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LA THÈSE A ÉTÉ
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PETROLEUM EFFECTS ON PLASMA ION BALANCE AND GILL ATPASE
ACTIVITIES IN RAINBOW TROUT,
SALMO GAIARDNERI
(RICHARDSON)

by

Michael Philip Wong

A thesis
presented to University of Ottawa
in partial fulfillment of the
requirements for the degree of
Master of Science
in
Department of Biology

OTTAWA, Ontario, 1982

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ABSTRACT

The present investigation determined the ionic and osmotic responses in fish exposed to petroleum hydrocarbons. Juvenile rainbow trout, Salmo gairdneri, in freshwater were exposed to 100 ul/L of micron-size, oil-in-water dispersions of fresh and weathered oils for 7 days. Three oils, a light weight Norman Wells crude, a medium weight Venezuelan crude, and a paraffin oil were tested. Significant decreases in plasma sodium and chloride ions, and osmolality, were found in the crude oil exposed fish. Major changes in plasma ionic status were not evident in the paraffin oil treatments. Pathological changes, such as epithelial lifting and chloride cell vacuolation, were evident in gills of fish exposed to crude oil dispersions. Exposure to the water-soluble fraction of weathered crude oils or intraperitoneal injections of weathered Norman Wells crude oil produced moderate alterations in hydromineral balance. Gill morphological changes were not evident in these fish. These experiments suggest plasma ionic and osmotic imbalance from exposure to petroleum hydrocarbons could result from cellular damage in the branchial tissue.

Decreases in plasma ionic and osmotic components were found in freshwater acclimated trout exposed for 7 days to paraffin oil dispersions spiked with an aromatic hydrocarbon. The five compounds studied were benzene, naphthalene, phenanthrene, benz(a)anthracene and benzo(a)pyrene. Only the benzene and benz(a)anthracene treatments resulted in significant changes in the plasma ionic profile. No correlation between the acute toxicity of the aromatic hydrocarbons and their molecular weight was found.

Salt water acclimated trout exposed to weathered crude oil dispersions for 7 days had significantly elevated plasma sodium and chloride concentrations, elevated osmolality, and decreased calcium concentrations. Mortality was evident in the Venezuelan crude oil exposures. No major changes in plasma ionic and osmotic composition were found in fish given intraperitoneal injections of weathered crude oil. In both freshwater and salt water fish, the changes in plasma ion and osmotic concentrations could have resulted from a breakdown of the ion transport mechanisms.

Activities of microsomal preparations of ion-stimulated adenosine triphosphatases ($\text{Na}^+\text{-K}^+$, $\text{Na}^+\text{-NH}_4^+$, HCO_3^- , Ca^{2+} , Mg^{2+} ATPases) from gills of freshwater and salt water trout were examined following 7 day exposures to weathered crude oil dispersions. Enzyme activities from control fish were

generally similar to those described in the published record and validated the suggested rôle these enzymes play in ion regulation. Crude oil dispersions were demonstrated to be capable of altering enzyme activities. The results suggest the ionic imbalance in the oil exposed fish can be attributed to changes in enzyme activities in the gill tissue.

Activities of the gill enzymes were assayed with 5.0 mg/L of the aromatic hydrocarbons. Generally, exposure to aromatic hydrocarbons resulted in inhibition of enzyme activities. Monovalent ion-stimulated ATPases were more adversely affected than calcium or magnesium-stimulated ATPases. The lower molecular weight hydrocarbons had the greatest effect on enzyme activities. These effects can be correlated to the observed ionic dysfunction of hydrocarbon exposed fish. This study has demonstrated that oil pollutants can disturb important physiological and biochemical processes involved in hydromineral regulation.

RESUME

Le but de cette étude était de déterminer la réponse ionique et osmotique de poissons téléostéens exposés à des hydrocarbures de pétrole. Des truites arc-en-ciel juvéniles, Salmo gairdneri, furent exposées pendant 7 jours à 100 µl/L d'émulsions microscopiques d'huile fraîche et altérée en eau douce. Les pétroles bruts de poids léger Norman Wells et de poids moyen de Vénézuéla, et une huile de paraffine furent testées. Les concentrations plasmiqes des ions de chlorure et de sodium ainsi que l'osmolalité furent plus faibles chez les poissons exposés aux pétroles bruts. De tels changements majeurs n'en furent pas enregistrés chez les poissons exposés à l'huile de paraffine. Des changements pathologiques, tels que la séparation épithéliale et la vacuolation des cellules chlorures, apparurent au niveau des branchies des poissons exposés aux émulsions de pétrole brut. La balance hydrominérale ne fut que faiblement affectée chez des poissons exposés aux fractions solubles dans l'eau des pétroles bruts altérée ou chez des poissons injectées intrapéritonéalement de pétrole brut altéré de Norman Wells. Ces poissons ne montrèrent aucun changement morphologique des branchies. Ces expériences suggèrent que le

déséquilibre ionique du plasma est dû à des dommages cellulaires dans les tissus des branchies.

Une diminution des concentrations des composantes ioniques et osmotiques du plasma fut observée chez des poissons acclimatés en eau douce et exposés à des émulsions d'huile de paraffine enrichit d'un hydrocarbure aromatique. Le benzène, la naphthaline, le phénanthrène, le benz(a)anthracène et le benzo(a)pyrène furent étudiés. Seul les traitements au benzène et au benz(a)anthracène aboutirent à des changements significatifs du profil ionique du plasma. Il n'y avait pas de corrélation entre la toxicité aigue des hydrocarbures aromatiques et leurs poids moléculaires.

Les truites acclimatées à l'eau salée et exposées pendant 7 jours aux émulsions de pétrole brut altéré avaient des concentrations plasmiques de sodium et de chlorure élevées, une osmolalité plus forte et une concentration de calcium plus faible. L'exposition au pétrole brut Vénézuéla aboutit à une mortalité. Aucun changement majeur de la composition ionique et osmotique ne fut enregistré chez les poissons injectés intrapéritonéalement de pétrole brut altéré. Des altérations pathologiques au niveau des branchies peuvent avoir contribué au déséquilibre ionique chez les poissons traités, mais le rôle d'autres tissus contrôlant la balance ne peut être exclu. Les changements des concentrations io-

niques et osmotiques du plasma autant chez les poissons en eau douce que chez les poissons en eau salée peuvent être le résultat d'un mauvais fonctionnement des mécanismes de transport d'ions.

L'activité de préparations microsomales d'ATPasiques stimulées par des ions ($\text{Na}^+\text{-K}^+$, $\text{Na}^+\text{-NH}_4^+$, HCO_3^- , Ca^{2+} , Mg^{2+} ATPasiques) provenant de branchies de truite en eau douce et en eau salée exposées pendant 7 jours à des émulsions de pétrole brut altéré fut examinée. L'activité des enzymes des poissons témoins était semblable aux valeurs déjà publiées et confirme le rôle probable que jouent ces enzymes dans la régulation des concentrations d'ions. Les émulsions de pétrole brut furent capable de modifier l'activité des enzymes. Ces données suggèrent que le déséquilibre ionique chez les poissons exposés à l'huile peut être dû à la modification de l'activité des enzymes des branchies.

L'activité de ces enzymes fut évaluée en présence de 5.0 mg/L d'un hydrocarbure aromatique. En général, l'exposition aux hydrocarbures aromatiques aboutit à une inhibition de l'activité des enzymes. Les composés de poids moléculaire faible eurent le plus d'effet. Les ATPasiques stimulées par des ions monovalents furent plus affectées que les ATPasiques stimulées par le calcium ou le magnésium. Ces effets sont en corrélation avec le déséquilibre ionique observé

chez les poissons exposés aux hydrocarbures. Cette étude démontre que les polluants petrolifères peuvent déranger des processus physiologiques et biochimiques importants pour l'équilibre hydrominéral.

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Chapter I
INTRODUCTION

The increase in demand for and the utilization of petroleum products in recent years has resulted in steadily increasing levels of hydrocarbons in the aquatic environment. A greater environmental pollutant load may be expected as higher volumes of petroleum are transported and new deposits are exploited. Estimates by the National Academy of Sciences (1973) indicate the influx of petroleum into the aquatic environment from all sources approximates 6.2 million metric tons per year. More recent evidence suggests the amount is as high as 10 million metric tons annually (Grossling, 1976). At present, hydrocarbons in the aquatic environment are already 100 times greater than their yearly production and the potential threat to organisms by this accumulation is obvious.

Petroleum is a naturally occurring mixture of organic compounds derived from the partial decomposition of animal and plant matter over geological time. It is composed of tens of thousands of different chemical compounds, with hy-

hydrocarbons being the most abundant. The hydrocarbon components can be divided into three general classes: (1) aliphatic, (2) alicyclic, and (3) aromatic (Clark and Brown, 1977). The aliphatic hydrocarbons consist of saturated or unsaturated open-chain compounds. Some hydrocarbons belonging in this group include the paraffins (alkanes), the olefins (alkenes), and the alkynes (acetylenes). Alicyclic hydrocarbons are saturated or unsaturated compounds having some or all of their carbon atoms in a ring structure. Aromatic hydrocarbons are compounds with one or more six-carbon benzenoid rings. It is important to realize the complex chemical composition of fossil fuels and different petroleum products have distinguishing chemical and physical characteristics. Variations in the chemistry of petroleum are common in samples from different wells in the same field or samples from the same well at different depths or time (Clark and Brown, 1977).

Hydrocarbons can enter the environment via three general pathways: (1) human activities, (2) geochemical processes and (3) biosynthesis (Clark and MacLeod, 1977). Anthropogenic activities are the major sources of petroleum hydrocarbons. Frequently, pollution by oil originates from discharge by barges and ships, accidental or careless handling of oil products in transportation, drilling and production activities, as well as surface runoff from land. Natural

oil seeps represent another important source of hydrocarbons, estimated to be as great in volume as the input from human activities (Wilson et al., 1974; Grossling, 1976). Biogenic hydrocarbons also occur in the environment. The classes of hydrocarbons from biosynthesis are mostly alkanes and cover a rather narrow molecular weight range. The question of whether aromatic hydrocarbons can be synthesized by organisms is still debated (Hites, 1976). Compared to other sources of pollutants, petroleum represents the substance which is discharged into the environment at the greatest volume and frequency (Fugaro and Boyd, 1978).

A considerable volume of literature exists on toxicity effects of petroleum hydrocarbons to aquatic organisms (Malins, 1977). It remains difficult, however, to evaluate the relative toxicity of petroleum products and the sensitivity of different species to oil. The main reason is that many of the different physical, chemical and biological interactions governing the possible impact of oil on organisms are often not considered (Moore and Dwyer, 1974). Oil bioassays differ from those involving other pollutants in that attempts are made to examine the effects of a mixture of toxicants combined rather than the effects of a single pure compound. In order to evaluate the results of such tests, one has to take into account the large number of interactions which occur and the many compounds that are present.

Most oil toxicity studies expose organisms to fresh oil layered on top of the water. Under most natural situations, spilled oil is acted upon by numerous environmental factors. Processes that can affect the parent oil when it is released into water include: (1) spreading on the water surface to form a slick, (2) evaporation of volatile fractions, (3) dissolution of water soluble components in the water, (4) emulsification (dispersion), (5) adsorption into tar balls, (6) modification of the petroleum mixture by microorganisms, and (7) photochemical reactions (Clark and MacLeod, 1977). Of these processes, dissolution and dispersion appear to represent the most important mechanisms by which organisms can encounter oil in the water column. Low molecular weight aliphatic and aromatic hydrocarbons are often removed from an oil slick by dissolution. The aromatic content of oil may be the most toxic fraction because of its preferential leaching into the water and its ability to be accumulated selectively by organisms (Moore and Dwyer, 1974). The physical and chemical characteristics of oil-in-water dispersions (also termed emulsions or particulate oil) have been studied previously (Shaw and Reidy, 1979), but there is a paucity of information on its toxicity. Oil dispersed in water as micron-size droplets results in a million fold increase in the surface area of the oil (Berridge et al., 1968). Parker et al. (1971) investigated the stability of fine dispersions of oil and found they can persist in water

for several weeks. . Emulsified oil can also contribute to long-term pollution as oil refinery effluent (van Gelder-Ottway, 1976). Oil-in-water dispersions may cause biological damage because of their rapid dispersibility and persistence in water. Additionally, some workers have suggested that suspended oil particles beneath a slick can have a greater impact on organisms than dissolved hydrocarbons (Parker et al., 1971; Nelson-Smith, 1972).

The weathering of petroleum can substantially modify its aqueous solubility, dispersibility and chemical composition in the water (Lysyj and Russell, 1974; MacKay and Shui, 1976; Shaw and Reidy, 1979). Upon release into the aquatic environment various physical forces immediately interact with the hydrocarbons beginning the process of weathering. A large proportion of hydrocarbons, particularly the aromatic fraction, can be retained despite considerable weathering action. Blumer and Sass (1972) found that oil stranded on beach sediment for over a year preserved its initial aromatic hydrocarbon ratio. Similarly, Vandermeulan and Gordon (1976) reported that five years after a spill, the oil in interstitial water environments had a sizable fraction of aromatics remaining, while the n-alkanes were completely degraded. They stated that the remaining hydrocarbons could eventually re-enter the water column by wave action and shifting sand. Such re-entry of hydrocarbons can contribute

to more adverse effects years after the initial oil discharge (Sanders et al., 1980).

The identification of organismic responses to oil is complicated by the nature of petroleum. The dominant constituents are hydrocarbons and almost all biological responses are attributed to these compounds (Moore and Dwyer, 1974). Volatile paraffinic hydrocarbons containing ten carbon atoms or less are generally insignificant in the environment because of their low boiling point. Some soluble alkanes can cause cell damage and narcosis in protozoans (Goldacre, 1968), but the high concentrations required to bring about such effects are unlikely to occur under spill conditions. Non-volatile paraffins occur naturally in many aquatic organisms and are not directly toxic (Clark and Brown, 1977). Petroleum often contains a small mixture of alicyclic hydrocarbons or naphthenes. These compounds have been isolated from marine algae and are prominent in some terrestrial plants (Clark and Brown, 1977). Given the low concentrations at which these compounds exist in petroleum, it is unlikely these hydrocarbons are responsible for the toxic effects (Moore and Dwyer, 1974). Recent work indicates the toxicity of petroleum products may be directly correlated to its content of aromatic hydrocarbons (Moore and Dwyer, 1974; Neff, 1979). Neff (1979) reviewed the sources and occurrence of aromatic hydrocarbons in the aquatic environment and re-

ported these compounds to be ubiquitous. Aromatic hydrocarbons, such as benzene, naphthalene and their derivatives, have higher water solubility than paraffins of equal molecular weight (McAuliffe, 1966). These lower molecular weight aromatics are believed to be the toxic components of oil. However, higher molecular weight aromatics with four and five benzenoid rings are cumulative toxicants and can be carcinogenic and mutagenic inducers in test animals (Hodgins et al., 1977; Neff, 1979).

Despite the large data base available on petroleum toxicity, most of the information is of limited value from a comparative standpoint. Generally, these studies are reported in the form of LD50s or the concentration of hydrocarbons required to produce fifty percent mortality in test animals over a given time. Considering the complex nature of petroleum products, it is impossible to evaluate the toxicity of different oil products. The validity of using such studies in oil toxicity investigations has been questioned (LaRoche, 1973; cited in Moore and Dwyer, 1974). These studies, however, are useful for examining the differential sensitivity of various species or life stages within the same species to the pollutant. In general, larval and juvenile life stages are more sensitive to oil than adult stages. The latter usually show lethal effects at higher concentrations. Estuarine and coastal species are believed to be more tolerant to

oil pollutant stress than pelagic species, because the former are adapted to a normally more stressful physical environment (eg. temperature, salinity) (Neff et al., 1976a).

Recent investigations have focused on sublethal effects of petroleum hydrocarbons. These physiological, behavioral and growth studies have acknowledged that oil and its constituents can affect many key biological processes in organisms. These responses are considered to be sensitive indicators of possible deleterious effects on long-term success of a population and may provide an early indication of increased environmental contamination. Two approaches can be taken when performing such studies. One can expose the test organism chronically to low levels of the stressful stimulus and measure its effects on growth and reproduction over a long period of time. The second approach is to expose the fish briefly and record the effects for possible mode of action of the pollutant (Duval and Fink, 1981).

A large number of physiological responses have been employed to study the influence of environmental stress on fish. One of these is the measurement of metabolic rate, or respiration. Sheepshead minnows, Cyprinodon variegatus, exhibited an increase in respiratory rate when exposed to the water-soluble fractions (WSF) of a No. 2 fuel oil, but reduced their oxygen consumption when exposed to a Bunker C

residual oil (Anderson et al., 1974a). Suppression of respiratory rates was observed in pinfish, Lagodon rhomboides, when challenged with a petrochemical waste (Anderson, 1979). Changes in respiratory rate of fishes have also been observed during exposure to crude oils and individual aromatic hydrocarbons. An increase in opercular ventilation was reported in pink salmon, Oncorhynchus gorbuscha, exposed to two Alaskan crude oils (Thomas and Rice, 1975; Rice et al., 1977b). Chinook salmon, O. tshawytscha, and striped bass, Morone saxatilis, exposed to benzene showed an immediate increase in respiratory rates, followed by a decrease upon prolonged exposure. These responses appear to be transitory, because the respiratory rates return to control levels upon release into clean water (Brocksen and Bailey, 1973). Studies performed with different fish species and many petroleum products indicate oxygen consumption can either increase, decrease or remain unchanged (Patten, 1977). Neff et al., (1976a) suggested that respiratory rates reflect the sum of several superimposed responses of the animal, thus accounting for the variability of the stress response. Therefore, oxygen consumption may not be useful as a general indicator of oil pollutant stress.

Several recent studies have dealt with behavioral reactions of fish to petroleum. Convulsive respiratory reactions were observed in Atlantic salmon, Salmo salar (Barnett

and Toews, 1978). The "cough" is actually a short reversal of normal water flow followed by a rapid extrusion of water through the operculum (Holeton and Jones, 1975). The amount of gas exchanged during this response is probably less than a normal respiratory movement because of an altered alignment of the gills. Morrow (1973) reported that coho (Oncorhynchus kisutch) and sockeye (O. nerka) salmon displayed abnormal behavioral reactions to crude oils at concentrations of 500 to 3000 $\mu\text{L/L}$. The fish swam on the water surface near the slick and lost their swimming coordination. Exposure of Fundulus similis to No. 2 fuel oil produced decreases in mobility and loss of equilibrium (Dixit and Anderson, 1977). These workers stated the degree of interference with locomotory capabilities in the fish was related to the naphthalene content accumulated in the brain. Several problems occur when behavioral responses are used as an index of sublethal pollutant stress. It is often difficult to distinguish pollutant-induced interactions from normal behavioral variations, which can be extremely complex and can be influenced by a wide spectrum of natural biotic and abiotic factors. In addition, the observed effects can not be interpreted with regard to long-term survival nor be used for predicting impact. Finally, in most cases, behavioral responses are no more sensitive to sublethal hydrocarbon stress than other physiological measurements.

Growth and reproduction have been used as means of measuring responses to sublethal concentrations of hydrocarbons. Rice et al. (1975) found lowered growth rates in pink salmon following a 10 day oil exposure. Delayed growth was also recorded in Pacific herring larvae, Clupea harengus pallasii, and striped bass, Morone saxatilis, after contamination by benzene (Struchsaker et al., 1974; Korn et al., 1976). In addition, weights and lengths of coho salmon fry decreased upon exposure to toluene and naphthalene (Moles et al., 1981). A decline in hatching success of ova from rainbow trout was reported following incorporation of crude oil in the diet (Hodgins et al., 1977b). The average survival of the resulting alevins decreased, but the authors stated the effects did not indicate a significant deleterious impact on reproductive potential of the trout. Struchsaker (1977), however, found a drastic reduction in survival of eggs and larvae of herring from a brief exposure to benzene. Similar adverse effects were reported on embryogenesis and larval development in winter flounder, Pseudopleuronectes americanus (Kuhnhold et al., 1978). These studies indicate that oil pollutants may have long-term detrimental impacts on fish populations.

Another physiological parameter employed as an index of pollutant stress is the ability of organisms to regulate internal ion balance. Water-electrolyte status in both fresh

and salt water adapted fish appears to be sensitive to environmental changes (Mazeaud et al., 1977). Reportedly, sublethal levels of copper resulted in loss of ionoregulatory capacity in brook trout, Salvelinus fontinalis, perch, Perca flavescens, bluegill, Lepomis macrochirus, and bullhead, Ictalurus nebulosus (McKim et al., 1970; Christensen et al., 1972). Similarly, exposure to copper and zinc resulted in a significant decrease in plasma osmolality in channel catfish, Ictalurus crysoleucas (Lewis and Lewis, 1971). McCarthy and Houston (1976) found osmoregulatory impairment in goldfish, Carassius auratus, as a functional consequence of exposure to sublethal cadmium concentrations. Changes in circulating plasma ion concentrations in fish exposed to chlorinated hydrocarbons have been documented (Janicki and Kinter, 1971a; 1971b; Leadem et al., 1974). Thus, osmoregulatory events are sensitive physiological measurements suitable for a variety of contaminants and species of test animals.

The branchial epithelium is the most important effector organ involved in the maintenance of salt balance in teleosts (Maetz, 1971; 1974). Freshwater fish loses salt to the hypotonic medium by diffusion and in the copious urine it excretes. Maintaining a constant and high internal osmotic environment require active uptake of ions through the gills. Comparatively, seawater fish compensates for the

large influx of ions from the hypertonic media across various epithelia and the reabsorption of water by the intestine by actively excreting electrolytes through the gills to maintain proper osmotic balance.

The gill, as one of the most delicate epithelia exposed to the environment, is prone to damage by pollutants. Hydrocarbons can cause such effects. For instance, seven species of fish collected near an oil discharge showed structural gill damage. Loss of epithelial and mucous cells, sloughing of the epithelia and reduction in the numbers of chloride cells (acidophilic cells or ionocytes) were evident (Blanton and Robinson, 1973). Eels, Anguilla anguilla, caught in waters near the wreck of the Amoco Cadiz off the coast of France, exhibited gill histopathology from oil exposure (Lopez et al., 1981). An abnormal proliferation of chloride cells and mucous cells was apparent in the oil exposed fish. Laboratory studies have validated these findings. Fresh and salt water acclimated rainbow trout, Salmo gairdneri, developed histopathological alterations in the gill as a consequence of Bunker C oil or Bunker C oil and dispersant exposures for 96 hr (McKeown and March, 1978a). The structural alterations included curling of lamellar tips, rupturing of cells, epithelial lifting and an increase in necrotic debris. Likewise, exposure to a Wyoming crude oil for 90 days

induced gill lesions in cutthroat trout, Salmo clarki (Woodward et al., 1981). The lesions were characterized by hyperplasia, edema and fusion of the lamellae. Similarly, Hawkes (1977) reported that coho salmon and starry flounder, Platichthys stellatus, exposed to the water-soluble fraction of a crude oil, showed cellular disruptions and sloughing of surface cells.

In conjunction with the above evidence, individual components of petroleum can also alter the gill architecture. The dinuclear aromatic hydrocarbon, naphthalene, at concentrations from 0.002 to 30 mg/L, caused gill hyperplasia and hemorrhaging in filaments of exposed F. heteroclitus (DiMichele and Taylor, 1978). Phenol, a minor component of crude oil, had severe pathological effects on gills of rainbow trout (Mitrovic et al., 1968), and fourteen species of fish collected in phenol polluted waters of the Rhine and Elbe Rivers (Reichenbach-Kline, 1965). Further, extracts of motor oil exhaust with high concentrations of monocyclic aromatic hydrocarbons caused alterations in gill structures of exposed goldfish (Brenniman et al., 1979). The gill area (2 to 10 cm²/g body weight) represents the largest permeable surface on a fish (Hughes, 1966) and appears to be a target site for water-borne hydrocarbons.

Branchial tissues of fish have been shown to accumulate substantial levels of crude oil and individual hydrocarbons (Neff et al., 1976b; Rice et al., 1977b; McKeown and March, 1978b). They are also the primary sites of hydrocarbon entrance, since the gill's micellar layer can adsorb the water-borne hydrocarbons (Lee et al., 1972). As stated earlier, acute exposures to petroleum products can cause cellular modifications in the gill epithelium (Blanton and Robinson, 1973; Hawkes, 1977; DiMichele and Taylor, 1978; Lopez et al., 1981). An attractive hypothesis is that the sensitivity of fish to petroleum hydrocarbons involves impairment of gill functions, specifically ion regulation, as a result of cellular changes in the gill tissue. Ionic dysfunction from oil exposure has been demonstrated in some organisms. Anderson and Anderson (1976) noted that oil-exposed oysters, Crassostrea virginica, had altered ion concentrations in the pericardial fluid. Ingestion of oil by ducklings, Anas platyrhynchos, inhibited salt and water transport in the intestine (Crocker et al., 1974). Similarly, a single oral dose of crude oil in herring gull chicks, Larus argentatus, caused impairment in sodium transport and nasal gland function (Miller et al., 1978). With these observations, it is of interest to test hydrocarbon stressed fish for osmoregulatory failure.

Roubal (1974) found hydrocarbons accumulating in lipid deposits -such as the lipoid portion of cellular membranes. This invasion of hydrocarbons, particularly aromatics, was postulated to be effective in altering membrane properties and perturbing membrane-bound enzymes. These enzymes -vital to many physiological processes- require precise lipid-protein interactions for their function. At present, very little is known concerning membrane-pollutant interactions. These influences may represent the molecular basis of physiological changes in chemically insulted organisms. A few studies indicate certain biochemical systems can be perturbed by petroleum. A great deal of attention has focused on the metabolism and discharge of petroleum products. This process is mediated by the mixed-function-oxidase enzymes located on the endoplasmic reticulum of animal cells. The topic of biotransformation of accumulated hydrocarbons has recently been the subject of several reviews (Varanasi and Malins, 1977; Nava, 1979; Connell and Miller, 1981). Fish collected from petroleum-contaminated areas had elevated levels of benzopyrene hydroxylase enzyme activities in the gill and liver tissues (Payne, 1976). Heitz et al. (1974) studied the acute effects of crude oil on enzymes in tissues of Mugil cephalus and found B-glucuronidase and leucine aminopeptidase had decreased activities while malate dehydrogenase and glutamic-pyruvic transaminase had augmented activities. Additionally, certain aspects of lipid metabolism

were altered in F. heteroclitus by low levels of No. 2 fuel oil (Stegeman and Sabo, 1976; Sabo and Stegeman, 1977). A major reduction in the rate of C^{14} -acetate incorporation into triglycerides occurred in oil exposed fish. As a result of decreased lipogenesis in the liver, a smaller number of lipid droplets was evident in this organ. Furthermore, a decrease in the rate of phospholipid synthesis and increased synthesis of cholesterol was found. This is a significant observation in that these products are major components of cellular membranes.

Recent studies clearly indicate chloride cells in the branchial epithelium of teleosts as the specific loci of electrolyte exchanges between internal and external media (Karnaky, 1980). Adenosine triphosphatase enzymes (ATPases) localized on the basolateral membrane in these specialized cells are closely associated with active ion exchange across the gill membrane. The fact ATPase enzyme activities vary with salinity adaptation emphasizes the important role these enzymes play in ion balance (Maetz and Bornancin, 1975).

Some evidence is available indicating organic pollutants can disrupt membrane transport (Kinter and Pritchard, 1977). Petroleum hydrocarbons, like many organic substances, have a high affinity for lipids. This association with the lipid portion of cellular membranes may alter the conformation of

the structure (Roubal, 1974; Roubal and Collier, 1974). Observations by Blanton and Robinson (1973) and Lopez et al. (1981), showed that petroleum can modify chloride cell morphology in gills of fish. One may postulate that the active ion transport mechanism in oil exposed fish can be disrupted by hydrocarbons. Examination of ATPase enzymes would provide insight into the effect of oil pollutants on this biochemical system.

The purpose of the present investigation is to examine the physiological responses, particularly ionic and osmotic balance, of rainbow trout following exposure to crude oils and their constituents in fresh and salt water. In addition, studies were performed to assess the effects of petroleum hydrocarbons on adenosine triphosphatase enzymes. Rainbow trout was selected as the test organism because the species occurs expansively in the temperate zone and much information concerning its physiology is currently available (Wells, 1977). It is a euryhaline teleost which survives well in both a fresh and salt water medium and is a good osmoregulator of internal body fluids (Houston, 1959; Gordon, 1963).

Chapter II

PETROLEUM EFFECTS ON OSMOREGULATION IN FRESHWATER ACCLIMATED TROUT

2.1 INTRODUCTION

Approximately 30% of the annual influx of petroleum into the aquatic environment (5 to 10 million metric tons) pollute freshwater ecosystems (Tarshis, 1981). Each of the Great Lakes has experienced oil pollution from accidental spills and refinery waste resulting in loss of fish (Wilber, 1969). Despite considerable research on the impact of petroleum on fish, the area is still poorly understood because of variability of oils and environmental factors affecting both the test species and the pollutant (Patten, 1977). Unless these variables are taken into consideration, predictions of potential environmental consequences by oil is impossible.

Information is sparse with respect to the effects of weathering processes upon the toxicity of spilled oil. Fresh and weathered oil have different chemical compositions, so one may expect different biological effects on exposed organisms. Weathered oil had greater toxicity effects on

hatchability of bird eggs (Macko and King, 1980) and on phytoplankton survival (Vandermeulan et al., 1979), than fresh oil. The probability of exposure of organisms, such as fish, to weathered oil is greater relative to fresh oil.

Oil-in-water dispersions can account for up to 98% of spilled oil (Boylan and Tripp, 1971; Gordon et al., 1973). Dispersed oil particles are often formed by wind and wave action, or by addition of chemical agents. These factors, by facilitating the formation of dispersions in the water column, may actually increase hydrocarbon exposure to aquatic organisms. In fact, oil in this form can be encountered by aquatic organisms, including fish (Conover, 1971; Kuhnhold, 1978). Finely dispersed oil may be more hazardous to aquatic life than water-soluble fractions of oil because of its bioavailability and persistence in the water (Clark and Findley, 1977). Yet, information on the toxicity of oil-in-water dispersions is lacking.

The acute toxicity of petroleum has been correlated with its content of soluble derivatives (Moore and Dwyer, 1974). The water-soluble fraction (WSF) of oil usually contains relatively high concentrations of mono- and dinuclear aromatic hydrocarbons and low concentrations of higher molecular weight aromatic hydrocarbons (Anderson et al., 1974b). No studies have been attempted to assess the toxic effects of WSF of oil following weathering.

Uptake of hydrocarbons from water or food occurs rapidly in fish (Lee et al., 1972; Roubal et al., 1977). There is evidence that relatively high concentrations (up to 1200x the media concentration) of these compounds can accumulate in various tissues such as / liver, brain and gills. Even though there has been extensive research on metabolism and biotransformation of assimilated hydrocarbons (Varánasi and Malins, 1977), there is little information on physiological consequences of this retention.

The purpose of the present study was to determine the toxicity of weathered crude oils, using plasma ionic and osmotic status of freshwater fish as an indicator of stress. Specifically, attempts were made to correlate effects to the chemical characteristics of the oils. Evaluation of the contribution to toxicity by various modes of hydrocarbon exposure, weathered oil was partitioned into (1) oil-in-water dispersions, (2) water-soluble fraction, (3) and systemic contamination (intraperitoneal injection). In addition, the toxicity of the aromatic fraction of oil was assessed by exposing the test animals to individual aromatic hydrocarbons in dispersed form.

2.2 METHODS AND MATERIALS

2.2.1 Experimental Animals and Holding Facilities

Juvenile rainbow trout, Salmo gairdneri (Richardson), from the same genetic strain were procured from Thistle Springs Trout Farm (Ashton, Ontario). The fish were held in large fibreglass tanks with a flow-through system supplied with dechlorinated Ottawa City tap water (8-12 °C; pH 7.4-7.8) and continuous aeration. Chlorine (American Public Health Association, 1971) and ammonia (Solorzano, 1969) were below detection limits. Dissolved oxygen was kept above 80% saturation (Yellow Springs Instruments). Photoperiod was held constant at 12L/12D, using wide spectrum fluorescent light. The trout, 50-150 g, were acclimated to these conditions for at least four weeks prior to experimentation. They were fed ad libitum once daily with dry commercial trout pellets (Purina Chow, No.4 Developer).

2.2.2 Experimental Procedures

Following acclimation, groups of 5 to 10 fish were placed in glass aquaria (60x60x80 cm) filled with dechlorinated water held at 10±1 °C. Water was continuously aerated and recirculated (Dynaflo Power Filter 150) at 4 to 6 L/min. Fish

were fed every other day and acclimated to these conditions for one additional week, before experimentation. Feeding ceased 48 h prior to start of the tests. Dissolved oxygen was maintained at 75-85% saturation (Yellow Springs Instruments). Ammonia was monitored regularly using the methods of Solorzano (1969) and the levels were always below the toxic limit recommended for salmonids (Wedemeyer and Yasutake, 1977).

A light weight Norman Wells and a medium weight Venezuelan crude oil were used in the toxicity tests. The Norman Wells crude oil from northern Canada (Northwest Territories) is representative of petroleum from the area, while Venezuelan crude oil is common to the type often transported to the east coast of Canada. Both crude oils were kept in sealed amber containers at -20 °C until use. A light weight paraffin oil (14 to 18 carbons) was used to test the effects of oil particles alone, without the presence of toxic components.

Standard 7-day toxicity tests were used to establish the influence of hydrocarbon treatments on trout. The methods employed were similar to those recommended by LaRoche *et al.* (1970) for oil toxicity evaluation. For weathered oil treatments, the three oil types were prepared at 1000 ul/L and aged for 3 days in aquarium water. When the concentrations

had reached approximately 100 ul/L, the fish were added. These concentrations were used to simulate those following a spill situation (1.0 to 200 ul/L) (Clark and Findley, 1977). For comparative purposes, fresh 100 ul/L doses of the three oils were studied in dispersed form. Fish were placed into the aquaria immediately after the oil addition. In both exposure regimes, the oil was emulsified mechanically into micron-size oil-in-water dispersions using a homogenizer. Simultaneous sets of non-exposed (control) fish accompanied the above tests. No food was administered to the fish during the exposure period.

To separate the effects of the water-soluble fractions of crude oils and dispersions, groups of fish were exposed to preparations of water-soluble components of the oils. Norman Wells and Venezuelan water-soluble fractions were prepared by placing the oil on top of the water in an enclosed container. The oil was in contact with the water but a micron-size mesh net prevented any particulate oil to enter the water column. The water-soluble fraction was weathered for 3 days, from a nominal concentration of 1000 ul/L, before addition of test animals.

The following design was employed to differentiate external oil effects from internal contamination. Groups of trout were treated with a 3 day weathered crude oil (Norman

Wells) by intraperitoneal injections (IP) at a dose of 100 ul/kg of fish per day for 7 days. Roubal et al.: (1977) demonstrated that IP injections of hydrocarbons in salmonids resulted in similar accumulative effects as those administered through diet. The fish were held in recirculating systems (Living Streams, Model LS 700, Frigid Units, Inc., Toledo, Ohio) equipped with a circulator (Min-o-cool circulator, Frigid Units, Inc.) and supplied with dechlorinated tap water (10 ± 1 °C). Charcoal filters were installed to remove any excreted hydrocarbons and metabolic products. Controls were kept in a similar system and were sham injected.

To test the effects of individual aromatic hydrocarbons, the following experimental design was employed. A nominal dose of paraffin oil (100 ul/L) was spiked with 3.0 mg/L of benzene, 0.3 mg/L of naphthalene, 0.03 mg/L of phenanthrene, 0.003 mg/L of benz(a)anthracene, or 0.0003 mg/L of benzo(a)pyrene, and made into a dispersion by homogenization (Figure 1). The concentrations were based on levels of these compounds one may detect in the water column after an oil discharge. Even though benzopyrene does not occur in crude oils, or is at very low concentration, it is often produced in the environment by various chemical reactions (Neff, 1979). The fish were exposed to these compounds for 7 days, by methods similar to those described above.

2.2.3 Sampling and Analytical Procedures

The fish were subjected to the hydrocarbon treatments for 7 days and were not fed during this period. Preliminary experiments indicated the fish ceased feeding upon exposure to oil particles at dosages as low as 50 $\mu\text{L/L}$. Following exposure, the trout were gently dipnetted from the water and anaesthetized with 0.1% ethyl-m-aminobenzoate methane sulfonate (MS-222, Sigma). The anaesthetic was prepared with dechlorinated water and kept at the same temperature as the test water. Neutralization of the pH of MS-222 (phosphate buffer, monobasic) was carried out to avoid any changes in the hematological profile (Wedemeyer, 1970; Soivio *et al.*, 1977). A high concentration of anesthetic was found to be more effective in that it reduced struggling time. At the above concentration the fish were sedated in less than one minute. Sampling was performed at the same time of day (14:00 h) to preclude any diurnal changes in blood parameters.

Blood was taken by severing the caudal peduncle of the anaesthetized fish and collected in ammonium heparinate (1000 I.U./ml) coated microtubules. Plasma was stored at -70°C until subsequent analysis.

Plasma sodium was determined by an internal standard flame photometer (Evans Electro Selenium, Ltd.). Plasma chloride was measured by potentiometric titration on a Buchler-Cotlove chloridometer (Buchler Instruments, Inc.). Calcium concentrations were estimated spectrofluorometrically by the methods of Wallach and Steck (1963) and Kepner and Hercules (1963) (G.K. Turner Associates). Plasma osmotic activity was determined by freezing point depression (Advanced Instruments, Inc.). If sufficient plasma was available, potassium concentration was determined by flame photometry. All ion and blank standards were made with doubly distilled and deionized water (0-1 mOsmole/kg).

Branchial tissues from control and experimental animals were sampled for histopathological examination. This work was performed by M.E. Duey (Duey, MS in preparation, University of Ottawa) and some of the results have been published (Engelhardt et al., 1981).

In some of the exposure regimes, groups of fish were sampled on days 1, 3, 5, and 7. These included exposures to weathered dispersions of the two crude oils (Norman Wells and Venezuelan) and Norman Wells oil IP injection (Appendix C). The purpose of these investigations was to examine the cortisol response as related to the ion profile. In addition, a study of the effects of cortisol injections, and

cortisol injections/oil exposure in combination, on ion balance was carried out (Appendix C, D). Cortisol was measured by the Immo-Phase method (Corning) as described previously for eel plasma (Nava, 1979). Since the corticosteroid response did not appear to have affected the ionic status in these fish, the results are discussed in Appendix C and D.

In addition to the plasma ions, several other physiological parameters were monitored in the above experiments. These included packed red blood cell volume (hematocrits) (Hesser, 1960) and packed white blood cell volume (leucocrits) (McLeay and Gordon, 1977). Blood hemoglobin concentration was determined by the cyanmethemoglobin reagent (Hycel). Plasma glucose was measured colorimetrically using the glucose oxidase and peroxidase enzyme reactions (Sigma). The plasma protein concentration was estimated by a modification of the procedures of Lowry et al. (1951). Liver somatic indices and percent water content of epaxial muscle were also determined. These results are in Appendix E.

Total hydrocarbon concentrations in test waters were measured spectrofluorometrically (G.K. Turner Associates) as described previously (Engelhardt et al., 1977). Optimal wavelengths of excitation at 290 and 365 nm and emission at 360 and 410 nm were employed, for Norman Wells and Venezuelan crude oils, respectively. Aliphatic and aromatic fractions


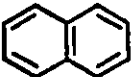
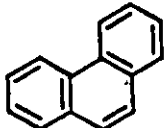
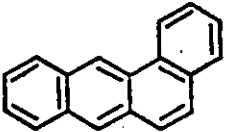
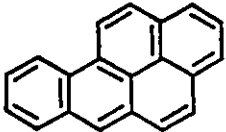
of the crude oils were also determined by gas chromatography (Appendix B). Total number of oil particles (dispersions) was measured using a Coulter Counter (Coulter Electronics, Inc.). A random selection of not less than 100 particles was measured microscopically with the use of a calibrated ocular grid to determine size distribution (Appendix A).

2.2.4 Statistical Analysis

Statistical comparisons of test and control data was made using one-way analysis of variance (ANOVA) following test for homogeneity. A significance level of $p \leq 0.05$ was employed.

The molecular weight and structure of selected aromatic hydrocarbons used in the study.

Figure 1: Aromatic hydrocarbons

Hydrocarbon	Structure	Molecular weight
Benzene (C ₆ H ₆)		78.11
Naphthalene (C ₁₀ H ₈)		128.16
Phenanthrene (C ₁₄ H ₁₀)		178.22
Benz(a)anthracene (C ₁₈ H ₁₂)		228.28
Benzo(a)pyrene (C ₂₀ H ₁₂)		252.30

2.3 RESULTS

No mortality was found in any of the exposure regimes, but changes in plasma water-electrolyte status were apparent. The effects of fresh and weathered paraffin, Norman Wells and Venezuelan oils on plasma sodium and chloride ion concentrations are illustrated in Figure 2 and 3, respectively. Significant decreases were found in levels of these two major electrolytes in the plasma of fish exposed to fresh and weathered crude oils, but ion levels in paraffin oil treated trout were not compromised. Plasma calcium concentrations did not change significantly in fish from any of the exposure regimes. Decreases in plasma osmolality was evident in fish from fresh and weathered crude oil exposures, except the fresh Venezuelan exposure. In addition, the 1000 ul/L paraffin oil exposed fish showed a decline in plasma osmolality (Figure 4).

Comparisons between fresh and weathered oil effects showed sodium concentrations were significantly lower in fresh Norman Wells oil exposed fish than those exposed to weathered oil. Fish exposed to fresh Norman Wells oil also had lower sodium levels than those exposed to fresh Venezuelan crude oil. No differences in plasma sodium concentrations were found in fish from the fresh and weathered Venezuelan crude oil exposures. Similarly, the sodium

concentrations from fish exposed to the two weathered oils showed no significant differences. Furthermore, the plasma chloride levels did not differ between fish exposed to fresh and weathered Norman Wells and Venezuelan oils. Fish from the weathered Venezuelan crude oil exposures, however, had significantly lower plasma osmolality than fish from fresh oil exposures. No differences were observed for fresh and weathered Norman Wells exposed fish. Comparisons between oils showed no differences in the weathered exposures but a significantly lower plasma osmotic activity was found in fresh Norman Wells relative to fresh Venezuelan oil exposures.

The water-soluble fractions (WSF) of the crude oils had only limited effects on ion balance. A significant decrease was found in plasma chloride and potassium levels in fish exposed to the WSF of Norman Wells crude oil. The sodium and calcium levels did not differ from controls. Plasma osmolality of fish exposed to the WSF of Venezuelan crude oil showed a significant decline (Figure 5). In the intraperitoneal injection experiment, weathered Norman Wells crude oil caused a decrease in plasma chloride ion levels. No changes were evident in the other plasma ion parameters (Figure 5).

External exposures to aromatic hydrocarbons resulted in some changes in plasma ion concentrations. However, only the monocyclic aromatic hydrocarbon, benzene and the four benzenoid ring compound, benz(a)anthracene, had significant effects. Exposure to benzene caused decreases in plasma sodium and chloride, and concomitant declines in plasma osmotic pressure. Benz(a)anthracene exposed fish had significantly lower plasma chloride ion concentrations compared to controls (Figure 6).

The external exposures to weathered crude oil over 1,3,5 and 7 days demonstrated the plasma ionic status in the fish over time (Appendix C). Changes in plasma sodium and chloride, as well as osmolality, were evident. The intraperitoneal experiment over the same time frame did not produce similar findings (Appendix C).

Changes in other hematological parameters were evident in the oil exposed fish. While hematocrits did not vary significantly in the external oil exposures, the packed white blood cell volumes, or leucocrits showed dramatic declines in the oil exposed fish (Appendix E).

Plasma sodium ion concentrations in freshwater acclimated rainbow trout following exposure to fresh (100 ul/L) and weathered (1000 ul/L) oil dispersions for 7 days.

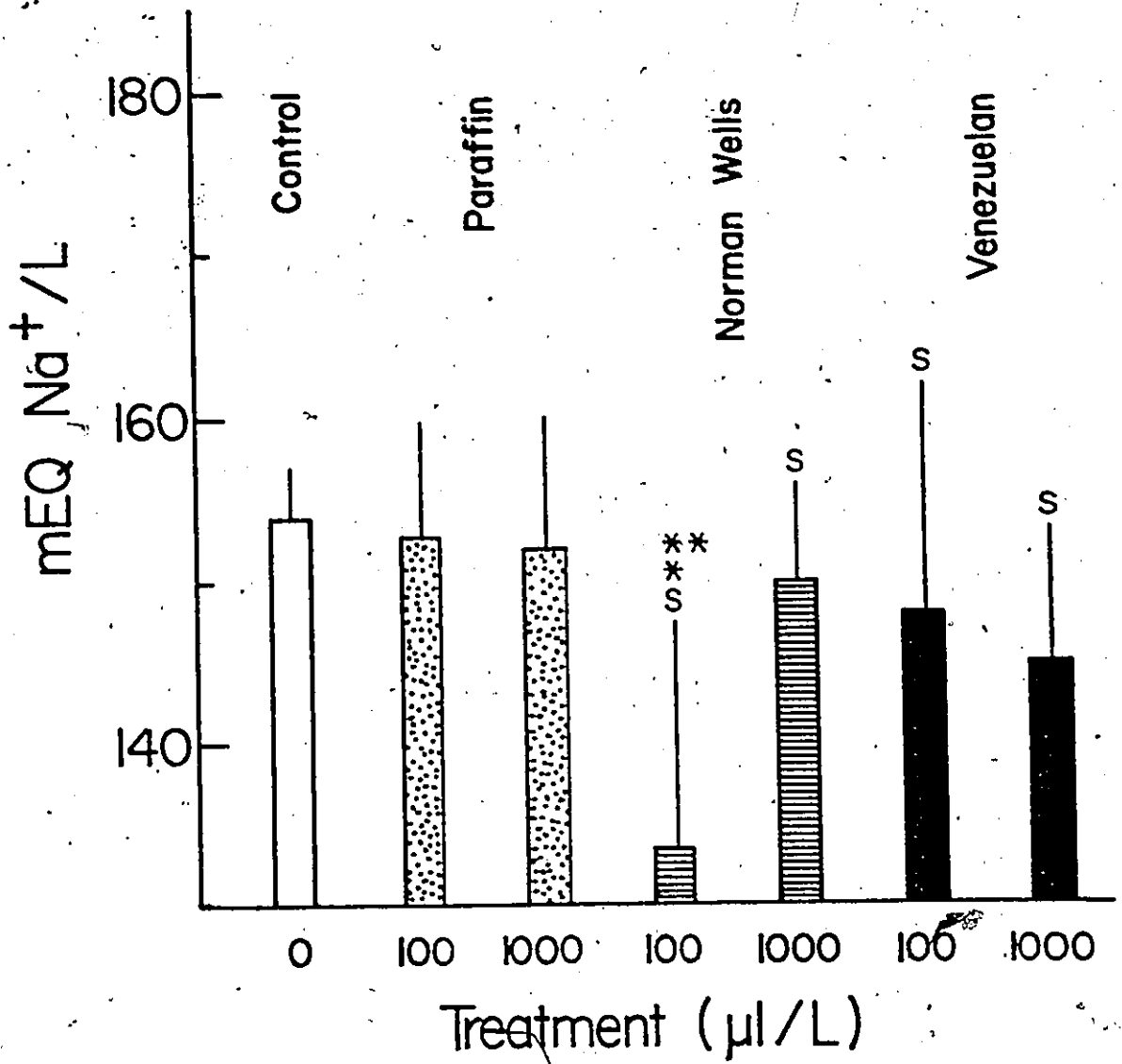
"S" denotes a statistically significant difference ($p \leq 0.05$) from controls.

"*" denotes a statistically significant difference ($p \leq 0.05$) between fresh and weathered crude oil treatments.

"**" denotes a statistically significant difference ($p \leq 0.05$) between fresh crude oil treatments.

All values are means of 10 fish, except control N=20. Vertical bars represent one standard deviation.

Figure 2: Sodium levels in oil dispersion exposed fish

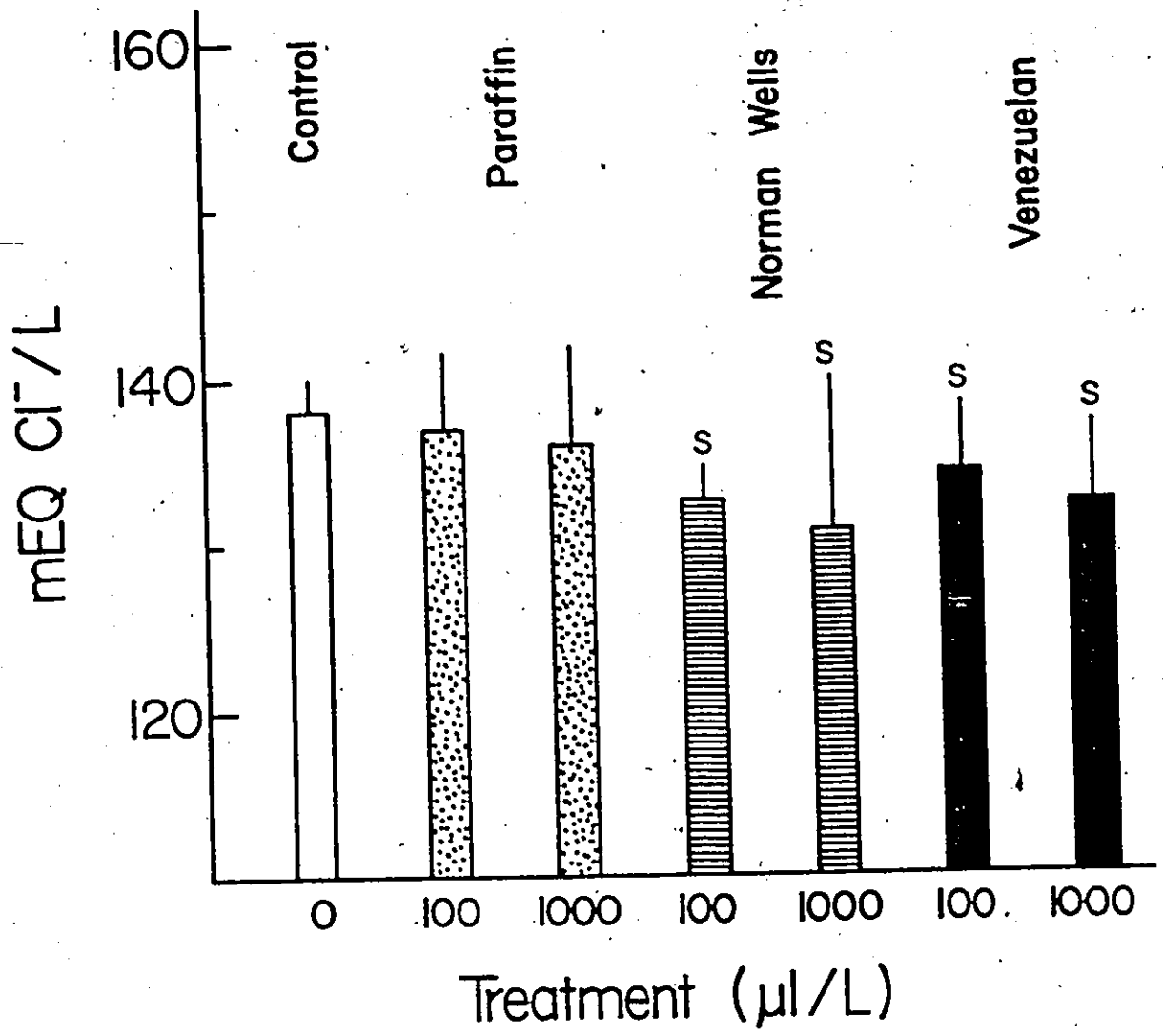


Plasma chloride ion concentrations in freshwater acclimated rainbow trout following exposure to fresh (100 ul/L) and weathered (1000 ul/L) oils for 7 days.

"S" denotes a statistically significant difference ($p \leq 0.05$) from controls.

All values are means of 10 fish, except control N=20. Vertical bars represent one standard deviation.

Figure 3: Chloride levels in oil dispersion exposed fish



Plasma osmolality in freshwater acclimated rainbow trout following exposure to fresh (100 ul/L) and weathered (1000 ul/L) oil dispersions for 7 days.

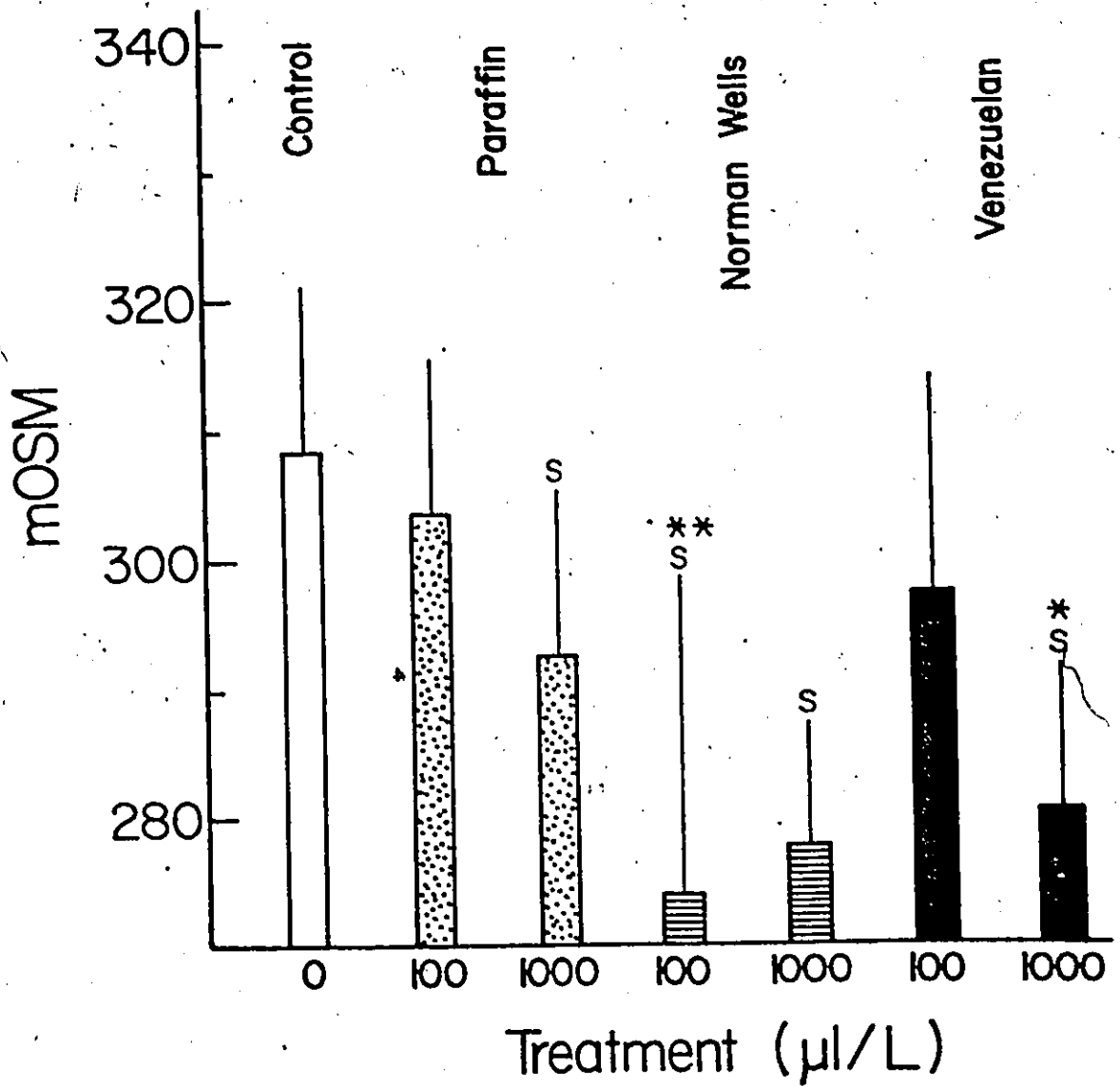
"S" denotes a statistically significant difference ($p \leq 0.05$) from controls.

"*" denotes a statistically significant difference ($p \leq 0.05$) between fresh and weathered crude oil treatments.

"**" denotes a statistically significant difference ($p \leq 0.05$) between fresh crude oil treatments.

All values are means of 10 fish, except control N=20. Vertical bars represent one standard deviation.

Figure 4: Plasma osmolality in oil dispersion exposed fish

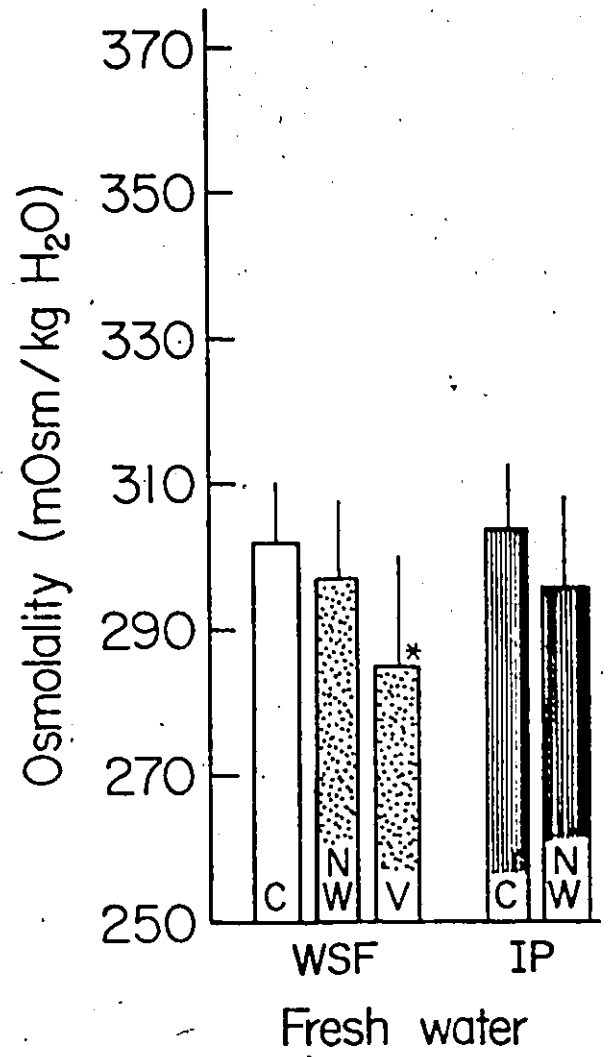
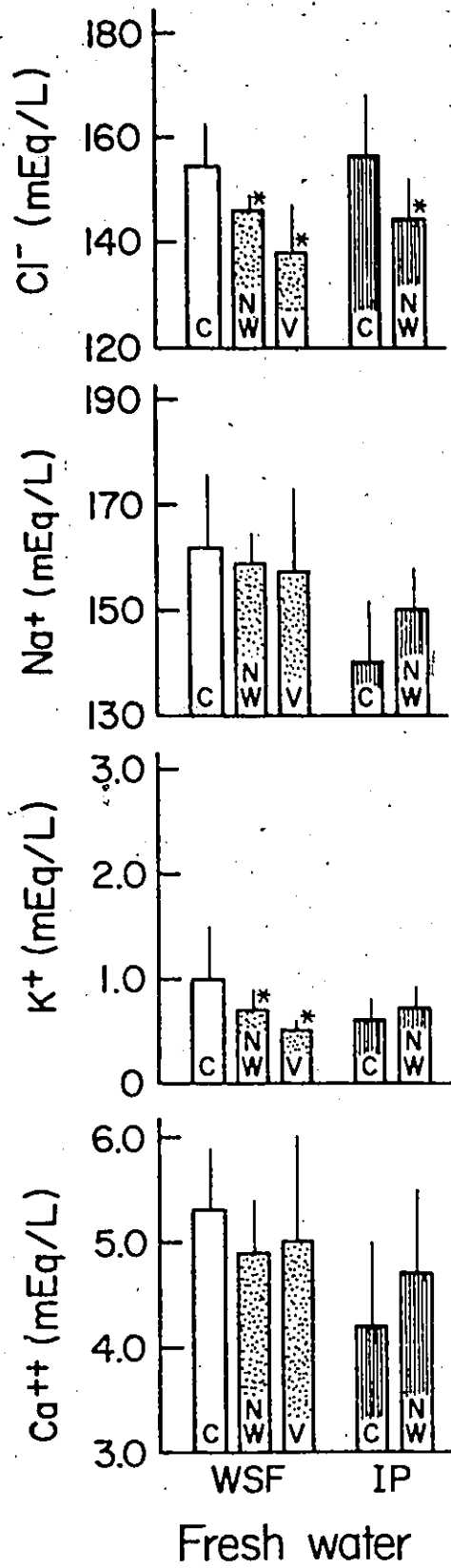


Plasma ion concentrations and plasma osmolality in fresh-water acclimated rainbow trout following exposure to weathered water-soluble fractions (WSF) of Norman Wells (NW) and Venezuelan (V) crude oils and intraperitoneal injections (IP) of weathered Norman Wells crude oil for 7 days.

"*" denotes a statistically significant difference ($p \leq 0.05$) from the respective controls.

All values are means of 8 fish and vertical bars represent one standard deviation.

Figure 5: Osmoregulatory effects in oil injected and WSF exposed fish



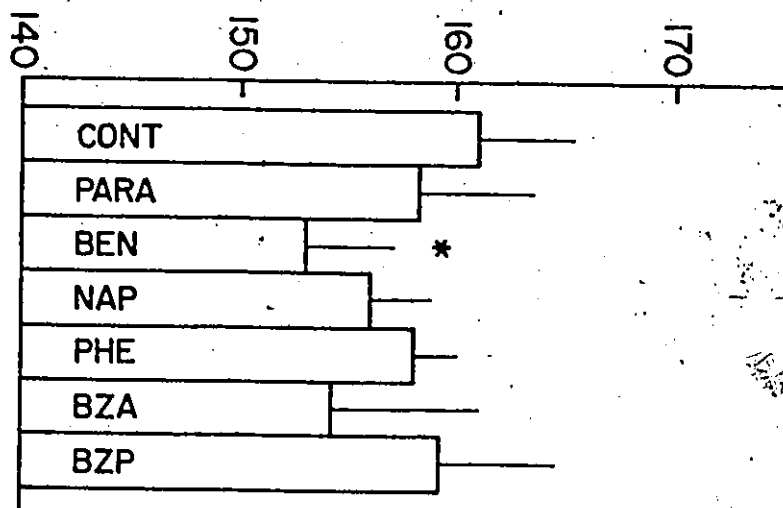
Plasma sodium and chloride ion concentrations and plasma osmolality in freshwater rainbow trout following exposure to individual aromatic hydrocarbons in (100 $\mu\text{l/L}$) paraffin oil (PARA) dispersions. The aromatic hydrocarbons used were: benzene (BEN), naphthalene (NAP), phenanthrene (PHE), benz(a)anthracene (BZA) and benzo(a)pyrene (BZP).

"*" denotes a statistically significant difference ($p \leq 0.05$) from controls (CONT).

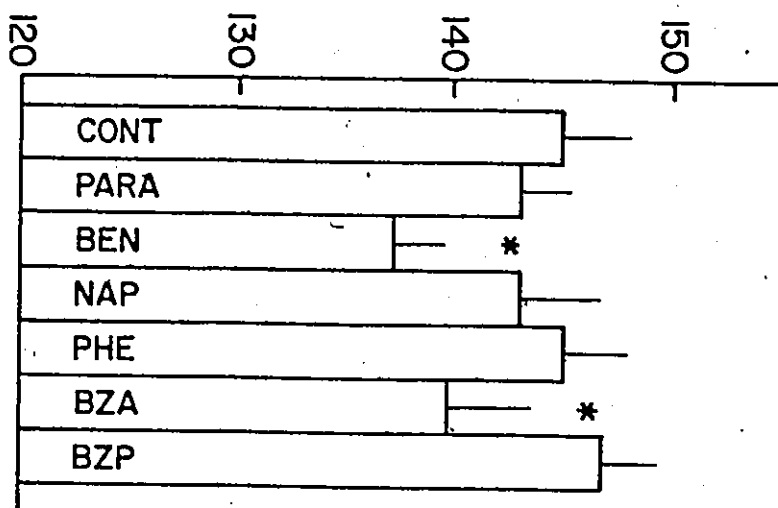
All values are means of 8 fish and vertical bars represent one standard deviation.

Figure 6: Osmoregulatory effects of aromatic hydrocarbon exposures

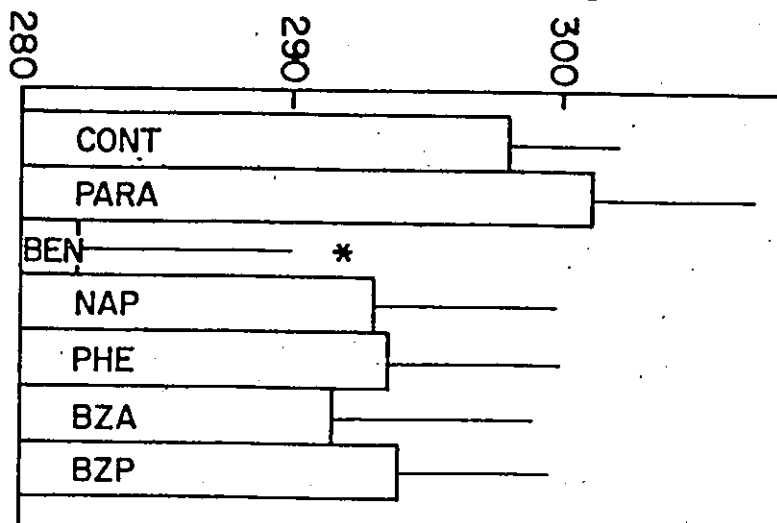
Na⁺ (mEq/L)



Cl⁻ (mEq/L)



Osmolality (mOsmo/kg)



2.4 DISCUSSION

Acute hydrocarbon exposure disrupted the ionic and osmoregulatory processes in trout (Figures 2 to 6). Reduced plasma ion and osmotic concentrations may be a non-specific reaction to various noxious compounds. A large number of water-borne substances are capable of inducing morphological changes in branchial tissue and plasma ionic imbalances. Exposure to vanadium or nickel can result in gill histopathology and shifts in plasma ion concentrations (Anderson et al., 1979). Similar observations were reported following treatment with formalin (Wedemeyer, 1971; Wedemeyer and Yasutake, 1974) and ozone (Wedemeyer et al., 1979) in hatchery fish. These two compounds are often used in fish cultures to control diseases. The alterations in gill lamellar epithelium, found in these studies, were similar to those described in fish from the present study (Engelhardt et al., 1981). Pathological changes included epithelial lifting and cellular damage. These morphological changes likely contributed to the hydromineral imbalance in the plasma.

Sublethal exposures to other toxicants can induce osmoregulatory impairment in freshwater fish. Some of these include copper (McKim et al., 1970; Christensen et al., 1972), zinc (Lewis and Lewis, 1971), cadmium (McCarty and Houston, 1976), and mercury (Schmidt-Nielson, 1974). Most of these

workers reported decreases in plasma electrolytes, agreeing with the present study. In contrast, exposure to endrin, a chlorinated hydrocarbon, resulted in higher serum electrolyte concentrations and osmolality of rainbow trout (Grant and Mehrle, 1973). A breakdown of the branchial uptake and transport mechanism in freshwater fish would cause a decrease in the animal's ability to replenish ions in its body fluids. This would result in a continuous loss of ions to the hypotonic medium. Such a dysfunction appears to have occurred in the test animals of this study. Interestingly, ionic stress from handling or pollutant exposure in fresh water can be reduced by addition of 0.3% salt or by increasing the calcium concentration in the water (Wedemeyer, 1972; Lewis and Lewis, 1971).

Both fresh and weathered crude oils caused osmoregulatory failure in the exposed fish (Figures 2, 3, 4). Different toxic fractions of the oil, however, may be responsible. The volatile, low-boiling hydrocarbons in fresh oil can rapidly penetrate and solubilize in the lipid components of the gill membrane, while the higher-boiling hydrocarbons in weathered oil would penetrate more slowly. The action of toxic oils in plant cells is by disrupting lipid molecules in cellular membranes (van Overbeek and Blondeau, 1954). The changes observed in gill morphology and plasma ionic/osmotic levels in fish from the different crude oil exposures can be

correlated to the specific chemical composition of the oils employed (Appendix B). The Norman Wells oil is a light-weight crude with high concentrations of volatile and soluble components. Upon weathering, one would expect most of these compounds to be eliminated from the water column. The Venezuelan crude oil, on the other hand, has a high proportion of medium and high molecular weight aromatic hydrocarbons, which would persist in the water. Bieri et al. (1977) found aromatic hydrocarbons accumulated in fish more rapidly from weathered crude oil. In addition, they observed more aromatics were accommodated in the water from weathered than fresh oil. It was somewhat surprising that the weathered Norman Wells oil had similar effects on the exposed fish as the fresh oil treatment. One would expect the more volatile components to evaporate from the medium. Possibly, when oil is formed into micron-size dispersions, the soluble and volatile hydrocarbons are retained in the water. Anderson et al., (1974b) found oil-in-water dispersions often retain the chemical composition of the parent oil. This notion is supported by a study showing weathered crude oil dispersions to cause increased coughing rates in Atlantic salmon, Salmo salar, similar to fresh dispersions (Barnett and Toews, 1978). Others demonstrated that oil layered on the water surface rapidly lose their toxic components (Morrow, 1973). This suggests crude oil dispersions can persist in the environment and can retain their toxic potential.

Paraffin oil particles did not induce significant changes in plasma ionic status (Figures 2,3,4) or gill morphology (Engelhardt et al., 1981). This observation is consistent with previous studies which showed paraffinic fractions of petroleum to have few toxic effects (Nelson-Smith, 1972; van Gelder-Ottway, 1976). This indicates the plasma ionic imbalance and gill pathology in crude oil exposures were a result of toxic hydrocarbons in the oil particles and not due to irritation of the gill epithelium by oil particles alone. Other workers, however, reported suspended inert particles, such as kaolin and diatomaceous earth, can alter fish gill morphology (Herbert and Merkens, 1961).

No major impact on ion balance was apparent from exposure to the weathered water-soluble fraction of the crude oils (Figure 5). In contrast, others reported many toxic effects attributable to this fraction of petroleum. Some of these effects included gill histopathology (Hawkes, 1977), altered respiratory rates (Thomas and Rice, 1975; Rice et al., 1977b), and loss of swimming coordination (Dixit and Anderson, 1977). All these studies, however, used fresh water-soluble fractions of petroleum and the concentrations were maintained by continuous additions. Such conditions are unlikely to occur during oil discharges in the environment unless the amount of oil spilled is extremely large. This study indicates that weathering of the water-soluble frac-

tion of oil results in a loss of toxic components. Yet, these components appear to be accommodated in the water column -even after weathering- when oil is dispersed into particulates.

Differences were evident in external and internal vectors of exposure. The absence of major disruptions in hydromineral regulatory processes in the IP injection experiments (Figure 5) may be attributed to the animal's ability to excrete the hydrocarbons before significant quantities can accumulate in the gills. It has previously been demonstrated that hydrocarbons taken up through the gut, versus those absorbed from the water, preferentially accumulate in various tissues (Mackie et al., 1974; Teal, 1977). These authors reported hydrocarbons from the gut were deposited in the liver, while those absorbed from water accumulated in muscle and other tissues. Hydrocarbons in the liver would be metabolized and excreted more rapidly than those in other tissues. In external exposures, however, the gills are the primary site of entry (Lee et al., 1972), and significant quantities of hydrocarbons can accumulate in the lipid portions of this organ. This is reportedly the case for rainbow trout exposed to Bunker C oil (McKeown and March, 1978b) and for other salmonids exposed to crude oils (Rice et al., 1977b). Interestingly, Coleman and co-workers (1977) found injections of the organochlorine pesticide, DDT (1,1,1, tri-

chloro-2,2-bis^c (p-chlorophenyl)ethane), into fresh and salt water adapted Tilapia aurea did not cause any appreciable changes in chloride cell ultrastructure in the gills, yet, this compound can disrupt plasma ion balance upon external application (Janicki and Kinter, 1971b; Leadem et al., 1974; Weisbart and Feiner, 1974). One can speculate that significant changes in the plasma ion profile would only occur during external exposure to oil and possibly other pollutants. While other routes of entry would have little or no effect on this physiological parameter.

There is general accord that aromatic hydrocarbons are responsible for the major toxic actions of petroleum products (Dwyer and Moore, 1974). The relative contributions of individual aromatics to toxicity are yet to be defined. Several studies have attempted to evaluate the impacts of these hydrocarbons in fish (Table I). Some workers concluded that much of the toxicity of petroleum is attributable to the mono- and dinuclear aromatics, while others found the toxicity to increase with greater ring number up to the four and five ring compounds (Neff, 1979). The results of this study do not contribute evidence to support either one of these arguments. Though moderate changes in plasma ions concentrations and osmolality were found for all five aromatic hydrocarbons (Figure 6), only benzene and benz(a)anthracene exposures resulted in major disruptions.

In addition, there were no significant trends with respect to molecular weight. The reason for this observation is not completely clear. Possibly, the paraffin oil could have adhered to the aromatic particles and made them unavailable to the fish. Boehm and Quinn (1976) reported that aromatic hydrocarbons can adsorb to organic matter in solution and become biologically inactive. More work is required before the relative toxicity of the various aromatic hydrocarbons to fish can be ascertained.

TABLE 1

Physiological effects of aromatic hydrocarbons

- References: (1) Struchsaker, 1977;
(2) Weber et al., 1981;
(3) Maynard and Weber, 1981;
(4) Korn et al., 1976;
(5) Brocksen and Bailey, 1973;
(6) Brenniman et al., 1979;
(7) Thomas and Rice, 1979;
(8) Moles et al., 1981;
(9) Levitan and Taylor, 1979;
(10) DiMichele and Taylor, 1978;
(11) Gerhart et al., 1981.

AROMATIC HYDROCARBON	FISH SPECIES	EFFECTS	REFERENCES
Benzene	<u>Clupea harengus pallasi</u>	Increased oxygen consumption	(1)
Benzene	<u>Oncorhynchus sp.</u>	Disrupted spawning migration	(2)
Benzene	<u>O. kisutch</u>	Avoided contaminated waters	(3)
Benzene	<u>Morone saxatilis</u>	Decreased fat content, caloric content and growth	(4)
Benzene	<u>M. saxatilis</u> <u>O. tshawytscha</u>	Increased respiratory rates	(5)
Toluene	<u>Carassius auratus</u>	Decreased blood pH, PO ₂ and increased PCO ₂	(6)
Naphthalene	<u>O. gorbuscha</u>	Increased oxygen consumption	(7)
Naphthalene	<u>O. kisutch</u>	Reduced growth	(8)
Naphthalene	<u>Fundulus heteroclitus</u>	Increased cortisol levels and oxygen consumption	(9)
Naphthalene	<u>E. heteroclitus</u>	Gill hyperplasia and hemorrhages in filaments	(10)
Phenanthrene	<u>Pimephales promelas</u>	Proliferation of mucous globules in intestine	(11)

It is not clear if the petroleum-induced disruptions in ion status and gill histopathology were deleterious to the trout. These effects may indicate the fish would require a greater energy expenditure to compensate for the osmoregulatory failure. Although only a few studies have focused on the precise metabolic cost of ionoregulatory processes, it appears to be an energetically expensive function (Rao, 1968). In freshwater acclimated rainbow trout, about 20% of total metabolism is used in osmoregulation (Rao, 1968). An elevated osmoregulatory cost would occur during periods of stress, as seen in trout experiencing ionic imbalance during exercise (Wood and Randall, 1973).

The reported increase in oxygen consumption in oil-exposed fish (Thomas and Rice, 1975; Rice et al., 1977) may involve the use of more energy to recover lost ions. This increase in metabolism for osmoregulation would result in a reduced scope for activity (Fry, 1971). This could explain the curtailed activity observed in some oil stressed fish (Morrow, 1973; Dixit and Anderson, 1977). Cyanide poisoning in trout results in osmoregulatory impairment, increased oxygen consumption and reduced swimming stamina (Leduc and Chan, 1975). These could also be the consequences of oil exposure. In addition, depending of the duration of the stress, there could be less energy available to channel into

other functions, such as growth and reproduction, thereby decreasing fitness.

Laboratory toxicity tests in which the measured effect is death do not adequately reflect the potential ecological impact of a pollutant (Perkins, 1979). Sublethal responses are considered to be more sensitive indicators of pollutant stress and survival potential. At present, there is a need for reliable indices of environmental contamination. The results of this study showed the homeostatic mechanisms involved in electrolyte metabolism are suitable responses. Hopefully the results would encourage further work with sublethal dosages and provide a better understanding of the effects of pollutant stress. Such studies are important in that small physiological perturbations may decrease viability or even survival of organisms in the environment. Furthermore, mortalities from such physiological dysfunctions can be regarded as even more serious than massive die-offs. Since such occurrences are less obvious, the situations may not be corrected in a short enough time span. The monitored effects in this study did not bring about immediate mortalities, but the fish may be incapable of withstanding further stress. For example, salmonids exposed to sublethal levels of copper developed osmoregulatory impairments. These fish were found to be less tolerant of salt water and none survived the transition to sea (Lorz and McPherson, 1976).

Similar findings were reported for chromium exposed fish (Sugata, 1980). Possibly, oil exposed fish will suffer the same fate.

In summary, trout exposed to crude oils and selected hydrocarbons developed osmoregulatory impairment. This was most evident in exposures to oil dispersions from fresh and weathered oil. The effects were attributed to toxic hydrocarbons on the oil particles, since paraffin oil had no major impact. Weathered water-soluble fraction exposures and oil injections of weathered oil did not appear to have significant effects on electrolyte metabolism. Aromatic hydrocarbons may be the toxic components in petroleum products, however, their relative contributions to toxicity is still poorly understood. Monitoring of ionic/osmotic status in fish subjected to environmental contamination is a sensitive index of water quality.

Chapter III

PETROLEUM EFFECTS ON OSMOREGULATION IN SALTWATER ACCLIMATED TROUT

3.1 INTRODUCTION

Salt water adapted fish possess different ionoregulatory strategies compared to their counterparts in freshwater. Salt water teleosts achieve mineral balance through active extrusion of electrolytes by the gills to compensate for the large influx of ions from the hypertonic media. To replace the water that is lost across the gills by osmosis, the fish drink salt water and excrete the excess salt via the gills. As in freshwater, two independent ion pumping mechanisms for sodium and chloride extrusion are found in salt water fish. Sodium efflux is believed to be mediated by a sodium-potassium exchange. An electrogenic pump is involved in chloride extrusion (Maetz, 1971).

Since fresh and salt water fish display different ion transport mechanisms, one may observe different effects of petroleum on fish adapted to different salinities. The purpose of this study is to assess the toxicity of external

weathered crude oil exposures and intraperitoneal oil injections on plasma ion balance in fish acclimated to a hypertonic medium.

3.2 METHODS AND MATERIALS

3.2.1 Experimental Animals

Rainbow trout, 100-150 g in size, were purchased from a local fish hatchery and acclimated to laboratory conditions for 4 weeks. Following this period, the fish were placed in large recirculating aquaria (Living Streams, Inc, Toledo, Ohio) containing 21 o/oo (ppt) salt water made from commercial sea salt (Instant Ocean, Inc.). The system was equipped with high capacity charcoal filters to remove metabolic waste products. The temperature was maintained at 10 ± 2 °C and photoperiod was held constant at 12L/12D. One third of the volume of the water was removed and replaced weekly with media of the same salinity. Oxygen was maintained at 75-85% saturation. Ammonia was not detected in significant amounts (Solorzano, 1969). The trout were kept in this system for four weeks before experimentation. They were fed ad libitum daily on dry commercial trout feed (Purina Chow, No.4 Developer).

3.2.2 Experimental Procedures

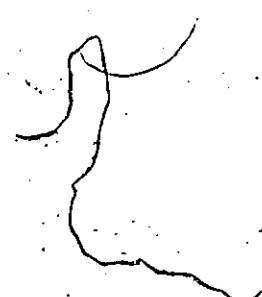
Following acclimation, groups of 5 to 10 fish were transferred to glass aquaria and held at 10 ± 1 °C with 21 o/oo

salt water similar to the test system described for freshwater exposure (section 2.2.2, pages 22-24). The water was continuously aerated and recirculated. Fish were acclimated for an additional week before the start of the tests. They were fed every other day until 48 h before experiments. The groups of fish were tested for the effects of weathered crude oils dispersions (Norman Wells and Venezuelan) in salt water. The concentrations and duration of the exposure were the same as those previously described (section 2.2.2, pages 22-24). Simultaneous sets of controls were employed.

To differentiate the effects of external and systemic contamination by petroleum hydrocarbons, groups of fish were treated with 3 day weathered Norman Wells crude oil by intraperitoneal (IP) injections at a dosage of 10 ul/kg fish per day for 7 days. Controls groups were sham injected. The fish were not fed during the 7 day test. The trout were held in recirculating systems (Living Streams, Frigid Units, Inc.) containing 21 o/oo salt water.

Branchial tissue from these fish were removed for histological examination following the experiment (Engelhardt et al., 1981). In addition, an experiment was performed to monitor the ionic status of oil injected trout over 1, 3, 5 and 7 days (Appendix C).

The sampling and analytical procedures were similar to those described for the freshwater exposures (section 2.2.3, pages 26-29). The neutralized anaesthetic (MS-222) was mixed with 21 o/oo salt water and kept at the same temperature as the test water.



3.3 RESULTS

Seven day exposure to weathered Norman Wells and Venezuelan crude oil dispersions in 21 o/oo salt water resulted in plasma ionic disturbances. Elevated sodium, chloride and potassium concentrations, as well as significantly higher plasma osmolality were found in the crude oil exposed fish (Figure 7). External oil exposures also caused reductions in circulating calcium concentrations. No significant differences were observed in plasma ions or osmolality between the weathered Norman Wells and Venezuelan oil exposed fish. In addition to the ionic and osmotic changes in the plasma of Venezuelan oil exposed fish, mortality was recorded in this exposure. The experiment was repeated with similar findings (40 to 50% mortality). Plasma from the surviving fish of the two experiments were used for ion analysis. Examination of the moribund fish showed significant quantities of oil particles in the alimentary tract.

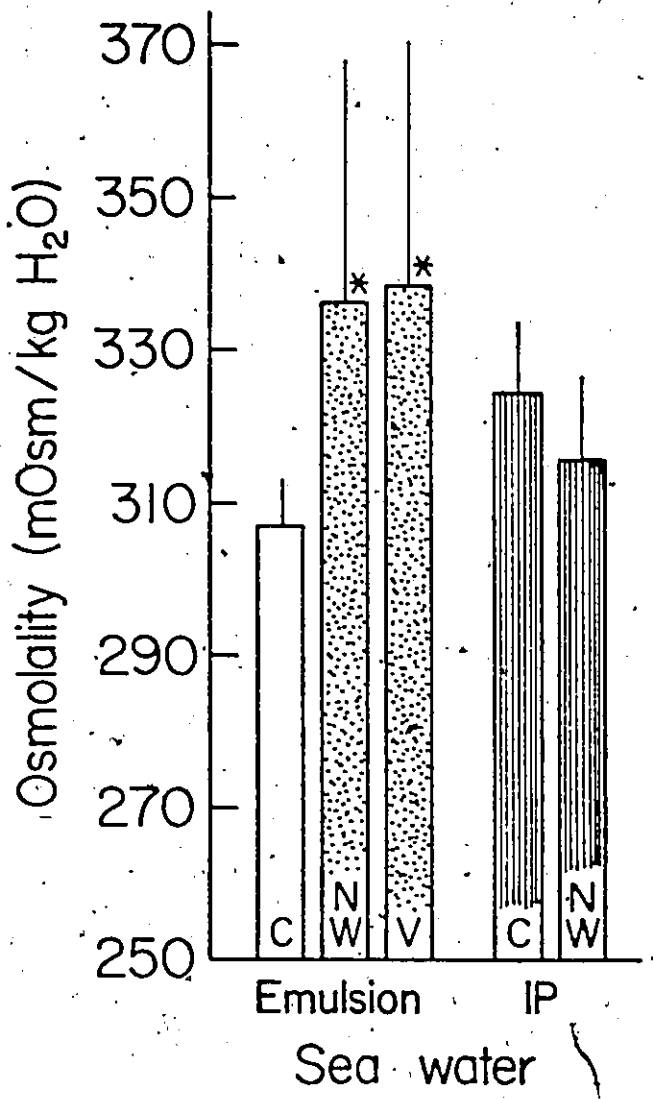
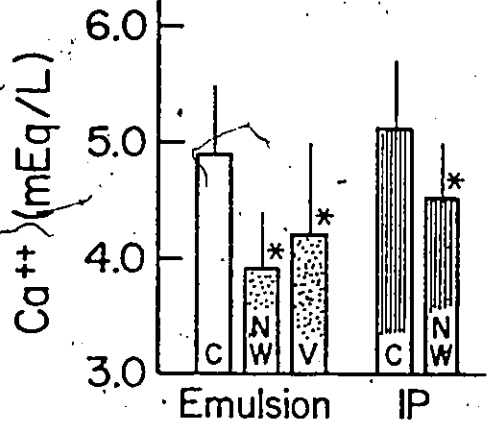
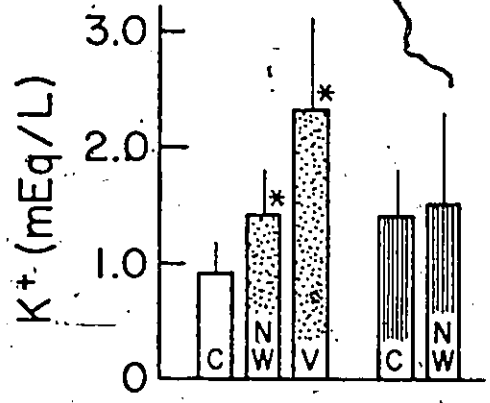
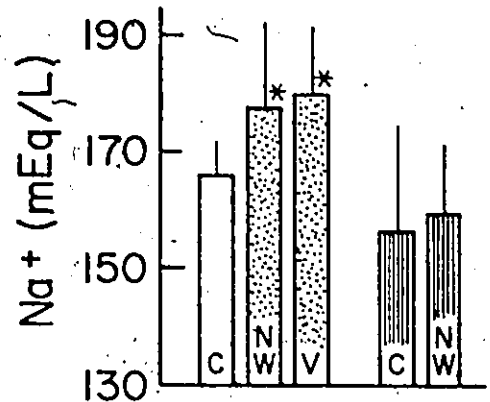
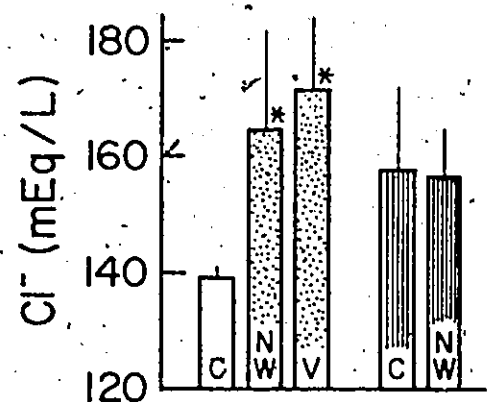
Intraperitoneal injections of weathered crude oil (Norman Wells) did not result in any major alterations in plasma ionic profile compared to controls. Only calcium showed a significant decline (Figure 7).

Plasma ion concentrations and plasma osmolality in salt water acclimated rainbow trout following exposure to weathered Norman Wells (NW) and Venezuelan (V) crude oil dispersions and intraperitoneal (IP) injections of weathered Norman Wells (NW) crude oil for 7 days.

"*" denotes a statistically significant difference ($p \leq 0.05$) from controls (C).

All values are means of 8 fish and vertical bars represent one standard deviation.

Figure 7: Plasma ion and osmolality in salt water acclimated trout



Emulsion IP
Sea water

3.4 DISCUSSION

Hydromineral disruptions were apparent in rainbow trout acclimated to a hypertonic medium. The increase in plasma monovalent ion concentrations and osmolality found in salt water acclimated fish (Figure 7) is reversed with respect to the trend observed in fish exposed to oil in freshwater (Chapter II). As a result of the large influx of ions from the external medium, alterations in ion transport processes in the gill epithelium would result in higher ion concentrations in the blood. This is consistent with the ionoregulatory mechanisms proposed for salt water teleosts (Maetz, 1971).

Elevated plasma osmotic components in fish from hyperosmotic salines may be a general effect of organic pollutant stress. Petroleum products, such as Bunker C oil (McKeown and March, 1978a), crude oil (Payne et al., 1978), monocyclic aromatic hydrocarbons (Morrow et al., 1975), and naphthalene (Levitan and Taylor, 1979), can effect such a response. Organochlorine hydrocarbons can also induce ionic imbalance in salt water fish (Eisler and Edmunds, 1966; Leadem et al., 1974). This study showed hydromineral balance was perturbed by external exposures to weathered oil-in-water dispersions. Cellular alterations in gills of these fish suggest these lesions were responsible for the

observed ionic dysfunction (Engelhardt et al., 1981). Chloride cell damage could result in a breakdown of ion transporting processes. In addition, decreased plasma calcium concentrations can cause changes in gill membrane permeability. This divalent ion is an important mediator of membrane permeability (Isaia and Masoni, 1976).

Injections of weathered crude oil did not produce significant alterations in ionic/osmotic balance in fish (Figure 7). This is consistent with the freshwater study (Chapter II), in which no major changes were observed in plasma ionic components. No gill damage was found in the IP injected trout of this study (Engelhardt et al., 1981). Further, Coleman et al. (1977) found no significant alterations in gill morphology when salt water fish were injected with an organochlorine hydrocarbon. Perhaps, as in freshwater, such a mode of entry of hydrocarbons does not result in physiological changes in ion balance because the fish are capable of metabolizing and excreting the toxic compounds. Roubal et al. (1977) stated that coho salmon were able to transform IP injected aromatic hydrocarbons within 24 hr into water-soluble, excretable products. It is well established that hydrocarbon hydroxylase enzymes are responsible for the metabolism and excretion of hydrocarbons from body tissues (Varanasi and Malins, 1977). These enzymes can reduce the hydrocarbon load of the fish, therefore, the ion regulatory processes were not compromised.

Tissues responsible for ion regulation, other than the gills, could have contributed to the observed osmotic failure. The intestine plays a role in osmotic balance in salt water acclimated fish (Maetz, 1971; 1974). The gut epithelium absorbs salt water along with its complement of monovalent ions. Perturbing this process would effect an increase in salt uptake or a loss of free water. Alterations in the permeability of the gut epithelium by the influx of hydrocarbons could result in an increased salt load which the gills are unable to excrete. The increased plasma ion levels and osmolality would support this. The function of the kidneys in these fish should also be considered. In marine teleosts, the kidney secretes a urine which is hypotonic to the external medium. If the function of this organ is disrupted, a further loss of water from the animals would be expected. This could result in dehydration.

Stresses requiring adjustment beyond the means of the organism would be lethal, as observed in the Venezuelan crude oil exposure. Mortality in these fish could have resulted from disruption of physiological processes other than ion regulation in the branchial tissue. No significant differences were found in plasma ion parameters of fish surviving the Venezuelan oil exposure and fish exposed to Norman Wells oil. Therefore, one can speculate that the lethal effects

were consequences of the fish's inability to cope with osmoregulatory impairment.

The mortality recorded in the Venezuelan oil exposure in salt water was not found with the same treatment in freshwater. This observation supports the suggestion that variation in environmental factors such as salinity can substantially affect the sensitivity of an organism to pollutants (Kinne, 1964). In this case, both survival and physiological responses were mediated by a change in physical parameters. This further emphasizes that, in order to monitor the sensitivity of an organism to petroleum or other pollutants, the many environmental variables governing the possible impact must be considered.

Interestingly, several studies have reported pollutant-salinity interactions in fish (Lorz and McPherson, 1976; Levitan and Taylor, 1979; Linden et al., 1979; Sugatt, 1980). Levitan and Taylor (1979) reported salinity-dependent mortalities in naphthalene exposed fish. Survival was greater at hypo- or isoosmotic salt concentrations than at hyperosmotic salinities. Further, sublethal exposure to chromium (Sugatt, 1980), or copper (Lorz and McPherson, 1976) induced osmoregulatory impairment in fish in freshwater which resulted in decreased survival following transfer to salt water. The ability of fish exposed to oil in fresh-

water to tolerate salt water or fish exposed to oil in salt water to tolerate freshwater remains to be evaluated. Such transitions from one salinity to another are often part of the life cycle of many anadromous or catadromous species.

Although the cost of osmoregulation does not increase proportionally, with increasing salinity, metabolic rates have been found to be higher in greater osmotic gradients. Rao (1968) reported that the energy needs attributable to osmoregulation in rainbow trout was 27% of total metabolism at 30 o/oo salt water compared to 20% in freshwater. In the present study, trout were acclimated to 21 o/oo salt water. The metabolic rates would probably be higher in fish from this salinity than those from freshwater. If this is the case, one can speculate that increased osmoregulatory stress in these fish would further tax the limited scope for activity in the oil exposed animals. This could have contributed to the lethal effects found in some fish. It would be of interest to test the effect of oil pollutants on ion balance in fish acclimated to water isoosmotic (15 o/oo) to blood concentrations. Since the fish may expend less energy for hydromineral balance, the effects of oil could be different from exposures in fresh and salt water.

The ecological significance of laboratory results is difficult to evaluate in terms of natural conditions. It is,

however, relevant to draw comparisons between these observations with those reported from field studies. For example, one oil spill which had adverse effects on fishery resources occurred during the West Falmouth incident off Woods Hole, Massachusetts (Sanders et al., 1980). A barge carrying fuel oil with a high aromatic hydrocarbon content (41%) released a relatively small load (600 to 700 tons) into the estuarine waters. The oil was quickly accommodated in the water column and spread over a wide area by wind and wave actions. This resulted in massive mortalities of fish and other benthic organisms. Several years following the original incident, re-entry of oil caused additional mortalities in the biota (Sanders, 1978; Sanders et al., 1980). The present study showed that weathered oil dispersions accommodated in water can have significant effects on fish and suggested that persistent aromatic compounds may be the toxic agents of petroleum. In addition, environmental factors, such as salinity, were found to be able to modify the sensitivity of organisms to pollutant stress. Organisms in estuarine conditions are subjected to wide fluctuations in environmental parameters (salinity, temperature) and these factors can increase the susceptibility of the animals to oil pollutants (Linden et al., 1979). These fluctuations and toxic hydrocarbons may have contributed to the adverse effects reported in the above field study.

In summary, salt water acclimated fish developed hydromineral imbalances upon exposure to crude oil dispersions. Elevated plasma electrolyte concentrations were found in these fish. The responses in salt water acclimated fish appear to be a reflection of the ion transport processes and the metabolic cost of these animals.

Chapter IV.

HYDROCARBON EFFECTS ON GILL ATPASES

4.1 INTRODUCTION

The majority of teleosts are found in waters ranging from 5 to 1000 mOsm/kg corresponding to fresh and sea water (Schmidt-Nielsen, 1974). All teleosts regulate the osmotic concentration of their internal environment to a level different from the external medium. The gill epithelium of teleosts is one of the most important effector organs for the maintenance of hydromineral balance (Maetz, 1971).

Due to its inherent morphological complexity, one cannot consider the gill epithelium as a simple tissue in ion transport studies, as is done with the intestine, the urinary bladder, or the flat skin from the operculum (Karnaky, 1980). The branchial tissue consists of respiratory cells, mucous cells, and at the basal portion of the secondary lamellae, chloride cells (Conte, 1969; Laurent and Dunel, 1980). In addition to osmoregulatory functions, the teleost gill performs other vital roles such as respiratory exchange (Randall et al., 1967), waste excretion (Maetz, 1973) and acid-base regulation (deRenzis and Maetz, 1973).

Keys and Willmer (1932) found acidophilic cells in teleost gills resembling the oxyntic cells of frog intestinal mucosa responsible for hydrochloric acid (HCl) secretion. They suggested these acidophilic cells were responsible for chloride secretion across the gills. Since their pioneering work, chloride cells are generally accepted as the sites of active ion exchange across the gill epithelium (Conte, 1969; Maetz and Bornancin, 1975). The chloride cell theory has been criticized on many occasions by various workers (Parry, 1966). Although most of the skepticism has been resolved by numerous physiological, morphological, and biochemical studies, certain aspects of the chloride cell function remain uncertain (Maetz Memorial Symposium, 1980).

Morphological observations show chloride cells to have numerous mitochondria and a dense aggregation of glycogen granules, reflecting the high energy requirement of the structure. Another important feature is the considerable development of an agranular endoplasmic reticulum. Electron micrographs have shown that a physical continuity exists between this tubular system and the basolateral membrane (Philpott, 1965; Shirai and Utida, 1970), suggesting these organelles are situated in a position suitable for a role in active ion transport. These structures may be enzyme-arrays associated with ion transporting activities of the cell.

(Ritch and Philpott, 1969). Radiolabelled sodium and chloride accumulated in chloride cells following intracardiac injection (Masoni and Garcia Romeu, 1973). Brown (1973; cited in Maetz and Bornancin, 1975) reported similar findings when electrolytes were added into the external media. Further, these ions were found to concentrate in vesicles of the tubular system of chloride cells (Shirai, 1972).

Several studies examined alterations in these cells following experimental manipulations. For instance, Pacific salmon, Oncorhynchus sp., developed chloride cell damage following irradiation and resulted in plasma osmotic imbalance (Conte, 1965; Conte and Lin, 1967). Maetz et al. (1969) found a breakdown in ion excreting mechanisms following injections of actinomycin D - a drug which prevents protein synthesis and cell regeneration. They attributed the ion dysfunction to blockage of chloride cell replacement. A major impairment of branchial osmoregulatory processes was evident in salt water while a slight perturbation was observed in freshwater. These findings suggest that osmotic disruptions are attributable to failure of the branchial salt transporting cells. -

Investigations with euryhaline fish subjected to varying salinities have implied chloride cells as sites of ion-transporting activity. Transfer of fish from fresh to salt

water results in an increase in ion exchange rates, an increase in number and size of chloride cells, and a greater development of mitochondria and endoplasmic reticulum in these cells. (Shirai and Utida, 1970). Transfer back to freshwater results in both a decrease in number and a degeneration of the cells.

The suggestion that ion-activated enzymes play a fundamental role in transepithelial salt transport has received support from numerous studies with the teleost gill (Maetz and Bornancin, 1975). Yet, in order to impart a direct role to these enzymes in ion regulatory processes, one must show they occur as a component of transporting cells. There is sufficient evidence showing chloride cells to be rich in $\text{Na}^+\text{-K}^+$ ATPase activity, indicating this enzyme plays an important role in the ion exchange mechanism (Sargent et al., 1978). Autoradiography with labelled ouabain - a potent inhibitor of $\text{Na}^+\text{-K}^+$ ATPase and sodium transport - demonstrated the enzyme is located in the basolateral membrane of chloride cells, on the numerous invaginations of the tubular system (Karnaky et al., 1976; Karnaky, 1980). Studies showing increases in chloride cell number and ultrastructural development during salinity adaptation have found similar increases in $\text{Na}^+\text{-K}^+$ ATPase enzyme activities (Shirai, 1972; Stirling et al., 1974; Sargent et al., 1975; Sargent and Thomson, 1978).

Since the work of Jens Skou (1957; see review in Skou, 1965) with crab nerve, the ouabain-sensitive, sodium and potassium stimulated, magnesium dependent adenosine triphosphatase (EC 3.6.1.3 ATPase) has been intimately associated with active cation transport across biomembranes. Included are such epithelial membranes as nerve cells, erythrocytes, kidney tubules, frog skin, avian nasal glands and rectal glands of elasmobranchs (Bonting, 1971). The presence of this enzyme in specialized cells from teleost branchial tissue was demonstrated independently by Epstein *et al.* (1967) and Kamiya and Utida (1968) in Fundulus heteroclitus and Anguilla japonica, respectively. These fish exhibited higher specific enzyme activities in salt water and the authors attributed this to increased salt load pumped across the gills in a hypertonic medium. Their findings are consistent with existing ion exchange mechanisms (Maetz, 1969). Gill ATPases have since been studied in numerous species of fish, including various salmonids (Kamiya and Utida, 1969; Jampol and Epstein, 1970; Lasserre, 1971; Butler and Carmichael, 1972; Pfeiler and Kirschner, 1972; Evans and Mallory, 1975). The relative importance of these gill enzymes to ion balance is reflected by the changes in their activities in relation to varying salinities. Reportedly, enzyme activities can also be affected by a variety of environmental factors, including temperature (Zaugg *et al.*, 1972; Adams *et al.*, 1975; McCarty and Houston, 1977), hydrostatic pressure (Pfeiler,

1981; Pequette and Gilles, 1978; Moon, 1975), and photoperiod (Zaugg and Wagner, 1973; Ewing et al., 1980). Further, many environmental toxicants can influence activities of ATPases isolated from branchial tissue (Leadem et al., 1974; Poston, 1979; Watson and Beamish, 1980; 1981). Thus it appears gill ATPases are sensitive to a variety of natural and anthropogenic environmental changes.

Hydrocarbons can traverse the gill epithelium (Rice et al., 1977b) and disrupt the structural integrity of the branchial tissue (Blanton and Robinson, 1973; McKeown and March, 1978a; Lopez et al., 1981). Morphological changes in gill architecture included alterations in chloride cell structures. In the present study, such changes caused ionic dysfunction in the oil-exposed fish. With this evidence, one can hypothesize that alterations in activities of membrane-bound enzymes (ATPases) can result from exposure to petroleum hydrocarbons.

A sodium-potassium stimulated, magnesium dependent, ouabain-sensitive ATPase ($\text{Na}^+\text{-K}^+$ ATPase) has been demonstrated in rainbow trout (Pfeiler and Kirschner, 1972; Zaugg and McLain, 1972; Watson and Beamish, 1980, 1981). Other ion activated ATPases also occur, including a bicarbonate stimulated, ouabain-insensitive, magnesium dependent, thiocyanate-sensitive ATPase (HCO_3^- ATPase) (Bornancin et al.,

1980; McCarthy and Houston, 1977), a sodium-ammonium stimulated, magnesium dependent, ouabain-sensitive ATPase ($\text{Na}^+ \text{NH}_4^+$ ATPase) (Watson and Beamish, 1980; 1981), and a calcium dependent ATPase (Ca^{2+} ATPase) (Ma et al., 1974; Watson and Beamish, 1980; 1981). A residual ATPase or ouabain-insensitive magnesium ATPase (Mg^{2+} ATPase) has been reported, but usually in relation to other ATPases (Zaugg and McLain, 1972; Watson and Beamish, 1980; 1981).

The present study consists of two separate phases. In the first part, in vivo experiments were conducted similar to the previous sections in freshwater (Chapter II) and salt water (Chapter III), to determine if ATPase enzymes activities are affected by petroleum hydrocarbons. In the second part, attempts were made to determine if individual aromatic hydrocarbons can influence the same enzymes in vitro. In addition, the relative toxicity of these hydrocarbons on enzyme activity was evaluated.

4.2 METHODS AND MATERIALS

4.2.1 Experimental Animals

Juvenile rainbow trout, 100-200 g in size, were obtained from a local commercial hatchery (Thistle Springs Trout Farm, Ashton, Ontario). They were acclimated in the laboratory to freshwater (section 2.2, pages 22-25) and salt water (section 3.2, pages 52-53) conditions as described previously.

4.2.2 Petroleum Effects on Gill ATPases In Vivo

The fish were exposed to weathered Norman Wells and Venezuelan crude oil dispersions in fresh and 21 o/oo salt water as described previously. Simultaneous sets of controls accompanied these tests. The exposure period was for 7 days. Following exposure, fish were removed from the aquaria, stunned with a blow to the head, weighed and exsanguinated. This was performed at the same time of day (11:00 h) to preclude any possible diurnal changes in enzyme activities. Procedures for extraction, isolation and determination of gill microsomal enzymes were modified from those of Watson and Beamish (1981) and summarized in Table 2. The branchial

apparatus from 4 to 6 fish were dissected individually and placed in ice-cold 0.9% NaCl. Gill filaments were homogenized (10% w/v) in solution (0-4 °C; pH 7.4) containing 0.25 M sucrose, 5 mM disodium EDTA (ethylenediamine tetraacetic acid, disodium salt), and 40 mM Tris-HCl (Tris(hydroxymethyl)aminomethane Hydrochloride) buffer. The tissue was ground in glass with a teflon pestle for 15 strokes, with the addition of sodium deoxycholate (0.1%) after 8 strokes. Treatment with detergent is required to increase accessible ATP-binding sites (Sargent et al., 1978). The suspension was centrifuged at 900 xg for 10 minutes, and then at 10,000 xg for 30 minutes, to remove the cellular debris and mitochondrial fraction, respectively. The remaining supernatant was centrifuged at 38,000 xg for 120 minutes to sediment the microsomal fraction. The resulting pellet was washed with ice-cold homogenizing medium and recentrifuged at 38,000 xg for another 30 minutes. The final pellet was resuspended with ice-cold homogenizing medium without deoxycholate. All the above operations were performed at 0 to 4 °C. Aliquots (0.1) of the final suspension were assayed in duplicate with 1.8 ml of incubation media (Table 3). The levels of substrate (ATP), ions and pH employed yielded maximal specific activities (Watson, personal communication; Watson, 1978). The assays were carried out on the same day of enzyme preparation to avoid loss of enzyme activities after freezing (Ma et al., 1974). Protein concen-

tration was estimated by a modification of the methods of Lowry et al. (1951) using bovine serum albumen as the standard. Enzyme reactions were initiated following preincubation for 15 minutes at the assay temperature (18 °C), by the addition of 0.1 ml 100 mM vanadium free adenosine triphosphate (ATP) (Sigma Product No. A-5394). It has recently been shown that some commercial preparations of ATP contain vanadium, a potent inhibitor of ouabain sensitive $\text{Na}^+\text{-K}^+$ ATPase (Cantley et al., 1978; Beauge and Glynn, 1978). The pH of the ATP solution was adjusted to 7.4 with 1 N NaOH. After 30 to 60 minutes of incubation, reactions were terminated by placing the incubation tubes on ice and by adding 0.5 ml of 30% (w/v) trichloroacetic acid (TCA). Following centrifugation at 3000 xg for 10 minutes, aliquots of the supernatant were removed for duplicate determination of inorganic phosphate (Pi) by an automated modification of the Fiske and Subbarow (1925) procedure using a Technicon AutoAnalyzer II (Technicon method No. SE2-004F3).

Activities of $\text{Na}^+\text{-K}^+$, and $\text{Na}^+\text{-NH}_4^+$ stimulated ATPase were calculated from the difference between assays performed in the presence and absence of 1 mM ouabain. Activities of calcium and magnesium dependent ATPases were estimated by the difference between blank determinations and those containing the respective cations. The HCO_3^- stimulated ATPase activity was calculated from the difference between assays per-

formed in the presence and absence of 5 mM SCN^- (thiocyanate). All activities are expressed as the number of micromoles of inorganic phosphate liberated per milligram of protein per hour ($\mu\text{M Pi/mg/hr}$).

TABLE 2
Procedures for ATPase determination

GILLS DISSECTED INTO 0.9% NaCl (0-4°C)

↓
FILAMENTS REMOVED, WEIGHED, AND PLACED IN 0.25M SUCROSE,
5M EDTA, 40 mM TRIS-HCL MEDIUM (10% W/V; 0-4°C; PH 7.4)

↓
HOMOGENIZED IN SORVALL (OMNI-MIXER) 90 s

↓
GROUND FOR 15 STROKES (TEFLON GRINDER), WITH 0.1% Na-DEOXYCHOLATE
ADDED AFTER 8 STROKES

↓
CENTRIFUGED (900X G) 15 MIN. (0-4°C) IN A SORVALL RC-2B

↓
SUPERNATANT CENTRIFUGED (10,000X G) 30 MIN. (0-4°C)

↓
SUPERNATANT CENTRIFUGED (38,000X G) 2 HR. (0-4°C)

↓
FINAL SUPERNATANT DISCARDED, MICROSMAL PELLET WASHED,
RE-CENTRIFUGED (38,000X G) 30 MIN. (0-4°C)

↓
FINAL PELLET RESUSPENDED IN 3-6 ML OF HOMOGENIZING MEDIUM
(0-4°C) WITHOUT DEOXYCHOLATE

↓
0.1 ML OF MICROSMAL SUSPENSION PLACED IN 1.8 ML OF INCUBATION
MEDIA, INCUBATED (18°C, 30-60 MIN.). REACTION STARTED BY
ADDITION OF 0.1 ML 100 nM VANADIUM-FREE Na₂ATP

↓
REACTION STOPPED WITH 0.5 ML OF 30% TCA (0°C)

↓
ATP HYDROLYSIS OF P_I DETERMINED BY AN AUTOMATED MODIFICATION
OF FISKE AND SUBBAROW (1925) PROCEDURE (TECHNICON METHOD,
No. SE2-004F3).

TABLE 3

Composition of branchial ATPase incubation media

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	$\text{Na}^+ - \text{K}^+$	$\text{Na}^+ - \text{NH}_4^+$	HCO_3^-	Mg^{++}	Ca^{++}
Tris-HCl	20 mM	20 mM	20 mM	20 mM	20 mM
pH	7.4	7.4	7.4	7.4	7.8
Mg^{++}	5 mM	5 mM	5 mM	5 mM	—
Na^{++}	100 mM	100 mM	—	—	—
NH_4^+	—	40 mM	—	—	—
K^+	20 mM	—	—	—	—
HCO_3^-	—	—	50 mM	—	—
Ca^{++}	—	—	—	—	3 mM
Ouabain	—	—	1 mM	1 mM	—
SCN^-	—	—	—	5 mM	—

4.2.3 Aromatic Hydrocarbon Effects on Gill ATPases In Vitro

The gill apparatus of six freshwater and 21 o/oo salt water acclimated trout were used in each test. Control enzyme activities were determined in non-exposed fish using the same extraction, isolation and assay procedures described in the previous section (4.2.2, pages 74-77). Test enzyme activities were assayed using the same microsomal suspension, but with the addition of 5.0 mg/L benzene, naphthalene, phenanthrene, benz(a)anthracene or benzo(a)pyrene (Figure 1). These compounds were added to the incubation media at their maximum water solubility and as finely dispersed particulates. No solvent carriers were used because most of these chemicals have been found to cause unacceptable inhibitory effects on ATPase enzymes (Janicki and Kinter, 1971b; Desai and Koch, 1977). The use of an in vitro design allowed the same fish to be assayed for control enzyme activity as well as for effects of one of the aromatic hydrocarbons.

4.2.4 Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) following test for homogeneity. Significance was accepted at $p=0.05$ or better.

4.3 RESULTS

4.3.1 Crude Oil Effects In Vivo

Specific activities of $\text{Na}^+\text{-K}^+$, $\text{Na}^+\text{-NH}_4^+$, HCO_3^- , Mg^{2+} and Ca^{2+} ATPase from control fish are presented in Table 4. Higher $\text{Na}^+\text{-K}^+$ ATPase activities and lower Mg^{2+} ATPase activities were observed in salt water acclimated fish relative to those in freshwater. No significant differences were found in the other enzyme activities which were attributable to salinity change.

Following exposure to the crude oils, significant alterations were observed in enzyme activities (Figures 8 to 12). In freshwater, significant reductions in $\text{Na}^+\text{-K}^+$ and HCO_3^- ATPase activities were found in the Norman Wells (NW) and Venezuelan (VEN) crude oil exposed fish. The petroleum hydrocarbons also produced elevations in Mg^{2+} ATPase activities. In addition, Norman Wells crude oil caused major reductions in $\text{Na}^+\text{-NH}_4^+$ and Ca^{2+} ATPase activities. In salt water, the Norman Wells crude oil caused significant increases in HCO_3^- and Ca^{2+} ATPase activities. The Venezuelan crude oil exposure resulted in a significant decrease in activities of $\text{Na}^+\text{-K}^+$ ATPase only. The other enzyme activities were altered moderately, but were not found to differ from control values.

The microsomal protein concentrations did not differ significantly between control, Norman Wells or Venezuelan oil exposed fish.

TABLE 4

Specific activities of control gill ATPases (I)

All values represent the means of 6 fish \pm one standard deviation.

Activities are expressed as $\mu\text{M Pi/mg/hr}$.

"*" denotes a statistically significant difference ($p \leq 0.05$) between enzyme activities of fresh and salt water acclimated fish.

	$\text{Na}^+ - \text{K}^+$	$\text{Na}^+ - \text{NH}_4^+$	HCO_3^-	Mg^{++}	Ca^{++}
Freshwater	2.32 ± 0.66	1.13 ± 0.44	4.72 ± 0.49	$9.79 \pm 1.66^*$	5.64 ± 0.72
Saltwater	$3.20 \pm 0.68^*$	1.49 ± 0.70	5.24 ± 0.43	7.11 ± 0.87	5.89 ± 1.44

Sodium-potassium stimulated ATPase activities in gills of fresh and salt water acclimated rainbow trout following exposure to weathered Norman Wells (NW) and Venezuelan (VEN) crude oil dispersions for 7 days.

"*" denotes a statistically significant difference ($p \leq 0.05$) from controls.

All values are means of 6 fish, except VEN in salt water $N=4$.

Vertical bars represent one standard deviation.

Figure 8: Sodium-potassium ATPase activities

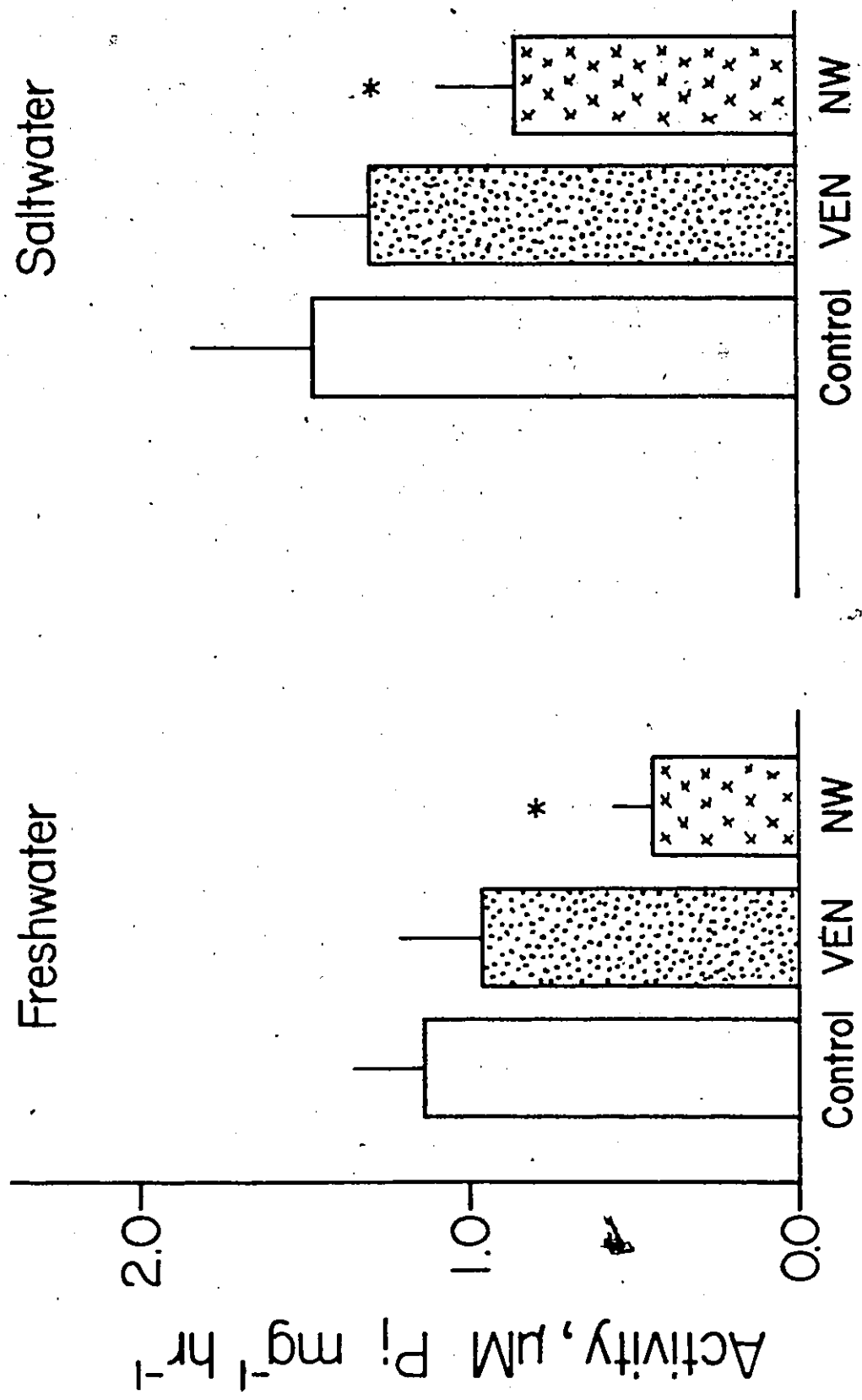
Sodium-ammonium stimulated ATPase activities in gills of fresh and salt water acclimated rainbow trout following exposure to weathered Norman Wells (NW) and Venezuelan (VEN) crude oil dispersions for 7 days.

"*" denotes a statistically significant difference ($p \leq 0.05$) from controls.

All values are means of 6 fish, except VEN in salt water $N=4$. Vertical bars represent one standard deviation.

Figure 9: Sodium-ammonium ATPase activities

$\text{Na}^+ - \text{NH}_4^+$ ATPase in vivo



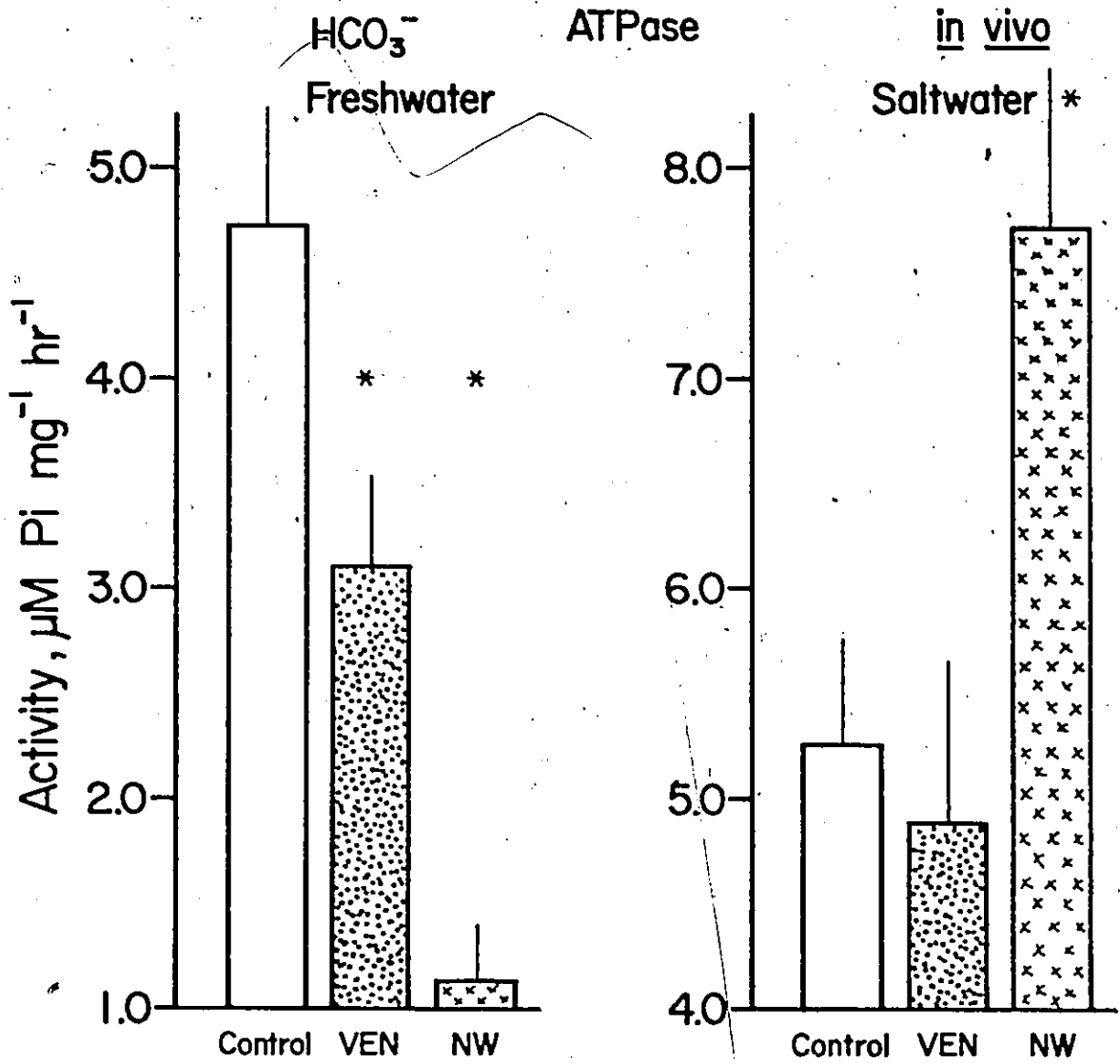
Bicarbonate stimulated ATPase activities in gills of fresh and salt water acclimated rainbow trout following exposure to weathered Norman Wells (NW) and Venezuelan (VEN) crude oil dispersions for 7 days.

"*" denotes a statistically significant difference ($p \leq 0.05$) from controls.

All values are means of 6 fish, except VEN in salt water $N=4$.

Vertical bars represent one standard deviation.

Figure 10: Bicarbonate ATPase activities



Magnesium stimulated ATPase activities in gills of fresh and salt water acclimated rainbow trout following exposure to weathered Norman Wells (NW) and Venezuelan (VEN) crude oil dispersions for 7 days.

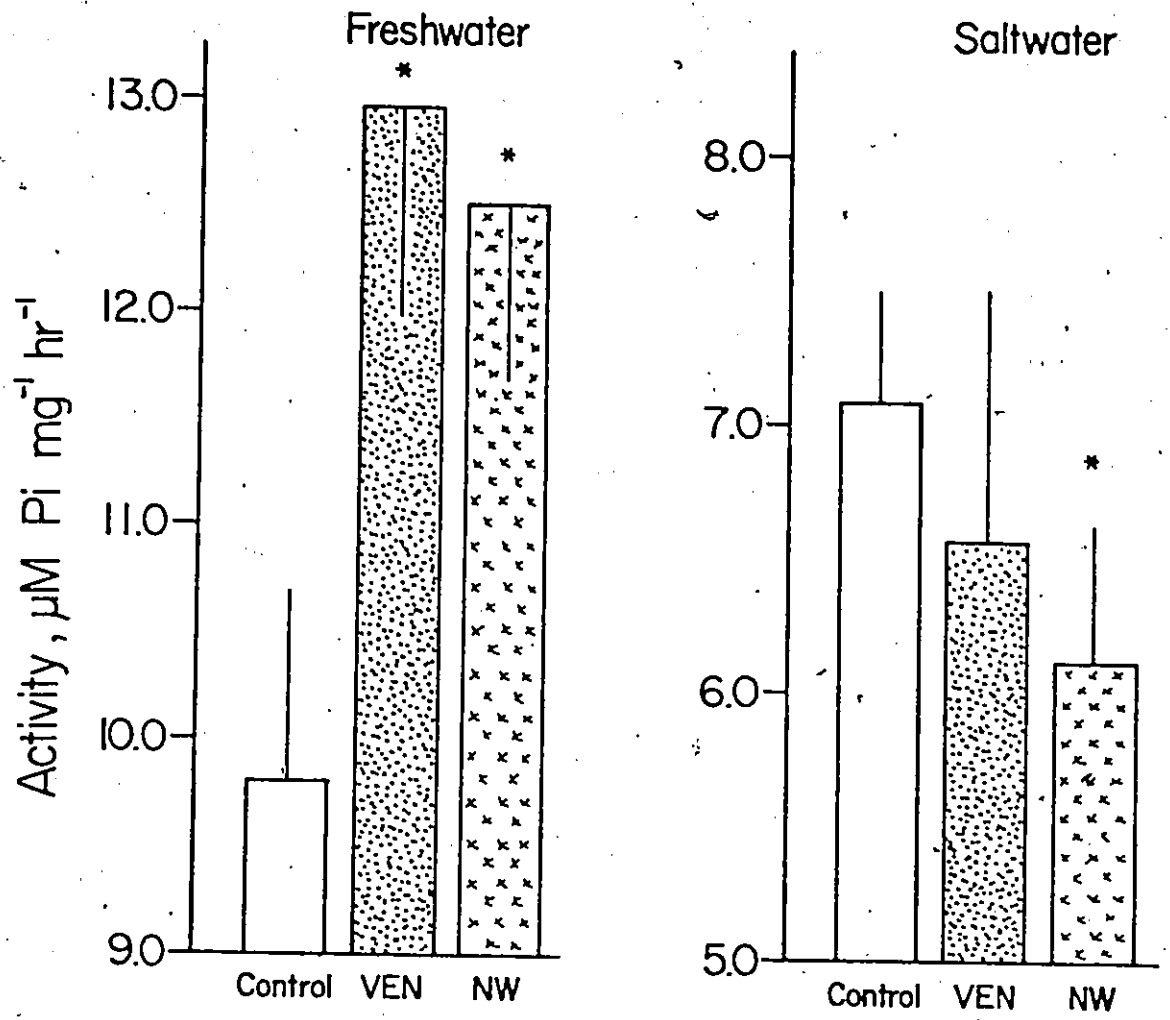
"*" denotes a statistically significant difference ($p \leq 0.05$) from controls.

All values are means of 6 fish, except VEN in salt water N=4.

Vertical bars represent one standard deviation.

Figure 11: Magnesium ATPase activities

Mg⁺⁺ ATPase in vivo



Calcium stimulated ATPase activities in gills of fresh and salt water acclimated rainbow trout following exposure to weathered Norman Wells (NW) and Venezuelan (VEN) crude oil dispersions for 7 days:

"*" denotes a statistically significant difference ($p \leq 0.05$) from controls.

All values are means of 6 fish, except VEN in salt water N=4.

Vertical bars represent one standard deviation.

Figure 12: Calcium ATPase activities

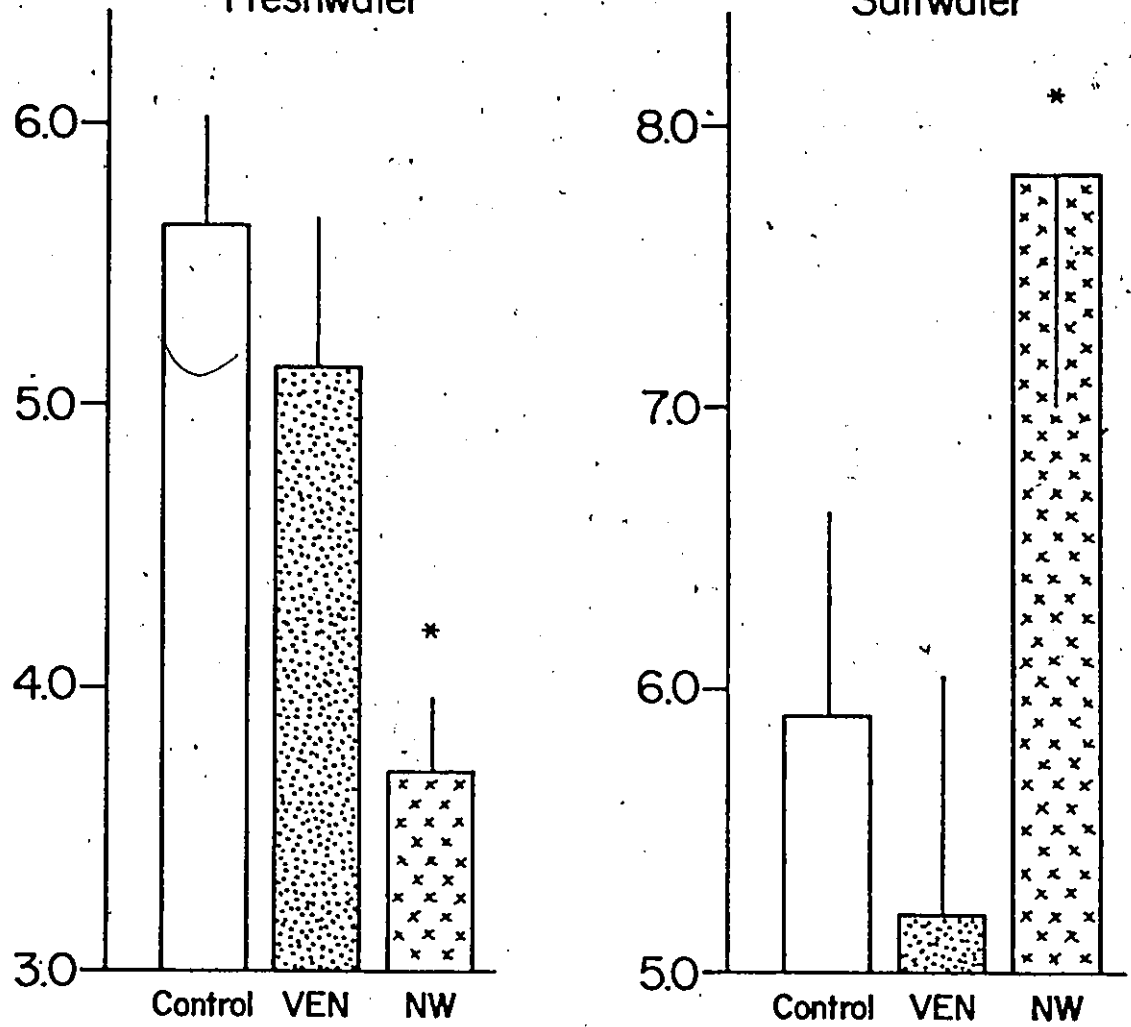
Ca⁺⁺ ATPase

in vivo

Freshwater

Saltwater

Activity, $\mu\text{M Pi mg}^{-1} \text{hr}^{-1}$



4.3.2 Aromatic Hydrocarbon Effects In Vitro

Fresh and salt water control ATPase activities are shown in Table 5. Higher $\text{Na}^+\text{-K}^+$ and $\text{Na}^+\text{-NH}_4^+$ ATPase activities were found in salt water acclimated trout compared to fresh-water trout. Activities of Mg^{2+} ATPase decreased in salt water. No significant changes were found in HCO_3^- and Ca^{2+} ATPase activities in fish from the two salinities.

Changes induced by aromatic hydrocarbons were more prominent in freshwater fish compared to those from salt water (Figures 13, 14). Benzene additions in the incubation media resulted in reduction in activities of all enzyme measured in both salinities, except Ca^{2+} ATPase from salt water. Naphthalene caused depressed activities in all enzymes except Mg^{2+} ATPase from freshwater fish. The three benzenoid ring compound, phenanthrene, caused significantly elevated $\text{Na}^+\text{-K}^+$ and HCO_3^- ATPase activities and no changes in $\text{Na}^+\text{-NH}_4^+$, Ca^{2+} and Mg^{2+} ATPase activities in freshwater. In salt water, its presence resulted in lowered $\text{Na}^+\text{-K}^+$ activity. Benz(a)anthracene induced lower $\text{Na}^+\text{-K}^+$, $\text{Na}^+\text{-NH}_4^+$ and HCO_3^- ATPase activities in freshwater, but did not affect enzyme activities from salt water fish. The five-benzenoid ring aromatic hydrocarbon, benzo(a)pyrene did not appear to have any significant effects on enzyme activities from the two salinities.

TABLE 5

Specific activities of control gill ATPases (II)

All values represent the means of 36 fish \pm one standard deviation.
Activities are expressed as $\mu\text{M Pi/mg/hr}$.

"*" denotes a statistically significant difference ($p \leq 0.05$)
between enzyme activities of fresh and salt water fish.

	$\text{Na}^+ - \text{K}^+$	$\text{Na}^+ - \text{NH}_4^+$	HCO_3^-	Mg^{++}	Ca^{++}
Freshwater	1.45 ± 0.56	1.24 ± 0.57	4.61 ± 1.53	$9.31 \pm 1.95^*$	3.36 ± 0.38
Saltwater	$2.48 \pm 0.72^*$	$1.95 \pm 0.82^*$	4.02 ± 1.71	5.22 ± 1.79	3.06 ± 0.79



Percentage change from control activities of branchial sodium-potassium, sodium-ammonium and bicarbonate stimulated ATPase activities of fresh and salt water acclimated trout, as affected by benzene (BEN), naphthalene (NAP), phenanthrene (PHE), benz(a)anthracene (BZA) and benzo(a)pyrene (BZP).

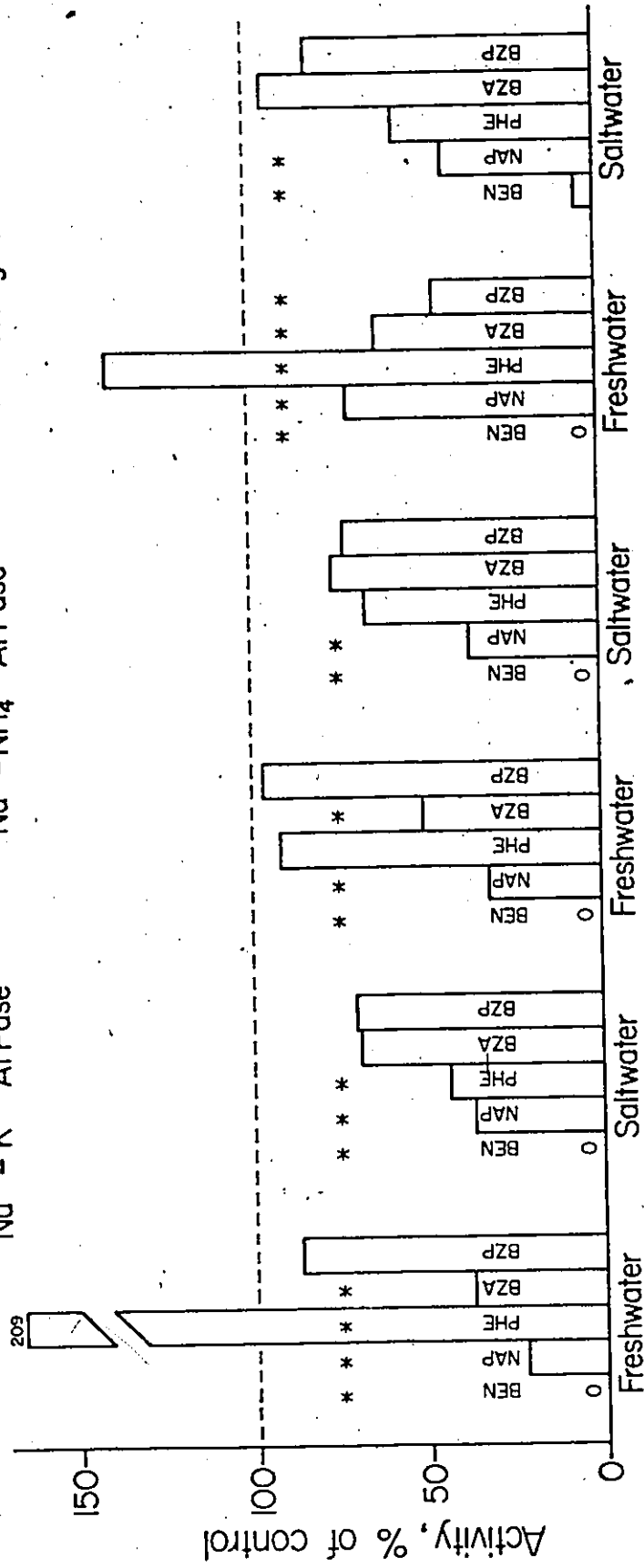
"*" denotes a statistically significant difference ($p \leq 0.05$) from the respective controls. All $N=6$.

Figure 13: Aromatic hydrocarbon effects on ATPase activities (I)

HCO_3^- ATPase

$\text{Na}^+ - \text{NH}_4^+$ ATPase

$\text{Na}^+ - \text{K}^+$ ATPase

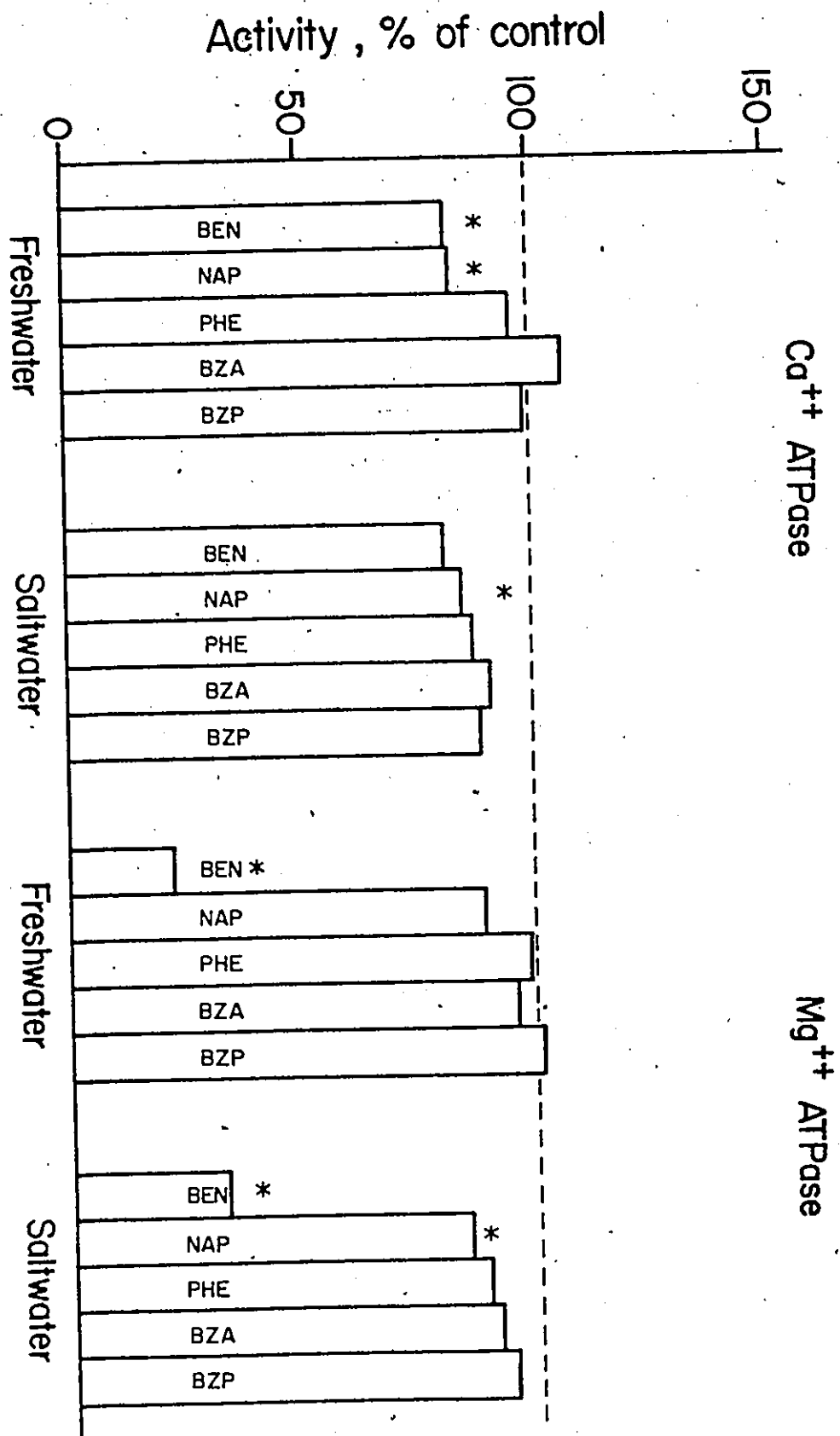


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Percentage change from control activities of branchial calcium and magnesium stimulated ATPase activities of fresh and salt water acclimated trout, as affected by benzene (BEN), naphthalene (NAP), phenanthrene (PHE), benz(a)anthracene (BZA) and benzo(a)pyrene (BZP).

"**" denotes a statistically significant difference ($p \leq 0.05$) from the respective controls. All N=6.

Figure 14: Aromatic hydrocarbon effects on ATPase activities (II)



4.4 DISCUSSION

4.4.1 ATPases and Osmoregulation

According to the published record, $\text{Na}^+\text{-K}^+$ ATPase is found in a variety of teleosts and generally exhibit higher enzyme activities in salt water species compared to their freshwater counterparts (Bornancin and Maetz, 1975). A few euryhaline labrid species, which are actually true marine fish (Lasserre, 1971; Gallis and Bourdichon, 1976; Gallis *et al.*, 1979) provide the exception to this rule. They display higher enzyme activities when transferred to freshwater from a marine environment. In addition, elevated $\text{Na}^+\text{-K}^+$ ATPase activities occur during parr-smolt transformation in some salmonids prior to seaward migration (Zaugg and McLain, 1970; 1972; Giles and Vanstone, 1976). This response may reflect physiological changes in these fish for a different mode of ion transport - one that is more suitable in salt water (Zaugg and McLain, 1970; 1972).

The higher $\text{Na}^+\text{-K}^+$ ATPase activities reported here in control salt water trout relative to those from freshwater (Tables 4, 5) agrees with similar studies using rainbow trout (Kamiya and Utida, 1969; Zaugg and McLain, 1970; 1972; Pfeiler and Kirschner, 1972). This increase in activity is

associated with an increase chloride cell number as well as increased organelle development in these cells (Shirai, 1972; Karnaky et al., 1976). There is a suggestion that fresh and salt water adapted fish may have different forms of $\text{Na}^+\text{-K}^+$ ATPase enzymes (Motais, 1970; Pfeiler and Kirschner, 1972). More research is required in this area, however, before one could say with certainty that two different $\text{Na}^+\text{-K}^+$ activated enzymes are operating at each salinity.

It is difficult to compare specific activities of enzymes in this study with values found in the literature. This can be attributed mainly to the diversity of techniques utilized. Some workers measure activities of whole tissue homogenates, while others eliminate the cellular debris and use the subcellular fractions. The subcellular fractions consistently exhibit higher enzyme activities (Bornancin et al., 1980; Ho and Chan, 1980). Some studies make no attempts at purifying the enzyme extracts, while others use deoxycholate treatment. This process of purification has been shown to be essential in many cases (Zaugg and McLain, 1970; 1972; Sargent et al., 1980). The assay temperatures employed in different studies have varied from 10 to 45 °C. The importance of using a temperature within the thermal range in which the fish inhabits has been emphasized (McCarty and Houston, 1977; Watson and Beamish, 1980). The temperature used in this study (18 °C) is well within the thermal tolerance lim-

its of rainbow trout (0 to 25 °C). As a result of the variety of methods employed in ATPase assays, only cautious qualitative comparisons of enzyme activities can be made. In addition to the increase in Na⁺-K⁺ ATPase activities reported for salt water acclimated fish and for salmonids undergoing smoltification stated earlier, a variety of environmental influences have been demonstrated to affect activities of this enzyme (Table 6).

TABLE 6

Environmental influences on gill ATPase activities

- References: (1) Moon, 1975;
(2) Pequette and Giles, 1978;
(3) Pfeiler, 1980;
(4) Murphy and Houston, 1974;
(5) Zaugg and McLain, 1976;
(6) Zaugg et al., 1972; Adams et al., 1973; 1975;
Zaugg and Wagner, 1973;
(7) McCarty and Houston, 1977;
(8) Ewing et al., 1980; Zaugg, 1981.

ENZYME	FISH SPECIES	ENVIRONMENTAL INFLUENCE	EFFECTS	REFERENCES
Na-K	<u>O. kisutch</u> <u>Antimora rostrata</u> in SW	Increased hydrostatic pressure	↓	(1)
Na-K Mg	<u>Ameiurus nebulosus</u> in FW <u>Cottus scorpius</u> <u>Anguilla anguilla</u> in SW	Increased hydrostatic pressure	↓	(2)
Na-K Mg	<u>Scorpaena guttata</u> <u>Anoploma fimbria</u> in SW	Increased hydrostatic pressure	↑	(3)
Na-K	<u>Carassius auratus</u> in FW	Increased water temperature	↑	(4)
Na-K	<u>O. kisutch</u> in FW	Increased water temperature	↑ at 6°C.	(5)
Na-K	<u>S. gairdneri</u> in FW	Increased water temperature	no change at 20°C ↑ at 10°C no change at 20°C	(6)
Na-K HCO ₃ Mg	<u>S. gairdneri</u> in FW	Increased water temperature	↑	(7)
Na-K	<u>S. gairdneri</u> in FW	Advanced photoperiod	↑	(8)

A $\text{Na}^+\text{-NH}_4^+$ ATPase was found in branchial tissue of trout (Tables 4, 5). Several groups of workers reported that ammonium ion can substitute for potassium in the $\text{Na}^+\text{-K}^+$ ATPase reaction. This has been demonstrated in *F. heteroclitus* (Towle et al., 1977) and *A. rostrata* (Butler and Carmichael, 1972). Further, a variety of monovalent cations, including Rb^+ , Cs^+ , Tl^+ , Li^+ , and NH_4^+ have been reported to be capable of replacing K^+ in the dephosphorylation reaction in $\text{Na}^+\text{-K}^+$ ATPase extracts from salt water *A. anguilla* (Bell et al., 1977; Sargent et al., 1980). This dephosphorylation step and resultant enzyme activity can be blocked by the cardiac glycoside, ouabain. Ammonium ions have long been labelled as the physiological counterion for sodium exchange in teleosts (Maetz and Garcia-Romeu, 1966; Maetz, 1971). The fact that ouabain can inhibit sodium uptake and ammonium excretion in rainbow trout (Kerstetter et al., 1970; Payan et al., 1975) further supports the presence of such an enzyme.

The reason for the moderate, but significant, increase in $\text{Na}^+\text{-NH}_4^+$ ATPase activity in salt water control fish from the second series of experiments (Table 5) is not immediately obvious. No such increase was observed in the first series (Table 4). Towle and coworkers (1977) reported ammonium ion inhibition of ouabain binding to gill microsomes can be

slightly higher or the same in salt water fish compared to those from freshwater. Other than this, the question of salinity effects on this enzyme has not been addressed. The increased $\text{Na}^+\text{-NH}_4^+$ ATPase activities in salt water could be an accessory mode of sodium transfer out of the blood. This mechanism may only be operative during periods requiring rapid removal of sodium, when $\text{Na}^+\text{-K}^+$ ATPases are not capable of handling the excess ion load. The turnover of chloride cells, and therefore ATPase enzymes, is greater in salt water fish than freshwater fish (Conte and Lin, 1967), so there could be periods when the fish will have fewer $\text{Na}^+\text{-K}^+$ enzyme units operating. Ammonia-sodium exchanges have been reported in a number of salt water acclimated teleosts (Evans, 1977) and as stated earlier, ammonium ions can substitute for potassium in the $\text{Na}^+\text{-K}^+$ ATPase reaction (Bell et al., 1977; Sargent et al., 1980).

An ATPase stimulated by anions, especially bicarbonate, was previously reported in oxyntic cells of Necturus sp. (Wiebelhaus et al., 1971) which was believed to be responsible for HCl secretion. Such an enzyme in fish gills has been found, but its cellular distribution has been debated. Van Amelsvoort et al. (1977) claimed the anion-dependent ATPase in trout gills is exclusive to the mitochondrial fraction. Yet, in order for it to participate in the ion transfer process, it must be localized in the microsomal fractions which

contain plasma membranes. Ho and Chan (1981) also argued for a mitochondrial distribution of this enzyme in Japanese eel branchial tissue, though activity was detectable in the microsomal fraction. Others have shown that an ATPase exists in the microsomal fraction of trout gills which is stimulated by anions in the presence of magnesium (Bornancin et al., 1980). Similar findings were reported for goldfish (deRenzis and Bornancin, 1977) and American eel (Soloman et al., 1975). Furthermore, these workers postulated that activity from this enzyme can function in active transport of chloride and bicarbonate in a similar manner that Na⁺-K⁺ ATPase is responsible for Na⁺/K⁺ exchange. The fact that thiocyanate can inhibit HCO₃⁻ ATPase agrees with physiological studies showing inhibition of chloride secretion and absorption by thiocyanate (Kerstetter and Kirschner, 1972; Epstein et al., 1973; deRenzis, 1975).

The method of HCO₃⁻ ATPase extraction in the present study from trout gills agrees with the microsomal localization of this enzyme (Bornancin et al., 1980). A similar extraction procedure for isolating ATPase enzymes from eel tissues showed mitochondrial contamination was not a significant factor (Fenwick, 1981). A problem occurred in this study during attempts to establish a baseline or residual activity for HCO₃⁻ ATPase. It was finally shown that magnesium ATPase in the presence of both ouabain and thiocyanate

provided acceptable results (Table 3). Addition of thiocyanate was required because residual activity (Mg^{2+} ATPase activity) was apparently subjected to activation by chloride ions in the incubation media (Tris-HCl, $MgCl_2$). Chloride stimulation can occur in various tissues responsible for Cl^-/HCO_3^- exchange (Bornancin *et al.*, 1980) and chloride can act as a substrate with weak affinity for the enzyme (deRenzis and Bornancin, 1977). In effect, the HCO_3^- ATPase in the present study should actually be called a bicarbonate-chloride stimulated ATPase.

Activities of branchial HCO_3^- ATPase showed no adaptive changes in fresh or salt water acclimated trout (Tables 4, 5). This is at variance with the work of Morisawa and Utida (1976) who reported higher enzyme activities in gills and intestine of salt water Japanese eels. The present results, however, agree with several studies for trout (Kerstetter and Kirschner, 1974; Bornancin *et al.*, 1980) and other fish (Soloman *et al.*, 1975; Naon *et al.*, 1981; Ho and Chan, 1981) reporting no changes in enzyme activity with variation in external salinity. Thus, it does not appear that this enzyme is involved in osmoregulatory chloride exchanges. Since the enzyme can be stimulated by bicarbonate and chloride, an involvement in acid-base regulation of the internal medium would be more reasonable. Indeed, external or internal modifications in pH can bring about changes in pumping rates of

sodium and chloride (deRenzis and Maetz, 1973; Cameron, 1976).

Microsomal extracts from trout adapted to fresh and salt water possess enzyme activities which are activated by magnesium and are insensitive to ouabain and thiocyanate. This enzyme has often been reported as "baseline" or "residual enzyme activity" in trout and other salmonids (Zaugg and McLain, 1969; 1970; 1971; Pfeiler and Kirschner, 1972). The specific activities of the enzyme have been found to vary with salinity. In coho salmon, magnesium ATPase activity decreased in salt water fish or in freshwater fish fed a NaCl-enriched diet (Zaugg and McLain, 1969; 1970). Similar results were found in this study after acclimation to a hyperosmotic medium (Tables 4, 5). Reportedly, salt water acclimated salmonids can convert Mg^{2+} ATPase to a form of Na^+-K^+ ATPase, since much of the elevation in Na^+-K^+ ATPase can be accounted for by a drop in Mg^{2+} ATPase activities (Zaugg and McLain, 1969; 1970). No decrease in Mg^{2+} ATPase activity was found, however, in other species of fish even though there was a significant increase in Na^+-K^+ ATPase activities (Kamiya and Utida, 1968). More work is required in this area to provide insight into the significance of these changes and to resolve the observed differences.

Calcium dependent ATPase extracted from gills of rainbow trout has previously been characterized by several workers (Ma et al., 1974; Watson and Beamish, 1980; 1981). This enzyme shows ouabain insensitivity and monovalent ion independence. Magnesium can activate this enzyme, but affinity is higher for calcium ions (Ma et al., 1974). A similar enzyme system has been described in other teleosts including A. rostrata (Fenwick, 1979), A. japonica (Ho and Chan, 1980), Rutilus rutilus (Shepherd and Simkiss, 1978; Shepherd, 1981), F. heteroclitus (Burdick et al., 1976) and Osteoglossum bicirrhosum (Moon, 1978). An important association of this enzyme with calcium metabolism is suggested by changes in enzyme activity with removal of the corpuscles of Stannius, which results in hypercalcemia (Fenwick, 1976).

The picture is less clear in attempts to correlate enzyme activities with salinity of the external media. Fenwick (1979) reported higher Ca^{2+} ATPase activities in freshwater eels compared to those from salt water. Conversely, others have reported increased enzyme activities in salt water adapted fish (Burdick et al., 1976; Ho and Chan, 1980). Furthermore, Ma (1976; cited in Fenwick, 1979) did not observe any significant differences in gill Ca^{2+} ATPase activity of fresh and salt water acclimated trout. This is consistent with the results of the present study (Tables 4, 5). The absolute levels of gill Ca^{2+} ATPase activity may be re-

lated to long-term adaptation to varying external calcium concentrations (Shepherd, 1981). Enzyme activities in R. rutilus showed little change following exposure for seven weeks to high and low levels of calcium in freshwater. Fish captured from ponds with high calcium concentrations exhibited high Ca^{2+} ATPase activity, and those from ponds with low external calcium had lower enzyme activity. It has yet to be established if the enzyme is responsible for uptake or excretion of calcium (Shepherd and Simkiss, 1978).

4.4.2 Hydrocarbon Effects

The effect of crude oil dispersions on ATPase activities correlated with the ionic imbalance in hydrocarbon intoxicated trout (Chapter II and III). Inhibition of enzymes can be correlated to a breakdown of the ion transport mechanism. Disturbances in plasma ion levels were found after blockage of salt transporting functions (Conte and Lin, 1967; Maetz et al., 1969). Such perturbations would result in decreased and increased plasma ion levels in fresh and salt water fish, respectively. These effects were evident in the hydrocarbon treated fish. Inhibition of $\text{Na}^+\text{-K}^+$ ATPase activity would cause a breakdown of active ion uptake and excretion mechanisms. Bonting (1970) found inhibition of this enzyme by ouabain is accompanied by a parallel reduction in sodium transport. Alterations in Ca^{2+} and Mg^{2+} ATPase activ-

ities could result in modifications in gill permeability since calcium and magnesium are essential for maintenance of cellular membrane integrity and for stabilization of branchial permeability (Potts and Fleming, 1971; Isaia and Maso- ni, 1976). Changes in HCO_3^- ATPase activity may interfere with the ability of the fish to regulate the pH of body fluids and disrupt chloride transport. In fact, lowered blood pH has been reported in hydrocarbon treated goldfish in freshwater (Brenniman et al., 1979). Perturbation of Na^+ - NH_4^+ stimulated enzyme activities can result in decreased active sodium movement and ammonia excretion. Some of these effects are speculative as information on the actual physiological actions of some of these enzymes is sparse. Although Na^+ - K^+ ATPase has been studied extensively, little is known about the other enzymes.

Other studies have also demonstrated ATPases to be sensitive to various environmental contaminants in vivo (Table 7) and in vitro (Table 8). Most have involved only Na^+ - K^+ ATPase. Furthermore, the xenobiotics tested were often lipophilic substances -such as DDT. Knowing the lipophilic nature of some petroleum compounds, including aromatic hydrocarbons, it may not be surprising that activities of membrane-bound enzymes were disrupted. In addition, a recent report showed petroleum refinery effluent is capable of inhibiting Na^+ - K^+ ATPase activities in the marine fish, Lep-

tocottus armatus (Boese et al., 1982). The refinery wastewater contained a high concentration of naphthalene, a compound shown effective in inhibiting enzyme activities in this study (Figures 13, 14). Interestingly, the Norman Wells crude oil was shown to have greater disruptive effects on gill enzyme activities than the Venezuelan crude oil (Figures 8 to 12). Chemical analysis of the two oils showed Norman Wells to have a higher proportion of lower molecular weight aromatic hydrocarbons than Venezuelan crude oil (Appendix B). The in vitro experiments demonstrated that these aromatic hydrocarbons had greater inhibitory effects on ATPase activities than the medium and high molecular weight aromatic compounds (Figures 13, 14). Furthermore, the effects on enzyme activities in the Norman Wells exposed fish can be correlated to the observed chloride cell damage (Engelhardt et al., 1981).

TABLE 7

In vivo effects of contaminants on gill ATPase activities

- References: (1) Bouquegneau, 1977;
(2) Lorz and McPherson, 1976;
(3) Campbell et al., 1974;
(4) Kuhnert et al., 1976;
(5) Kinter et al., 1972;
(6) Desai et al., 1975;
(7) Watson and Beamish, 1980;
(8) Shepherd and Simkiss, 1978.

ENZYME	FISH SPECIES	ENVIRONMENTAL CONTAMINANT	EFFECTS	REFERENCES
Na-K	<u>Anguilla anguilla</u> in SW	Mercury	↓	(1)
Na-K	<u>O. kisutch</u> in FW	Copper	↓	(2)
Na-K	<u>S. gairdneri</u> in FW and SW	DDT	↓	(3)
Na-K	<u>S. gairdneri</u> in FW	Chromium	no change	(4)
Na-K Mg	<u>A. rostrata</u> in SW	DDT PCB	↓ Mg less sensitive than Na-K	(5)
Na-K Mg	<u>Pimephales promelas</u> in FW	DDT	↓	(6)
Na-K Na-NH ₄ Mg Ca	<u>S. gairdneri</u> in FW	Zinc	↑	(7)
Ca	<u>Rutilus rutilus</u> in FW	Copper	no change	(8)

TABLE 8

In vitro effects of contaminants on gill ATPase activities

- References: (1) Primor et al., 1980;
(2) Davis and Wedemeyer, 1971;
(3) Janicki and Kinter, 1971b;
(4) Poston, 1979;
(5) Watson and Beamish, 1981;
(6) Shepherd and Simkiss, 1978.

ENZYME	FISH SPECIES	ENVIRONMENTAL CONTAMINANT	EFFECTS	REFERENCES
Na-K	<u>Aphanius dispar</u> in SW	Pardoxin (ichthyotoxin)	↑ at low concentrations ↓ at high concentrations	(1)
Na-K	<u>S. gairdneri</u> in FW	DDT	↓	(2)
Na-K Mg	<u>Pseudopleuronectes americanus</u> in SW	DDT	↓	(3)
Na-K Mg	<u>S. gairdneri</u> in FW	Phenol	↓ Mg less sensitive than Na-K	(4)
Na-K Na-NH ₄ Mg Ca	<u>S. gairdneri</u> in FW	Zinc	↓ Ca and Mg least inhibited	(5)
Ca	<u>Rutilus rutilus</u> in FW	Copper Lead Mercury Zinc	↓	(6)

Lethal effects were found in the Venezuelan crude oil exposure in salt water, but only minor changes occurred in gill enzyme activities. This indicates that although the disrupted enzyme activities can explain the ionic changes, other factors could be involved in the observed mortalities. Respiratory disturbances could have caused the lethal effects (Meyerhoff, 1975; Rice et al., 1977b). Possibly, hydrocarbons were able to disrupt carbonic anhydrase activity. This enzyme is known to play an important role in gas exchange (Haswell et al., 1980) and ion regulation (Maetz, 1971).

Generally, inhibitory effects were observed in the aromatic hydrocarbon in vitro exposures. The lower molecular weight compounds had greater effects on enzyme activities at the concentrations used (Figures 13, 14). Other results might have been evident had the hydrocarbons not been tested on a mass/volume basis. By using this method for comparison, the benzene concentration (0.064 mM) is higher than the benzo(a)pyrene concentration (0.020 mM). The consequences of using the same molar concentrations of each aromatic hydrocarbon remain to be evaluated. The measured effects may be due to the ability of these compounds to be rapidly incorporated into membrane layers. This is discussed in detail later in the text. To the knowledge of the author,

only one study has compared the toxicity of a homologous series of aromatic hydrocarbons. Rossi and Neff (1978) found the toxicity of polynuclear aromatic hydrocarbons was directly related to their solubility and residence time in the test solution. These workers reported the lower molecular weight hydrocarbons had greater toxic effects on the marine worm, Neanthes arenaceodentata, than those with higher molecular weights. The latter groups of hydrocarbons may also affect enzyme activities, but their slow mobility across biological membranes would modify their apparent toxicity. These compounds can form biologically inactive micelles with dissolved organic matter (Boehm and Quinn, 1976). This could have occurred with the microsomes in the present study.

The stimulatory effects of phenanthrene on $\text{Na}^+\text{-K}^+$ and HCO_3^- ATPase from freshwater trout (Figure 13) is not fully understood at the present time. Increased activities of ATPase enzymes have been reported in enzyme preparations contaminated with zinc (Watson and Beamish, 1980). Possibly, phenanthrene was capable of changing the enzyme conformation to cause induction of activity. At present, no evidence of such an occurrence can be advanced.

Differences in enzyme sensitivity to hydrocarbons were found between the in vivo and in vitro tests (Figures 8 to

14). Several possible explanations can account for this, aside from the obvious employment of different chemical compounds. In the in vivo exposures, one would expect the fish to be capable of metabolizing some of the petroleum hydrocarbons to a non-toxic or a rapidly excretable product. The ability of fish, including trout, to biotransform and discharge petroleum hydrocarbons has been well documented (Varanasi and Malins, 1977). Another possibility is that some components in blood can bind the hydrocarbons following their entry into the fish and render them biologically inactive. Roubal (1974) found non-polar hydrocarbons associating with low viscosity molecules such as lipids, while polar hydrocarbons interact with non-polar blood proteins like albumen. The concentration of hydrocarbons at the sensitive site would certainly influence the apparent toxicity of these substances. In live animals, the concentration in tissues may be much higher than the levels in the external medium, but the bioavailability of these compounds is unknown. Gill tissues can accumulate a substantial amount of hydrocarbons (Rice et al., 1977b), yet, the sensitive sites of the enzymes may be protected by the intact membrane. In the in vitro tests, enzyme units are directly exposed to the chemicals. Even in this case, the actual concentrations found in the microenvironment is unknown. The toxicants may reach the immediate area of the enzyme, but binding to other membrane components could limit their access to the enzyme

unit. Another possible explanation is that a live animal can synthesize new enzyme units to replace the inactivated forms. Since no significant differences were found in microsomal protein concentrations, this last possibility seems unlikely. Yet, de novo synthesis cannot be dismissed on this result alone.

4.4.3 Possible Mechanisms of Toxicity

Little is known concerning the mechanism of toxicity of petroleum, but hydrocarbons tend to associate with biomembranes and can interfere with cellular structure and function. Aromatic hydrocarbons can cause swelling and disruptions of plasma membranes of protozoans (Goldacre, 1968). Further, spin-labelling techniques were employed to study the in vivo and in vitro interactions of petroleum hydrocarbons in coho salmon. These compounds were shown to preferentially bind to cellular membranes (Roubal, 1974; Roubal and Collier, 1975). Specifically, aromatic hydrocarbons were bound to various non-polar and electron-interactive sites on the membrane surface resulting in perturbations in membrane organization. Similar findings were reported by Stegeman (1977). Lipophilic hydrocarbons preferentially associated with such lipid structures as plasma membrane, mitochondrial membrane, nuclear envelope and endoplasmic reticulum. Disturbances of plasma or mitochondrial membranes in the liver

by hydrocarbons caused alterations in normal intermediary metabolism (Sabo and Stegeman, 1976; Stegeman and Sabo, 1977). Many physiological functions -such as neurotransmission, muscle contraction, and ion/osmotic/exchange- are directly dependent on the structural properties of biological membranes. The results of the present study clearly showed crude oils and aromatic hydrocarbons can interfere with membrane-bound enzymes responsible for osmotic and ionic exchange in branchial tissue of fish.

Enzyme inhibition by DDT and other chlorinated hydrocarbon pesticides has been the focus of considerable research. These lipophilic compounds are potent inhibitors of $\text{Na}^+\text{-K}^+$ ATPase extracted from fish gills and can disrupt osmoregulation (Janicki and Kinter, 1971a; 1971b; Leadem et al., 1974; Miller and Kinter, 1977). ATPase enzymes have a requirement for lipids and contamination by lipid-soluble substances such as hydrocarbons can result in altered enzyme function. Schneider (1975) showed that DDT in particulate or solubilized form can inhibit $\text{Na}^+\text{-K}^+$ ATPase activity from rat brain. The toxicant can affect the enzyme directly, independent of changes in sodium or potassium concentrations, and does not cause inhibitory effects by classical binding to specific sites. The mechanism of toxicity was suggested to be mediated by the ability of DDT to dissolve in the lipid phase of the membrane and disrupt enzyme function by alter-

ing the properties of the membrane. Changes in the lipid composition of membrane can result in modifications in the allosteric properties of $\text{Na}^+\text{-K}^+$ ATPase and other membrane-bound enzymes (Moreno et al., 1972). According to the fluid mosaic model of membrane structure (Singer, 1972; Singer and Nicholson, 1972), membrane phospholipids are aligned in a bilayer. The polar heads of the phospholipids are at the surface of the membrane and the fatty acid tails extend into the fluid center. Globular proteins are embedded in this phospholipid matrix. Any alterations in the protein-lipid interactions may affect transport rates and enzyme activities (Kinter and Pritchard, 1977). Thus, it seems reasonable to postulate that DDT induces its toxic effects by decreasing the fluidity of membrane phospholipids, which in turn would cause alterations in allosteric transitions required for enzyme activity (Schneider, 1975; Miller and Kinter, 1977).

Such a mechanism has also been proposed for enzyme inhibition by cholesterol (Kimelberg and Papahadjopoulos, 1974). These workers investigated the relationship between $\text{Na}^+\text{-K}^+$ ATPase, membrane fluidity and cholesterol. Cholesterol was found to reduce the fluidity of membrane phospholipids, resulting in inhibition of $\text{Na}^+\text{-K}^+$ ATPase activity. In addition, some anaesthetics (n-hexanol) can have disruptive effects on membrane fluidity and promote inactivation of

Na⁺-K⁺ ATPase (Cossins et al., 1981). The above hypothesis of enzyme inhibition receives further support from investigations demonstrating that addition of phospholipids into Na⁺-K⁺ ATPase preparations can prevent or reverse inactivation of enzymes by chlorinated hydrocarbons, detergents and other organic compounds (Wilson and Wilson, 1972; Sharp et al., 1974). This non-competitive nature of enzyme inhibition may also be appropriate for lipophilic petroleum hydrocarbons. The water soluble (polar) components of petroleum would interact with structures on the surface of the membrane, such as the phospholipid heads and extrinsic proteins. While the lipid soluble (non-polar) hydrocarbons can move into the membrane and interact with the hydrophobic interior, with such structures as the phospholipid tails and intrinsic proteins. Both types of interactions would result in disrupted membrane integrity.

The present study showed divalent cations stimulated ATPases to be less affected by hydrocarbon treatment than enzymes stimulated by monovalent ions (Figure 14). Similar results were obtained by others (Watson and Beamish, 1980; 1981). This could in part be related to the molecular structure and the lipid-dependence of the different enzymes. Two recent reports (Kyte, 1981; Klingenberg, 1981) showed Na⁺-K⁺ ATPase and Ca²⁺ ATPase to have distinct structural conformations. Stable tetramers, dimers, and monomers of Ca²⁺ ATPase

have been identified, while $\text{Na}^+\text{-K}^+$ ATPase exists only as a dimer. With this evidence, one may postulate that since Ca^{2+} ATPase can function in three physical units, disruption in activity would be less pronounced. In addition, Ca^{2+} ATPase does not appear to require a counterion for proper function. The binding sites may only be located on one side of the enzyme, whereas in the $\text{Na}^+\text{-K}^+$ ATPase enzyme, binding sites are required on both sides, one for sodium and another for potassium. Therefore, alterations on any one site would impair enzyme function. This, of course, is strictly speculative due to the paucity of information on the molecular structure and mechanism of these enzymes.

4.4.4 General Discussion

Results of this study showed that the plasma imbalance caused by petroleum has a biochemical basis. Oil-exposed fish, especially anadromous and catadromous species, challenged by changing salinities in the environment may consequently be greatly affected. Salmonids undergoing parr-smolt transformation, in preparation for seaward migration, can also be affected. For example, Zaugg *et al.* (1972) found an increase in water temperature can affect $\text{Na}^+\text{-K}^+$ ATPase activity in rainbow trout, resulting in loss of migrating disposition in these fish. In addition, depressed gill

Na⁺-K⁺ ATPase activity after copper-zinc contamination reduced the number of coho salmon successfully completing the downstream migration, and increased mortality in these fish upon exposure to salt water (Lorz and McPherson, 1976). Similarly, this type of pollution interrupted the upstream migration of Atlantic salmon in the Miramichi River (Sprague *et al.*, 1965; Sprague and Saunders, 1967). The salmon populations declined in the polluted river. Finally, a recent study found migrating species of Pacific salmon returned downstream upon exposure to water contaminated by monocyclic aromatic hydrocarbons (Weber *et al.*, 1981). This shows an interesting correlation with the present work in that benzene had the most severe effect on gill enzymes (Figures 13, 14).

Since crude oil and refined petroleum products are transported in large quantities over many waterways, the risk of oil pollution in the aquatic environment increases with rising demands for fossil fuels. For example, in order to bring oil produced in fields in Alaska's North Slope (an estimated amount of 2 million barrels a day) to refineries on the west coast of North America, large supertankers (45,000 to 150,000 tons) will be used. The potential for accidents along the northwestern shipping lanes is presumed to be high due to navigational hazards along the routes and severe climatic conditions (Clark and Findley, 1977). In addition,

these tankers are allowed under regulation to release oily ballast water into the Gulf of Alaska beyond a specified distance from the shore (Clark and Findley, 1977). These incidents could lead to gradual accumulation of hydrocarbons in the area, and may represent a hazard to the marine ecosystem. This study clearly showed petroleum exposure can have significant physiological consequences in fish. It can be postulated that oil discharged into biologically productive areas, such as coastlines and estuaries, could contribute to a loss of commercially valuable fish species.

In summary, petroleum hydrocarbons were capable of disrupting branchial ATPase activities in both fresh and salt water acclimated trout. These effects could have played a major contributory role in the ionic/osmotic dysfunction found in hydrocarbon stressed fish.

Chapter V

SUMMARY AND CONCLUSIONS

Fresh and weathered crude oil dispersions were found to affect plasma ion balance in rainbow trout. Oil accommodated in the water column in the particulate form can have an impact on important physiological processes in fish. Very little work has been done on the toxicity of oil-in-water dispersions, despite the fact that most of the oil found in water is in this form. Even after weathering, oil dispersions can retain their toxic components. No major changes were observed in the plasma ion levels of paraffin oil dispersion exposed fish. This would indicate that the disruptions in electrolyte metabolism were not a result of exposure to oil dispersions, but from toxic hydrocarbons in the oil particles. The results of the weathered crude oil experiments showed that these compounds are still in the water even after undergoing weathering.

Other modes of weathered oil exposure did not result in major changes in the plasma ion profile of trout. The water-soluble fraction of weathered crude oils does not contribute to the acute toxicity of petroleum to fish. Fur-

ther, oil introduced to the fish by intraperitoneal injections (systemic contamination) did not compromise the mechanisms for ionic balance -possibly because the oil is quickly eliminated from body tissues.

The changes in hydromineral balance in trout exposed to aromatic hydrocarbons adds evidence to the postulation that these compounds are responsible for the toxic effects of petroleum. The results from the experiments, however, did not show any correlation between effects on ion balance and molecular weight. Any trends could have been masked by the addition of the paraffin oil dispersions.

Salt water acclimated trout exposed to weathered crude oil dispersions had increased plasma ion levels and osmolality. This effect is converse to that observed in freshwater acclimated fish. This can be attributed to the difference in salinity of the external media, and the different ion transport mechanisms in the animals.

The functional consequences of exposure to crude oil dispersions in both freshwater and salt water acclimated trout appeared to have resulted from a breakdown of the respective ion transport mechanisms. The observed changes in gill morphology, especially damage to the chloride cells, may account for this. Other ion-transporting tissues could also

have contributed to the ionic dysfunction, but they have yet to be examined.

The disruption in gill ATPase activities following exposure to weathered crude oil dispersions support the hypothesis that hydrocarbons can have serious effects on cellular membrane structures which can result in impairment of enzyme function. This series of experiments showed disruption of enzyme activities could have played a major role in the plasma ion imbalance in hydrocarbon exposed fish. Other vital physiological processes can be altered by oil contamination. The possibility that other enzyme systems are affected remains to be evaluated.

Information is sparse on the acute toxicity of aromatic hydrocarbons. The in vitro experiments showed lower molecular weight aromatic hydrocarbons had greater effects on branchial ATPase enzymes of trout than higher molecular weight compounds on a mass/volume basis. The observation that monovalent ion stimulated ATPases were more severely affected than divalent ion stimulated enzymes may have resulted from their structural differences and lipid-dependence. But little is known about the structure and mechanisms of ATPase enzymes.


Finally, this study showed that the water-electrolyte status in fish is a reliable indicator of oil pollutant stress. It is clear that the study could be expanded to examine the possible role of other ion-regulating tissues. In addition, an evaluation of the dose response of the enzymes to various petroleum hydrocarbons should be undertaken. The results of this study provide evidence that the mode of action of petroleum may be one of pollutant-cellular interaction. This area of research deserves attention.

Appendix A
OIL-IN-WATER DISPERSIONS

The particles in the oil-in-water dispersions were found to be from 1 μ m to 2 mm in diameter, with the majority at 5 μ m. The initial particle number of fresh dispersions was 1×10^8 particles/ml for the 1000 μ l/L and 3×10^4 particles/ml for the 100 μ l/L treatments (Figure 15). This decreased steadily to about 2×10^4 /ml and 8×10^3 /ml at the end of the experiments. Following three days of weathering, the 1000 μ l/L oil dispersion declined in particle number to $3.0-4.5 \times 10^4$ /ml, at which time the tests of weathered oil effects started.

Total hydrocarbon concentrations in the water also decreased from the initial concentration. At the end of the tests, hydrocarbon concentrations were found to be at 60-100 μ l/L for the 1000 μ l/L dose and 5-10 μ l/L for the 100 μ l/L dose. The rate of decline over the course of the experiment was similar for both fresh and weathered oil exposure regimes (Figure 16). At both exposures, the heavier Venezuelan crude oil was found to be more persistent in the water than the lighter Norman Wells crude oil.

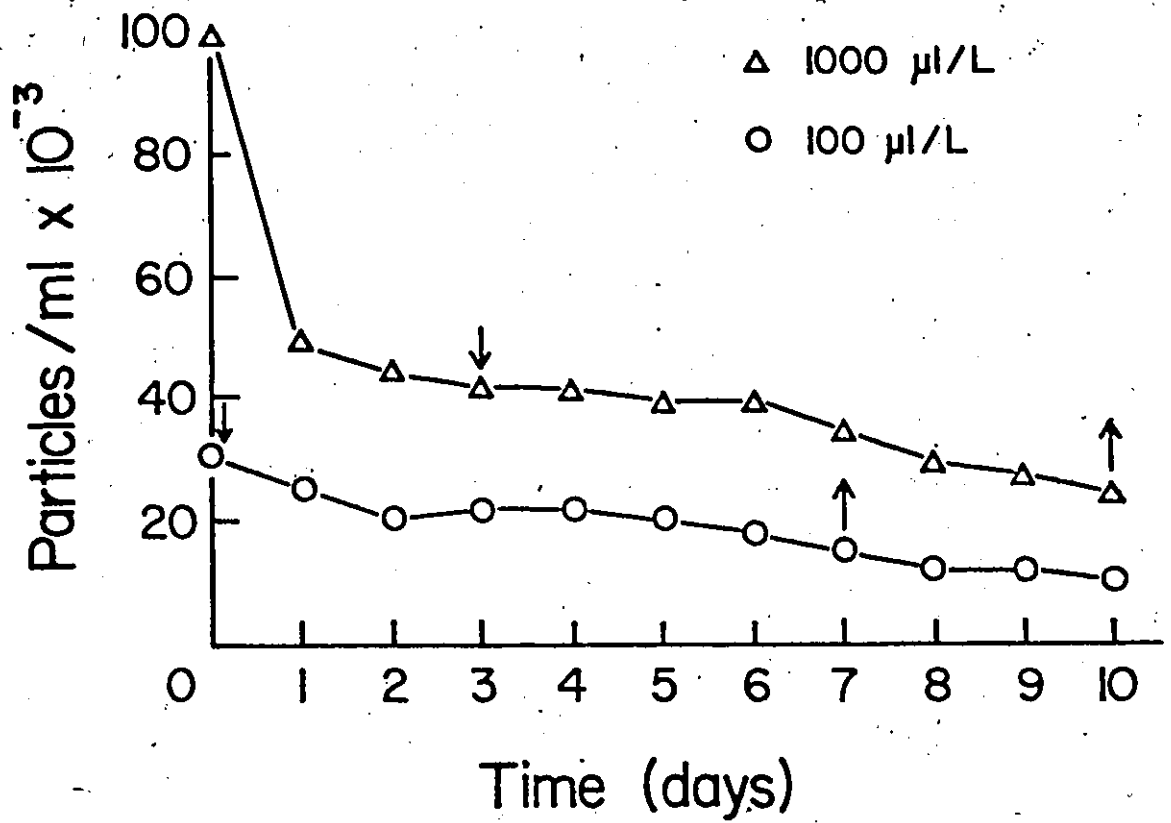
The physical characteristics of the oil dispersions used in the present investigation were similar to those described for spills occurring in the field (Forrester, 1971) or experimental spills under laboratory conditions (Shaw, 1979). It was reported that micron-size oil particles formed by natural water movements could be detected in the water column to depths of 80 m and distances of 250 km from the original spill site (Forrester, 1971). It has also been stated that in areas of strong water movements, such as the St. Lawrence River, it is impossible to either recover or disperse the oil (Drapeau et al., 1974). It is reasonable to assume that oil accommodated in the water column in this form can encounter and affect aquatic organisms.



Number of paraffin oil particles in 1000 ul/L (weathered) and 100 ul/L (fresh) treatments over a 10 day period in aquaria. Similar graphs were found for the two crude oils.

Arrows denote the time of addition (↓) and and removal (↑) of test fish.

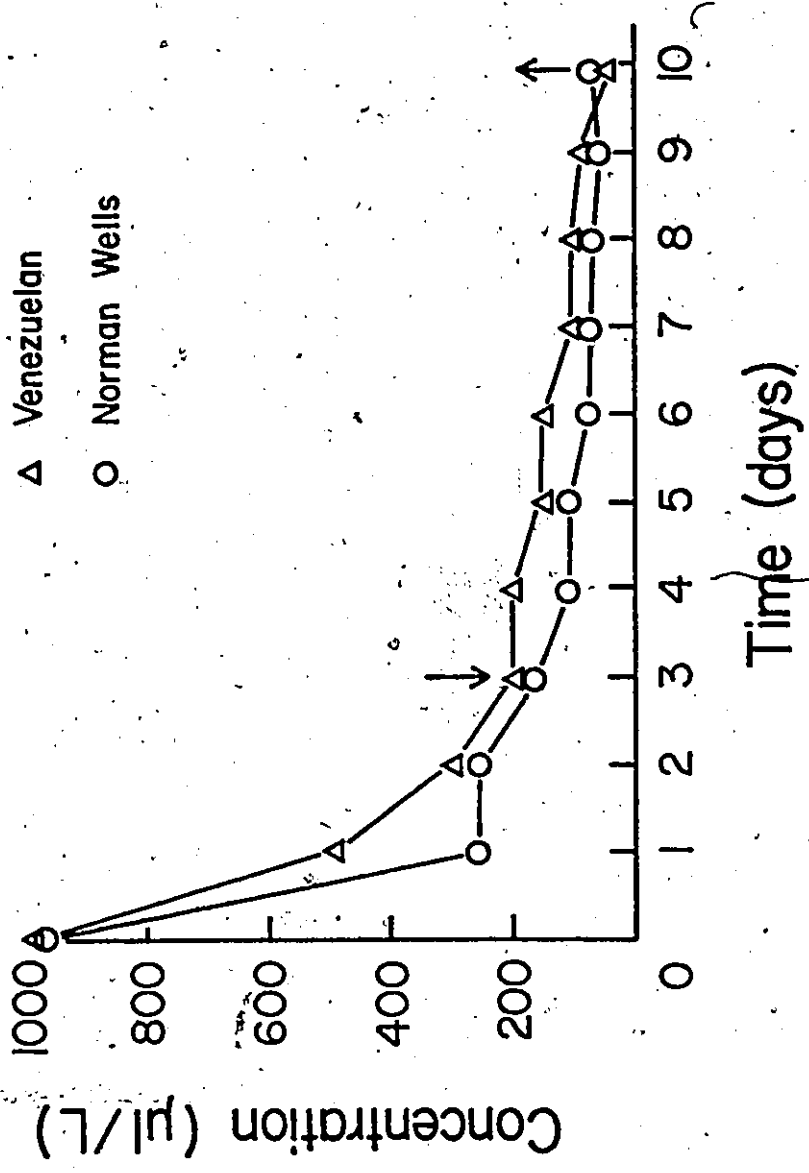
Figure 15: Oil particle number



Total hydrocarbon concentrations in water during the weathered (1000 ul/L) treatments of Venezuelan and Norman Wells crude oils over a 10 day period. Similar weathering patterns were found for the fresh (100 ul/L) dose.

Arrows denote the time of addition (↓) and removal (↑) of test fish.

Figure 16: Hydrocarbon concentrations in water



Appendix B
CHEMICAL ANALYSIS OF CRUDE OILS

The crude oils were fractionated into aliphatic and aromatic fractions on neutral alumina column (5% deactivated) prior to analysis. Aliphatic and aromatic hydrocarbons were eluted with hexane and benzene, respectively. Aliphatic fractions were analyzed by gas chromatography (Hewlett packard 5840 GC). Aromatic hydrocarbons were analyzed on GC/MS. Scan integration was used and dedicated mass plots were performed to identify the structures of individual aromatic compounds.

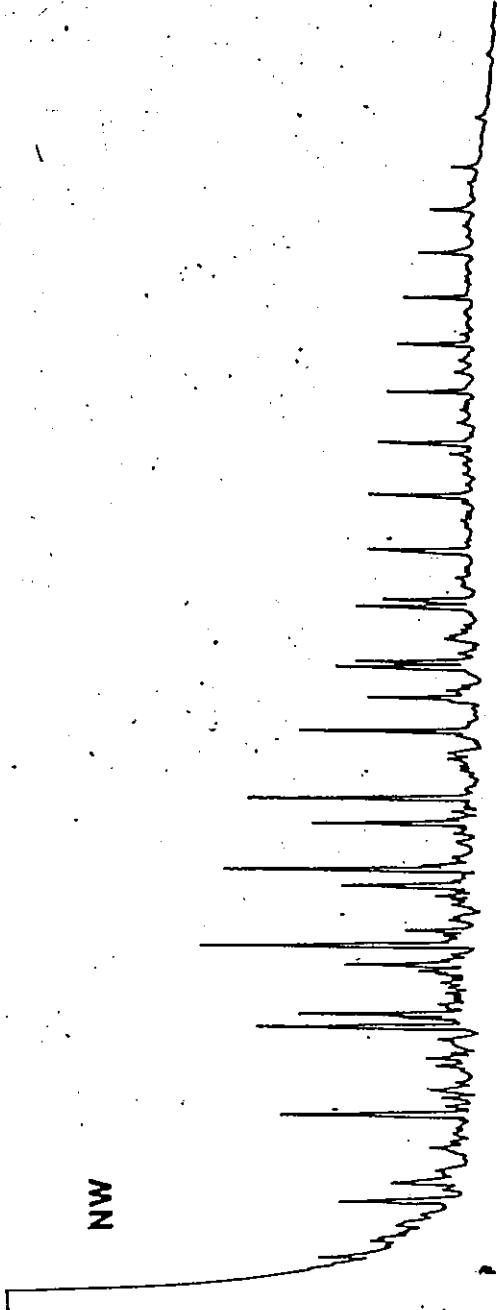
The chromatograms of the two oils showed that the Norman Wells and Venezuelan crude oils differ substantially in their chemical composition (Figures 17, 18). The Norman Wells crude oil contained high concentrations of aliphatic and low molecular weight aromatic hydrocarbons. The Venezuelan crude oil contained very low concentrations of aliphatic and low molecular weight hydrocarbons, but had high concentrations of medium to high molecular weight aromatic hydrocarbons.

Gas chromatograms of Norman Wells (NW) and Venezuelan (VEN) crude oils.

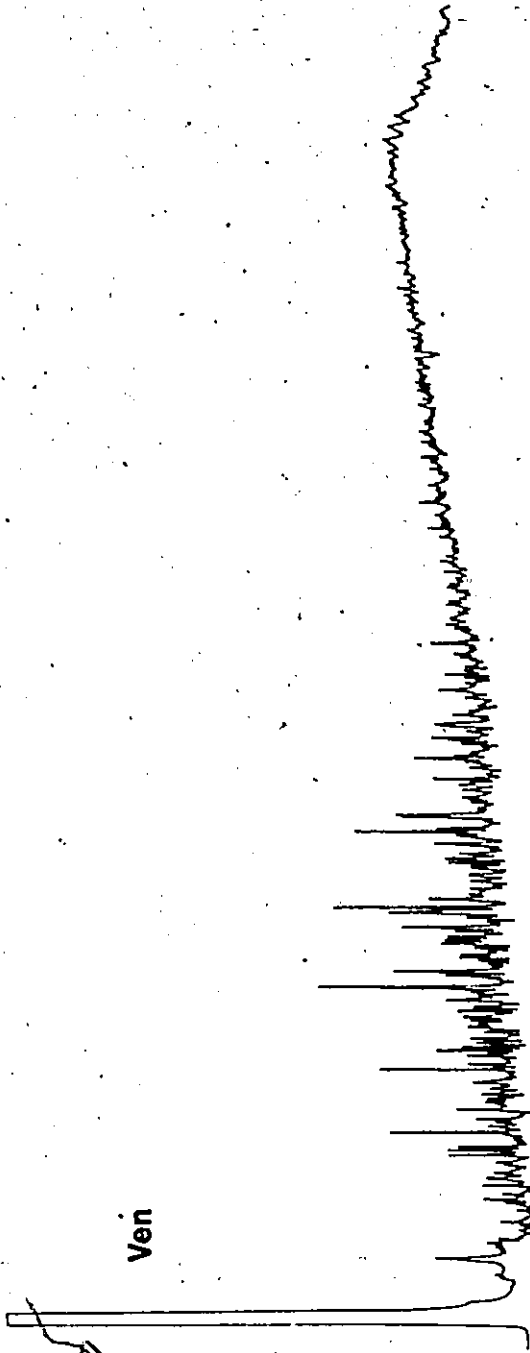
Aliphatic fraction.

Figure 17: Aliphatic fraction of crude oils

NW



Ven



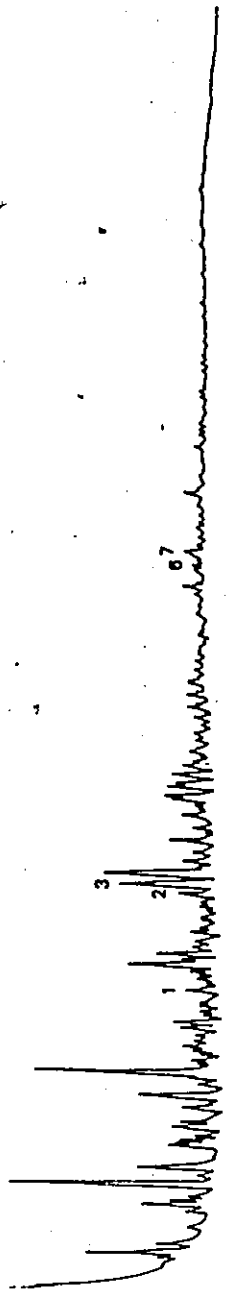
Gas chromatogram of Norman Wells (NW) and Venezuelan (VEN) crude oils.

Aromatic fraction.

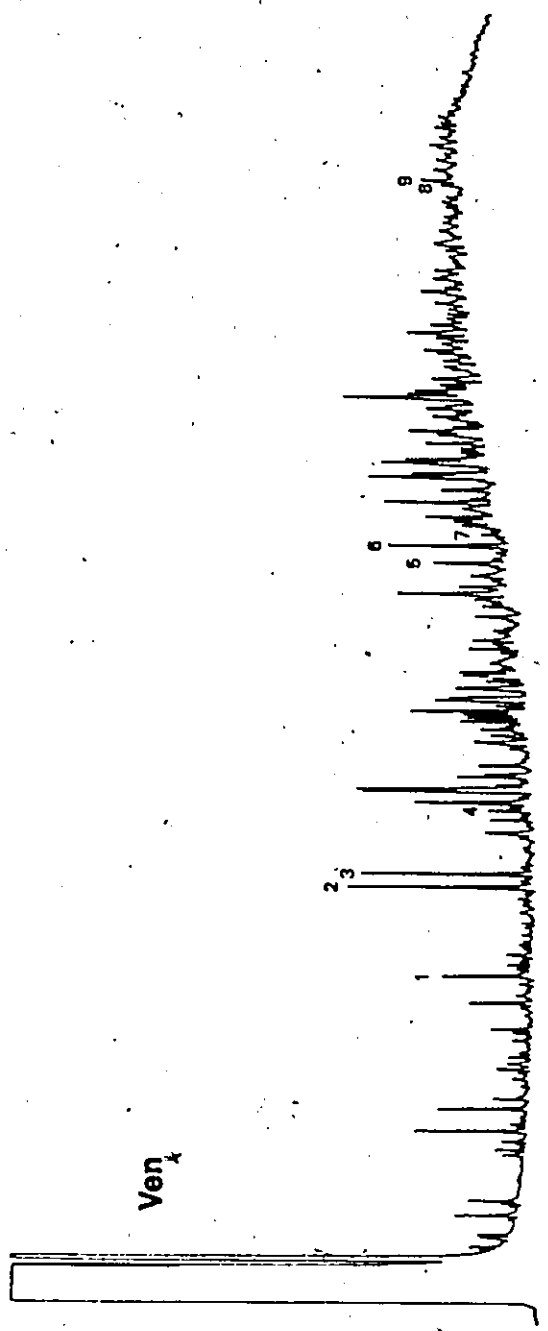
Figure 18: Aromatic fraction of crude oils

4

NW



Ven₁



Appendix C

PLASMA ION CONCENTRATIONS AND OSMOLALITY

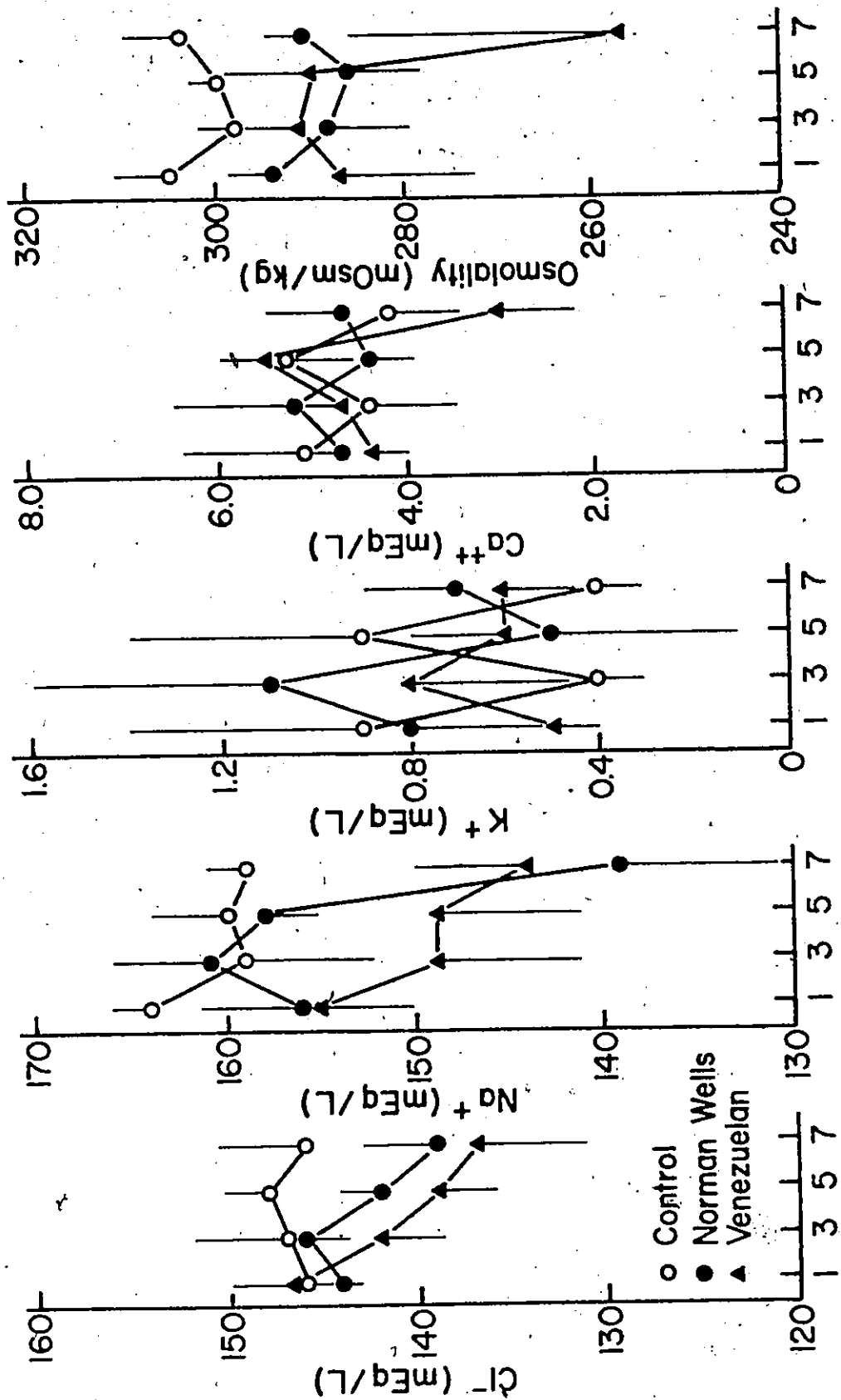
The temporal response of plasma ions in fish sampled over 1, 3, 5 and 7 days are illustrated in Figures 19, 20, and 21. The oil exposure regimes were similar to that described in Chapters II and III. All sampling and analytical procedures were also the same.

The plasma ionic response as affected by cortisol injections was studied. Freshwater acclimated trout were sham injected, exposed to weathered crude oil dispersions, cortisol injected, and cortisol injected/oil exposed for 7 days. The concentration of the IP injected cortisol was 80 ng/100 g fish. The changes in plasma ion concentrations and osmolality are documented in Table 9.

Changes in plasma ion concentrations and osmolality in freshwater acclimated rainbow trout during the course of 7 days of exposure to dispersions of weathered crude oils.

All values means of 8 fish.
Vertical bars represent one standard deviation.

Figure 19: Osmoregulatory responses over 1, 3, 5 and 7 days

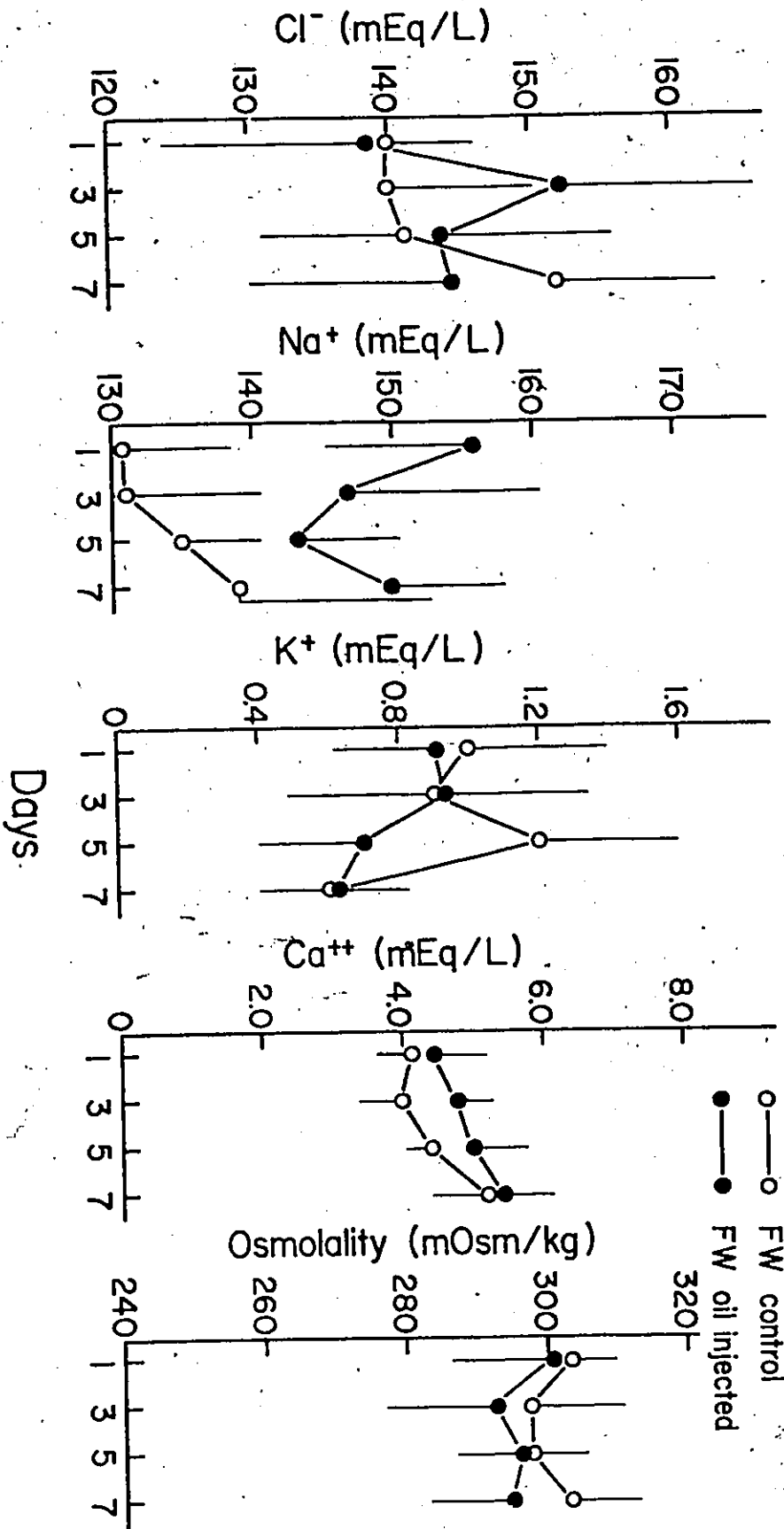



Days

Changes in plasma ion concentrations and osmolality in freshwater acclimated rainbow trout during the course of 7 days of IP injections of weathered crude oil.

All values are means of 8 fish.
Vertical bars represent one standard deviation.

Figure 20: Plasma ion profile over 1, 3, 5, and 7 days (I)





Changes in plasma ion concentrations and osmolality in salt water acclimated rainbow trout during the course of 7 days of IP injections of weathered crude oil.

All values are means of 8 fish.
Vertical bars represent one standard deviation.

Figure 21: Plasma ion profile over 1, 3, 5, and 7 days (II)

5

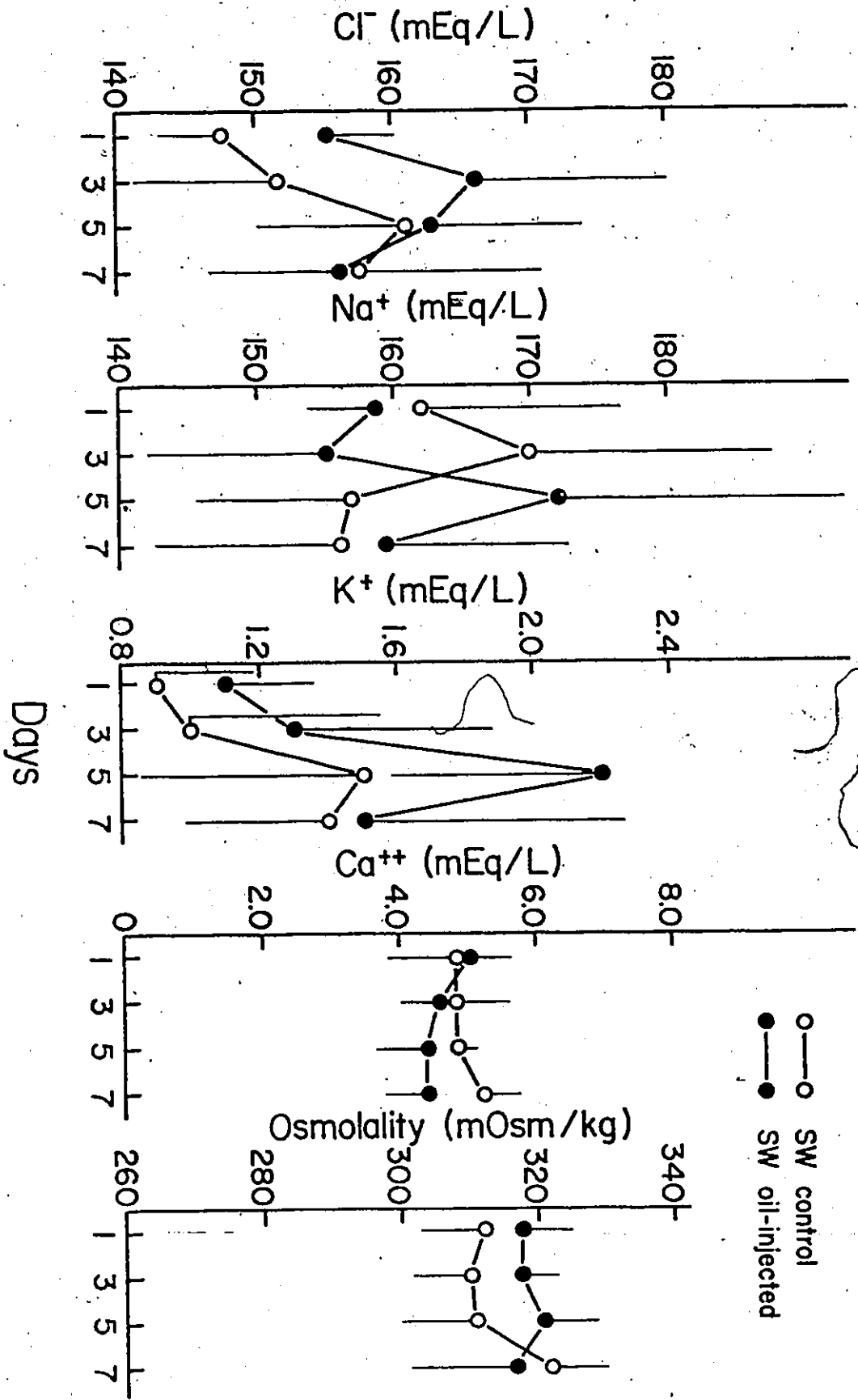


TABLE 9

Cortisol effects on plasma ion concentrations

All values are means of 8 fish and numbers in the brackets represent one standard deviation.

"*" indicates a statistically significant difference ($p \leq 0.05$) from control.

Plasma parameters

Experimental condition	Cl ⁻ (mEq/L)	Na ⁺ (mEq/L)	K ⁺ (mEq/L)	Ca ⁺⁺ (mEq/L)	Osmolality (mOsm/kg H ₂ O)
Control	148 (7)	157 (3)	0.6 (0.3)	3.1 (0.7)	295 (5)
Sham	146 (6)	156 (3)	0.8 (0.5)	4.7 (0.8)	297 (5)
Cortisol (I. P.)	139 (4)	151 (4)	0.5 (0.1)	3.9 (1.0)	295 (11)
Norman Wells	134 (4)*	139 (10)*	0.7 (0.2)	4.7 (0.8)*	291 (4)
Norman Wells + Cortisol (I. P.)	144 (3)	148 (8)*	0.7 (0.4)	3.3 (1.1)	295 (11)
Venezuelan	137 (6)*	144 (6)*	0.6 (0.4)	4.6 (0.8)	257 (30)*
Venezuelan + Cortisol (I. P.)	147 (3)	151 (4)*	0.9 (0.3)	3.5 (0.8)	294 (6)

Appendix D

PLASMA CORTISOL LEVELS

Plasma cortisol levels were measured in rainbow trout following exposure to various oil treatments. Cortisol was assayed by RIA, using Corning Immo-Phase methods and reagents.

Cortisol has been implicated to play a role in hydromineral balance in fish (Forrest et al., 1973). No correlations, however, were found between the plasma ionic response and the plasma cortisol levels in the various exposures. The lack of correlative effects is not clear at the present time. A more detailed analysis of this question is warranted.

TABLE 10

Plasma cortisol levels (1)

All values are mean of N fish and numbers in brackets are standard deviations.

PLASMA CORTISOL (NG/ML)

N

CONTROLS	21.85 (28.39)	8
	15.00 (11.21)	10
PARAFFIN (FRESH)	10.40 (6.08)	8
PARAFFIN (WEATHERED)	12.58 (4.11)	8
NORMAN WELLS (FRESH)	4.40 (2.58)	7
NORMAN WELLS (WEATHERED)	69.80 (49.02)	10
VENEZUELAN (FRESH)	12.58 (4.19)	8
VENEZUELAN (WEATHERED)	69.88 (37.91)	10
NORMAN WELLS (WSF)	23.82 (11.66)	8
VENEZUELAN (WSF)	26.03 (9.47)	8

TABLE 11
Plasma cortisol levels (II)

All values represent the mean of 8 fish and numbers in brackets are standard deviations.

PLASMA CORTISOL (NG/ML)

CONTROLS		NORMAN WELLS (WEATHERED)	VENEZUELAN (WEATHERED)
DAY 1	9.87 (4.95)	21.39 (11.61)	94.23 (63.59)
3	13.97 (6.85)	11.83 (6.20)	49.49 (15.64)
5	9.92 (6.90)	11.28 (4.76)	77.48 (41.27)
7	11.16 (7.07)	12.66 (4.69)	17.47 (14.09)

TABLE 12

Plasma cortisol levels (III)

All values represent the mean of 8 fish and numbers in brackets are standard deviations.

PLASMA CORTISOL (NG/ML)

CONTROL (FRESHWATER) DAY 0	OIL-INJECTED (FRESHWATER) DAY 0
2.70 (2.75)	4.87 (3.89)
1 2.14 (1.53)	1 6.26 (4.56)
3 1.66 (2.06)	3 15.36 (15.59)
7 4.49 (2.80)	7 21.62 (8.99)

CONTROL (SALT WATER) DAY 0	OIL-INJECTED (SALT WATER) DAY 0
3.22 (4.30)	1.54 (2.18)
1 5.63 (5.62)	1 2.32 (4.90)
3 2.01 (1.49)	3 0.84 (0.71)
7 26.08 (39.34)	7 13.09 (8.62)

TABLE 13.

Plasma cortisol levels (IV)

All values represent the mean of 8 fish and numbers in brackets are standard deviations.

PLASMA CORTISOL (NG/ML)


CONTROL	8.06	(4.70)
SHAM-INJECTED	8.97	(9.50)
CORTISOL-INJECTED	12.70	(6.34)
CORTISOL AND NW EXPOSED	11.60	(7.27)
CORTISOL AND VEN EXPOSED	24.57	(17.20)

Appendix E

PHYSIOLOGICAL PARAMETERS IN OIL EXPOSED TROUT

Some of the physiological responses measured in trout under the various experimental conditions were not included in the main text because of their peripheral importance to ion regulation. They are illustrated in the following tables (14 to 19) to demonstrate that other physiological parameters are disturbed by oil exposure.

TABLE 14
Physiological parameters (I)



	HEMATOCRITS (%)	HEMOGLOBIN (mg/dL)	LEUCOCYTES (%)	LIVER SOMATIC INDEX (%)	MUSCLE (% WATER)
CONTROLS	36.25 (4.00)	5.91 (0.68)	0.68 (0.26)	0.77 (0.09)	76.00 (0.58)
PARAFFIN (FRESH)	39.78 (4.40)	5.96 (0.72)	0.45 (0.26)	0.87 (0.15)	76.95 (1.05)
PARAFFIN (WEATHERED)	37.25 (3.30)	6.17 (0.24)	0.50 (0.18)	0.75 (0.07)	76.62 (0.96)
NORMAN WELLS (FRESH)	38.07 (6.83)	5.60 (0.75)	0.33 (0.08)	1.13 (0.06)	75.53 (2.69)
NORMAN WELLS (WEATHERED)	36.71 (3.54)	6.25 (1.11)	0.28 (0.09)	1.08 (0.27)	77.90 (1.90)
VENEZUELAN (FRESH)	38.25 (3.53)	5.73 (0.58)	0.55 (0.17)	1.07 (0.11)	76.63 (0.96)
VENEZUELAN (WEATHERED)	38.00 (3.50)	6.28 (0.74)	0.33 (0.09)	1.18 (0.13)	78.33 (2.49)
NORMAN WELLS (MSF)	33.60 (3.54)	5.93 (1.11)	0.40 (0.13)	0.95 (0.06)	77.43 (1.19)
VENEZUELAN (MSF)	36.10 (5.06)	6.24 (0.60)	0.53 (0.11)	0.96 (0.09)	76.47 (1.15)

TABLE 15
Physiological parameters (II)

	HEMATOCRITS (%)	HEMOGLOBIN (MG/DL)	LEUCOCRIT (%)	PLASMA GLUCOSE (MG/ML)	PLASMA PROTEIN (MG/ML)	LIVER SOMATIC INDEX (%)	MUSCLE (% WATER)
CONTROL							
DAY 1	33.96 (4.66)	5.66 (1.02)	0.51 (0.11)	0.79 (0.18)	271.99 (28.27)	0.85 (0.11)	78.80 (1.61)
DAY 3	41.43 (9.25)	7.32 (0.70)	0.63 (0.27)	0.84 (0.21)	245.79 (20.51)	0.98 (0.15)	78.10 (1.77)
DAY 5	36.63 (7.52)	6.57 (0.95)	0.51 (0.11)	0.67 (0.14)	253.85 (72.46)	0.78 (0.07)	78.45 (1.29)
DAY 7	34.83 (5.34)	7.00 (1.16)	0.63 (0.24)	0.73 (0.15)	269.74 (33.71)	0.77 (0.09)	78.95 (1.64)

	HEMATOCRITS (%)	HEMOGLOBIN (MG/DL)	LEUCOCRIT (%)	PLASMA GLUCOSE (MG/ML)	PLASMA PROTEIN (MG/ML)	LIVER SOMATIC INDEX (%)	MUSCLE (% WATER)
NORMAN WELLS							
DAY 1	42.87 (4.68)	7.48 (0.84)	0.41 (0.16)	0.62 (0.10)	213.43 (20.74)	0.73 (0.06)	77.01 (1.60)
DAY 3	32.66 (5.71)	5.92 (1.12)	0.46 (0.18)	0.65 (0.10)	171.73 (29.52)	0.77 (0.11)	77.59 (1.66)
DAY 5	30.25 (7.89)	5.76 (1.13)	0.43 (0.13)	0.62 (0.09)	166.26 (30.44)	0.87 (0.10)	79.71 (1.09)
DAY 7	36.70 (3.54)	6.25 (1.11)	0.28 (0.09)	0.61 (0.12)	238.12 (25.06)	1.08 (0.27)	77.90 (1.89)

	HEMATOCRITS (%)	HEMOGLOBIN (MG/DL)	LEUCOCRIT (%)	PLASMA GLUCOSE (MG/ML)	PLASMA PROTEIN (MG/ML)	LIVER SOMATIC INDEX (%)	MUSCLE (% WATER)
VENEZUELAN							
DAY 1	44.91 (6.67)	8.76 (2.38)	0.56 (0.08)	0.66 (0.11)	267.25 (58.80)	0.81 (0.10)	78.86 (1.11)
DAY 3	42.96 (4.52)	8.49 (1.16)	0.46 (0.17)	0.63 (0.13)	276.41 (35.59)	0.88 (0.06)	78.60 (1.11)
DAY 5	40.92 (4.18)	7.53 (1.45)	0.24 (0.10)	0.82 (0.22)	266.41 (64.69)	0.98 (0.13)	77.45 (3.07)
DAY 7	38.00 (3.50)	6.30 (1.12)	0.32 (0.09)	0.66 (0.16)	260.00 (41.43)	1.18 (0.13)	78.34 (2.49)

TABLE 16

Physiological parameters (III)

	HEMATOCRITS (%)	HEMOGLOBIN (MG/DL)	LEUCOCRITS (%)	PLASMA GLUCOSE (MG/ML)	PLASMA PROTEIN (UG/ML)	LIVER SOMATIC INDEX (%)	MUSCLE (% WATER)
CONTROL	36.3 (2.10)	6.66 (0.75)	0.53 (0.12)	0.69 (0.08)	268.29 (52.87)	1.02 (0.12)	78.00 (1.13)
SHAM- INJECTED	36.2 (4.88)	7.15 (1.01)	0.47 (0.11)	0.65 (0.13)	283.12 (30.20)	0.90 (0.11)	78.53 (1.99)
CORTISOL- INJECTED	39.1 (6.42)	6.72 (1.35)	0.43 (0.07)	0.93 (0.27)	265.31 (32.30)	1.12 (0.20)	78.65 (1.95)
CORTISOL AND M OIL EXPOSED	33.7 (2.81)	4.45 (1.24)	0.38 (0.10)	0.67 (0.15)	237.5 (55.50)	1.22 (0.16)	78.01 (2.94)
CORTISOL AND VEN OIL EXPOSED	35.1 (5.78)	5.05 (1.75)	0.36 (0.08)	0.52 (0.10)	278.34 (60.85)	1.11 (0.10)	78.30 (1.55)

TABLE 17
Physiological parameters (IV)

	HEMATOCRITS (%)	HEMOGLOBIN (mg/dL)	LEUCOCRITS (%)	LIVER SOMATIC INDEX (%)	MUSCLE (% WATER)
CONTROL	38.87 (4.62)	6.62 (0.57)	0.54 (0.14)	0.77 (0.12)	77.21 (1.95)
PARAFFIN	35.06 (4.09)	---	0.54 (0.20)	0.64 (0.06)	75.45 (1.29)
BENZENE	40.43 (4.03)	6.15 (0.72)	0.34 (0.12)	0.64 (0.09)	76.68 (0.76)
NAPHTHALENE	37.38 (5.00)	6.14 (0.56)	0.39 (0.10)	0.81 (0.11)	77.28 (1.56)
PHENANTHRENE	36.33 (4.85)	5.69 (1.04)	0.36 (0.13)	0.80 (0.16)	79.44 (1.80)
BENZ(A)ANTHRACENE	34.12 (4.44)	6.04 (0.30)	0.42 (0.15)	0.72 (0.09)	79.66 (2.14)
BENZO(A)PYRENE	31.81 (3.64)	5.55 (0.68)	0.25 (0.06)	0.75 (0.08)	79.68 (1.65)

TABLE 18
Physiological parameters (V)

HEMATOCRITS (%) HEMOGLOBIN (MG/DL) LEUCOCRITS (%) LIVER SOMATIC INDEX (%)

CONTROL
N=8

34.9 (5.89) 5.56 (0.81) 0.62 (0.18) 0.60 (0.08)

NORMAN WELLS
OIL (WEATHERED)
N=8

30.9 (5.15) 5.01 (0.63) 0.30 (0.14) 1.43 (0.22)

VENEZUELAN
OIL (WEATHERED)
N=9

28.7 (5.10) 4.76 (0.82) 0.42 (0.20) 1.43 (0.22)

TABLE 19
Physiological parameters (VI)

	HEMATOCRITS (%)	HEMOGLOBIN (mg/dl)	LEUCOCRITS (%)	LIVER SOMATIC INDEX (%)	MUSCLE (% WATER)	
CONTROLS (freshwater)	Day 0	38.81 (2.34)	6.50 (0.63)	0.46 (0.15)	1.16 (0.49)	77.08 (0.91)
	1	36.75 (5.71)	6.08 (0.72)	0.55 (0.14)	1.29 (0.50)	74.41 (2.41)
	3	39.81 (4.46)	6.08 (0.52)	0.46 (0.14)	1.37 (0.44)	73.87 (1.44)
	7	39.93 (2.92)	6.23 (0.60)	0.44 (0.08)	1.02 (0.26)	74.49 (2.23)
OIL-INJECTED (freshwater)	Day 0	37.38 (5.03)	6.43 (1.35)	0.48 (0.09)	1.00 (0.20)	76.29 (0.91)
	1	41.75 (3.36)	6.90 (0.69)	0.42 (0.12)	1.62 (1.05)	76.76 (1.34)
	3	42.81 (3.14)	7.12 (0.92)	0.33 (0.15)	1.44 (0.42)	76.09 (0.96)
	7	38.75 (5.05)	6.26 (0.54)	0.18 (0.09)	1.02 (0.18)	76.12 (0.57)
CONTROLS (salt water)	Day 0	36.19 (4.08)	6.00 (0.59)	0.39 (0.11)	1.14 (0.10)	76.55 (1.74)
	1	34.31 (3.79)	5.88 (0.41)	0.42 (0.11)	1.32 (0.29)	77.37 (1.74)
	3	37.26 (4.99)	6.63 (1.13)	0.39 (0.13)	1.15 (0.19)	76.58 (0.63)
	7	37.82 (4.63)	6.78 (1.08)	0.42 (0.14)	1.17 (0.20)	73.15 (2.38)
OIL-INJECTED (salt water)	Day 0	38.00 (5.20)	6.88 (0.44)	0.57 (0.12)	1.08 (0.12)	76.21 (1.69)
	1	37.50 (5.17)	6.49 (0.79)	0.36 (0.16)	1.17 (0.21)	76.67 (1.83)
	3	36.69 (3.94)	6.01 (0.75)	0.31 (0.06)	1.17 (0.23)	77.77 (1.14)
	7	39.56 (8.22)	6.33 (1.23)	0.21 (0.19)	1.22 (0.18)	74.06 (2.00)

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