by coho predators compared to the consumption of controls.

Similarly, exposure of coho salmon predators to 340 ppb of the SWSF of CICO for over 3 days resulted in a reduction in numbers of salmonid fry eaten. Concentrations of parent hydrocarbons were higher in both liver and brain of actively eating oil-exposed predators compared to those not eating. This suggests that the parent hydrocarbons were not the compounds primarily influencing a decline in feeding behavior.

Avoidance Behavior of Flatfish Exposed to Oil-Contaminated Sediment

Although there is evidence of initial avoidance of oil-contaminant sediment by juvenile English sole, generally the fish did not avoid oil-sediment mixtures at concentrations up to 10,000 ppm - the sediment's apparent oil-carrying capacity. At these concentrations, some mortality did occur over a 15-day period, but the majority of flatfish were active and fed readily.

Effect of Petroleum Hydrocarbons on Development of Embryos and Larvae

Embryos of chum salmon, English sole, and sand sole exposed to water-borne hydrocarbon concentrations ranging from 100 to 500 ppb showed either high embryonic mortality, abnormal development, or delayed larval mortality. In addition, hydrocarbon concentrations of less than 100 ppb severely reduced the larval survival of surf smelt.

Effect of Crude Oil Ingestion on Salmonid Reproductive Success

Long-term peroral exposure of maturing male and female rainbow trout to high levels of PBCO had no marked effects on reproduction, as measured by hatching success and alevin survival.

8.3.2 Invertebrate Studies

Defense Behavior of Sea Urchins Exposed to Petroleum Hydrocarbons

Results of studies on the sea urchins' defense behavior following exposure to the SWSF of PBCO and its major aromatic components indicate that no single principal component of the SWSF is responsible for the total inhibition observed by exposure to the SWSF alone; that at low ppb concentrations the pedicellarial defense inhibition resulting from hydrocarbon exposure is apparently a chemosensory specific reaction and not general narcosis; and that SWSF-exposed sea urchins are preyed upon by Pycnopodia to a greater extent that non-exposed urchins.

Reproductive Behavior of Dorid Nudibranchs Exposed to Petroleum Hydrocarbons

Interference with the reproductive processes of dorid nudibranchs by the SWSF of PBCO occurred at several different levels. First, there was disruption of the chemotatic responses necessary to form mating

aggregations. Second, there was disruption of the egg laying process as noted by the lack of egg encapsulation, and delay in egg deposition. Third, eggs exposed to the SWSF showed retarded development and increases in abnormalities. Disruptive effects on the reproductive biology of dorid nudibranchs occurred at hydrocarbon concentrations as low as 15 ppb.

Feeding Behavior of Shrimp Exposed to Petroleum Hydrocarbons

Exposure of spot shrimp to the SWSF of PBCO resulted in a reduction in feeding activity. At 25 ppb there was a 50% reduction in overt behavioral "activity elicited in response to food stimuli, and at higher concentrations of the SWSF there was a still further decline in feeding responses, with some loss of equilibrium and mortality occurring at 500 ppb.

9. PAPERS PUBLISHED, DISSERTATIONS, THESES, AND PRESENTATIONS GIVEN UNDER RU73 OCSEAP SPONSORSHIP

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John S. Finley
Andrew J. Friedman
Donald D. Gennero*
Kirsten M. Gleim*
Dennis J. Gmur*
Lucinda Grant*
Victor D. Henry
Eric Henderson*
Tom Horn
Dr. Frederick G. Johnson*
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Kate King*
Dr. Vyacheslav Konchin*
Daniel Lazuran*
Dr. William D. MacLeod, Jr.
Dennis Markey*
Desmond J. Maynard*
Mark S. Meyers
James L. Mighell
Stephen E. Miller*
David A. Misitano
Laura M. Mumaw*
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Kenneth V. Pierce*
Patty G. Prohaska*
L. Scott Ramos*
Linda D. Rhodes
Paul A. Robisch
Philip Scordelis*
George F. Slusser*
Carla M. Stehr
Susan I. Stranahan*
Dr. Lawrence C. Thomas*
Patrick A. Treseler*
Michael Uhler*
Marianne Y. Uyeda*
Ellen M. Warinsky*
Dr. Marleen M. Wekell*
Keith K. Wong*

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