SOME EFFECTS OF DEHYDROABIETIC ACID (DHA) ON
HYDROMINERAL BALANCE AND OTHER PHYSIOLOGICAL
PARAMETERS IN JUVENILE SOCKEYE SALMON
<u>ONCORHYNCHUS</u> <u>NERKA</u>

by

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ABSTRACT

Laboratory experiments were conducted to study the effects of dehydro-abietic acid (DHA) on the physiology of the adaptation of sockeye salmon smolts (Oncorhynchus nerka) to sea water.

Dehydroabietic acid occurs in the rosin of commercially important coniferous trees and is found in the untreated effluents of the pulp and paper industry at concentrations acutely toxic to salmonids. As this resin acid is known to be one of the more persistent toxic components of kraft mill effluent (KME) and although its concentrations are greatly reduced by biological treatment, DHA is nevertheless discharged in the effluents of the pulp mills situated on the Fraser River system as well as of those located on the coast of British Columbia. As sockeye salmon utilize both the Fraser and Thompson Rivers during their downstream migration, this species may be exposed to DHA before entering the sea.

An attempt was made to simulate this situation in the laboratory by exposing sockeye salmon smolts to a sublethal concentration of DHA (0.65 mg/L) in fresh water for 120 h and then transferring them into sea water (28 ^{o}/oo) containing no DHA.

Hydromineral balance was studied by monitoring changes in plasma osmolality, plasma Na^+, K^+, Ca^{++}, Mg^{++} and Cl^-, blood hematocrit and muscle water content at the end of the freshwater DHA exposure and at 24 h intervals during the adaptation to sea water (120 h). After 24 h in sea water the gill permeability to water and the water transport ability of the gut were also determined. Supportive experiments measured changes in the size of red blood cells, the levels of plasma bilirubin as well as the uptake and tissue distribution of DHA in sockeye salmon smolts. Lipid extracts of various tissues were analyzed for DHA residues by gas chromatography

coupled with mass spectrometry (GC-MS).

The exposure of sockeye salmon to DHA in fresh water resulted in a hydromineral disturbance characterized by a drop in plasma osmolality, sodium, and chloride, indicating a general hydration which was reflected by increased muscle water content. A lowering of dissolved oxygen to 75% saturation markedly increased the toxicity of DHA and the osmotic imbalance may have been a secondary result of an adaptive respiratory response to a hypoxic stress brought on by DHA exposure. Increases in blood hematocrit were caused by a swelling of the red blood cells related to lowered plasma osmolality.

When these fish were transferred to sea water, the hydration was replaced by dehydration and a rise in osmolality was caused by abnormally elevated levels of all the plasma ions. The added salinity stress resulted in some mortality and considerably greater excursions in plasma electrolytes occurred in fish which were experiencing locomotor difficulty. Plasma magnesium showed the greatest elevation and took the longest (96 h) to return to normal levels. Prior DHA exposure increased the permeability of the gill.

During acute DHA exposure in fresh water a gradual deterioration in schooling and fright response was followed by hypersensitivity to mechanical stimuli and abnormal swimming behavior. After sublethal exposure, the reduction in schooling and fright response generally became most evident during the first 24 h of sea water adaptation. These results of the study are discussed in terms of the possible roles played by the gills, gut and kidney in the DHA-induced perturbations of hydromineral balance. The implications of the accompanying alterations in behavior are discussed

in the context of the ecological survival of sockeye salmon smolts during adaptation to sea water.

Residue analyses showed that sockeye salmon accumulated DHA from the water to high levels in the brain (954 x), liver (428 x) and kidney (404 x) as well as in other tissues. The presence of DHA metabolites in the bile, which also contained the highest DHA residues (647.3 µg/g), indicates that the hepatobiliary route is important in the excretion of DHA by fish.

The possibility of the bioaccumulation of DHA by fish in the wild is discussed in relation to the setting of water quality criteria for pulp mill effluent.

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GENERAL INTRODUCTION

Wastes from the pulp and paper industry form the largest single source
of industrial effluent being discharged into rivers and estuarine waters
of British Columbia. At the same time, all five species[1] of the anadromous
Pacific salmon must spend varying lengths of time in rivers and estuaries
containing this potentially toxic waste. Based on available information it
appears that in the field, salmon do not markedly avoid dilute kraft mill
waste and, in fact, may sometimes be attracted to it (Holland et al., 1960).

In the Fraser River, the fry of chum and pink salmon as well as yearling
chinook, coho and sockeye salmon are exposed to dilute concentrations of
kraft mill effluent (KME) during the period of seaward migration. Sub-
sequently, chum fry and fingerling chinook and coho salmon feed in estuarine
areas for periods of up to several months (Williams et al., 1953; Dorcey
et al., 1978); whereas sockeye salmon smolts may spend relatively little time
in brackish water before moving out towards the open sea (Williams, 1969).

In the adult stage, all five species of salmon must move through estuar-
ies where they spend variable lengths of time on their way to natal streams.
For example, the Adams River sockeye run delays approximately three weeks off
the mouth of the Fraser River prior to the freshwater transition. After
entry into fresh water, some races of adult migrants spend up to three weeks
in the river before reaching their spawning grounds: e.g. Stuart and
Bowron stocks (Figure 1) (Killick, 1955).

[1] Oncorhynchus nerka sockeye

    O.kisutch   coho
    O.keta   chum
    O.gorbuscha   pink
    O.tshawytscha   chinook
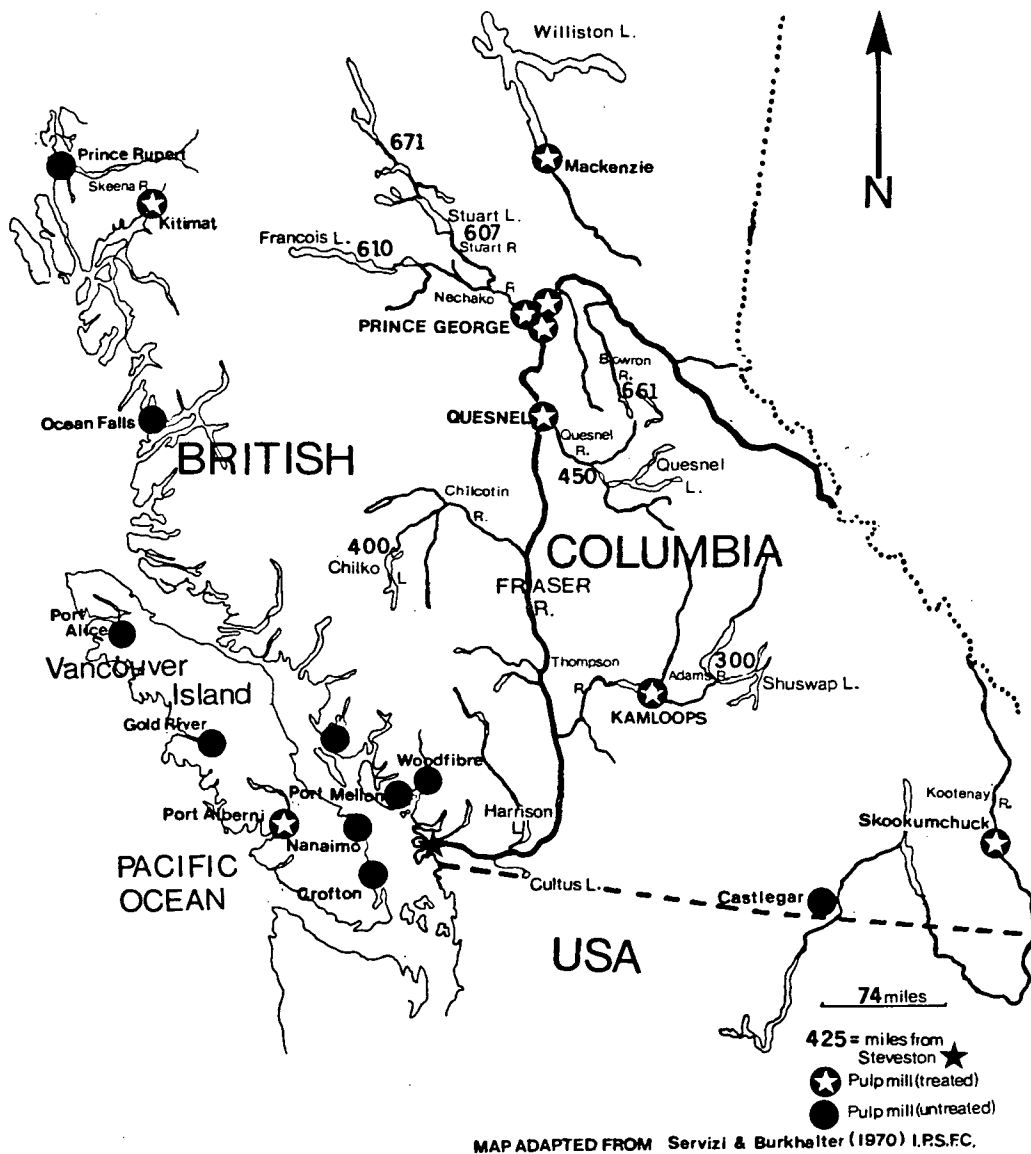
Figure 1. Map showing the location of pulp mills in British
Columbia and the major sockeye salmon migration
routes of the Fraser River system.

During the period of river migration, whether as adults or smolts, these fish must pass through waters receiving a total of 4×10^5 m^3 per day of waste from the three pulp mills at Prince George and one at Quesnel. The maximum concentrations of effluent estimated to be present in the Fraser mainstem in the region of Prince George are illustrated in Figure 2. During its passage through the Thompson River, the Adams River run, which provides 50-75% of the total commercial catch of Fraser sockeye (Gilhousen, 1960), would encounter the estimated effluent concentrations shown in Figure 3.

Even though all the kraft mills on the Fraser River system provide biological (secondary) waste treatment, a study by Gordon and Servizi (1974) showed that treated effluent discharged at Prince George was acutely lethal to salmon 90% of the time. Substandard treatment was also found at the Kamloops mill on the Thompson River (Servizi and Gordon, 1973). Some toxic components of kraft mill waste are now known to survive biological treatment and in spite of continuing improvements in these waste treatment systems, economic factors preclude the complete removal of these toxicants from the effluent. Thus while the chronic dicharge of the more persistent waste components is likely to continue, little is known about their sublethal effects on salmon in the river. On the B.C. coast however, only 2 of the 11 mills practice secondary treatment of effluent, consequently much higher levels of toxicants can be expected to reach estuarine or marine regions utilized by salmon.

Because of the extreme complexity and variability in the composition of whole kraft mill effluent, the present study was restricted to the consideration of a single component. Although this necessarily represents a gross simplification of the possible biological effects of whole KME in the field, the component chosen (dehydroabietic acid-DHA) is known to be one of the major toxic constituents found in a wide variety of wastes originating from
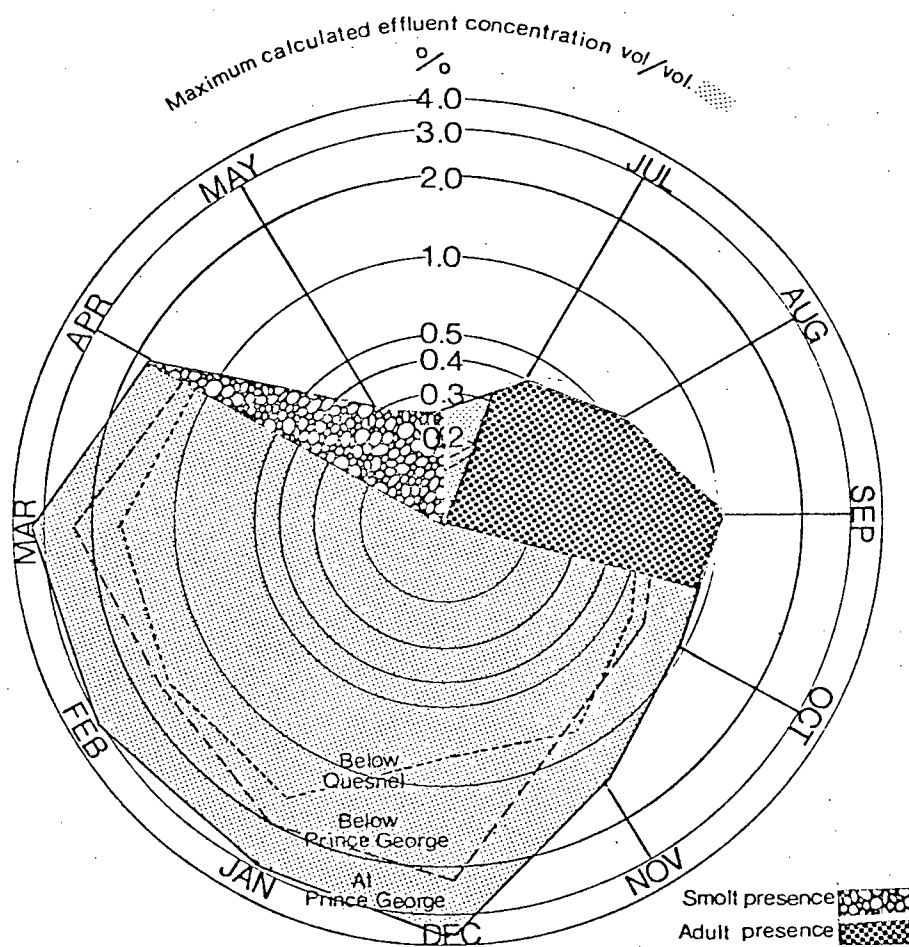
Figure 2. The seasonal presence of sockeye salmon in the Fraser
River in the region of the kraft pulp mills at Prince
George and Quesnel. The maximum theoretical
concentrations of effluent to which these migrating
salmon would be exposed , were calculated on the
basis of mill discharge relative to minimum mean
monthly river volume flow (1950-1970), assuming
rapid and complete mixing.
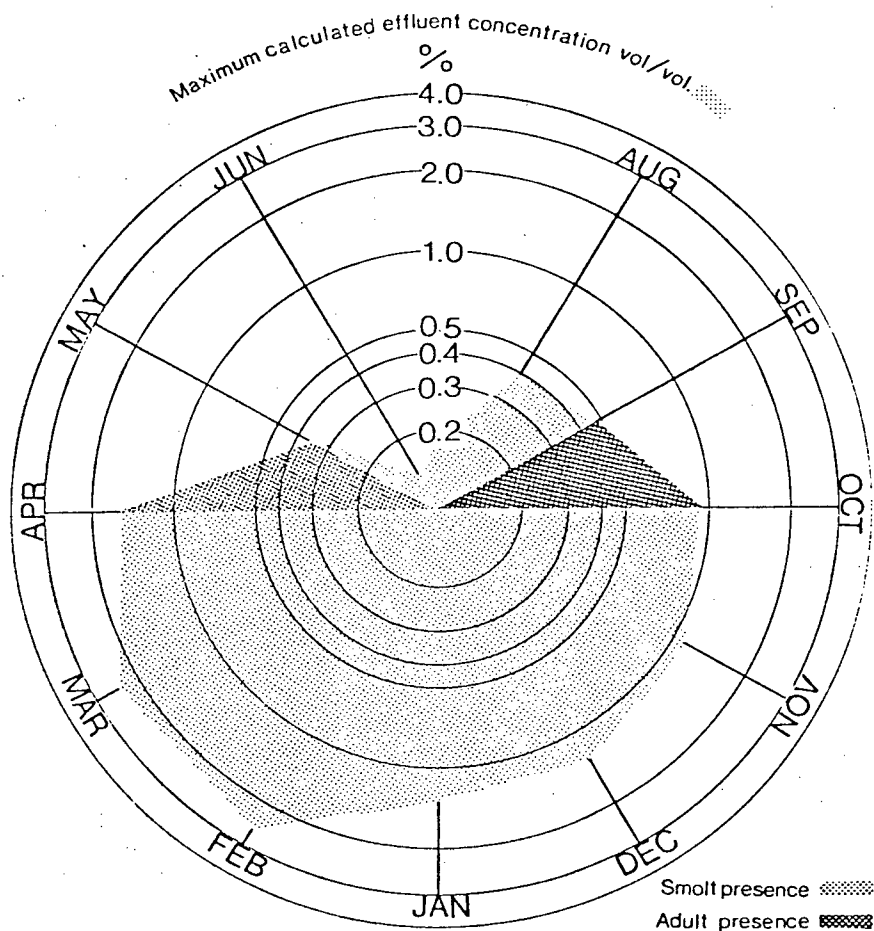
Figure 3. The seasonal presence of sockeye salmon in the Thompson River downstream from the kraft pulp mill at Kamloops. The maximum theoretical concentrations of effluent to which these migrating salmon would be exposed were calculated on the basis of mill discharge as diluted by the minimum mean monthly river volume flow (1911-1958), assuming rapid and complete mixing.

the forest products industry and is currently being discharged in significant amounts into waters inhabited by migrating salmon. Brownlee et al. (1977) determined the half-life of DHA to be close to the 8 week value defining a "persistent" organic compound (International Joint Commission, 1975) and stressed the need for chronic effects studies with a view of establishing safe limits.

For the present study, a perspective of the problem researched can be gained by considering the conflict of interest that exists in the use of the Fraser River. In essence, this river system remains one of the world's largest producers of sockeye salmon but at the same time it must assimilate a continuous input of industrial wastes from five pulp mills. It is known that appreciable quantities of DHA can survive the waste treatments practiced by these mills, furthermore there continues to be a lack of information on the possible effects of sublethal DHA exposure on salmon. Since sockeye salmon smolts must pass through these waters on their downstream migration, their ability to cope with the normal stress experienced during movement into saline waters may be altered by exposure to pulp mill effluent. Although we are ignorant of the actual behavior of salmon during this fresh water (FW) to sea water (SW) transition in the wild, laboratory experiments have shown that it is accompanied by changes in water and electrolyte balance. Therefore the present study investigated the effects of sublethal DHA exposure on the hydromineral balance of sockeye smolts in fresh water and during adaptation to sea water.

The experimental design was based on the premise that if DHA interferes with the mechanisms of osmotic and ionic homeostasis, the net result will be reflected in altered levels of the main plasma electrolytes. Thus hydromineral balance was studied by measurement of plasma osmotic pressure and

plasma sodium, potassium, calcium, magnesium and chloride concentrations in the blood of salmon during DHA exposure in fresh water and after transfer into sea water; muscle water content was measured as an indicator of tissue water balance. In addition, the gut water transport ability and the gill water permeability were determined as these organs are intimately involved in the physiology of salmonid adaptation to sea water. Finally, observations were made on DHA-induced changes in salmon behavior which may be of importance to survival during the transition from a freshwater to a marine existence.

To keep the experimental design relevant to the natural situation, attempts were made to simulate within the constraints of a laboratory study, several of the conditions that occur in the wild. The fish were exposed to DHA while actively swimming for a period of time that the Adams River smolt migration is estimated to take to reach the sea (5 days), and subsequent transfer into sea water was done at a rate thought to be representative of the transition from river to the sea during normal migration.

Nature of the Toxicant

The kraft process, in essence, involves the alkaline digestion of wood chips which breaks down the lignin and releases the fibers. This is followed by bleaching, usually by chlorination, to obtain the desired brightness of the final pulp products. During this process a number of natural products synthesized by the living tree are solubilized, extracted and washed out of the pulp. Common among these are the resin acids and their derivatives (alcohols, aldehydes and ketones-termed "unsaponifiables") which together with fatty acids occur in the wood rosin of commercially important conifers. Depending on their abundance, these products may be recovered as tall oil soap. In the USA, resin acid from pine wood tall oil forms the basis of an industry valued at $138 million; in Canada, where the lower

rosin content of pulpwood and high unsaponifiable yield preclude economical

recovery, resin acids are usually discharged as wastes (Swan, 1973). Wash

water from the bleaching process yields chlorinated lignin derivatives such

as tetrachloroguaiacol and tetrachlorocatechol which are highly toxic to

fish and whose structural similarity to pentachlorophenol (Fig. 4), a well-

known uncoupler of oxidative phosphorylation, may suggest a similar mode of

toxic action. Servizi et al. (1968) observed increased oxygen consumption in

sockeye salmon exposed to 0.1 mg/L of tetrachlorocatechol. Davis (1973) and

Webb and Brett (1972) observed an increase in oxygen requirements of salmon

exposed to sublethal doses of kraft mill wastes. Food conversion efficiency

was lowered and maintenance costs elevated by KME in juvenile salmon in

studies by Ellis (1967) and Webb and Brett (1972), while Servizi et al. (1966)

observed a reduction in the efficiency of yolk utilization in alevins of

sockeye and pink salmon exposed to 1% neutralized bleach waste. However, as

the composition of the mixed waste from a kraft pulp mill is extremely

variable and depends to a large extent on the wood species being pulped, the

precise chemical state and interaction of these compounds in receiving

waters remain largely unknown.

Although reliable gas liquid chromatographic (GLC) methods for identifi-

cation of resin acids were developed 20 years ago (Hudy, 1959), they were

not applied to environmental studies until 1968 by Maenpaa et al. in Finland,

and still later in North America (NCASI 1972, 1975; Rogers, 1973; Leach and

Thakore, 1973). These techniques more recently combined with mass

spectrometry (GC-MS), have confirmed the presence of DHA in effluents from

a variety of forest products processes: sulfite and kraft mills (Maenpaa

et al., 1968; Rogers, 1973; Leach and Thakore, 1973; Rogers et al., 1975),

mechanical pulping (Row and Cook, 1971; Leach and Thakore, 1976), hardboard
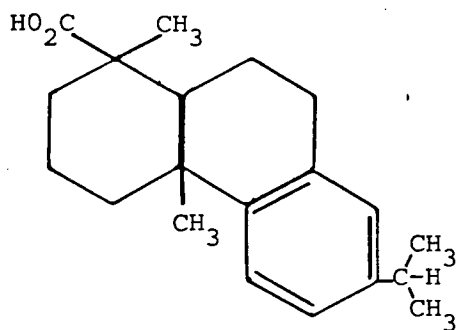
DEHYDROABIETIC ACID
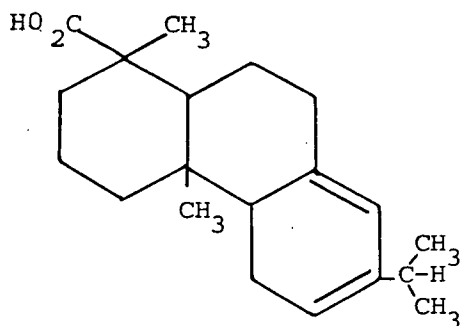
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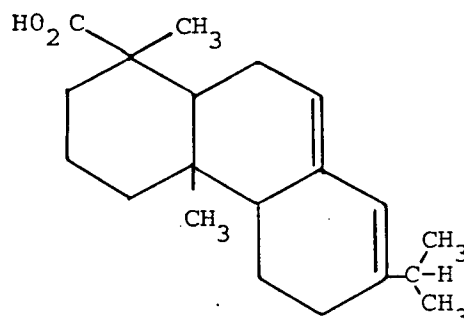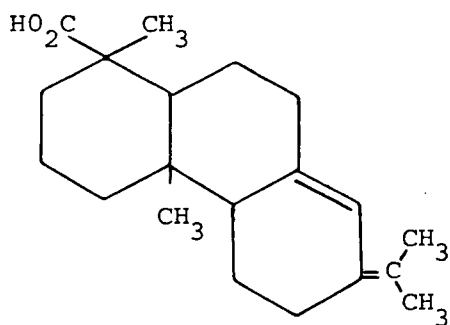
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NEOABIETIC ACID
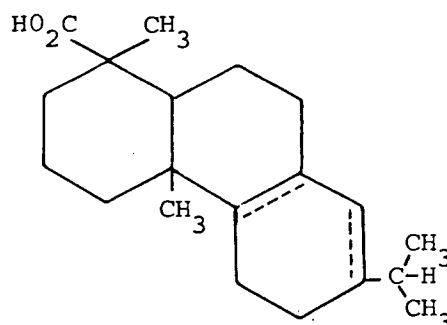
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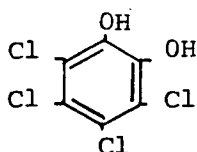
TETRACHLOROGUAIACOL

PENTACHLOROPHENOL
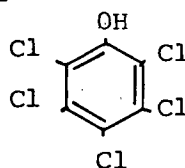
TETRACHLOROCATECHOL

Figure 4. Structures of some isolated components of kraft mill waste toxic to salmon. Pentachlorophenol is not found in KME and its structure is shown for purposes of comparison only.

plants (Row and Cook, 1971; Rogers et al., 1977), and in water from log storage areas (Fox, 1977). Only very recently have techniques been available to precisely quantify traces of resin acids in environmental samples.

A kraft mill of average size and practicing secondary treatment can emit 50 kg of mixed resin acids daily because of the high (87 x 10^3 m^3/day) effluent discharge rates (Hrutfiord et al., 1975), and still meet the regulatory standards for toxic emissions. In spite of such high discharge rates, relatively little is known about the subsequent biological fate of resin acids in the environment. However, Maenpaa et al. (1968) found 0.17 mg/L resin acids in lake waters at a distance of 4 miles from a kraft pulp mill in Finland and the recovery of resin acids from the Fraser River 70 miles downstream from the three pulp mills at Prince George (I.H. Rogers, personal communication) provides additional significance to the present investigation.

The chemical reactions of resin acids have formed a significant part of the larger study of terpenes in classical natural products chemistry. Some of the more important resin acids such as palustric, levopimaric, and neoabietic (Fig. 4) are chemically unstable and either oxidize or isomerize spontaneously to more stable forms such as abietic and dehydroabietic acid (DHA) during wood chip storage (Zinkel, 1975), or with heat and chemical treatment during the various pulping processes (Lawrence, 1959). The stability of DHA can be attributed to the presence of an aromatic ring which also can be predicted to make this compound more water soluble than the other resin acids.

Recent studies indicate that DHA may be the most persistent of the resin acids after discharge into the environment, both from natural and man-made sources. Simoneit (1977) reported DHA to be the most common resin acid found in lipid extracts of ocean sediment samples and suggested that DHA may

be an excellent natural biological marker of resinous higher plants for

geochemical studies. Canadian studies in a Lake Superior ecosystem have

established DHA as being the most important persistent organic contaminant

in the sediment and water of a 25 km^2 zone influenced by the discharge of a

mixed-groundwood, kraft pulping plant (Brownlee and Strachan, 1977; Fox,

1977). Brownlee et al. (1977) estimate that 340 kg of DHA may be discharged

daily into Nipigon Bay on Lake Superior. Disappearance due to bacterial

degradation appears slow and dilution was suggested as the most significant

short-term removal mechanism (Fox, 1977).

As resin acids generally possess a low aqueous solubility but are

freely soluble in fat solvents, one would expect these compounds to pass

readily across the fish gill epithelium into the blood and thus become

distributed throughout the body. Preliminary studies indicate that this in

fact does happen both in the laboratory and in the field. In the Lake

Superior study, rainbow trout exposed to dilutions of whole kraft mill

effluents in the laboratory accumulated DHA to a level 20 times that in the

water (Fox et al., 1977) and DHA was isolated from native fish captured at a

distance of 3 km from the mill discharge point (Brownlee and Strachan, 1977).

The distribution and the biological significance of DHA residues within

the fish are unknown. The biological magnification of lipid soluble

organochlorine insecticides is now well known and their action is often

delayed until lipid reserves are utilized. Such an action could be of

significance to migrating adult Pacific salmon which cease feeding upon

entry into fresh water and rely on lipid reserves as a major energy source.

Conceivably chlorinated lignin derivatives or resin acids could follow

similar pathways in fish. Storage of these toxicants in cellular lipids may

occur with resulting disruption of cellular function. Based on widespread

histological damage in fish exposed to kraft mill waste in field experiments (Fujiya, 1961; 1965) Warner and Tomiyama (in Fujiya, 1965) suggested such a possible mode of action for resin acids.

An early clue to the possible fate of absorbed components of kraft mill waste was provided by Hagman (1936) who analyzed various organs of moribund fish collected from a river below a kraft mill. Resin acids were found in liver, kidney, pancreas, and brain tissue, with highest levels in the "liquid which surrounds the brain in the skull cavity". Although the actual concentrations found are not given, the observation is significant. This author also described a variety of biochemical changes which he attributed to resin acid buildup.

The acute toxicity of mixed resin acids to aquatic organisms has been known for many years. Hagman (1936) and Ebeling (1931) reported toxicity to fish as being in the 1-2 mg/L range. More recent work has shown that mixed resin acids are responsible for much of the residual toxicity in effluents from the bleached kraft (Rogers, 1973), sulfite (Maenpaa et al., 1968) and mechanical pulping processes (Leach and Thakore, 1976). Continuous-flow bioassays with purified individual resin acids have established 96 h LC50's[2/] for juvenile sockeye salmon to be less than 1 mg/L (Rogers et al., 1975; G.M. Kruzynski, unpublished data). Based on acute toxicity alone, these compounds can be classified as "highly toxic" contaminants (Warner 1967; GESAMP, 1973).

Salinity Stress

All anadromous salmonids have the capacity of maintaining a relatively constant blood osmotic pressure in both fresh and saline waters, depending on

[2/] 96 h LC50. Concentration lethal to 50% of the fish in 96 h.

the stage in their life cycles. During migration and often within a short time, a complete reversal in ionic and osmotic regulatory function must take place both in smolts moving into sea water and in adults entering fresh water.

In fresh water, osmotic and ionic gradients are such that fish are faced with continual endosmosis of water and loss of salts, as blood is maintained hyperosmotic to the surrounding water. As a result of these unavoidable passive movements of electrolytes, a salmon in fresh water must actively absorb salts, primarily Na^+ and Cl^- from the water. Water is continuously excreted in large volumes via the kidneys as dilute urine along with a slight loss of salts. The renal and branchial loss of Na^+ and Cl^- is compensated by active ion uptake by the gills as well as in the diet.

In sea water, the ionic and osmotic gradients are reversed from the freshwater situation and water is lost across the gills while Na^+ and Cl^- diffuse into the blood down a concentration gradient. To compensate for the water lost, the fish swallows sea water which is absorbed by the gut along with the salt which must be excreted by a process involving the transport of Na^+ and Cl^- by the gills against a concentration gradient (active transport) (Maetz, 1971). The primary roles of the kidney are the conservation of water and the excretion of the divalent ions (Mg^{++}, $SO_4^=$, Ca^{++}) which are absorbed by the gut.

The anadromous life cycle of salmon has necessitated the development of mechanisms to counter the passive movements of ions and water in both media. In summary (Wood and Randall, 1973a) these comprise:

a) Active transport mechanisms in the gills which can pump ions against their net diffusional fluxes.

b) Efficient kidneys to eliminate osmotic gains in fresh water and to limit water loss in sea water.

c) Intestinal mechanisms to replace water lost to the hypertonic (SW) environment.

Thus a successful transition from one medium to the other requires a series of fundamental modifications in the physiological function of these organ systems. Prior to the completion of these adjustments, blood and tissue water and electrolyte levels depart significantly from the steady state; these are then returned to close to pre-transition values and are subsequently maintained by a variety of regulative functions. The period of departure from steady state values together with the time span required to bring electrolyte and water levels to a new steady state has been termed the "adjustive phase" while the maintenance of the new steady state is termed the "regulative phase" (Houston, 1959a). The adjustive phase has been shown to last approximately 36 hours (h) in coho salmon (Smith et al., 1971; Conte et al., 1966) and chum fry (Black, 1951; Houston, 1959b) and 60-100 h in rainbow trout Salmo gairdneri (Conte et al., 1966). Atlantic salmon (Salmo salar) smolts required 100 h to adjust plasma Na^+ back to normal levels (Koch and Evans, 1959) although plasma osmotic pressure was adjusted in only 4 h (Parry, 1960).

During these adjustive periods, plasma Na^+, K^+, Cl^- and Mg^{++} concentrations increase rapidly due to salt influx and loss of plasma water across the gills. Continued dehydration stimulates the ingestion of sea water, which leads to a further rise in plasma ionic levels imposing an added load on the newly activated branchial salt excretory mechanisms. Finally, the kidney which has had to sharply reduce its activity as an organ of water excretion and salt retention begins to excrete the divalent ions absorbed by the gut (Smith et al., 1971).

These profound changes in hydromineral control as well as the departure of both blood and tissue ion levels from the steady state during the adaptive phase are not without consequence. Houston (1959b) found that the activity

and cruising speed of chum salmon fry dropped sharply upon transfer into sea water. This reduction lasted approximately 36 hours and was found to correspond to increases in body chloride and decreases in body water during the adjustive phase. When these levels returned to the characteristic sea water values, swimming performance returned to normal. The author interpreted these results as an inhibition of neuromuscular function caused by electrolyte imbalance during the adaptive phase.

Gill permeability has been described as a compromise between respiratory and osmoregulatory needs (Steen and Kruysse, 1964; Randall et al., 1967). As the maintenance of a large respiratory surface area for gas exchange also provides a large surface for passive osmotic and ionic movements, any factor which serves to increase the perfusion of blood through the respiratory lamellae can be expected to increase these passive movements, disturbing hydromineral balance. Wood and Randall (1973 a,b,c.) observed an increased Na^+ efflux and water influx in rainbow trout during exercise in fresh water, to compensate for the osmotic water gain, urine flows then increased. As trout respond to hypoxia by increasing ventilation volume and probably the proportion of blood passing through the respiratory lamellae (Randall et al., 1967; Randall, 1970), concurrent hydromineral alterations could therefore be expected as a result of the response to hypoxic conditions. In a study of the effects of handling and anesthesia on brook trout in fresh water, decreases in plasma and tissue ion levels were the results of in-completely compensated endosmosis and increased branchial and renal electrolyte efflux derived from a primary response to vascular hypoxia (Houston et al., 1971).

Kraft Mill Waste and Hydromineral Balance

Respiratory distress has been shown to be one of the most common manifestations of exposure to kraft pulp mill wastes in salmonids (Alderdice and Brett, 1957; Schaumburg et al., 1967; Walden et al., 1970). Davis (1973) has shown that salmon will sharply increase ventilation volume in response to sub-lethal exposure to KME. As reduced arterial oxygen was observed, the fish would probably attempt to increase lamellar flow to make up for the oxygen demand as blood is usually maintained at 85-95% saturation (Randall, 1970). Such responses associated with enhancement of gas exchange will lead to increase of branchial fluxes and result in alterations of water and electrolyte balance.

Responses such as increased ventilation volume can have serious toxicological as well as physiological implications. Lloyd (1961) has suggested that the majority of the increase in toxicity of poisons in water of low dissolved oxygen is caused by the increase in the rate of respiratory flow, with a consequent increase in the rate at which toxic substances reach the gill epithelium. This applies to any environmental or physiological factor which increases ventilation volume; a direct response of the fish to oxygen deficiency.

Changes in gill permeability and kidney function can also occur as a result of the direct effect of toxicant action, especially if the toxicant combines with membranes or accumulates to high concentrations. Such changes can lead to an increase in the rate of passive salt and water movements across the branchial epithelium, or the fish may become diuretic and lose electrolytes in the urine. Subsequent departures of plasma and tissue electrolyte levels from normal values would be governed by the direction of osmotic and ionic gradients between body fluids and the surrounding medium

once the homeostatic mechanisms were overwhelmed.

Smith et al. (1971) suggested that any major decrease in the rate of kidney function could cause accumulation of divalent ions Ca^{++}, Mg^{++} in marine salmon, or water in freshwater-adapted stages. The accumulation of divalent ions can interfere with swimming performance (Houston 1959b); a function of critical importance to migrating salmon. Preliminary observations of Smith et al. (1971) showed a drop in urine production in salmon upon exposure to lowered dissolved oxygen conditions. In view of the observed interference of some kraft mill waste components with respiration in salmonids, the maintenance of water balance may be affected in a similar way.

To summarize, sockeye salmon can encounter pulp mill wastes during three critical phases of their life cycle: smolt migration and entry into sea water, entry of sea water-adapted adults into fresh water, and subsequent upstream migration to the spawning grounds. The transition between hypertonic ⇄ hypotonic media requires adjustment and regulation by a variety of osmo-regulatory systems. The interference with the optimum function of these systems could lead to hydromineral imbalance and thereby reduce the efficiency of such ecologically critical activities as swimming, feeding and predator evasion. Thompson (1945) indicated that adult migrant sockeye, delaying more than 12 days would fail to reach the spawning grounds. Thus for a migrating salmon, the net result of a sufficient reduction in scope for activity (Fry, 1971) by an indiscriminate stress such as pulp mill waste could be equivalent to outright mortality caused by acute toxicity in the river (Brett, 1958). The experiments which follow were designed to investigate some effects of DHA, a major toxic component of pulp mill waste, on the hydromineral balance and related physiology of sockeye salmon smolts.

GENERAL MATERIALS AND METHODS

Water Supply

Experiments were conducted in the laboratories of the Pacific Environment Institute (PEI), West Vancouver, B.C., from 1973-1978. Fresh water supplied to the laboratory from a well was processed as shown in Fig. 5. Water passed through a cartridge filter (Cuno-Micro Kleen II, 125 µm) and then through a bank of aspirators into a series of constant level foam-insulated header tanks equipped with refrigeration units (Frigid Units, Toledo, Ohio) where it was vigorously aerated and chilled. Gas supersaturation was eliminated by means of a stripping column in which a counter-current of oil-free air passed through a bed of glass marbles. Air-stones were used to bring the water supply to saturation. The chemical composition of the well water during the study period is shown in Table I. The laboratory sea water supply is drawn from a depth of 18 m in Burrard Inlet and was also filtered, chilled and aerated before use. Sea water varied in salinity from 26-29 ^{o}/oo ($\sim$795-890 mOsM/kg) had a pH range of 7.6-7.9 and a temperature range of 11.5 $\pm$0.5^{o}C.

The system illustrated in Fig. 5 supplied filtered, air equilibrated water to the experimental tanks at constant temperature. The bottom header tanks provided each annular tank (donut) with SW and FW through separate lines and flowmeters (Manostat Predictability Flowmeter, 36-541-31) so that a change from one water supply to the other could be done separately at each tank by closing one flowmeter and opening the other. Thus "transfer" experiments were conducted without disturbance of the fish and at the water flow rates used, (500 mL/min) 95% replacement of FW by SW was accomplished within $\sim$ 5.8 h (Sprague, 1969).

1 VALVE
2 CARTRIDGE FILTER
3 ASPIRATORS
4 HEADER TANK
5 1 HP REFRIGERATION UNIT
6 OVERFLOW
7 GAS STRIPPING COLUMN
8 AIR SUPPLY TO SINTERED GLASS FUNNEL
9 AIR SUPPLY TO AIR-STONE
10 ⅓ HP REFRIGERATION UNIT
11 WATER SUPPLY TO FLOWMETERS
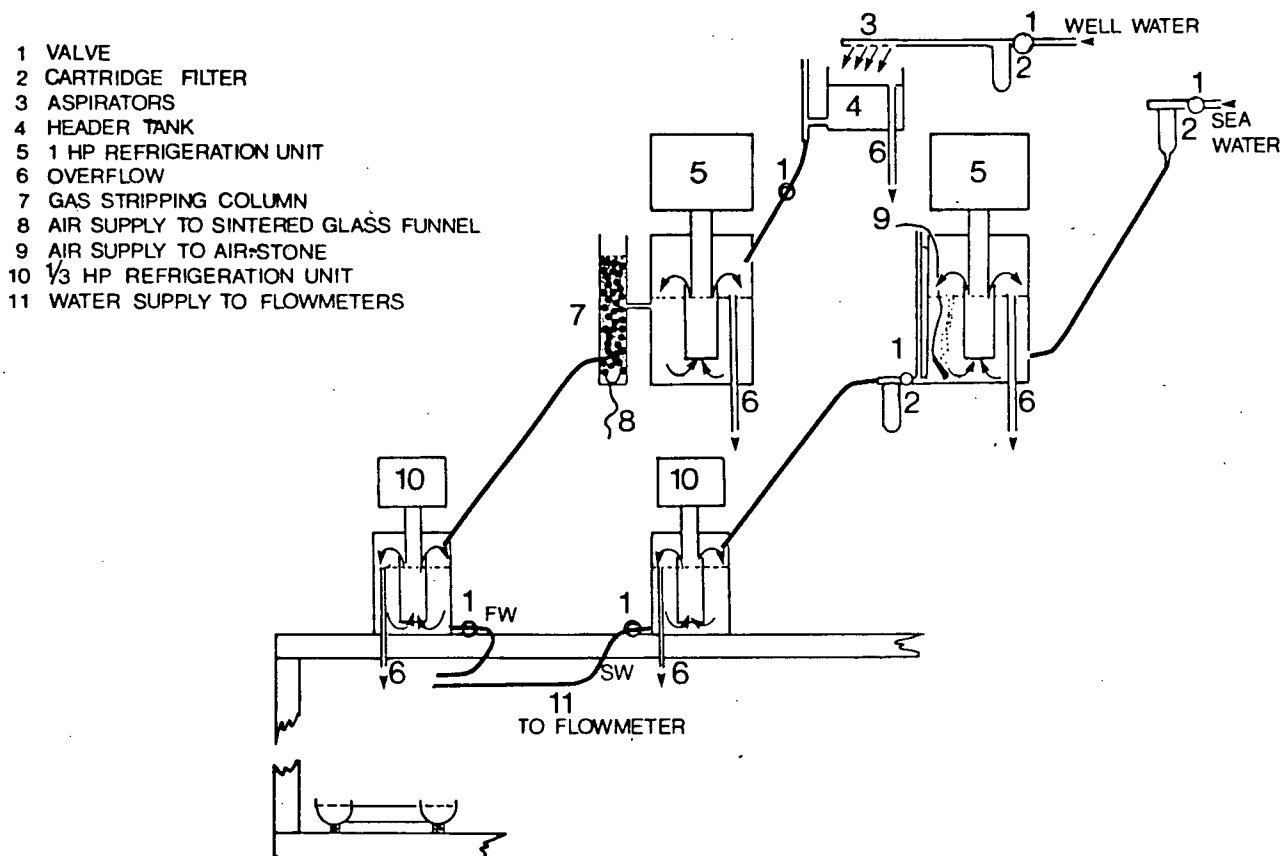
Figure 5. Water supply system as used for continuing flow bioassays with DHA.

Table I. Chemical and physical characteristics of well water
used in continuous flow bioassays with DHA.

Mean (Range)

	mEq/L		mg/L	
Na^+	0.79	(0.52-1.26)	18.2	(12.0-29.0)
K^+	0.04	(0.02-0.05)	1.43	(0.81-2.10)
Ca^{++}	0.78	(0.56-1.25)	15.6	(11.3-25.0)
Mg^{++}	0.22	(0.13-0.34)	2.68	(1.60-4.11)
Cl^-	1.00	(0.71-1.71)	35.4	(25.0-60.7)
Hardness mg/L $CaCO_3$			49.2	(41.0-60.1)
Alkalinity mg/L $CaCO_3$			27.4	(26.6-28.0)
Conductivity µmho/cm			218.2	(163-302)
pH[1] range			6.86-6.96	
Temperature °C			11.5 ±0.5	

[1]Range of pH in experimental tank water   6.71-6.96

The Annular Bioassay Tank System

All experiments were conducted using a continuous-flow system. A series of 12 annular fiberglass tanks was supported on a two-tiered wooden frame in a U-shaped array (Fig. 6) and supplied with water as shown in Fig. 7. The tanks were a modification of the "annular growth chambers" described by Kruzynski (1972). The mold was based on a radial tire inner tube (50 cm id, 90 cm od) and was manufactured to specifications by Everlast Plastics, North Vancouver, B.C. Urethane foam was sprayed on the outside for insulation and the tank stood on wooden blocks as illustrated in Fig. 8, which also shows cross-sectional view of the drain area.

Details of a tank module are shown in composite form in Fig. 7. The toxicant metering system is illustrated at the top and consisted of a 25 L glass Mariotte bottle delivering a concentrated stock solution to the tank through Teflon tubing (1.5 mm id, 0.4 mm wall) supported by glass tubing held in a biuret clamp (Leduc, 1966). A toxicant flow of 3 mL/min was mixed with a well water flow of 500 mL/min. The bottom of Fig. 7 illustrates the re-circulating pump connection to the stand pipe/water drive unit, the waste trap tubing in the waste collecting position, and fish schooling under the dark-ened screen portion (right). A water pump (March MDX-3) recirculated the water at ∿ 25L/min and provided a water current around the tank with a velocity determined by the stand pipe/water propulsion unit used. For experiments in which fish were free swimming, the stand pipe unit shown in Fig. 8b provided a current of ∿15 cm/sec with the 70 L tank filled to a volume of 58L. In the gill permeability experiments (p.83) in which fish were restricted to tubes placed within the tank, a modified unit illustrated in Fig. 8c was used to maintain adequate water movement against the added resistance of the tubes.

Figure 6.   Illustration of the arrangement of the donut tanks used
for continuous flow bioassays with DHA.
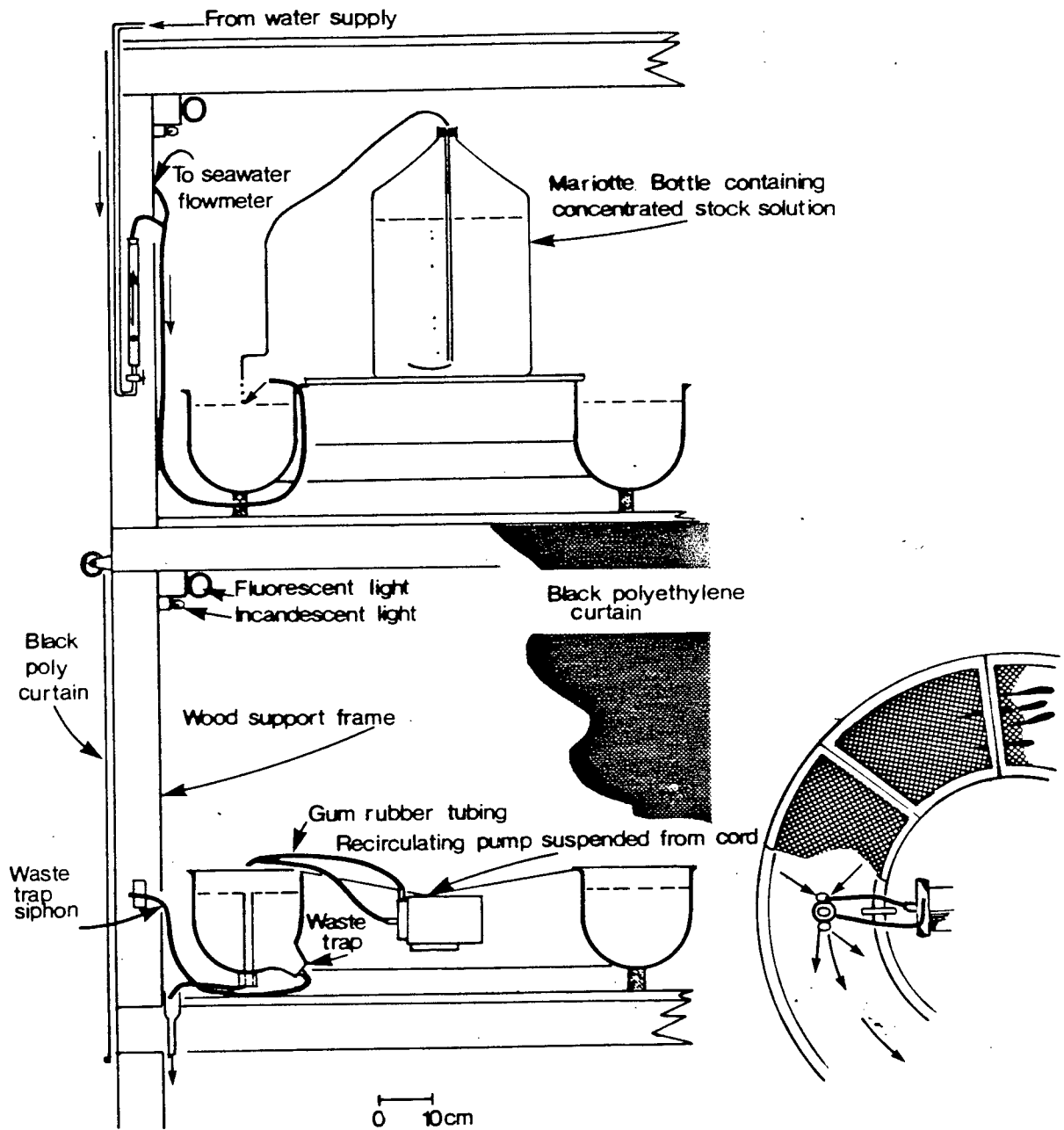
Figure 7. A section of two donut tanks showing details of the water supply and the DHA metering system.
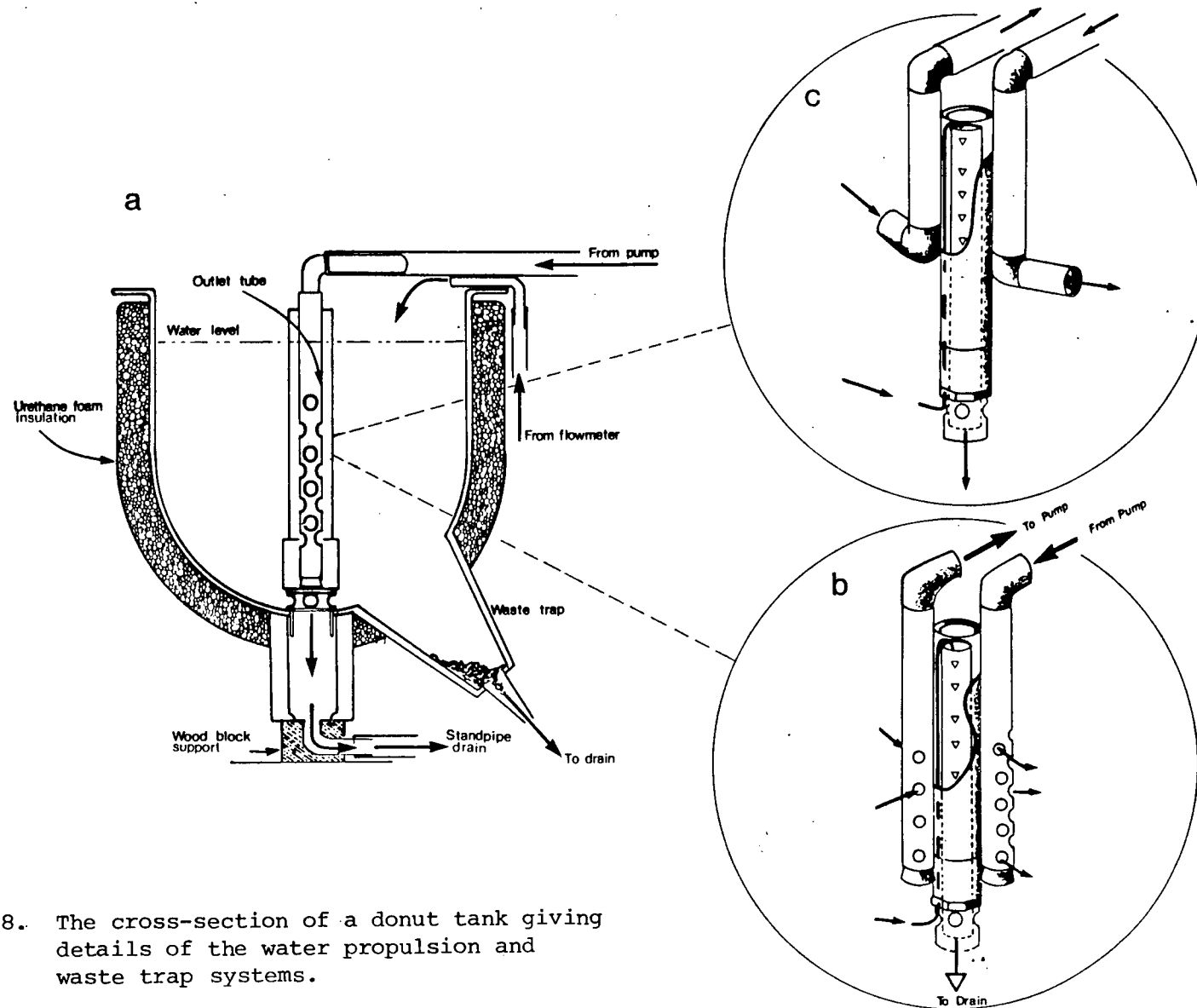
Figure 8. The cross-section of a donut tank giving details of the water propulsion and waste trap systems.

The recirculating pump was suspended from a nylon cord across the internal diameter of the tank to eliminate vibration. Each tank was equipped with an air-stone to maintain dissolved oxygen levels above 90% saturation after it appeared that the toxicant increased O_2 consumption in exposed fish.

Covers made of fiberglass mosquito screening kept fish from jumping out, while a piece of black plastic provided a shaded area. Salmon maintained position against the current under this covered area and then schooled tightly when disturbed. At feeding, pellets of OMP (Oregon Moist Pellet) were dropped just downstream from the stand pipe outlet and were quickly consumed by the fish as the pellets were carried around the tank by the current. The tank incorporated a waste trap (Fig. 8a) which collected un-eaten food and feces drifting around the interior circumference of the tank wall. The trap was drained by a length of Tygon tubing which was kept at water level for collection (Fig. 7) and lowered to the drain to siphon the accumulated waste. This was done immediately before the daily morning feed-ing to drain waste accumulated overnight and about five minutes after feed-ing to remove remaining food.

Adjacent tank modules were separated by black plastic curtains to prevent visual disturbance of the fish. Each module was equipped with a 20W fluorescent light (Duro Test Vita Lite) providing an illumination of 30-50 lx at the water surface. Natural photoperiod was maintained by a light sensor mounted on the roof of the laboratory building. An incan-descent light bulb was left on 24 h a day to provide a dim light simulating night-time outdoor lighting conditions and to facilitate observations.

No metal except for stainless steel came into contact with the water supply. Header tanks were of polyethylene (Nalgene), piping was of PVC and all tubing delivering water to the glass flow meters was Tygon. The

refrigeration units had Teflon coated components. The magnetic drive pump

housings were polypropylene and latex rubber tubing supplied water to the

PVC stand pipe units. The tanks were epoxy reinforced fiberglass while the

toxicant was delivered from glass bottles through Teflon tubing.

Fish Holding

Sockeye salmon stocks were kept in the outdoor fish-holding facility

at the Pacific Environment Institute. As fish were obtained from a variety

of sources and at various stages of development, details are given in the

Materials and Methods section of each experiment. The outdoor holding tanks

were of fiberglass construction, measuring 3.1 x 1.2 m and were filled to a

volume of 4000 L with well water whose chemical characteristics have been

described in Table I. Water temperatures ranged seasonally from $10-12^{o}C$ and

the fish were fed twice daily on a diet of Oregon Moist Pellets.

Fish Transfer

Fish were dip-netted from the outside holding tanks in groups of $\sim$ 20

and transferred into a polyethylene bucket containing 33 mg/L MS-222

(Tricaine methanesulfonate, Sandoz) dissolved in water of salinity $\sim$ 10 $^{o}/oo$

at $11^{o}C$. Fish were then placed into the laboratory donut tanks where they

were held for a minimum of 96 h for acclimatization to the flowing water

conditions in the laboratory prior to the start of an experiment. As the

anesthesia was very light, fish were swimming normally and schooling under

cover within 5 min and would feed within 1 h of introduction into the tank.

Feeding was done once daily with OMP and was discontinued 24 h prior to the

start of experiments.

Anesthesia and Blood Sampling

Fish were anesthetized in two stages. A light anesthesia was brought

on by the addition of 2g of MS-222 to the tank after shutting off the water

supply and adding an air-stone lightly bubbling O_2 into the water. The anesthetic was dissolved in 1L SW and then slowly added to the tank where thorough mixing was ensured by the recirculating pump.

As soon as the fish began to drift with the current, two fish at a time were dip-netted into a polyethylene bucket containing 200 mg/L MS-222 for rapid terminal anesthesia. This solution was prepared by the addition of 1.2 g MS-222 to an oxygenated mixture of 3L SW and 4L FW, yielding a salinity of 10-12 °/oo at a pH ∿6.3. This method ensured rapid anesthesia, minimized hypoxia and was meant to minimize the osmoregulatory stress involved in un-buffered MS-222 anesthesia (Wedemeyer, 1970) and the physiological stress caused by the handling of fish in soft water (Wedemeyer, 1972).

After immobilization, the fish were rinsed with deionized water, measured to the nearest mm, blotted and weighed to the nearest 10 mg on a Mettler P1200 or to 100 mg on a PS1200 top loading balance. The caudal peduncle was severed and blood was collected into Natelson heparinized capillary tubes. The tubes contained 6 USP ammonium heparin and were chilled on ice prior to blood collection. Depending on the size of each fish, from 200-500 μL of blood was collected and the tubes were sealed with Critocaps (Sherwood Medical Industries) and stored on ice until all the fish had been processed.

The tubes were placed in chilled balsa-wood liners to minimize warming and centrifugated at 1300G for 20 minutes in a DAMON/IEC Model CS centrifuge. At other times, blood was collected in microhematocrit tubes and centrifugated in an IEC microhematocrit centrifuge Model MB for 3 minutes at 13,000G. The hematocrit was recorded, plasma was separated, transferred to 2 mL disposable conical polystyrene sample cups (Technicon

Auto Analyzer) and analyzed immediately or stored frozen.

In cases where the water content of the fish was determined, each carcass was blotted dry with an absorbent wiper, wet-weighed in a tared aluminum dish and dried in a forced air oven at 110^{0}C to constant weight. In cases where the "muscle" water content was determined, a cross-section of the fish was used. After the caudal peduncle had been transected and the blood sampling completed, a second transverse cut was made immediately posterior to the vent. This section consisted primarily of muscle but included the anal and adipose fin, a section of the vertebral column as well as the skin and scales. This preparation was then dried as described above. The % water calculated was termed "muscle" water.

Blood Electrolyte Determination

Plasma chloride was measured on a Buchler-Cotlove Direct Reading Chloridometer (Buchler Instruments Division, Nuclear-Chicago, N.J.) adapted for 10 µL samples with a rheostat provided by the manufacturer. A variable volume Buchler Micropipet fitted with a short length of PE-90 tubing was used to transfer the plasma sample to the instrument and de-ionized water was used as the wash-out solvent. The Chloridometer was calibrated using NaCl solutions following manufacturer's instructions and read directly in mEq/L Cl^{-}.

Plasma osmolality was determined on a 1:1 dilution with deionized water. Using an Eppendorf Micropipet, a 100 µL aliquot of plasma was transferred to an osmometer vial followed by 100 µL deionized water using the same pipette tip. An Osmette-S-Semi-Automatic Osmometer (Precision Systems Inc., Mass.) calibrated with manufacturer's standards and operated in the small-sample (200 µL) mode, was used to determine plasma osmolality (milliosmol (mOsm)/kg water). After a determination, the sample

was thawed and 100 µL of the mixture was transferred with an Eppendorf

Micropipet to a 4 ml conical sample cup containing 2 mL 0.25% strontium

chloride ($SrCl_2$) dispensed with an Oxford Pipettor Model R, then capped

and mixed on a vortex stirrer prior to cation analysis by Atomic Absorption

Spectrophotometry (AAS). In cases where there was not enough plasma for

osmometry, 50 µL of undiluted plasma was added directly to the $SrCl_2$

solution. Strontium chloride was used according to the method of Paschen

and Fuchs (1971) for suppression of anionic interferences during plasma

analysis by AAS. A single dilution sufficed for analysis of the four

cations Na^+, K^+, Ca^{++}, Mg^{++}.

Plasma cations were analyzed on a Perkin Elmer Atomic Absorption

Spectrophotometer Model 403, utilizing an air/acetylene flame. Na^+, K^+,

Ca^{++}, Mg^{++} were analyzed at wavelengths (nm) 330 UV, 385 VIS., 211 VIS., and

285 UV respectively. A standard solution containing (in mEq/L) 140 Na^+,

5.0 K^+, 5.0 Ca^{++}, and 1.97 Mg^{++} was prepared according to methods described

in the instrument manual and then diluted in a fashion identical to the

unknown samples. Absorbance was read and the concentration of the various

ions was calculated and expressed in mEq/L. Periodic checks of instrument

performance were made using Hyland I and II and Dade Lab-trol and Patho-trol

Chemistry Control Sera.

Preparation of DHA for Fish Bioassays

Dehydroabietic acid (DHA) was prepared by the method of Halbrook and

Lawrence (1966) to a purity of 95.7% as determined by gas liquid

chromatography (GLC). A concentrated stock solution was made by dissolving

the required amount of DHA in 100 mL ethyl alcohol, adding 2.5 mL 5N NaOH

followed by 100 mL distilled water. Light stirring with a magnetic stir bar

and slow addition of the water ensured complete solution. The mixture was then slowly added to the Mariotte bottle containing approximately 20 L distilled water and 2.5 mL 5N NaOH, again stirring continuously. This concentrated stock solution ($\sim$ 100-200 mg/L) was then made up to 25 L with distilled water. The bottle was then connected to a water-operated vacuum pump (aspirator) for 10-15 min. This evacuation procedure combined with vigorous stirring effectively de-gassed the solution. As the Mariotte bottle system operates under partial vacuum, this procedure eliminated subsequent problems arising from the formation of microbubbles which would coalesce in the fine bore of the toxicant delivery tubing disrupting the flow. Using this degassing procedure, the toxicant flows when once established, required little or no adjustment. The bottle was placed on a board at the center of the donut (Fig. 7) and at a flow rate of 3 mL/min provided toxicant for the duration of the 120 h exposure period with no further disturbance to the fish. In preliminary experiments, measurements (by GLC) of the concentrations of DHA actually present in the water showed that $\sim$ 90% of the theoretical dosage had been attained (Appendix 1).

SYNOPSIS OF STUDIES ON DHA

During preliminary experiments to establish the acute toxicity of DHA to

sockeye salmon it became necessary to develop chemical methods to determine

the actual concentrations of DHA present in the water. These methods (the

extraction of DHA from the water and its quantification by gas-liquid-

chromatography (GLC)) were used to refine DHA solubilization techniques and

subsequently to monitor DHA concentrations in flow-through bioassays. When

it became confirmed that fish were removing DHA from the water during bio-

assays, extraction techniques were also developed to measure DHA residues in

fish tissue and finally in fish food organisms. In all, five experiments

were done and the main findings are summarized below; the details are given

in Appendix I.

The survival of fish in what should have been an acutely toxic con-

centration of DHA during a static bioassay was due to a rapid reduction of

the actual amount of DHA present in the water (Appendix I-1). Although

this was largely attributable to the presence of fish, some adsorption onto

the walls of the test aquarium was also indicated. On the basis of these

results, all subsequent experiments were conducted under continuous flow

conditions exceeding the toxicant/water replacement guidelines given in

Sprague (1969). In addition, a check of actual DHA concentrations present

in the water during these bioassays showed that 90-95% of the theoretical

dose was maintained during the duration of the exposure period.

When DHA was prepared and mixed with well water in the form used for

continuous-flow bioassays (as the sodium salt), filtration had no effect on

its recovery from the water (Appendix I-2). This experiment showed that the

resin acid was in solution, and as such should be available to the fish.

Another experiment (Appendix I-3) determined the direct aqueous solubility of DHA to be 3.3 mg/L, indicating that the free acid can dissolve directly in the water to concentrations exceeding those found to be acutely toxic to salmonids.

As preliminary studies had shown that DHA was taken up by salmon during sublethal exposure in fresh water, an experiment was conducted to determine whether accumulation in the body did occur and if so, to determine the tissue distribution of the toxicant (Appendix I-4). The results confirmed that DHA was taken up by fish and accumulated to a level 30 times higher than that available in the water. Much higher bioconcentration was measured in individual organs such as the brain (954 x), kidney (428 x), liver (404 x); the bile contained the highest overall concentration of DHA (996 x). Gas chromatography coupled with mass spectrometry (GC-MS) was used to detect several metabolic derivatives of the parent DHA molecule in the bile, indicating that the hepatobiliary route is involved in DHA excretion in sockeye salmon.

The exposure of a representative fish food organism (the amphipod Anisogammarus confervicolus) to DHA resulted in a bioconcentration of 21 x that present in the water (Appendix I-5). These results indicate that salmon may accumulate DHA through the food chain as well as directly from the water. The Discussion will go into the biological significance of these high DHA residues in relation to feeding behavior and physiological function of the salmon.

This concludes the summary of studies which were done on the toxicant and the next section will cover experiments which investigated the direct effects of DHA exposure on sockeye salmon.

PART I. PRELIMINARY EXPERIMENTS

A. ACUTE TOXICITY OF DHA TO JUVENILE SOCKEYE SALMON

INTRODUCTION

The acute toxicity of DHA to sockeye salmon was determined on three

separate occasions under continuous-flow conditions with the purpose of

establishing the 96 h LC50 in fresh water. The toxicity curves thus

generated were then used to estimate a concentration of DHA which would

cause negligible mortality during the subsequent sublethal electrolyte

balance experiments. Of the three acute bioassays, the first in March 1974

(Expt. 74) utilized a broad range of 6 concentrations (0.47 to 3.13 mg/L

DHA) while the second in March 1976 (Expt. 76) and third in April 1977

(Expt. 77) employed a more restricted range of concentrations which was

expected to bracket the 96 h LC50.

During the course of acute bioassays, salmon appeared to be under a

respiratory stress, as manifested by frequent coughing and ventilatory

changes. Subsequent work showed that an elevation of hematocrit occurred

during sublethal exposure to DHA, a response that is known to occur during

hypoxia (Doudoroff and Shumway, 1970). If DHA was interfering with normal

gas exchange or with the transport of oxygen by the blood, then a lowering

of dissolved oxygen levels in the water could increase the toxicity of the

resin acid. To test if this was the case, an experiment was conducted on

two groups of salmon smolts exposed to a normally sublethal exposure to DHA.

In one group, dissolved oxygen (D.O.) levels were maintained at $\sim$ 75%

saturation, while in the other, dissolved oxygen was maintained $\sim$ 90%

saturation. Both groups of fish were exposed to a normally sublethal

concentration of DHA (0.65 mg/L) for 120 h in fresh water.

MATERIALS AND METHODS

The sockeye salmon used in all three acute bioassays were of the Cultus Lake stock. Expt. 74 was conducted with fish which had been raised at PEI from eggs obtained from Cultus Lake in November 1973. The 1976 fish were obtained as smolts in January 1976 and had been kept at PEI for 2 months prior to the experiment, while the 1977 fish were obtained as yearlings and had been at PEI for 5 months prior to use. At the time of the acute bioassays all fish were 17-18 months old and had the silvery coloration characteristic of sockeye smolts.

After transfer from outdoor holding tanks according to procedures described in General Methods, the fish were given 48 h to acclimatize to the laboratory tanks before the toxicant exposure was started. In Expt. 74, the fish had been in the laboratory tanks for 2 months prior to the bioassay. Feeding was discontinued 48 h prior to the start of the exposure and fish were not fed during the experiments. Test conditions and fish size are given in Table II. One control tank was used in each experiment and received the solvent carrier at the same rate as the test tanks but no DHA. Continuous observations were made during the day while at night, mortalities were recorded at approximately 4 h intervals. Death was judged by the absence of all movement upon handling. The time to death was recorded, the fish was measured to the nearest mm (fork length), blotted and weighed to the nearest 10 mg on a top-loading balance.

Log probit paper was used to plot cumulative % mortality against time and the TL50 (time to 50% mortality) for each concentration was determined graphically (Litchfield, 1949). Acute toxicity curves were then plotted using TL50's vs concentration on log-log paper and the 96 h LC50's were

Table II. Fish size and acute bioassay operating parameters.

Experiment	Fish Size Mean ±SE(n) Fork length cm	Wet weight g	Number per tank	Loading density L/g fish/day	Water Characteristics (range) Temperature °C	pH	Dissolved oxygen %	DHA mg/L Concentrations tested	Estimate of 96h LC50 [1]
74	11.7 ±0.06(141)	17.74 ±0.26	20	2.03	11.2 ±0.3	6.32 – 6.40	70–85 [2]	3.13, 1.92, 1.65, 1.16, 0.61, 0.47	0.50
76	12.0 ±0.15(44)	17.97 ±0.52	12	3.34	10.5 ±0.5	7.04 – 7.13	90–95	1.00, 0.87, 0.65	0.79
77	13.0 ±0.15(59)	27.06 ±0.93	15	1.79	11.2 ±0.5	6.80 – 6.87	90–95	1.29, 1.00, 0.79, 0.63	0.88

[1] By interpolation from toxicity curve.

[2] Dissolved oxygen dropped to 70% during the first 24 h of exposure and was returned by supplemental aeration to >85% for the remaining 96 h.

estimated graphically by interpolation from the toxicity curves. The absence of partial mortalities at 96 h precluded the calculation of a more precise LC50 estimate and confidence limits as recommended by Sprague (1969).

In Expt. 74 the toxicant exposure period extended to 170 h while in Expts. 76 and 77 toxicant flow was discontinued after 120 h. At the conclusion of the exposure in Expt. 77 the water supply to the 0.63 mg/L tank was switched from FW to SW to determine whether. The surviving DHA-exposed fish would tolerate the additional osmotic stress. This result would have to be taken into consideration when choosing a "sublethal" exposure regimen for subsequent electrolyte studies.

For the hypoxia experiment, sockeye salmon smolts were obtained from the Great Central Lake (Vancouver Island) run at the beginning of March 1978. After 2-1/2 months in the outdoor holding tanks at PEI, 21 fish were transferred to each of 2 laboratory tanks by standardized methods previously described where they were kept for 1 week prior to the experiment. Well water temperature was maintained at 11.4 ±0.1 ($\bar{X}$ ±SE) during the 5-day exposure period to 0.65 mg/L DHA. After a 24 h starvation period, one tank was switched to hypoxic water ($\sim$ 75% saturation) and the fish were given an additional 24 h acclimation to these conditions before the toxicant exposure was started. After 120 h, the toxicant exposure was discontinued, and the normoxic tank (90-95% saturation) was switched to hypoxic water to investigate the possibility of any latent synergistic toxicity.

Fish size during this experiment was 13.7 ±0.17 cm and 26.3 ±1.09 g ($\bar{X}$ ±SE).

RESULTS AND DISCUSSION

Behavioral Symptoms

Frequent observations of fish during the course of the acute bioassays made it possible to detect certain behavioral changes induced by the toxicant exposure. The rate of progression of these behavioral alterations appeared to be dose dependent. Control fish maintained position against the current in a school which was centered under the shaded area of the tank, covering at most about 1/6th of the circumference of the donut. If disturbed, the school would immediately tighten up so that all the fish were under cover. At night, under the continuous dim illumination provided, the size of the school expanded to cover about 1/2 of the circumference with individual fish occassionally turning to swim with the current but rarely for more than one circuit.

In fish exposed to DHA, the first behavioral symptom to be observed was a reduction in the compactness of the school resulting in a gradual increase in the sector occupied by the salmon. This schooling breakup occurred in ∿ 20 h in fish exposed to 1 mg/L DHA. The normal cover response upon visual disturbance became progressively diminished until it was totally eliminated. At this time a tap on the tank resulted in a somewhat confused effort to accelerate forward and school, but the fish appeared to be having problems with muscular coordination. At this point, individual fish could no longer maintain position against the current and began to drift down-stream. At no point, however, did such fish abandon their rheotactic response and appeared to be attempting to head upstream. A tap on the tank often resulted in a spasmotic, undirected movement. Eventually a fish manifesting this behavior would lose equilibrium and be swept around the tank

with the current. Attempts at movement at this stage resulted in muscular

tremors and death followed approximately 3 h after equilibrium loss. If

the toxicant exposure was discontinued when the fish could no longer maintain

station, they appeared to recover gradually and in one case would accept

food after ∿ 24 h in clean fresh water; however, no attempt was made to

assess the recovery of fish which were at a more advanced stage of debility.

The LC50

The results of the three acute bioassays are illustrated in Fig. 9

showing 96 h LC50's of 0.50, 0.79 and 0.88 mg/L DHA for Expts. 74, 76 and

77 respectively. In Expt. 74, the shape of the toxicity curve suggests the

presence of an acute toxicity threshold in the 0.4 mg/L range; however the

lowest concentration tested was 0.47 mg/L DHA. In Expt. 76 and 77 the

toxicity curves generated by the limited number of concentrations used

remained in the linear range. In Expt. 76 the toxicant exposure was

continued until complete mortality occurred in the lowest (0.65 mg/L)

concentration; the last fish died at 262 h. In Expt. 77, toxicant

exposure was discontinued at 120 h and the water supply was switched from

fresh to sea water (∿ 27 °/oo). In this case the slope of the log probit

line used to determine the LT50[3]/ for the 0.79 mg/L group increased, suggesting

a latent and enhanced toxicity brought on by the added salinity stress. In

the lowest concentration tested (0.63 mg/L), no mortality occurred during the

120 h exposure period; however, by the time the experiment was discontinued

after 120 h in sea water a further 6/14 fish had died. These results

indicated that the prior (sublethal) exposure to 0.63 mg/L DHA reduced the

subsequent survival of some sockeye salmon smolts in clean sea water. There

were no control mortalities in any of the bioassays and in Expt. 77 no

behavioral changes could be detected in control fish which encountered the

3/ Time to 50% mortality (LT 50)
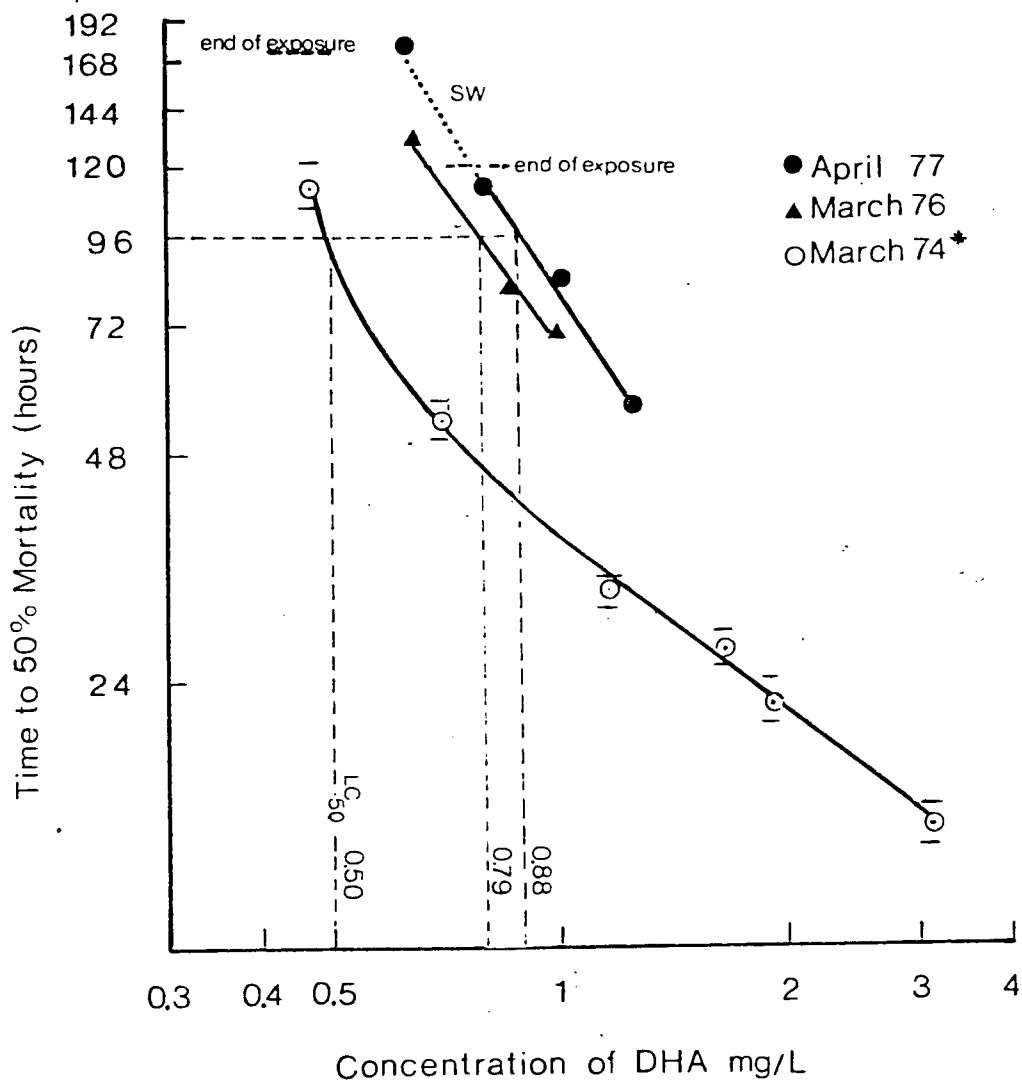
Figure 9. Toxicity curves illustrating 96 h LC50 values for DHA to juvenile sockeye salmon in fresh water. *Confidence limits (95%) are given by ⊙.

rapid (5.8 h for 95% replacement) FW→SW transition.

The 96 h LC50 value of 0.50 mg/L DHA which was obtained in Expt. 74 is somewhat lower than that obtained in Expt. 76 and 77. Measurements of dissolved oxygen during the course of Expt. 74 suggested an elevation in oxygen consumption in exposed fish. After 24 h of exposure, dissolved oxygen levels in the tanks receiving the highest dose of DHA had dropped to 70% saturation while the controls remained above 90% saturation. Supplemental aeration was added and dissolved oxygen levels remained above 85% for the rest of the bioassay. Nevertheless, as the subsequent experiment showed that a reduction in dissolved oxygen in the water led to a marked increase in DHA toxicity, the LC50 value of 0.50 mg/L DHA obtained during Expt. 74 was probably somewhat depressed by reduced O_2. Expts. 76 and 77 were conducted with oxygen levels in excess of 90% saturation and in spite of differences in fish stock and size, test temperature and pH, the 96 h LC50's (0.79 and 0.88 mg/L) were remarkably close.

Effects of Hypoxia

The experiment confirmed the hypothesis of a joint action of low dissolved oxygen and DHA toxicity. Fish exposed simultaneously to 0.65 mg/L DHA and reduced oxygen experienced 100% mortality while in the group exposed at normal oxygen levels, only one fish died within the 120 h exposure period. These relationships are illustrated in Fig. 10 in which the bottom heavy line depicts measured D.O. levels, with the accompanying mortality curve (hypoxic) showing 50% mortality at 76 h. The rise in D.O. levels from 78 to 120 h represents the reduction in O_2 consumption due to the reduction in the number of fish and once the D.O. level reaches ∿ 85% the mortality curve flattens out somewhat due to a reduction in the combined stress of hypoxia/toxicity. In the case of the group exposed under normal
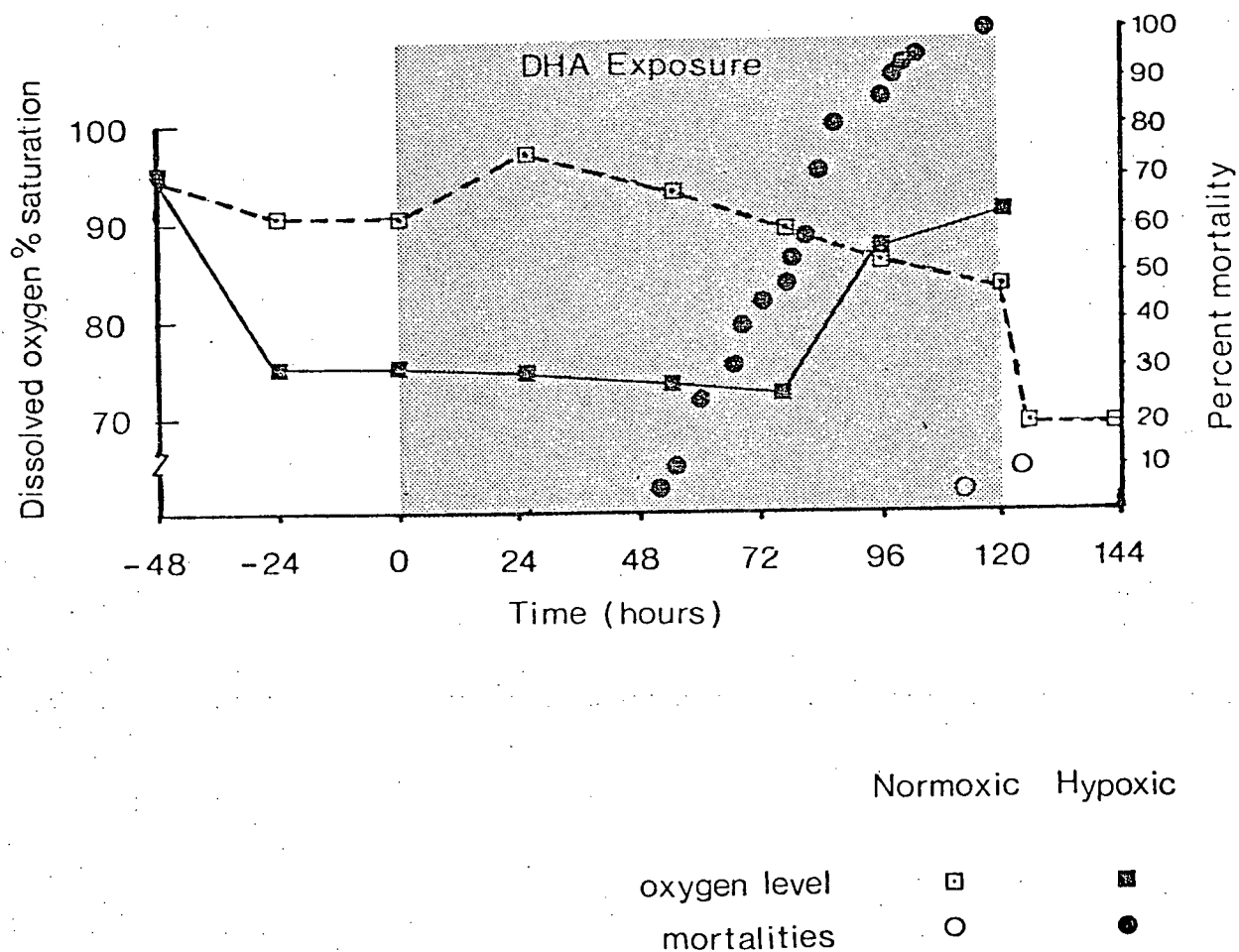
Figure 10. The effect of reduced dissolved oxygen in the water on the toxicity of DHA (0.65 mg/L) to sockeye salmon smolts.

D.O. conditions, a gradual increase in oxygen consumption by these fish can be seen by the slope of the upper D.O. curve. The first mortality occurred when the D.O. level reached $\sim$ 85% saturation. The sharp drop after 120 h represents the measurement 6 h after the toxicant flow had been discontinued and the water supply switched to hypoxic water. A second mortality occurred 3 h after the switch but no further mortalities followed. This indicates that it was the interaction of DHA with hypoxia which led to lethality. Once the toxicant was discontinued, the remaining fish appeared to recover well (behaviorally normal) even at 70% O_2 saturation and began feeding again within 24 h.

These results indicate that hypoxia acted as a loading stress since a concentration of DHA shown previously to be sublethal under O_2-saturated conditions proved lethal when combined with a 30% hypoxia. This hypoxia in itself was probably not deleterious to the salmon (Davis, 1976). In addition it should be noted that sublethal DHA exposure in a normoxic environment caused an increase in the rate of removal of O_2 from the water (dashed line, Fig.10) indicating an increase in the oxygen demand of the fish. These observations compare favorably with the findings by Hicks and DeWitt(1971) of a marked increase in the acute toxicity of whole KME to juvenile coho salmon at reduced levels of dissolved oxygen.

On the basis of these experiments, 0.65 mg/L was chosen to represent a concentration of DHA which would be sublethal to juvenile sockeye salmon during an exposure period of 120 h in well-oxygenated fresh water. Subsequent experiments were conducted to investigate the observed interaction between previous DHA exposure and salinity stress.

B. EFFECTS OF ACUTE DHA EXPOSURE ON OSMOTIC BALANCE

INTRODUCTION

During the course of acute toxicity bioassays, some sockeye salmon appeared normal in size and shape while others developed a swollen or bloated appearance (Fig.11a). Dissections of fish which were visibly swollen revealed an accumulation of fluid in the stomach, in some cases to such a degree that the organ was quite turgid (Fig.11b). As these symptoms were suggestive of a water balance problem, experiments were conducted to quantify this response to DHA poisoning. Condition factors ($K = \frac{weight}{length^3}$ x 100) were calculated as a measure of body "fatness" (Lagler, 1969) and measurements of total body water were made to determine whether a general hydration was occurring. Following these preliminary observations, experiments were conducted to establish whether this apparent osmotic imbalance extended to the muscle tissue. This being the case, the muscle of fish exposed to DHA in fresh water should gain weight (hydrate), whereas fish exposed in sea water should lose water (dehydrate).

MATERIALS AND METHODS

Bloating

During a preliminary bioassay (Expt. A) in which a wide range of fish size was used to investigate the relationship between fish size and acute DHA toxicity (1.1 mg/L),the bloated stomachs of five visibly swollen sockeye salmon (fish size range, 11.2 to 369 g) were excised by a cut distal to both sphincters. Each fluid-filled sac was then dried in a forced-air oven at 105°C and the % water was compared to that of stomachs taken from fish which had died during the bioassay but which had maintained an apparently normal body form.
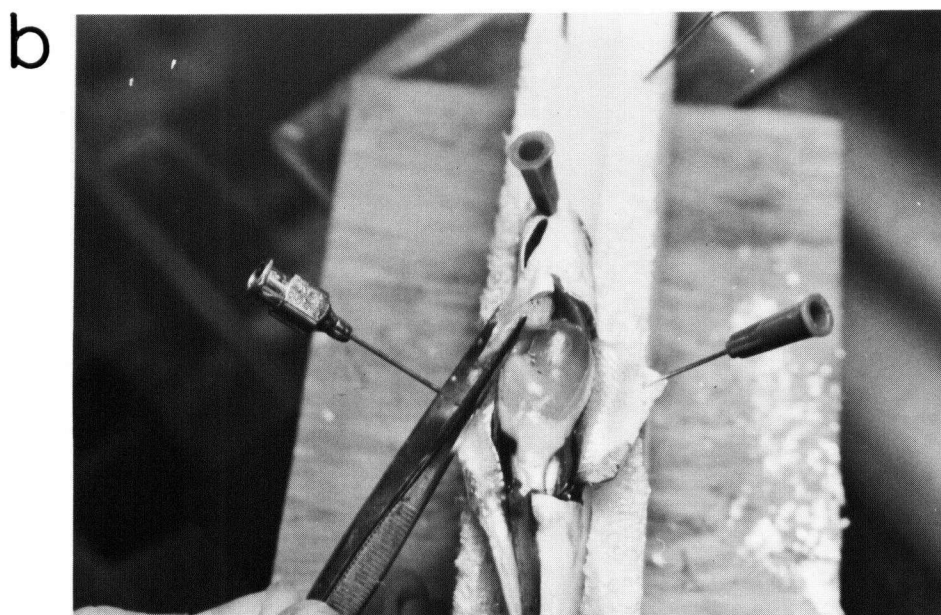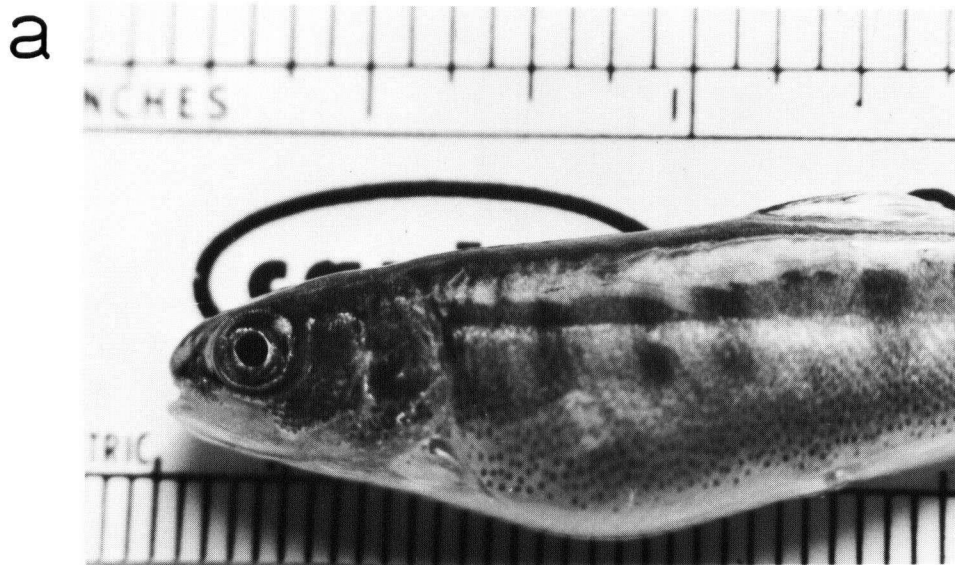
Figure 11.   Illustration of the swelling of sockeye salmon
caused by DHA exposure.

Condition factors were calculated for fish which had died in three preliminary bioassays (Expts. B, C and D): In addition, total body water was measured in Expt. D. Experimental details are outlined in Tables III and IV in the Results section.

Muscle Water

Following these preliminary observations, the percentage muscle water was determined in fish exposed to DHA in fresh water (Expt. E) and in sea water (Expt. F). In Expt. E, underyearling sockeye salmon were exposed to DHA in fresh water and each fish was collected at the stage when it could no longer maintain rheotaxis in the annular tanks. In the second experiment, underyearling chum salmon were exposed to DHA in sea water. Half of the fish were collected at death, while the other half were still alive when the bioassay was discontinued.

The sockeye salmon were of the Great Central Lake (Vancouver Island) stock and were raised from fertilized eggs at PEI. At the time of the experiment, these fish were 8 months old. As no sea-water adapted sockeye salmon were available, chum salmon from the Inches Creek stock (Dewdney, B.C.) were used and had been hatched at PEI where they were maintained in sea water (27-29 ^{o}/oo). In the case of survivors or drifting fish, sampling was done by gently dipnetting individuals as they drifted around the tank. Fish were killed by a sharp flick of the finger, blotted and a tissue sample was taken for determination of % water. A transverse cut was made through the body immediately posterior to the vent, with a second cut about 1 cm further posteriorly. The bulk of such a trunk section consisted of muscle but included several vertebrae and skin. The sample was weighed in an aluminum dish, dried as described previously, and the moisture content was expressed as % muscle water.

RESULTS AND DISCUSSION

Not all fish exposed to DHA developed a bloated appearance nor was the swelling a post-mortem development as it was frequently observed many hours before equilibrium loss in acute bioassays. In Expts. B, C, and D, condition factors of sockeye salmon which died during DHA exposure are significantly higher than the controls (Table III and IV). The progressive nature of this weight gain is reflected by a gradual increase in K factor before and after equilibrium loss leading to death (Table III, footnote 2).

Of the 24 fish exposed to DHA during Expt. A, 5 had visibly swollen abdomens. The accumulation of fluid in the stomachs of these fish is shown in the results in Table V. The stomach of one fish which appeared partially swollen contained an amount of water mid-way between that present in normal and swollen fish. In Expt. D, total body water measurements confirmed that DHA exposed fish contained significantly more water than the controls (Table IV).

These experiments showed that in some salmon DHA exposure in fresh water results in a dramatic increase in water ingestion, leading to a visible accumulation of water and that in others, although the swelling is not apparent, there occurs a general hydration which is reflected by elevated K-factors and increased total body water content. The muscle water experiments described below were conducted to determine whether this edematous condition extended to the tissues of fish exposed to DHA.

Muscle Water

The results of the sockeye salmon experiment are presented in Table VI and show that exposure to DHA resulted in a hydration of muscle tissue in fresh water. As these changes were observed at a time when the fish could no longer maintain position against the current perhaps this muscle

Table III. Condition factors of underyearling sockeye salmon exposed to acutely lethal and sublethal concentrations of DHA in fresh water (Expts. B and C).

Mean ±SE

Bioassay	(DHA) mg/L	N	length cm	weight g	condition factor K[1]
B	1.81	8	10.8 ±0.26	17.4 ±1.11	1.37*±0.03
	0.83	7	10.3 ±0.23	15.2 ±1.14	1.36*±0.02
	Control	9	10.9 ±0.16	15.6 ±1.09	1.07 ±0.02
C[2]	1.32	9	11.9 ±0.65	22.7 ±1.13	1.35*±0.02
	0.57[3]	6	11.2 ±0.18	19.0 ±0.91	1.37*±0.02
	Control	10	11.8 ±0.33	17.9 ±1.43	1.08 ±0.03

[1] $K = \dfrac{weight}{length^3} \times 100$

[2]
one fish sampled before loss of equilibrium   cm 12.0/g 19.6   K=1.13
one fish sampled after loss of equilibrium   11.8/20.5   K=1.25

*significantly different from controls p<0.05 Student's t-test

[3]
Time to 50% mortality (LT 50)=15.3 days

Table IV. Condition factor and total body water in underyearling sockeye
           salmon which died during exposure to 0.95 mg/L DHA[1] in fresh
           water (Expt. D).

	N	length cm	weight g	condition factor K	body water %
Exposed	19	9.6 ±0.24	9.2 ±0.60	1.04* ±0.02	78.55* ±0.35
Control	10	10.2 ±0.20	9.0 ±0.55	0.85 ±0.01	75.16 ±0.88

[1]
LT 50 = 62.5 h

*significantly different from controls $p<0.05$. Student's t-test

Table V. Percentage water of stomachs dissected from "swollen" and "normal" salmon exposed to 1.11 mg/L DHA (Expt. A).

	N	% Water (Mean ±SE)
Swollen	(5)	91.7[*]±1.2
Normal	(10)	79.0 ±1.7
Partly Swollen	(1)	84.8

*significantly different from controls $p < 0.05$. Student's t-test

Table VI. Muscle water in underyearling sockeye salmon exposed to DHA[1] in fresh water and sampled at the drifting stage.

Mean ±SE

	N	Length cm	Weight g	Muscle Water %
Exposed	20	11.2 ±0.27	13.96 ±0.94	77.05* ±0.31
Control	20	11.3 ±0.24	12.89 ±0.85	75.78 ±0.14

[1] 1.4 mg/L

*significantly different from controls p<0.05. Student's t-test

hydration interfered with normal contractile processes and thus contributed to a reduction in swimming performance.

The results of the exposure of chum salmon to DHA in sea water are shown in Table VII and illustrate a significant dehydration of muscle tissue when compared to the controls. The gradual development of dehydration is illustrated by the reduction in water content of salmon which had survived the 96 h exposure to DHA. This value suggests that the amount of dehydration increases with exposure time.

These preliminary experiments indicated that exposure to DHA led to an osmotic imbalance in a direction dictated by the osmotic gradient between body fluids and the surrounding water. As such an osmotic imbalance could be accompanied by an ionic disturbance, experiments were conducted to determine plasma ionic composition in sockeye salmon exposed to DHA in fresh water. Subsequently, experiments were performed to measure the hydromineral regulatory ability of salmon exposed sub-lethally to DHA and then trans-ferred to sea water.

Table VII.   Muscle water in underyearling chum salmon exposed to DHA[1] in sea water.

Mean ±SE

		N	Length cm	Weight g	Muscle Water %
Exposed	At death	10	10.0 ±0.23	7.70 ±0.53	77.09* ±0.36
	Survivors	10	10.0 ±0.16	7.62 ±0.43	79.53* ±0.38
Controls		20	10.1 ±0.13	7.92 ±0.33	80.72 ±0.14

[1]1.4 mg/L

*significantly different from controls p<0.05. Student's t-test

PART II. PRINCIPAL EXPERIMENTS

A. EFFECTS OF SUBLETHAL DHA EXPOSURE ON HYDROMINERAL BALANCE
    IN SOCKEYE SALMON SMOLTS

INTRODUCTION

The suggestion made from the previous experiments that osmotic imbalance was also accompanied by an electrolyte disturbance was confirmed in an exploratory experiment (unpublished observations) in which blood electrolyte levels were measured in sockeye salmon sublethally exposed to DHA in fresh water. Exposure to DHA resulted in a significant reduction in the concentrations of the main plasma electrolytes Na^+ and Cl^-. However, no changes were observed in plasma K^+ or Mg^{++} levels and plasma Ca^{++} concentrations increased. This complex response cannot be explained by a simple DHA-induced hydration.

To investigate these findings more fully, this experiment was repeated and the observations were also extended into the seawater phase. As discussed in the General Introduction, prior sublethal DHA exposure followed by the movement of fish into sea water was meant to simulate the exposure of sockeye salmon to pulp mill waste in a river during the course of smolt migration. Under these conditions, the extent of toxicant exposure would be limited to the migration time in fresh water and subsequent entry into the sea would involve a rapid transition from a hypotonic to a hypertonic medium. Although this seawater acclimation period comprises a "recovery phase", as the toxicant exposure has been discontinued, the interaction of the effects of prior exposure with natural salinity stress could have an effect on hydromineral homeostasis.

Three experiments were conducted (Expts. 1,2 and 3) in which sockeye salmon smolts were exposed to a sublethal dose of DHA in fresh water and subsequently "transferred" to sea water. Osmoregulatory performance was

gauged by measuring plasma electrolyte concentrations at the end of the toxicant exposure period and, subsequently, by following the time course of plasma electrolyte regulation during the transition to sea water. In view of the results obtained in fresh water, it was hypothesized that DHA exposure should lead to elevated plasma ionic levels in fish in sea water if the toxicant acted to cause a loss in ionoregulatory precision.

During Expt. 1 however, some of the fish were suspected of suffering from a chronic infection of bacterial kidney disease (BKD) and the presence of the infection was confirmed during Expt. 2 which followed immediately. Bacterial kidney disease involves a gradual destruction of the kidney which is intimately involved with hydromineral balance in fish. Thus, to reduce the possibility of the disease confounding the effects caused by the toxicant, it seemed imperative to devise a procedure to eliminate from the data fish in which the infection had reached an advanced stage; this was done using hematocrit values. Blood hematocrit is known to drop gradually as the disease progresses, therefore data collected from salmon with abnormally low hematocrits were deleted. A thorough discussion of bacterial kidney disease and its interaction with DHA toxicity, as well as the details of the screening method and tables containing the deleted data are presented in Appendix II.

At a later date, when a new stock of disease-free salmon became available, the electrolyte balance experiment was repeated a third time (Expt. 3) and confirmed the results which had been obtained in Expts. 1 and 2.

MATERIALS AND METHODS

Three similar but separate experiments were conducted in which sockeye smolts were exposed to 0.65 mg/L DHA for 5 days (120 h) in fresh water under continuous flow conditions. At the end of this sublethal exposure period, toxicant administration was discontinued, and the fresh water supply was

switched to sea water. A 95% replacement in 5.8 h yielded a salinity of ∿

26 $^\circ$/oo at the end of this time. The first blood sampling was conducted at

the end of the freshwater DHA exposure period (0 h) and subsequently for 120 h

at intervals of 24 h to follow the time course of adaptation to sea water.

Fish handling, blood sampling and analysis protocol was performed as described

in the General Methods section. The sizes of the fish used in the three

experiments are given in Table VIII.

The three experiments were conducted using two separate stocks of sockeye

salmon. Expts. 1 and 2 were performed in May 1977 utilizing fish which were

obtained as yearlings from the Cultus Lake stock and had been kept at PEI

for 6 months prior to use. This was the stock of fish which developed

bacterial kidney disease. In June 1977, sockeye smolts from the Great Central

Lake (Vancouver Island) run were brought to PEI but proved too small to conduct

the corroborative experiment (Expt. 3). As a result, Expt. 3 was conducted in

the first week of December 1977 at which time these fish were 21 months old.

Thus while the size of fish used in the three experiments was similar, the

sockeye in Expt. 3 were of a different stock and were three months older.

RESULTS

Latent Acute Toxicity

For the purposes of these experiments, "sublethal" was defined as a dose

(the product of concentration x time) causing negligible mortality in 120 h.

According to this definition 0.65 mg/L DHA was sublethal in Expts. 1 and 3.

In Expt. 2 however, mortality reached ∿ 7% during the exposure period. This

increase in toxicity was caused by the BKD infection and is discussed in

Appendix II.

Table VIII. Size of the sockeye salmon used in the electrolyte balance experiments: (Expts. 1,2 and 3).

Mean ± SE(n)

	Time in hours	Control Fork length cm		Wet weight g		Exposed Fork length cm		Wet weight g	
	0	14.9	0.38(10)	33.97	2.66	14.9	0.27(10)	34.42	1.83
	24	15.0	0.47(9)	33.93	3.24	14.3	0.21(10)	30.71	2.06
Experiment 1	48	14.8	0.30(10)	32.16	1.96	14.2	0.50(7)	29.44	2.75
	72	14.6	0.27(9)	31.21	1.98	15.0	0.26(8)	33.43	1.69
	96	15.2	0.48(9)	34.61	3.54	15.1	0.28(9).	34.02	1.92
	120	14.9	0.22(10)	32.48	1.53	15.1	0.40(7)	35.53	3.23
	0	15.7	0.38(10)	40.93	2.33	15.9	0.30(11)	40.65	2.27
	24	16.4	0.32(9)	44.09	2.43	15.4	0.33(9)	37.06	2.66
Experiment 2	48	15.8	0.60(6)	40.82	5.25	15.3	0.32(10)	36.75	2.10
	72	15.5	0.30(8)	37.06	2.43	15.6	0.24(7)	37.36	1.78
	96	15.8	0.33(10)	39.85	2.19	16.0	0.39(8)	39.69	2.97
	120	15.6	0.40(8)	38.15	2.46	15.3	0.42(8)	35.90	3.18
	0	15.9	0.35(11)	36.68	2.69	15.3	0.33(12)	31.74	2.18
	24	15.0	0.37(11)	29.07	2.25	15.3	0.41(12)	32.09	2.58
Experiment 3	48	15.8	0.35(12)	33.71	2.10	15.6	0.47(11)	32.56	2.87
	72	16.0	0.24(11)	35.44	1.75	15.5	0.52(10)	32.48	3.79
	96	15.2	0.45(12)	32.14	2.70	16.0	0.42(10)	36.15	2.56
	120	16.0	0.32(12)	36.04	2.58	15.6	0.43(12)	34.28	2.30

The presence of a latent acute toxicity of DHA became apparent when the previously exposed fish were faced with a salinity challenge. As illustrated in Fig.25 (p.155) this combination of stressors led to mortalities in Expt.1 only after the fish had been in sea water for 24 h. In Expt. 2, mortalities which started during the freshwater exposure period appeared to continue at the same rate for the first 24 h in sea water, with a subsequent break in the slope of the toxicity curve indicating an attenuation of latent acute toxicity after 48 h in clean sea water. Actual mortalities for Expts. 1, 2 and 3 were 7/60, 15/75 and 2/72. These figures include "moribund" fish for Expt. 2 but do not include the 3 "drifters" in Expt. 3 which were sampled in the early stages of intoxication. "Moribund" and "drifter" fish are discussed in the last part of the Results section.

Control mortalities were negligible, with only 1 of the 204 total dying of what appeared to be a fungus infection of the gills.

<div align="center">Sublethal Effects</div>

Behavioral Observations

Observations made during the course of the toxicant exposure indicated a slight reduction in the compactness of the school, a behavior which corresponds to the first stage of the sequence described in the Behavioral Symptoms portion of the Acute Toxicity section (p.37). The normal response to visual disturbance (cover response) was only slightly reduced in Expt. 1 and 3 whereas in Expt. 2 the fish generally reacted more slowly. In Expt. 2, 15 fish were more severely affected after 103 h exposure; these were sampled prior to the end of the exposure period and are discussed in a later section as "moribund" fish.

This slowness in response of DHA-exposed fish generally became more pronounced during the first 24 h of seawater adaptation, and many fish still did not display a normal cover response after 72 h in sea water. In contrast the behavior of control fish remained unchanged throughout the entire experiment.

Hydromineral Balance in Fresh Water

The plasma electrolyte levels in sockeye salmon smolts exposed for 120 h to 0.65 mg/L DHA in fresh water are presented in Table IX and illustrated in Fig.12. In addition to plasma ions, blood hematocrit was determined in all three experiments whereas % muscle water was measured only in Expts. 2 and 3. Table IX gives the means of the actual values measured in each of the three experiments, whereas Fig.12 combines these means to illustrate an overall "average response".

Statistical significance shown in Fig.12 was determined by combining the probabilities obtained from separate tests of significance between "control" and "exposed" means in each experiment. The Student's "t" test was used and was corrected when variances were unequal. Exact probabilities were determined for each calculated "t" and then pooled according to the method given in Sokal and Rohlf (1969). This treatment provides a single test of significance of the aggregate based on the product of the probabilities observed individually (Fisher, 1958). This approach was used because, although the three experiments were similar in design, they were not identical, so that pooling of all the data for joint statistical treatment was not warranted.

Fig.12 shows that a 120 h exposure to a sublethal dose of DHA caused a significant disturbance in the levels of all plasma electrolytes investigated, except for sodium. The direction of the change however, was not uniform.
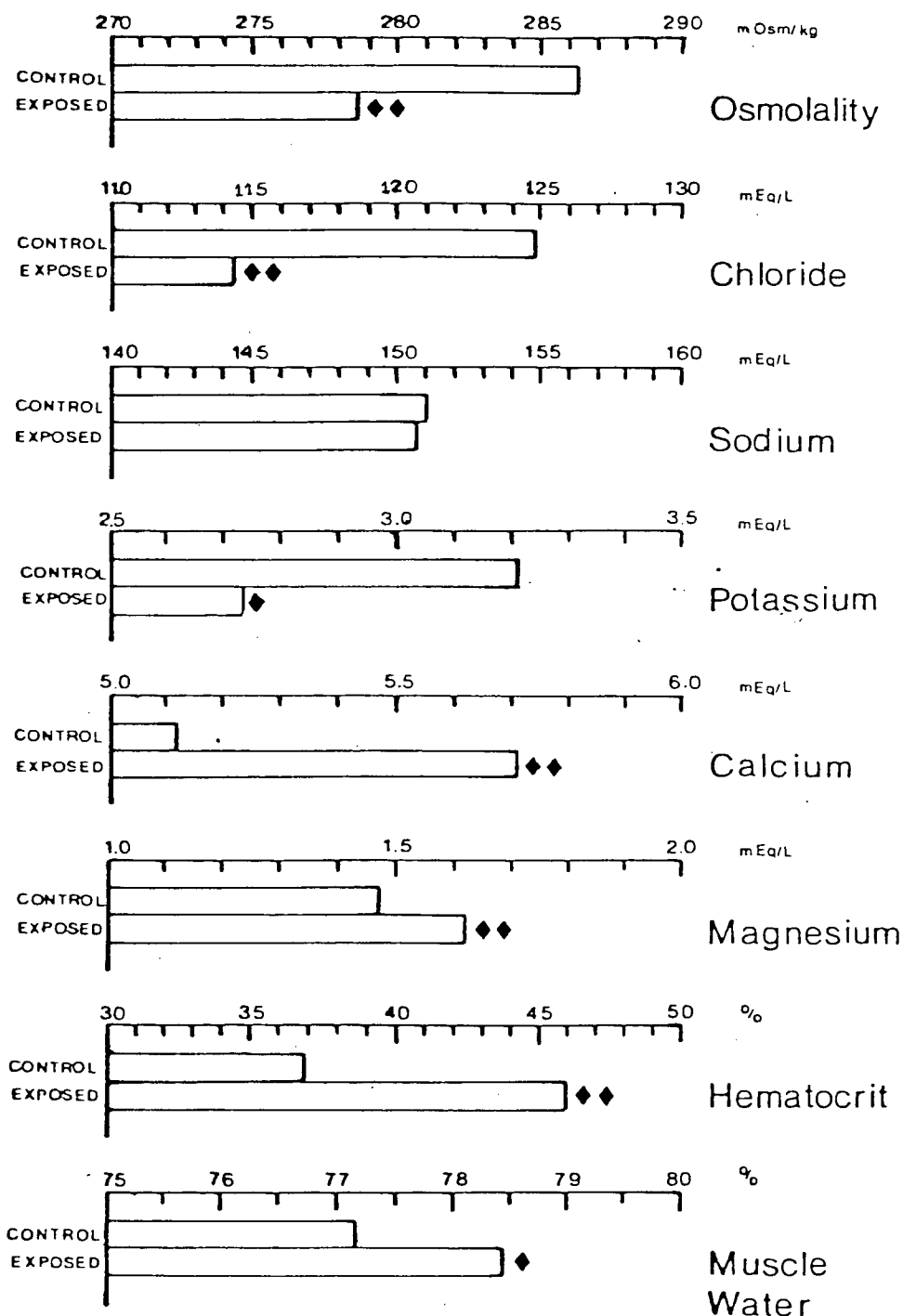
Figure 12.   Plasma electrolyte levels, hematocrit and muscle water
content in sockeye salmon exposed to 0.65 mg/L DHA for
120 h in fresh water.   Values are means for each
parameter based on Expts. 1, 2 and 3 except for muscle
water which was measured in Expt. 2 and 3 only.
Significance level p<0.05◆; p<0.01◆◆

Table IX. Plasma ionic composition, hematocrit and muscle
water content of sockeye salmon exposed to
0.65 mg/L DHA for 120 h in fresh water in
Expts. 1, 2 and 3.

		Experiment 1		Experiment 2		Experiment 3	
Osmolality mOsm/kg	Control	286.6	1.77(10)	287.6	3.11(10)	285.1[1]	3.23(11)
	Exposed	276.4[a]	1.63(9)	286.0	3.07(10)	273.8[b]	2.13(12)
Chloride mEq/L	Control	124.8	0.39(10)	123.9	1.51(10)	124.3	1.15(11)
	Exposed	114.4[a]	1.35(10)	114.5[a]	1.00(11)	110.6[a]	1.40(12)
Sodium mEq/L	Control	153.83	1.31(10)	147.29	1.13(10)	151.94	2.37(11)
	Exposed	152.01	1.71(10)	146.08	2.03(11)	154.14	1.61(12)
Potassium mEq/L	Control	2.81	0.21(10)	3.63	0.16(10)	3.20	0.14(11)
	Exposed	1.93[c]	0.24(10)	3.56	0.16(11)	2.68	0.26(12)
Calcium mEq/L	Control	5.69	0.12(10)	5.38	0.07(10)	4.29	0.08(11)
	Exposed	6.18[d]	0.15(10)	6.18[a]	0.07(11)	4.78[d]	0.21(12)
Magnesium mEq/L	Control	1.50	0.03(10)	1.55	0.03(10)	1.37	0.05(11)
	Exposed	1.52	0.04(10)	1.74[b]	0.04(11)	1.60[a]	0.03(12)
Hematocrit %	Control	33.97	1.63(10)	36.30	1.12(10)	40.56	0.73(11)
	Exposed	45.00[a]	2.00(10)	44.76[a]	1.19(11)	47.83[a]	1.04(12)
Muscle Water %	Control	-	-	74.79	0.42(10)	79.50	0.53(11)
	Exposed	-	-	75.69	0.18(11)	81.17[d]	0.59(12)

[1] Total sum of ions

[a] $p < 0.001$, [b] $p < 0.01$, [c] $p < 0.02$, [d] $p < 0.05$
differs significantly from control t- test

Overall, DHA exposure in fresh water led to most pronounced and consistent

changes in plasma levels of Cl^- and Ca^{++} and in blood hematocrit. In all

three experiments, plasma Ca^{++} and Mg^{++} increased whereas plasma Na^+ levels

remained remarkably stable. The decrease in plasma osmolality was accompanied

by an increase in muscle water levels.

Hydromineral Balance in Sea Water (Plasma Electrolytes)

The effects of the combined stress of prior toxicant exposure and

salinity on plasma electrolytes of sockeye salmon are presented in Table X

and illustrated in Fig.13.Table X gives the means of the various plasma

electrolyte determinations for Expts. 1, 2 and 3, while Fig.13 provides a

comparison of "exposed" relative to "control" values; controls are shown as

100. Each point represents an average of the combined responses obtained in

Expts. 1, 2 and 3, while the level of statistical significance of the departure

of each point from the control (dotted line) was determined from pooled

probabilities as described previously. Significance levels are shown in the

box below each graph. Note that the points for 0 hours SW represent the

various electrolyte values after 120 h DHA exposure in fresh water. Since

they are not part of the seawater phase, for reasons of clarity they are not

connected to the curves but have been shown to provide a relative indication

of the magnitude of the direct effect of DHA on electrolyte balance in fresh

water.

The results summarized in Fig.13 show that the balance of all the plasma

electrolytes except for K^+ was altered in fish which had previously been

exposed to DHA. The departures from the control values were all in the

positive direction and subsequently returned to normal levels after varying

intervals. Plasma Cl^- concentrations remained elevated ($p < 0.001$) for 96 h,
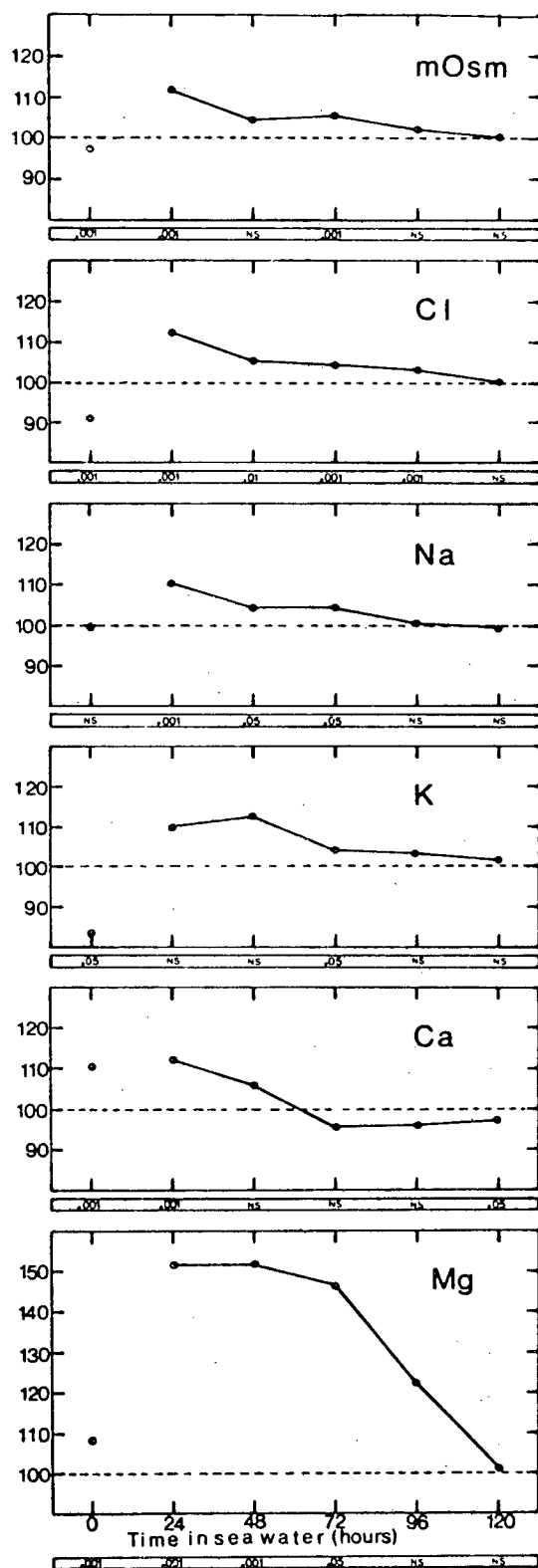
Figure 13. The change in plasma electrolyte levels in sockeye salmon during adaptation to sea water following a 120 h exposure to 0.65 mg/L DHA in fresh water. Points for each parameter represent the overall mean % change (relative to a control value of 100) derived from Experiments 1, 2 and 3. The level of statistical significance of the change is given at the base of each graph.

Table X. Plasma electrolyte levels [Mean $\pm$SE(N)] in sockeye salmon during sea-water adaptation following a 120 h exposure to 0.65 mg/L DHA in fresh water in Expts. 1, 2 and 3.

EXPT 1

| Time in sea water hours	Osmolality (mOsm/kg) Control	Osmolality (mOsm/kg) Exposed	Chloride (mEq/l.) Control	Chloride (mEq/l.) Exposed	Sodium (mEq/L) Control	Sodium (mEq/L) Exposed	Potassium (mEq/L) Control	Potassium (mEq/L) Exposed	Calcium (mEq/L) Control	Calcium (mEq/L) Exposed	Magnesium (mEq/L) Control	Magnesium (mEq/L) Exposed
0	286.6 1.77(10)	276.4[a] 1.63(9)	124.8 0.39(10)	114.4[a] 1.35(10)	153.83 1.31(10)	152.01 1.71(10)	2.81 0.21(10)	1.93[c] 0.24(10)	5.69 0.12(10)	6.18[d] 0.15(10)	1.50 0.03(10)	1.52 0.04(10)
24	299.5 1.64(8)	341.2[a] 2.06(5)	128.3 0.84(9)	146.7[a] 2.86(10)	162.36 2.49(9)	179.75[a] 3.33(10)	1.60 0.30(9)	1.84 0.17(10)	6.22 0.15(9)	7.35[a] 0.22(10)	2.19 0.08(9)	3.62[a] 0.26(10)
48	291.1 3.40(9)	316.7 10.69(6)	127.2 1.18(10)	137.1[b] 3.06(7)	154.26 0.92(10)	165.36[d] 4.21(7)	2.42 0.35(10)	2.83 0.24(7)	5.94 0.06(10)	6.26 0.19(7)	1.89 0.04(10)	3.53[d] 0.49(7)
72	299.1 3.59(7)	308.3 4.38(8)	133.2 1.48(9)	130.8 1.13(8)	159.44 2.23(9)	164.72 2.89(8)	3.95 0.14(9)	3.41 0.20(8)	5.88 0.07(9)	5.93 0.14(8)	1.99 0.08(9)	2.30 0.19(8)
96	295.1 2.06(9)	293.1 2.09(9)	131.3 1.64(9)	130.9 1.58(9)	160.65 2.21(9)	155.30 1.51(9)	3.44 0.09(9)	3.62 0.28(9)	5.86 0.07(9)	5.83 0.07(9)	1.47 0.09(9)	1.92 0.30(9)
120	295.4 2.48(10)	298.9 1.85(7)	129.7 0.34(10)	131.4 0.57(7)	160.82 1.09(10)	161.56 2.10(7)	3.67 0.11(10)	3.75 0.11(7)	5.89 0.08(10)	5.99 0.09(7)	1.66 0.04(10)	1.72 0.04(7)

EXPT 2

Time in sea water hours	Osmolality Control	Osmolality Exposed	Chloride Control	Chloride Exposed	Sodium Control	Sodium Exposed	Potassium Control	Potassium Exposed	Calcium Control	Calcium Exposed	Magnesium Control	Magnesium Exposed
0	287.6 3.11(10)	286.0 3.07(10)	123.9 1.51(10)	114.5[a] 1.00(11)	147.29 1.13(10)	146.08 2.03(11)	3.63 0.16(10)	3.56 0.16(11)	5.38 0.07(10)	6.18[a] 0.07(11)	1.55 0.03(10)	1.74[b] 0.04(11)
24	296.2 2.30(9)	325.5[b] 6.92(8)	130.3 1.78(9)	142.1[a] 2.78(8)	155.90 1.55(9)	170.21[a] 2.89(8)	3.39 0.20(9)	3.85 0.14(8)	5.52 0.07(9)	5.94[d] 0.15(8)	1.94 0.09(9)	3.18[b] 0.37(8)
48	306.0 3.72(6)	305.6 2.64(9)	132.4 0.91(6)	135.0 0.95(10)	160.13 2.84(6)	165.09 1.67(10)	3.31 0.18(6)	3.80 0.20(10)	5.45 0.26(6)	5.52 0.04(10)	1.83 0.04(6)	2.22 0.17(10)
72	289.4 2.68(7)	314.0[a] 3.27(6)	128.6 1.00(8)	140.3[a] 1.86(6)	154.46 1.83(8)	165.65[b] 3.11(6)	4.01 0.21(8)	4.17 0.25(6)	5.73 0.33(7)	5.56 0.08(6)	1.84 0.06(8)	2.92 0.46(6)
96	293.8 3.55(10)	301.1 2.09(7)	129.0 1.11(10)	135.1[a] 0.84(8)	155.04 1.74(10)	157.51 4.21(8)	3.61 0.18(10)	3.60 0.15(8)	5.69 0.10(10)	5.48 0.16(8)	1.65 0.05(10)	1.63 0.07(8)
120	295.0 2.24(8)	294.0 1.91(8)	129.8 0.86(8)	129.3 1.30(8)	152.03 1.92(8)	152.49 1.61(8)	4.02 0.19(8)	3.94 0.18(8)	5.68 0.14(8)	5.54 0.09(8)	1.64 0.05(8)	1.62 0.05(8)

EXPT 3

Time in sea water hours	Osmolality Control	Osmolality Exposed	Chloride Control	Chloride Exposed	Sodium Control	Sodium Exposed	Potassium Control	Potassium Exposed	Calcium Control	Calcium Exposed	Magnesium Control	Magnesium Exposed
0	285.1 3.23(11)	273.8[b] 2.13(12)	124.3 1.15(11)	110.6[a] 1.40(12)	151.94 2.37(11)	154.14 1.61(12)	3.20 0.14(11)	2.68 0.26(12)	4.29 0.08(11)	4.78[d] 0.21(12)	1.37 0.05(11)	1.60[a] 0.03(12)
24	290.2 2.07(11)	325.9[a] 7.00(12)	128.6 1.20(11)	146.3[a] 3.29(12)	151.76 1.21(11)	168.71[a] 3.58(12)	3.15 0.22(11)	3.19 0.16(12)	4.70 0.12(11)	5.18[c] 0.13(12)	2.00 0.14(11)	2.52[c] 0.14(12)
48	291.7 1.57(12)	305.6[d] 5.57(11)	129.3 1.13(12)	137.5[c] 2.56(11)	153.15 0.95(12)	157.54 3.11(11)	3.28 0.18(12)	3.48 0.14(11)	4.50 0.06(12)	4.57 0.09(11)	1.72 0.09(12)	2.53[b] 0.18(11)
72	299.0 3.17(11)	312.6 8.30(10)	130.5 2.22(11)	139.6[a] 3.70(10)	158.44 1.86(11)	161.65 4.19(10)	3.40 0.14(11)	4.14[c] 0.23(10)	4.99 0.14(11)	4.40[b] 0.12(10)	1.71 0.04(11)	2.83[d] 0.47(10)
96	288.6 2.37(12)	300.3 3.00(10)	129.2 1.49(12)	136.3[a] 1.47(10)	149.33 1.19(12)	153.51 2.14(10)	3.69 0.14(12)	3.88 0.23(10)	4.63 0.08(12)	4.27[c] 0.11(10)	1.78 0.08(12)	2.43 0.39(10)
120	292.3 2.09(12)	291.7 1.67(12)	130.7 1.55(12)	131.5 0.78(12)	151.87 0.87(12)	149.22 1.33(12)	3.48 0.15(12)	3.63 0.30(12)	4.38 0.07(12)	4.08[b] 0.07(12)	1.73 0.06(11)	1.76 0.11(12)

Significance Level P < [a] 0.001 [b] 0.01 [c] 0.02 [d] 0.05

Na^+ and Mg^{++} for 72 h (p<0.05) while plasma Ca^{++} levels had returned to the control range within 48 h. Of all the electrolytes investigated plasma Mg^{++} increased by far the most, reaching a maximum average of 3.10 mEq/L within 24 h as compared to 2.04 mEq/L for controls within 24 h (Table X). Plasma Mg^{++} levels still remained elevated at the 96 h sampling period however, the gradual return to control levels coupled with a high variation in the exposed groups rendered this difference statistically non-significant. In general, DHA exposure reduced the precision of regulation as indicated by an increase in the standard errors in Table X and illustrated in Fig. 16 for Expt. 3. Overall, these changes led to a general increase in the total plasma osmolality which persisted for 72 h after toxicant exposure.

Hydromineral Balance in Sea Water (Hematocrit, Muscle Water, Gut Water Content)

In addition to the determination of plasma electrolyte concentrations, measurements were made of blood hematocrit, muscle water and gut water content. If the fresh water DHA exposure led to a subsequent dehydration of salmon in sea water, this could be reflected in a lowering of muscle water content coupled with an increase in the rate of sea water ingestion as a counter-measure.

Blood hematocrit levels at the various sampling periods are presented in Table XI and a comparison between exposed and control groups is illustrated in Fig. 14 (top). Hematocrit was measured in Expts. 1, 2 and 3, whereas muscle water was determined in Expt. 2 and 3. The % water in the gut was measured only in Expt. 2; the results are presented in Table XII and are illustrated in Fig. 15 which also shows the relation of muscle and gut water determinations in "moribund" salmon to those of control and exposed groups.

Table XI. Hematocrit of sockeye salmon after a 5-day exposure to 0.65 mg/L DHA in fresh water followed by exposure to sea water in Expts. 1, 2 and 3.

	Time in sea water h	Hematocrit %	
		Control Mean ± SE(n)	Exposed Mean ± SE(n)
Experiment 1	0	33.97 1.63(10)	45.00 2.00(10)[a]
	24	45.19 2.19(9)	49.36 2.67(10)
	48	42.12 1.06(10)	41.74 1.24(7)
	72	38.33 0.89(9)	36.18 0.63(8)
	96	35.59 0.96(9)	35.57 1.22(9)
	120	36.58 1.06(10)	34.63 0.85(7)
Experiment 2	0	36.30 1.12(10)	44.76 1.19(11)[a]
	24	38.99 0.85(9)	38.34 2.01(8)
	48	37.28 2.26(6)	36.53 1.47(10)
	72	35.05 1.84(8)	31.93 1.26(7)
	96	35.35 1.46(10)	37.75 1.38(8)
	120	36.73 1.76(8)	31.60 1.44(7)[b]
Experiment 3	0	40.56 0.73(11)	47.83 1.04(12)[a]
	24	44.80 1.08(11)	42.30 1.36(12)
	48	41.28 1.10(12)	41.10 0.65(11)
	72	41.86 0.90(11)	40.55 1.40(10)
	96	40.48 0.90(12)	39.75 1.34(10)
	120	38.88 0.82(12)	36.58 0.92(12)

differs significantly from control
t-test [a] $p<0.001$, [b] $p<0.05$
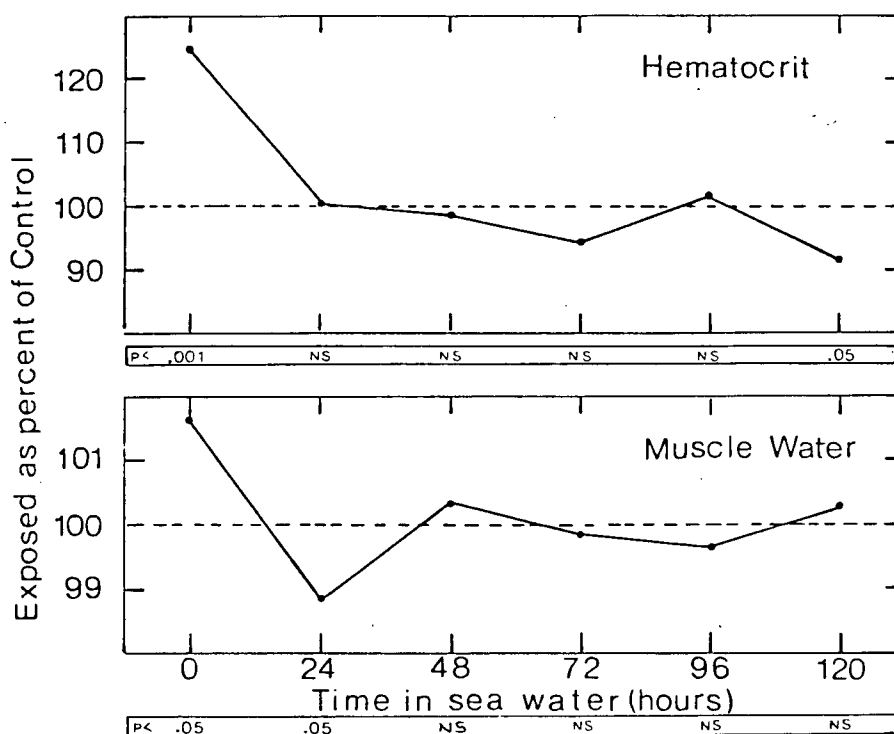
Figure 14. The change (relative to control = 100) of hematocrit
and muscle water in sockeye salmon during sea-water
adaptation. The fish had previously been exposed to
0.65 mg/L DHA for 120-h in fresh water. Points for
hematocrit are mean % based on Expts. 1, 2 and 3.
Points for muscle water are mean % based on Expts.
2 and 3. The level of statistical significance of the
change is given at the base of each graph.

Table XII. Percentage water in gut of sockeye salmon after
a 5-day exposure to 0.65 mg/L DHA in fresh water
followed by exposure to sea water (Expt. 2).

Percentage water in gut

Time in sea water hours	Control Mean $\mp$ SE(n)	Exposed Mean $\mp$ SE(n)
0	76.71 0.97(10)	77.86 0.74(11)
24	76.11 0.52(9)	80.24[a] 1.71(9)
48	76.96 1.20(6)	75.61 0.96(10)
72	77.60 0.70(8)	79.77 1.33(7)
96	76.88 0.65(10)	77.35 0.39(8)
120	77.68 0.52(8)	80.18[a] 0.57(8)

[a]differs significantly from control $p < 0.01$
 t-test

Figure 14 indicates that the blood hematocrit was consistently elevated at the end of the freshwater exposure phase (p<0.001) but dropped rapidly to control values after the fish had been in sea water for 24 h. Subsequently hematocrit remained close to or slightly below control values, although after 120 h in sea water, the hematocrits of the exposed group again became lower than in the controls (p<0.05).

Parallel muscle water determinations (Table XIII) confirmed previous results that the DHA exposure led to a hydration in fresh water; this was shown to be followed by a dehydration after the fish had been in sea water for 24 h (Fig. 14). By 48 h, however, the muscle water levels were restored to the control range although some fluctuation was evident. In Expts. 2 and 3 complete muscle water regulation was regained by 72 h as illustrated in Fig.14.

Measurements of the % water in the gut during Expt.2 (Table XII) showed that muscle dehydration (which approached but did not meet statistical significance in Expt. 2) was accompanied by an increase in gut water content at 24 h. The amount of water in the gut was again significantly increased (p<0.05) after 120 h in sea water although the muscle water content was by now back to control levels. Blood hematocrit, however was significantly reduced in exposed fish of Expt. 2 at this time (Table XI).

During Expt. 1 one fish was sampled when, having lost equilibrium, it appeared to be having serious problems with muscular coordination. These observations were made after the fish had been in sea water for ∿ 70 h. At the 72 h sampling period, this fish was still breathing regularly but was quite rigid. The results of plasma electrolyte analysis for this

Table XIII. Percentage water in muscle of sockeye salmon after a 5-day
exposure to 0.65 mg/L DHA in fresh water followed by
exposure to sea water (Expts. 2 and 3).

Percentage water in muscle

	Time in sea water hours	Control Mean SE(n)		Exposed Mean SE(n)	
	0	74.79	0.42(10)	75.69	0.18(11)
	24	73.27	0.30(9)	72.39	0.30(9)
Experiment 2	48	73.39	0.76(6)	74.52	0.20(10)
	72	74.91	0.16(8)	74.51	0.24(7)
	96	74.99	0.44(10)	74.46	0.19(8)
	120	74.92	0.23(8)	74.99	0.27(8)
	0	79.50	0.53(11)	81.17	0.59(12)[a]
	24	76.22	0.32(11)	75.34	0.43(12)
Experiment 3	48	76.86	0.20(12)	76.17	0.78(10)
	72	77.21	0.27(11)	77.38	0.49(10)
	96	77.18	0.57(12)	77.18	0.31(10)
	120	77.35	0.63(12)	77.74	0.22(12)

[a]
Differs significantly from control $p < 0.05$.

moribund fish are compared in Table XIV to mean values for controls and

exposed groups taken at the 72 h sampling period and clearly indicate that

this fish had lost the ability to regulate plasma ionic levels. The relation

of behavioral symptoms to hydromineral disturbance was further investigated

during Expts. 2 and 3.

Moribund and Drifting Fish

As the analysis of plasma ionic composition was not conducted until after

the completion of both Expts. 1 and 2, the presence or extent of ionic

disturbance was not known during the second experiment. Based on behavioral

observations, however 15 moribund fish were collected during the course of

Expt. 2 at various stages of debility and muscle and gut water determinations

were conducted. These results together with a description of symptoms are

presented in Table XV and illustrated in Fig. 15 for comparison with exposed

and control groups in Expt. 2.

The results illustrated in Figure 15 indicate that the muscle water content

during the freshwater exposure period was greatly elevated indicating hydration

which was subsequently followed by a tissue dehydration whose severity appeared

to be related to the time of seawater residence. Parallel measurements

revealed a concurrent rise in the amount of water in the stomach, although it

appears that this water ingestion may have been elicited in some fish several

hours prior to seawater entry (Fig. 15). Muscle water levels (Table XV)

in "inverted" fish (fish which had lost equilibrium) suggest that the de-

hydration may already be advanced at this point in the intoxication sequence.

The presence of much higher water content in the gut in one fish (3.0 h in

Table XV which was sampled immediately after equilibrium loss suggests

that the water accumulation may have been triggered by a combination of

Table XIV. The plasma ionic composition of an "exposed" fish
which lost equilibrium after 72 h in sea water
compared to mean values for controls and the
"normal" exposed fish sampled after 72 h in sea
water in Expt. 1.

	Control	Exposed	Inverted
Sum of ions mEq/L	304.4	307.1	387.6
Chloride mEq/L	133.2	130.8	177.5
Sodium mEq/L	159.4	164.7	187.3
Potassium mEq/L	3.95	3.41	4.47
Calcium mEq/L	5.88	5.93	7.33
Magnesium mEq/L	1.99	2.30	11.00
Hematocrit %	38.3	36.2	36.4

Table XV. Size, percentage muscle and gut water and description of symptoms in fish which appeared to be seriously affected during the course of Expt. 2.

	Time hours	Fork length cm	Wet weight g	Muscle Water %	Gut Water %	Observations[1]
Fresh water exposure	103.2	13.4	28.05	78.66	72.78	MB, YF
	103.2	13.5	28.80	78.36	75.32	MB, YP rigid
	115.1	16.1	48.47	77.16	85.05	YF
	115.1	15.5	36.42	77.97	81.56	Fungus on gills[2]
	120.0	15.4	36.80	78.14	85.67	MB
Time in sea water	2.0	15.2	35.20	78.34	89.67	MB
	3.0	15.1	35.20	76.29	88.05	IJ
	15.4	14.5	33.89	74.01	89.79	MB, SGG
	15.4	14.4	31.96	76.77	87.39	MT, SG, HM
	15.4	15.4	40.68	74.44	91.22	SG, HM
	15.4	14.9	35.72	74.13	87.24	SG
	19.6	15.3	33.15	73.61	90.06	MB, SGG, YP
	20.3	13.2	23.71	71.22	90.07	MT, SGG[3]
	24.4	15.2	36.10	72.91	80.23	I, SG, YP
	44.6	15.2	35.79	72.89	87.71	I, SG, HM, YP
	88.2	14.7	28.80	72.71	80.38	MT 1hr

[1]
HM hemorrhage in fin membranes
I(J) inverted (just)
MB moribund - still breathing
MT collected after death
SG swollen gut
SGG greatly swollen gut
YF yellow fish
YP yellow plasma

[2] Control fish

[3] Gut contents 780 mOsm/kg (SW 824 mOsm/kg)
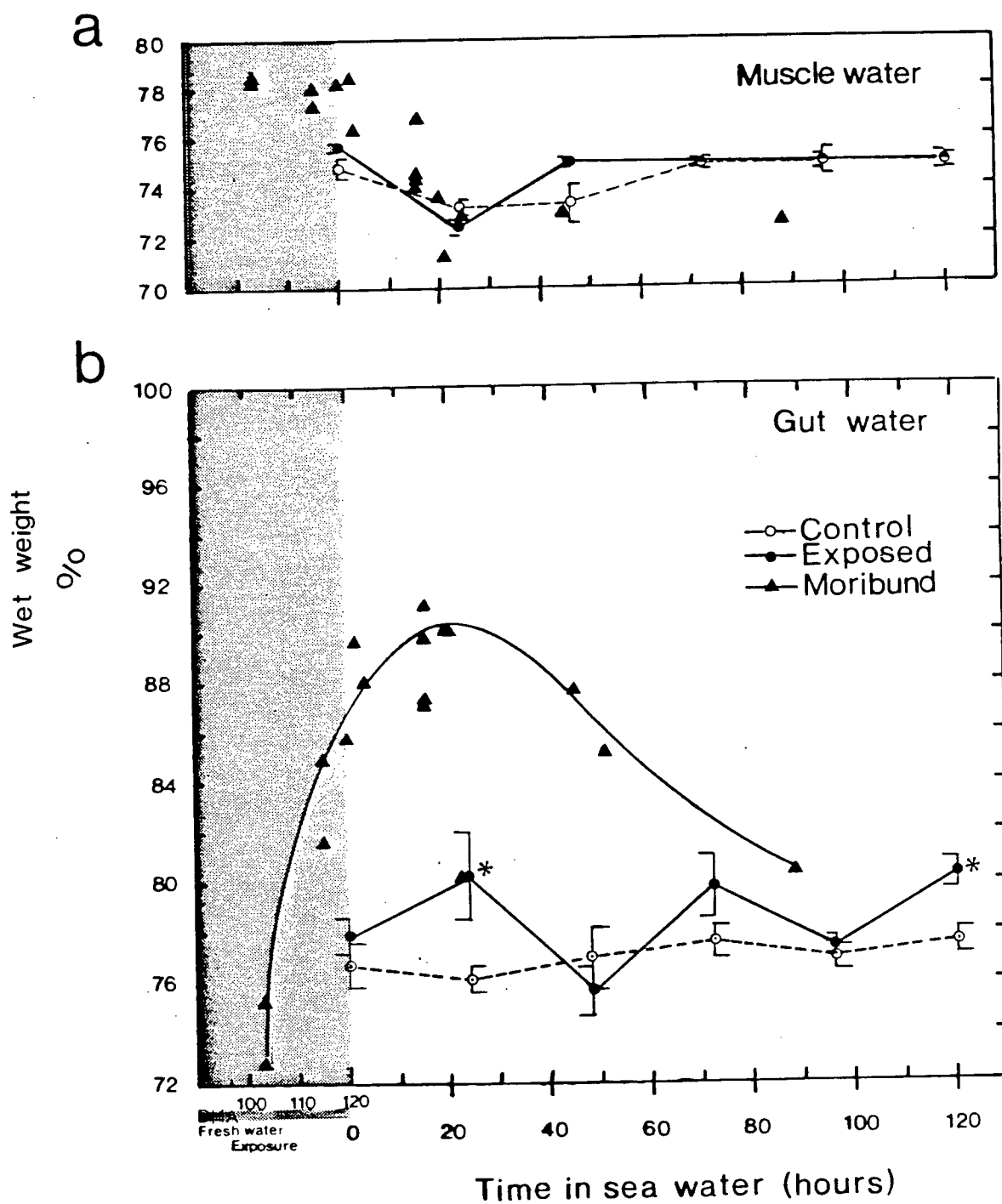
Figure 15. Relations between muscle (a) and gut water % (b) determinations on moribund fish and mean values for exposed and control groups in Expt. 2. Vertical bars indicate ±SE of the mean.

toxicant and seawater exposure, as the muscle water content of this fish (76.29%) was still higher than that of the control (74.79%) or of the exposed (75.69%) groups (Table XIII).

Among the symptoms listed in Table XV is that of a yellowish tinge of the fish which was accompanied by a yellowish plasma in those cases in which blood samples were taken. This color was later attributed to a form of toxicant-induced jaundice and is discussed in Appendix III.

As the observations made on moribund fish in Expt. 2 suggested a serious osmotic imbalance; during the course of Expt. 3 three fish were sampled at 48, 72 and 96 h when it became apparent that they could no longer maintain position against the current. These fish were termed "drifters" and were analyzed separately in the hope of establishing a link between their behavior and the degree of ionic imbalance. Based on past observations, "drifting" fish generally expired within 24-48 h, so that they would have probably added to the overall mortalities by the conclusion of Expt. 3.

The plasma electrolyte levels of drifting fish in Expt. 3 are compared to those of control and exposed groups in Figs.16 A to F and show that all the ions measured were considerably above their respective "exposed" means. The breakdown of total ions (Fig.16 A) indicates that the greatest changes appeared to have occurred in plasma Mg^{++} (Fig.16 F) which seemed to be increasing with time in sea water. Plasma Ca^{++} regulation (Fig.16 E) in exposed groups was restored within 48 h and there was some subsequent overcompensation when compared to controls. However, plasma Ca^{++} levels in drifting fish remained roughly double at this time. The major plasma cations Na^+ and Cl^-
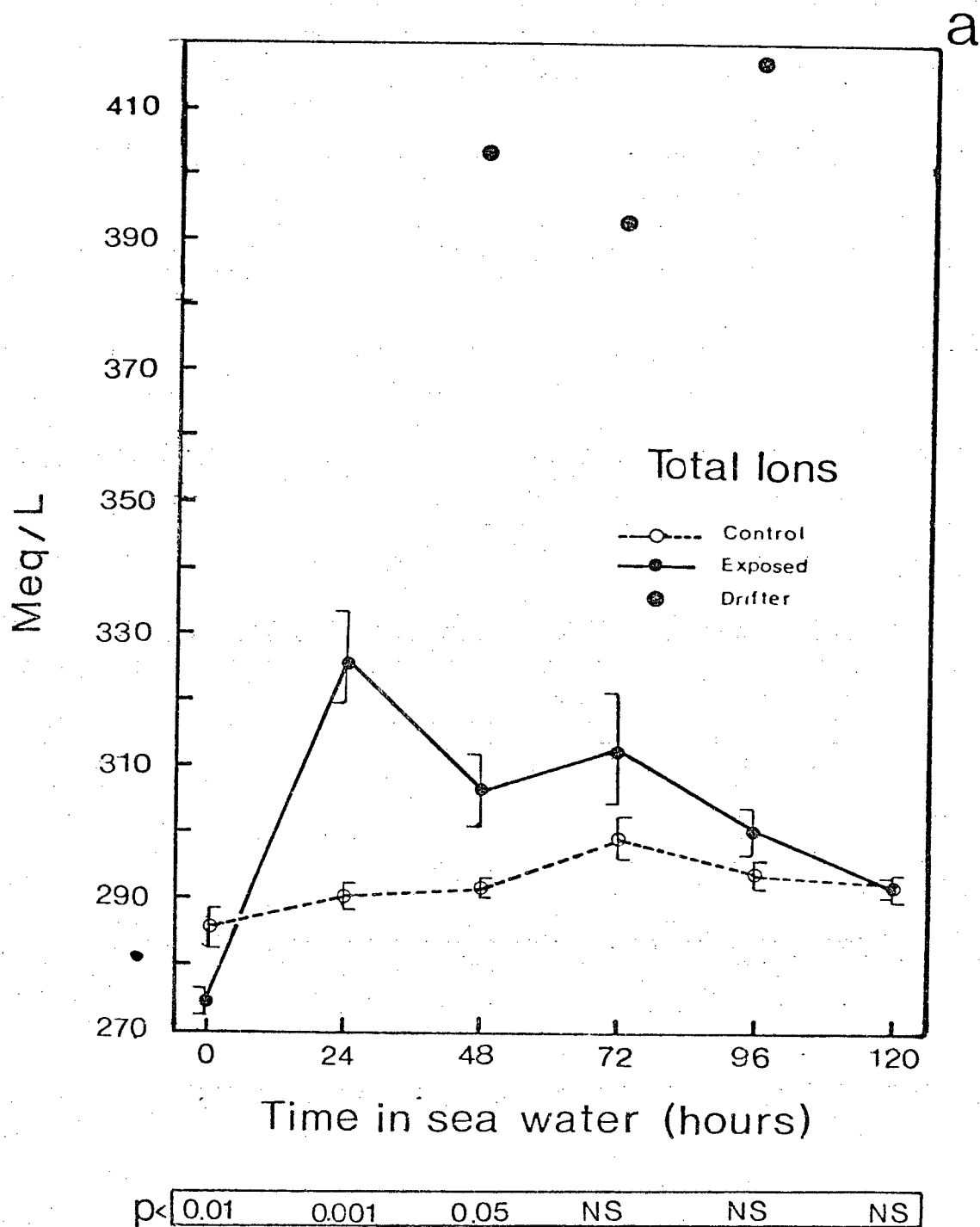
Figure 16. A comparison of plasma electrolyte concentrations
(a-f) measured in 3 drifting fish, with the means of control
and exposed groups during Expt. 2. Vertical bars
indicate ±SE of the mean and the level of significance
of the difference between control and exposed means is
given in the box below each graph.

b

Cl

c

Na

Control
Exposed
Drifter

Meq/L

Time in sea water (hours)

p<
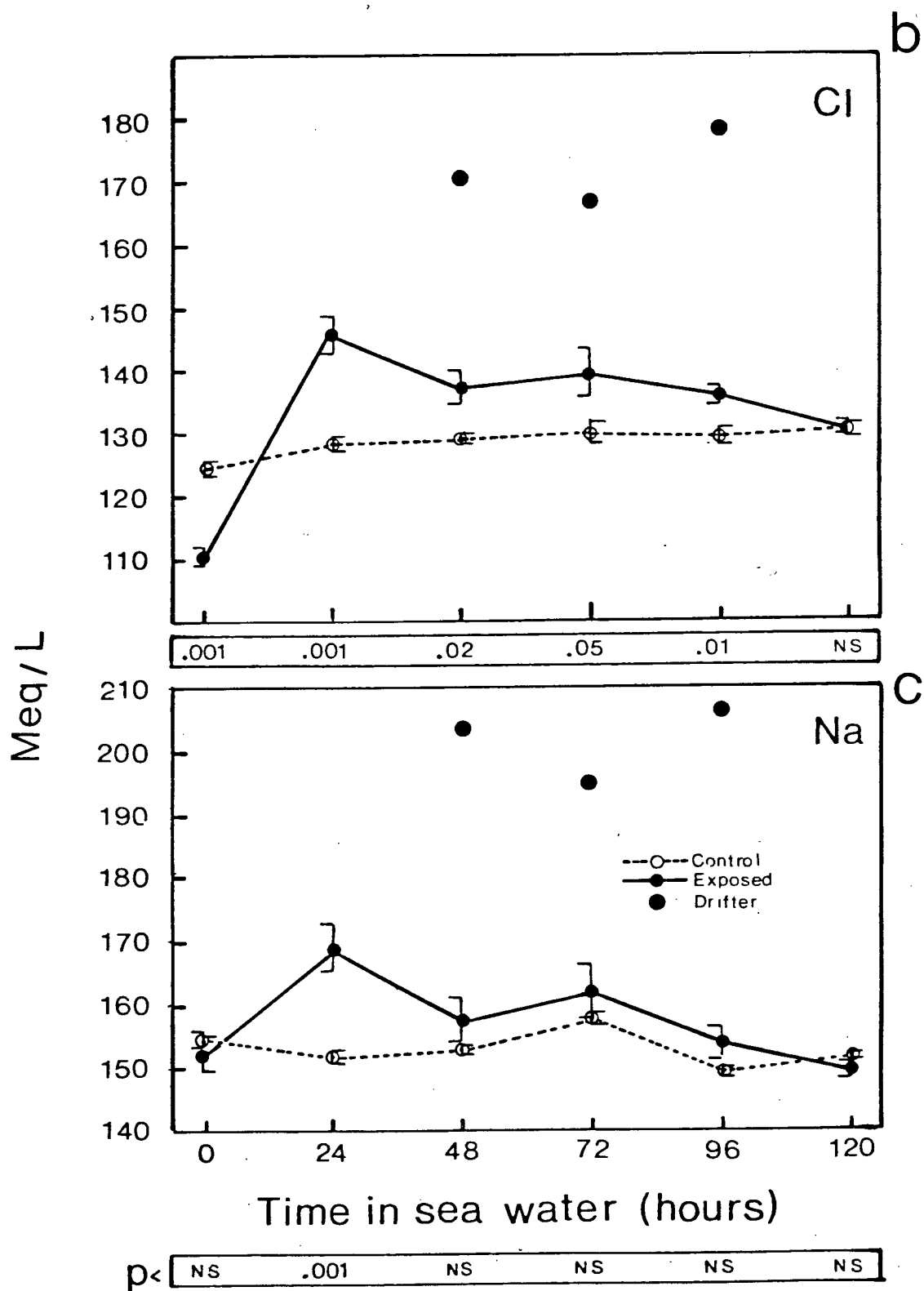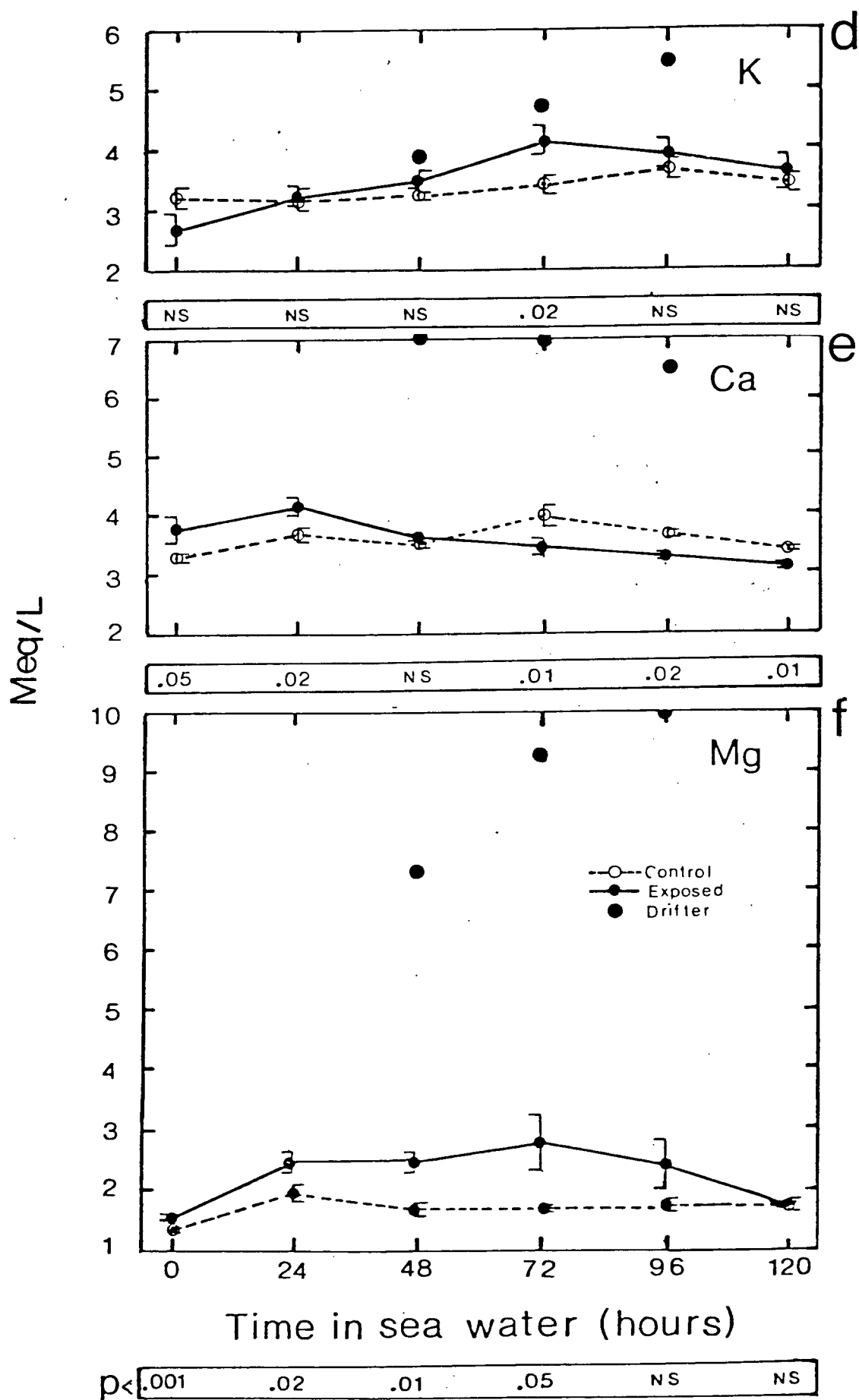
persisted at greatly elevated levels in drifting fish at a time when the previously exposed but behaviorally normal fish were regulating these ions towards control values (Fig. 16 B,C). While plasma K^+ levels remained closer to "exposed" means than any of the other ions, they did appear to be increasing with time in sea water.

These experiments confirmed that the osmotic imbalance brought on by sublethal exposure of salmon to DHA in fresh water was also accompanied by an electrolyte disturbance. Furthermore, the subsequent transfer of these fish into clean sea water resulted in a marked loss in the precision of plasma ion regulation and in a general dehydration.

As the maintenance of hydromineral balance in salmonids relies heavily on a reduction of the gill permeability and an increase in the gut permeability to water, an experiment was conducted to measure the effects of DHA exposure on this aspect of the function of these two osmoregulatory organs. Alterations in the permeability of either organ could contribute to the rapid rise in plasma electrolyte levels which was observed above.

B. EFFECTS OF DHA ON GILL AND GUT PERMEABILITY TO WATER

INTRODUCTION

The movement of euryhaline fish into sea water from the freshwater environment triggers a complex sequence of adaptive mechanisms which act in concert to maintain mineral homeostasis. Although rapid dehydration is avoided through a reduction in the permeability of the branchial epithelium to water (Maetz, 1970a) a net water loss still occurs and sea water is swallowed, absorbed across the gut and subsequently physiologically "distilled" to replace the free water lost by the gills (Smith, 1930). The reduction in gill permeability serves to limit this inevitable water loss while the mechanism of intestinal water transport enables the fish to replace branchial and renal water losses. Impediment in the reduction of free water movement across the gills would place an additional load on the gut to absorb more water and consequently on the branchial and renal ion pumps which dispose of the excess salt.

Since the electrolyte imbalance observed during the present study could have originated in a toxicant-related interference with normal permeability changes in the gill and/or gut, an experiment was conducted to test whether sublethal exposure of sockeye salmon to DHA in fresh water interfered with subsequent permeability changes in the gills and intestines during sea water adaptation. The water permeability of the isolated gill and the water transport ability of the isolated gut were measured in DHA-exposed fish after a 24 h recovery period in sea water.

Based on the isolated gill technique of Bellamy (1961) and Utida et al. (1967), a procedure was developed to measure the change in buoyancy of an isolated gill arch incubated in sea water for 1 h. This change in buoyancy

was assumed to be due to the loss of water across the gill epithelium making

the preparation more dense and therefore sinking deeper in the sea water and

registering an apparent weight increase on an electrobalance. The water

transport ability of the gut was measured by the weight changes of intestinal

sacs (Oide and Utida, 1967; Utida et al., 1967) filled with and incubated in

Cortland saline (Wolf, 1963). To enable sampling of individual fish without

disturbance of the others, an apparatus was designed in which electroshock

was used to rapidly stun a selected individual with no disturbance of the

other fish in the tank.

MATERIALS AND METHODS

Two similar experiments were conducted, one in June and the second in

July 1978. In both experiments, ten fish were exposed to 0.4 mg/L DHA in

fresh water for 120 h. An equal number of fish served as controls. The

toxicant was then discontinued, FW replaced by SW, and 24 h later individual

fish were removed and the gill and gut prepared for incubation. The exposure

to 0.65 mg/L DHA which was sublethal to sockeye salmon during the ionic

balance experiments proved to be lethal to fish confined in the tubes used

for the permeability study. The choice of 0.4 mg/L for the gill permeability

experiments was made on the basis of a comparison of the time to 50%

mortality (LT50) of the "tubed" fish exposed to 0.65 mg/L DHA,to the

toxicity curve (Fig. 9) obtained previously for free-swimming fish. By

extrapolation, 0.4 mg/L DHA was chosen to ensure 100% fish survival during

a 5-day exposure period.

Sockeye salmon smolts were obtained from the Great Central Lake run in

March 1978 and kept in outdoor holding tanks at PEI under conditions described

in the General Methods section. Approximately 25 fish were dip netted from

the outdoor tanks and transferred to the laboratory donut tanks in a bucket

containing 33 mg/L MS222 dissolved in water of 10 $^{\circ}$/oo salinity. Fish were

given a minimum of 96 h to acclimate to the flow conditions in the laboratory,

were fed once daily with OMP, and starved for 24 h prior to transfer to the

experimental tanks. At the time of transfer, a light anesthesia was brought

on by the addition of a seawater solution of 2 g MS222 to the tank (36 mg/L)

after shutting off the water supply and adding an air-stone lightly bubbling

oxygen into the water. As soon as the fish began to drift with the current,

they were netted and transferred to the tubes in the experimental tanks. Fish

of comparable size were selected and distributed alternately between the

control and test tanks. Each tank contained 10 fish which were placed in

individual plastic tubes suspended in the water current (Fig.17). The up-

stream end of each tube had a 6 mm mesh netting glued to it, while at the

downstream end, the netting was attached to a removable split plastic ring.

After the fish was inserted, the ring was replaced and following recovery

from anesthesia, the fish rested quietly on the tube bottom or swam slowly

against the current. Attached to the length of each tube were two strips of

2.5 cm aluminum tape (3M Scotch No. 425) forming electrodes on either side of

the fish (Fig.18). Each tube was wired to a Variac through a switching

circuit so that voltage could be applied to one tube at a time. Each tube was

also covered with black plastic to minimize visual disturbance. Using this

apparatus, individual fish could be instantaneously stunned in their tubes

and removed from the water without disturbing the upstream fish.

The fish were given an acclimation period of 72 h before the start of the

toxicant exposure. An air-stone ensured that dissolved oxygen levels remained

in excess of 90% saturation throughout the experiments and the temperature was

maintained at 11.5 $\pm$0.5°C.After the 120 h exposure period, followed by 24 h
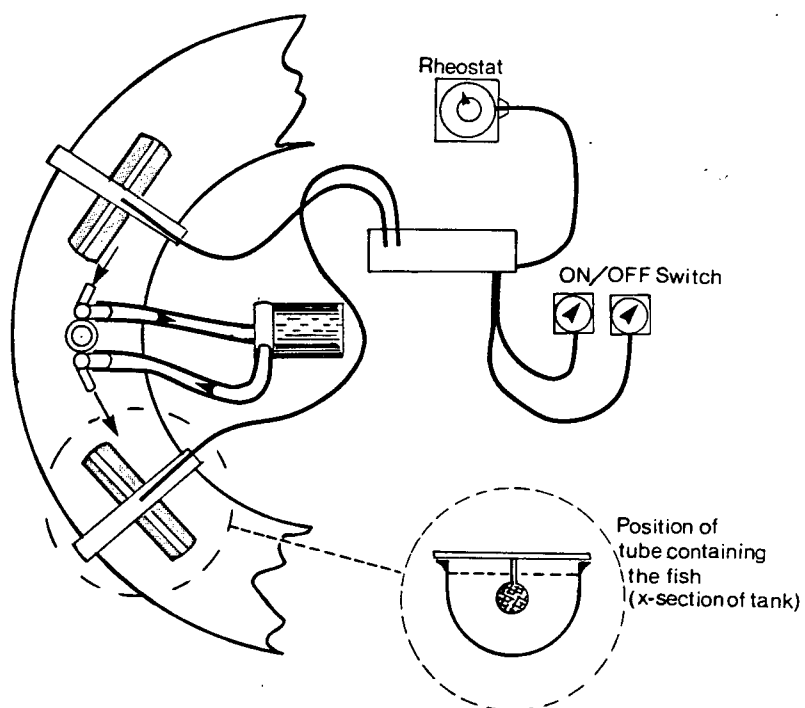
Figure 17. Illustration of a section of a donut tank showing
the positioning of plastic tubes to which sockeye
salmon were confined during a 120 h exposure to
0.4 mg/L DHA in fresh water.
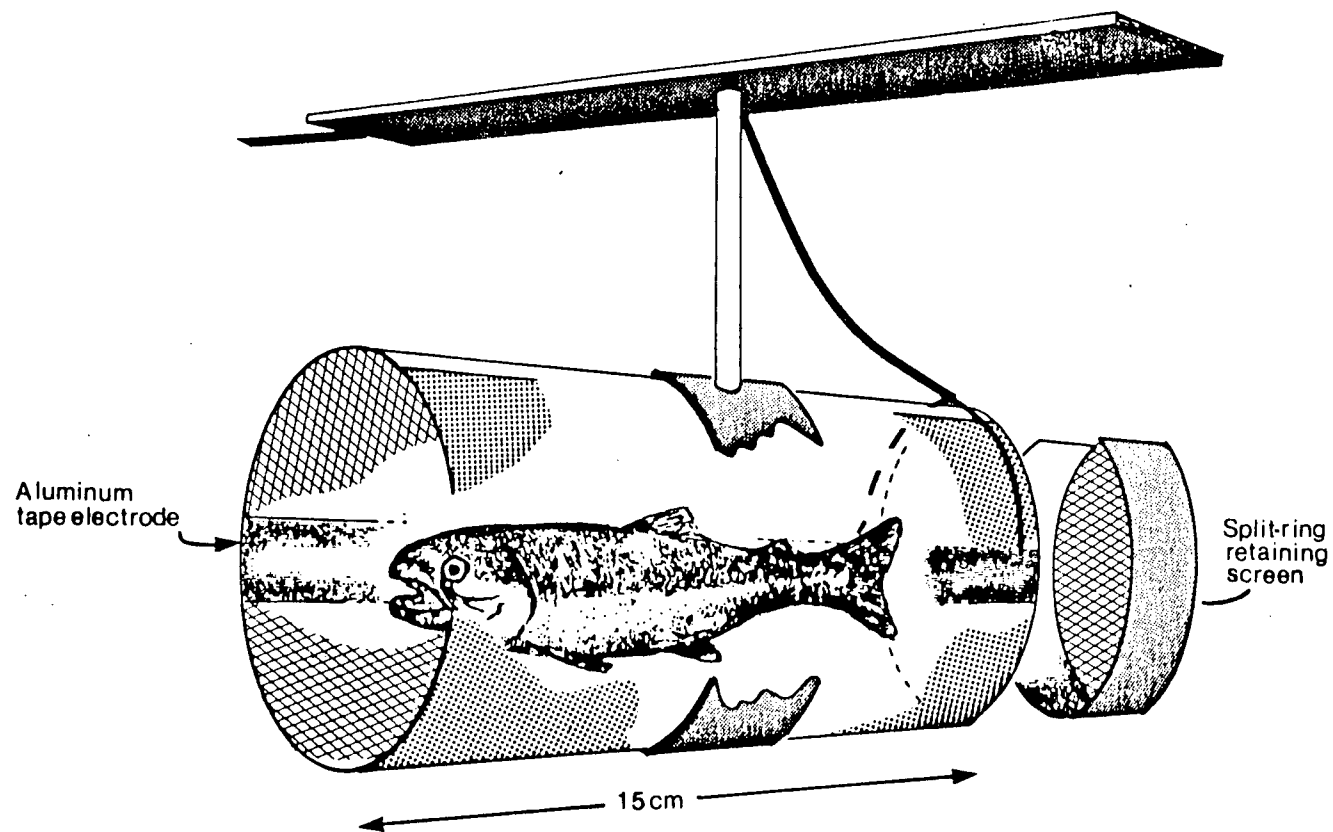
Figure 18. Detailed illustration of one of the plastic tubes used for the containment of sockeye salmon during their exposure to 0.4 mg/L DHA prior to the gill permeability experiments.

Aluminum tape electrode

Split-ring retaining screen

15 cm

in sea water, individual fish were stunned by electroshock (20V AC for 10s.).

The tube containing the immobilized fish was carefully lifted from the water

so as not to disturb the neighboring fish. The fish was then removed from

the tube, stunned by a blow on the head, measured, blotted and weighed. The

entire branchial basket was then rapidly removed as shown in Fig. 19, 1-12

with frequent rinsing with chilled (10^{0}C) Cortland saline. During step 3,

bleeding from the severed pseudobranchial artery was checked by means of

cautery. After removal, the first branchial arch was tied off with silk and

separated from the rest of the gill bars of the branchial basket. A stainless

steel hook (0.46 mm x 1.5 cm) was glued to the ventral inside surface of the

first gill bar with a drop of Eastman 910 cyanoacrylate adhesive. After a

rinse with deionized water, the entire preparation was transferred to the

beaker of the gill incubation apparatus (Fig. 20) in which water from the

constant temperature bath was circulated through the beaker water jacket. The

beaker was filled with aerated sea water (810 mOsM) and temperature equil-

ibrated while the dissections were carried out. The gill preparation was then

incubated for 1 h at 11.9 $\pm0.01^{0}$C ($\bar{X}$ $\pm$SE). The apparent change in gill weight

was monitored on a Sanyo 4092U Video Monitor using a Sanyo Video Camera VC1150

and recorded on a Sanyo Video Tape Recorder (VTR 1200) for future processing.

At the end of the gill incubation period, the preparation was removed

from the balance, the gill bars dissected away from the central cartilage

(Fig. 19, 13-14) placed in tared aluminum dishes and oven dried to constant

weight. The weight change of the gill was expressed in mg/100 mg dry gill

weight/hr.

The gut extraction was started as soon as the gill incubation was under

way ($\sim$ 5 min from fish electroshock). The abdominal cavity was opened and
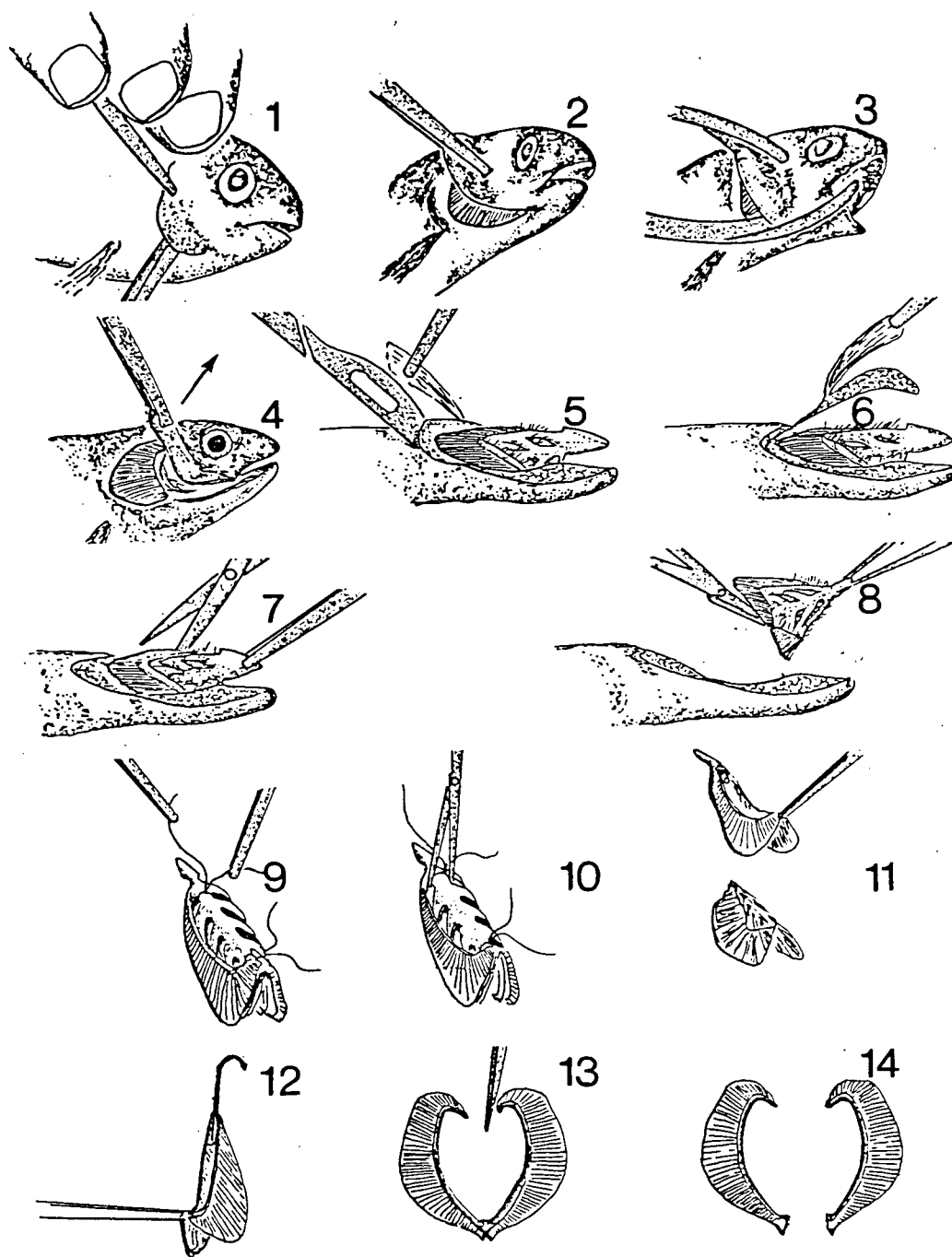
Figure 19. (1-14)

Dissection procedure followed to obtain the filament preparation used in the gill permeability experiment. Figure -12 shows the finished first branchial arch unit. After completion of the experiment, the cartilage was trimmed off (Figure -13 and -14) prior to the determination of dry weight.
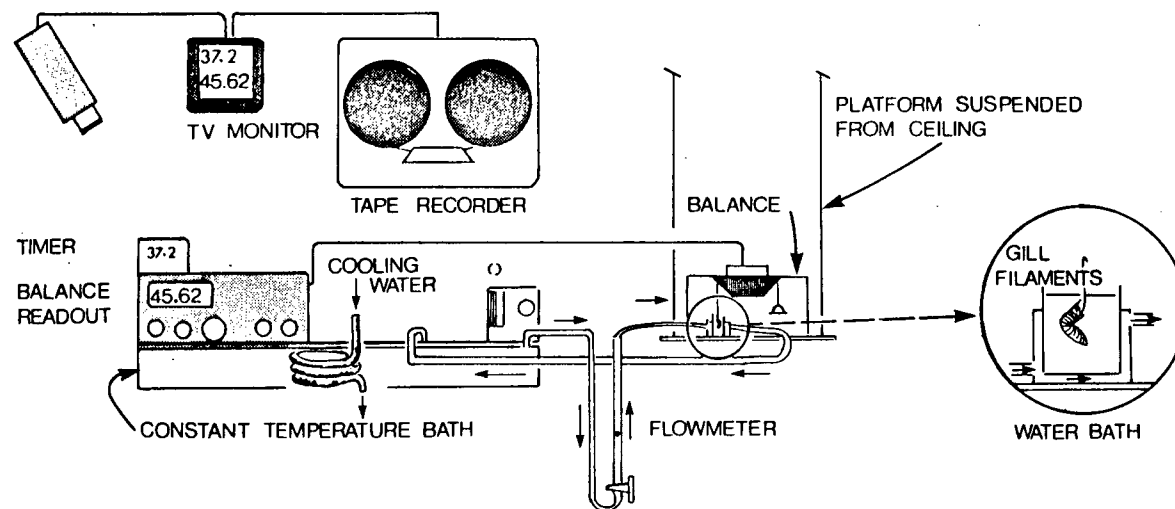
Figure 20. The apparatus used to measure the weight loss of isolated sockeye salmon gill filaments incubated in sea water. The fish had been previously exposed to 0.4 mg/L DHA for 120 h in fresh water.

the intestine was transected in the region of the vent, freed from the supporting mesentery anteriorly and cut just posterior to the last pyloric caecae. The gut was flushed clean with chilled (10°C), aerated Cortland saline (285 mOsM), then filled with 200 μl saline by means of a 1 ml syringe fitted with a 21 G needle tipped with 1 cm of PE 60 (Intramedic) polyethylene tubing. Both ends of the intestinal sac were tied off with surgical silk, adhering fat was gently teased off, and after blotting, the preparation was weighed to the nearest mg on a Mettler H 43 analytical balance.

It was then transferred to the incubation bath (Fig. 21) containing lightly aerated Cortland saline at 11.9 $\pm 0.1^{o}$C ($\bar{X} \pm SE$) for 1 h. At intervals of approximately 10 min, the sac was removed, blotted, weighed, and replaced in the saline. After the final weighing, the silk was snipped off, and the gut was dried to constant weight in a tared aluminum dish in a forced draft oven at 105°C. Weight change was expressed in mg/100 mg dry gut wt./h.

RESULTS

As the changes in weight of control gills in Expts. 1 and 2 were not significantly different, the data were pooled (Table XVI). The combined results indicate that the prior sublethal exposure of sockeye salmon to DHA in fresh water led to a significantly greater weight loss by their gills in sea water. The results of the gut incubation experiment indicate that while the amount of water transported by the intestines of previously exposed fish was considerably lower than the corresponding controls (Table XVII), the amount of variation in both groups was large and the difference was not significant.
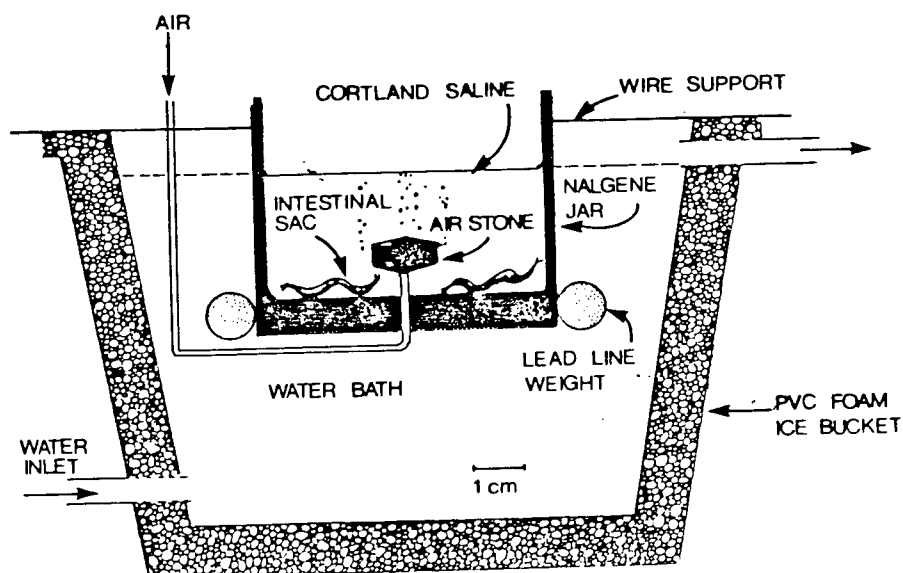
Figure 21. Diagram showing the apparatus used for the incubation of isolated intestinal sacs taken from sockeye salmon which had previously been exposed to 0.4 mg/L DHA for 120 h.

Table XVI.   Fish size and the loss in weight[a] (during sea
water incubation) of gill arches isolated from
juvenile sockeye salmon which had been
previously exposed to 0.4 mg/L DHA in fresh
water for 5 days[b].

(Mean ±SE)

	Fork length cm	Wet Weight g	Gill weight loss mg/100 mg dry weight/h
Control (19)	14.3 ±0.16	30.48 ±0.87	5.685 ±0.27
Exposed (17)	14.7 ±0.18	31.99 ±1.00	6.471 ±0.25[c]

[a] Weight of water lost.

[b] Gills were extracted from fish which had been in SW for 24
to 36 h.

[c] Differs significantly from control $p < 0.05$ (t-test).

Table XVII. Fish size and the loss in weight of isolated intestinal sacs taken from juvenile sockeye salmon which had previously been exposed to 0.4 mg/L DHA in fresh water for 5 days.[a,b]

	Fork length cm	Wet Weight g	Gut weight loss mg/100 mg dry weight/h
(Mean ±SE)			
Control (14)	14.1 ±0.17	29.44 ±0.87	45.18 ±4.97
Exposed (17)	14.7 ±0.18	31.99 ±1.00	37.73 ±4.31

[a]Intestines were extracted from fish which had been in SW for 24 to 36 h.

[b]Intestinal sacs filled with and incubated in Cortland saline.

DISCUSSION

Water and electrolyte homeostasis in fish is achieved through the coordinated functions of the gill, gut and kidney. Since plasma electrolyte levels normally are maintained within a relatively narrow range, their measurement provides a broad assessment of the performance of this integrated homeostatic function. General deviations from the normal range do not explain which control mechanisms have been disturbed but the patterns of osmotic and ionic change in relation to the external environment can suggest which mechanisms may be involved. However, the specific mechanisms by which the toxicant (DHA) affects hydromineral balance were not studied in the present investigation.

The present study established that sublethal exposure to DHA altered osmotic and ionic balance in sockeye salmon smolts in fresh water. In addition, when previously exposed fish were transferred into sea water, hydromineral balance was again disturbed for several days before returning to normal. Exposure to DHA also led to a change in gill permeability. An observed accumulation of fluid in the gut could not be attributed to a difference in the water absorptive function of the intestine but could represent an increase in the ingestion of sea water in response to dehydation in the hypertonic medium. Studies on DHA uptake showed that the resin acid accumulates several-hundred-fold in the tissues, notably the gill and the kidney; which are organs directly involved in electrolyte balance in salmon.

A consideration of the overall changes observed in hydromineral balance and in several hematological parameters suggests three possible patterns of toxic action: 1) DHA directly affects gill and gut permeability and increases the passive movements of water and electrolytes. 2) DHA exposure

leads to hypoxia and the observed changes in hematocrit and electrolytes

represent a secondary response; 3) DHA affects kidney function. Although

it is of course possible that all three of these actions occur simultaneously,

they nevertheless have been partitioned for the purposes of the discussion

and as a result some repetition has been unavoidable.

Changes in Permeability - Gill

Among the mechanisms utilized by euryhaline fish to maintain osmotic and

ionic balance in media differing widely in osmotic pressure and ionic compo-

sition is that of a change in gill permeability. The maintenance of low

osmotic permeability of the gill is essential to minimize water gain in fresh

water and thus reduces the amount of work required by the kidney to maintain

water balance. When euryhaline fish move from fresh water into the marine

environment, a reduction in gill permeability functions to diminish the

osmotic loss of water due to the gradient between the blood plasma ($\sim$ 300

mOsM/kg) and sea water ($\sim$ 1000 mOsM/kg). Evans (1969) reported a lower gill

water permeability in SW-adapted as compared to FW-adapted flounder and eels.

However, there exists a certain time lag before these adaptive permeability

changes are complete. The rate of branchial water loss in eels just after

transfer from FW to SW was almost triple that observed after 3 days in SW

(Oide and Utida, 1968) and Kamiya (1967) found that gills excised from eels

after 6-12 h adaptation to SW lost significantly less water than FW gills and

if the fish were given 24 h in SW, their gills behaved as SW gills. Because

of this time lag in the establishment of gill permeability characteristics,

in the present study sockeye salmon were given at least 24 h of sea water

adaptation prior to gill and gut extraction. The period in sea water varied

from 24 $\sim$ 36 h as it took about 75 min for a complete experiment to be

performed on one fish.

After entry of the fish into the sea, osmotic loss of water is recovered by ingestion of SW which is then absorbed across the intestine and subsequently physiologically "distilled" with the bulk of Na^+ and Cl^- excretion occurring across the gills. As these mechanisms take some time to become fully operational (Kirsch and Mayer-Gostan, 1973), the reduction in gill permeability may be an effective and energetically inexpensive method of limiting dehydration upon entry into sea water. The strategy used to accomplish adaptation to sea water appears to vary with species. Lahlou et al. (1975) showed that the trout Salmo irrideus depends primarily on reduction of peripheral water loss and therefore less on the active uptake of ions and water in the intestine. This energetically economical strategy was linked to the observation of a relatively slow start-up time for the gill Na^+ excretory mechanism. In this case, limiting the loss of water appears to be "cheaper" than replacing it after loss. The maintenance of an essentially homoiosmotic internal concentration during adaptation to sea water in the rainbow trout relies heavily on a rapid reduction of osmotic permeability (Gordon, 1963), much faster than some other euryhaline species such as the eel, flounder (Motais et al., 1966) and the killifish (Maetz et al., 1967).

In the present study, normal sockeye salmon were observed to maintain essentially homoiosmotic regulation during adaptation to sea water. On the other hand the salmon which had been pre-exposed to DHA in fresh water showed a considerable loss of plasma electrolyte regulatory precision after transfer to sea water. If sockeye salmon rely on a reduction in permeability to a similar extent as do trout, the observed ionic and osmotic disturbance may be linked in part to a toxicant-induced increase in gill permeability. The more pronounced water loss across isolated gills of salmon which had been previously exposed to DHA supports this hypothesis.

In many species, during the time that osmotic permeability is being reduced, there is a progressive augmentation of branchial permeability to Na^+ and Cl^- (Maetz, 1974) as well as an increase in the intestinal salt pumping efficiency and its attendant solute-linked water flow (Skadhauge, 1969). In three euryhaline species (the flounder, eel and killifish) the very low rate of sodium turnover in FW (0.01 to 0.55% internal Na^+/h) rose to 29.5 to 46.9% in sea water (Maetz, 1970b), with the rate of turnover generally directly proportional to salinity. These species rely on the rapid activation of a powerful salt pumping mechanism in the gills and the intestine to maintain salt and water balance; accordingly, their permeability to both salt and water is high and during adaptation to sea water, the electrolyte levels in the blood plasma depart considerably from FW levels. In marked contrast, the rainbow trout maintains a low Na^+ turnover in sea water (4-10%/h) as well as an only slightly increased Cl^- turnover rate (Lahlou et al., 1975; Gordon, 1963). Lahlou et al. (1975) also found that when trout were mildly stressed during seawater adaptation, Na^+ influx increased by 4-5 times while outflux only doubled, leading to a net gain of sodium until death. In the trout, the strategy appears to be primarily one of limiting salt loading and water loss during adaptation to sea water. Thus although energetically advantageous, such a strategy will render species using it more vulnerable to hydromineral disturbance by factors which directly or indirectly lead to increased gill permeability, as the homeostatic mechanisms which excrete salts and absorb water may be easily overloaded.

If the strategy followed by the rainbow trout is representative of salmonids in general, then a rapid salt loading of DHA-exposed sockeye salmon may occur, placing an excessive burden on osmoregulatory mechanisms which are

designed to avoid salt loading rather than to deal with it.

Although the permeability of the gills to salt was not determined in this study, an augmentation in branchial ionic permeability could explain the rapid rise in plasma electrolyte levels in DHA-exposed salmon undergoing adaptation to sea water. The mechanisms behind the apparent increase in gill permeability induced by DHA exposure are unknown. Residue analysis showed that DHA was accumulated by the gills (Appendix I-4) so a direct action on the permeability characteristics of the branchial epithelium may have occurred. Another possibility could be that in response to DHA-induced hypoxia a greater effective exchange area was perfused with blood at the time that the gills were extracted and incubation of gill arches in sea water led to a more rapid water loss.

Increases in the functional surface area of the gills have been shown to occur in response to hypoxic stress (Steen and Kruysse, 1964; Holeton and Randall, 1967a) and can result in an increase in passive water movements (Loretz, 1979). Both of these phenomena would be expected to render less effective the reduction in branchial permeability necessary for successful seawater adaptation. The effect of such a dysfunction would be to increase water loss and salt gain and may have contributed to the elevation in plasma electrolyte levels of sockeye salmon observed in the sea water stage of the present study. The time course of recovery of hydromineral balance as indicated by the gradual return of plasma electrolytes to control values suggests that gill permeability may be disturbed for $\sim$ 3 days after sublethal DHA exposure. All plasma electrolyte and muscle water levels had returned to normal by 120 h in sea water indicating that irreversible changes had not occurred.

The presence of calcium ions appears to be essential in fishes to ensure

successful adaptive permeability changes to both water and electrolytes

(Potts and Fleming, 1970, 1971). This effect may be largely a passive process

related to changes in the structural properties of the diffusion barrier of

the gills, such as stabilization of the mucus coat or the cell membrane

(Mashiko and Jozuka, 1962). The addition of calcium to fresh water in amounts

equivalent to seawater concentration reduced the urine flow in the brown trout

(Salmo trutta) by 32% (Oduleye, 1975). As urine flow is a measure of the

osmotic permeability in freshwater fish, this reduction in flow may represent

a significant decrease in the work required of the kidney.

The observed elevation of plasma Ca^{++} levels may be an adaptive response

in an effort to reduce the permeability of the gills to water influx. As

the calcium content of the fresh water used in the present study was very low

(0.8 mEq/L) it is doubtful that the rise in plasma Ca^{++} was due to an increased

uptake. More likely is the mobilization of calcium from reservoirs such as

scales and bone (Mashiko and Jozuka, 1962) which can serve as a calcium

source when required during times of stress (Ichikawa, 1953; Urist, 1966;

Weiss and Watabe, 1978). Although the reduction in gill permeability by the

presence of external calcium has been well established, I was not able to find

any literature on whether an elevation of plasma Ca^{++} levels from within has

a similar effect. Plasma Ca^{++} levels in freshwater fish are known to be

maintained by prolactin secretion, which in turn is controlled by environmental

Ca^{++} and Mg^{++} concentrations. Ogawa (1974) showed that prolactin injections

into eels and rainbow trout prior to sacrifice reduced the water permeability

of isolated gills incubated in deionized water. It appears probable that a

lowering of plasma Ca^{++} levels may trigger prolactin secretion leading to an

increase in plasma Ca^{++} concentration and possibly its binding at the gills
which then influences plasma ionic composition and osmolality (Wendelaar
Bonga, 1978).

Changes in Permeability - Gut

The ability of the isolated intestine to transport water appeared to be
little affected in fish exposed to DHA in fresh water and then transferred to
sea water for 24 h. However, the increased water content in the stomachs of
salmon which were dehydrated may indicate that the rate of water absorption
across the gut could not keep up with the rate of water loss across the gills
in these fish. Under normal circumstances, the process of seawater absorp-
tion involves an initial movement of plasma water into the gut (Utida et al.,
1967; Skadhauge, 1969); excessive ingestion may lead to a further dehydration
and salt loading of plasma (Kirsch and Mayer-Gostan, 1973). Dehydrated fish
cannot gain more water by drinking more as there is a limit to the amount of
salt and its attendant water flow that the intestine is capable of absorbing,
especially during the early stages of adaptation to sea water (Skadhauge,
1969). This emphasizes the importance of reducing osmotic permeability of the
gills during sea water adaptation (Kristensen and Skadhauge, 1974).

Although the ingestion of some fresh water by teleosts has been well
documented, (Maetz and Skadhauge, 1968; Gaitskell and Chester-Jones, 1971;
Potts and Fleming, 1970) and is not considered to be energetically dis-
advantageous (Shuttleworth and Freeman, 1974), the ingestion of abnormal
amounts of fresh water did lead to a rapid general hydration in eels (Kirsch
and Mayer-Goston, 1973). In the present experiment, the increased ingestion
of water was frequently observed during freshwater exposure of salmon to DHA.
The dissection of fish having a bloated appearance revealed a turgid stomach
filled with water. As the maintenance of water permeability, initiation of

branchial salt excretion, and the drinking reflex have been shown to be under

the control of the nervous system (Mayer-Gostan and Hirano, 1976), the

indicated stimulation of drinking in freshwater-adapted sockeye salmon may

be related to the accumulation of high concentrations of DHA in the brain or

other parts of the nervous system. On a wet weight basis, the brain tissue

contained the highest levels of DHA of all the organs analyzed in this study

(Appendix I-4). In addition, the stomach turgidity frequently observed during

DHA exposure could be expected to exert considerable pressure on internal

organs. Such a mechanical blockage could have interfered with the normal

flow of bile and contributed to a form of obstructive jaundice observed in

DHA-exposed fish and which is described in Appendix III.

Blood Hematocrit and Plasma Electrolyte Changes

The blood sampled from salmon exposed sublethally to DHA in fresh water

frequently appeared to be more viscous and darker in color than that taken

from control fish, indicating that some form of hemoconcentration had

occurred. Hemoconcentration was further indicated by the highly significant

increase in blood hematocrit observed in fish after 120 h of DHA exposure.

On the other hand, the concurrent reduction in plasma osmolality suggested

that hemodilution had occurred, as might be expected in fish suffering from

an osmotic imbalance in fresh water. A possible explanation of this apparent

discrepancy is offered after a discussion of three mechanisms which can act

to increase hematocrit in fish.

An increase of hematocrit is a well-known response of fish to hypoxia

(Doudoroff and Shumway, 1970) which can be an adaptive measure to increase

the oxygen carrying capacity of the blood. At least three mechanisms can

contribute to a rise in hematocrit: 1) a decrease in plasma volume,

2) an actual increase in red blood cell numbers (polycythemia) 3) an increase in red cell size due to swelling. Any one or combination of these changes will lead to an elevation in hematocrit.

A decrease in plasma volume in response to hypoxia can result from movement of water from the blood to tissues (Hall, 1928; Black et al., 1959; Soivio et al., 1974c) or an increased diuresis (Westfall, 1943; Hunn, 1969; Swift and Lloyd, 1974; Lloyd and Swift, 1976). These fluid shifts can result in hemoconcentration and a rise in hematocrit. In the present study, hemo- concentration due to fluid shifts from the plasma into the tissues can be ruled out since the plasma osmolality in DHA exposed fish in fresh water dropped (Fig.12) rather than increased as might be expected if plasma water were being removed from the circulation. After transfer to sea water, plasma osmolality was significantly elevated at 24 h but hematocrit was not. However, when plasma osmolality of both groups became essentially identical at 120 h, the hematocrits of exposed fish were significantly below those of controls.

An actual increase in the numbers of red blood cells can result from a stimulation of hematopoietic activity and/or release of erythrocytes from storage depots such as the head kidney (Ostroumova, 1957; Zanjani et al., 1969; Fromm, 1977). Some authors have suggested that the spleen may also serve as a storage organ for erythrocytes which can be released into the circulation in response to hypoxic stress (Dawson, 1935; Lloyd and Swift, 1976). It is doubtful however, that splenic contraction alone could contribute enough red blood cells to alter hematocrit significantly as Stevens (1968) determined the blood volume of the spleen in rainbow trout to be 70 μl/100 g body weight. Considering a blood volume of 5% of the body weight (Smith, 1966) and an

initial hematocrit of 30%, even a complete emptying of the splenic contents (assuming its entire blood volume comprised erythrocytes) would only raise the hematocrit to 31.3% in a 100 g trout. It is more likely then that the spleen is involved in the uptake and destruction of red blood cells and functions as a storage site for iron in the form of hemosiderin bodies (Grover, 1968; Yu et al., 1971) and responds to hypoxia by supplying the iron required for hemoglobin synthesis in the hematopoietic tissue of the kidney (Ostroumova, 1957). The evidence suggests that the contribution of the spleen to raising hematocrit is probably indirect and not immediate.

If as suggested, DHA exposure in fresh water led to hypoxia, a stimulation of erythropoiesis (red blood cell formation) may have occurred with red blood cells being added to the circulation in sufficient numbers to account for the higher hematocrit. Although red blood cells counts were not performed, it is unlikely that the observed rise in hematocrit (from 34% to 45%, typical) could have been due to polycythemia because within 24 h of recovery in sea water, the hematocrit of exposed and control fish was similar. This would have required a rapid removal of a large number of red blood cells from the circulation. Although such a function was implied for the liver and the spleen by Swift and Lloyd (1974), based on blood volume determinations for these organs (Stevens, 1968) it seems unlikely that such a storage of red blood cells would occur. Soivio et al. (1974a) could not find any particular area in the trout kidney specialized for blood storage nor any evidence for a release of erythrocytes from that organ during hypoxia.

The rise in hematocrit during toxicant exposure in fresh water followed by a rapid drop after the fish were transferred to sea water, when considered together with changes in plasma osmolality, rather suggests a swelling and

shrinking of erythrocytes. If indeed DHA exposure contributed to a hypoxic

condition in sockeye salmon through some interference with gas exchange

mechanisms, then a buildup of CO_2 could occur in the blood. In salmonids,

the swelling of red blood cells in response to hypoxic stress can be caused

by small increases in plasma CO_2 levels (Benditt et al., 1941 ; Irving et al.,

1941). Cellular swelling contributed to increased hematocrits in hypoxic

rainbow trout (Holeton and Randall, 1967a) and Soivio et al. (1974b,c)

confirmed that the swelling observed in vivo in trout could also be demon-

strated in vitro at reduced O_2 levels. The incubation of swollen erythrocytes

in O_2 did not result in complete volume regulation and these authors concluded

that the volume change was a complex response involving changes in pH, PCO_2 and

lactic acid as well as some unknown metabolite. Casillas and Smith (1977)

reported that a localized tissue hypoxia after muscular exertion in trout

resulted in a swelling of erythrocytes and led to a sharp rise in hematocrit.

The in vitro addition of lactic acid to the blood of the sucker (Catastomus

commersoni) led to a 30% increase in hematocrit also as a result of erythrocyte

swelling (Black and Irving, 1938). Hypoxic conditions may contribute to a

buildup of these metabolites in the tissues or in the red blood cells per se.

Eddy (1977) determined that the O_2 uptake by the blood formed a significant

portion (4.5% at $20^{o}C$) of the total energy consumption of trout, and may be

associated with the metabolic processes of the red blood cell.

Recent work has shown that volume changes in red blood cells of fish can

also be brought about by osmotic and ionic movements as part of the mechanism

of "isosmotic intracellular regulation". A decrease in plasma osmolality

resulted in swelling of flounder erythrocytes (Fugelli, 1967) and Cala (1977)

showed that subsequent cell volume regulation was accomplished by means of a

direct change in the permeability of the membrane to Na^+ or K^+. Net water

flow was secondary to net inorganic cation flux. Changes in the ionic

composition of plasma must be accompanied by ionic shifts within erythrocytes

to maintain ionic and osmotic equilibrium (Munroe and Poluhowich, 1974;

Oikari, 1978). There is also considerable evidence to indicate that even in

relatively stable osmoregulators, fluctuations in plasma osmolality result

in significant shrinking or swelling of red blood cells (Schmidt-Nielsen,

1975). Thus it appears that successful osmoregulation in teleosts undergoing

a change in salinity involves both extracellular and intracellular components.

In the present study, a drop in plasma osmolality at the end of the DHA

exposure period was accompanied by an increase in hematocrit. Measurements

of the dimensions of red blood cells showed that swelling had indeed occurred.

The response of sockeye salmon to sublethal DHA exposure in fresh water

probably represents a complex physiological reaction to the toxicant stress.

Although the actual mechanisms of toxicity were not investigated, the data

suggest that a form of hypoxic stress is involved which results in, or is

accompanied by an osmotic influx of water. Reductions in plasma osmolality

were accompanied by an increase in size of the red blood cells; a volume

increase which for some reason was not regulated. This could result from the

partitioning of DHA into the lipid components of the red cell membrane altering

its permeability and/or interfering with the ion pumping mechanism involved

in the process of volume regulation. After the toxicant exposure was dis-

continued and the fish transferred to sea water, the rise in plasma osmolality

would facilitate water outflux from the erythrocytes into the hypertonic

medium and thus account for a lowering of the hematocrit. The depression of

hematocrit measured in exposed fish after 120 h in sea water may represent a

shrinking of red blood cells. As plasma osmolality was in the normal range

at this time, this may be due to inadequate volume regulation after hyperosmotic

stress, perhaps due to an alteration in cell membrane permeability. Some evidence for DHA-induced hypoxia was provided during the course of acute DHA bioassays. Symptoms such as an increased breathing amplitude and frequent coughing indicated that the salmon were undergoing respiratory distress. This was confirmed during the hypoxia bioassay which demonstrated a dramatic increase in toxicity of a normally sublethal dose of DHA brought on by a reduction of dissolved oxygen levels to 75% saturation. These results suggested that DHA may interfere with the mechanism of oxygen uptake by the blood, leading to hypoxemia. Additional support for this view was provided during the gill permeability experiments when it became apparent that the confinement of fish to the tubes increased their susceptibility to DHA. As dissolved oxygen levels were maintained in excess of 90% saturation in these experiments, it is unlikely that the apparent increase in toxicity was due to reduced oxygen availability. However, if DHA were to interfere with normal transfer of oxygen over the gills, the reduction in swimming activity of fish confined to the tubes would have reduced ram ventilation and could have led to hypoxemia. Because of the added resistance of the fish retaining screens, the velocity of the water current passing through the tubes was about half of that against which sockeye salmon maintained position when swimming free in the donut tanks. Iwama et al. (1976) observed an increase in toxicity of DHA to juvenile coho salmon at reduced activity levels. In all the experiments conducted, sockeye salmon attempted to maintain positive rheotaxis during acute stages of DHA toxicity. Although one would assume that swimming with the current would have been energetically much easier, this did not occur. A concerted effort to head into the current may be an adaptive response to increase passively the volume of water irrigating the gills.

Although no information is available on a hypoxia-induced increase of resin acid toxicity,Davis (1973) found that sockeye salmon exposed to sublethal levels of bleached kraft mill effluent (BKME) responded with an increase in ventilation volume and coughing rate. In spite of an increase in oxygen consumption, there was an average 20% reduction in the oxygen saturation of arterial blood. The effluent used in that study had been aerated, neutralized, and filtered so that the residual toxicity was probably attributable to the more persistent toxic components such as resin acids and chlorinated organics. I.H. Rogers (personal communication) later confirmed the presence of DHA (1-2 mg/L) in effluent from the same pulp mill.

Davis (1973) suggested that part of the observed reduction in the efficiency of gas exchange may have been due to an elaboration of mucus by the gill epithelium,as Walden and Howard (1968) reported an increase in mucus production at the gills of underyearling salmon exposed to neutralized BKME.

The effects of an increase in diffusion distance on fish respiration have recently been estimated. Based on the assumption that the thickness of the lamellar diffusion barrier was $\sim$ 5 μm (Steen, 1971) the elaboration of a 5 μm thick layer of mucus on a secondary lamella was calculated to lead to an 81% increase of the diffusion resistance to oxygen (Ultsch and Gros, 1979). They also suggested that the presence of this mucus would result in the reduction of the effective respiratory water flow by half. The compensatory hyperventilation with its attendant oxygen demand at a time when O_2 flux into the blood was being restricted by additional mucus corresponds quite closely to the observations of increased ventilation, O_2 consumption and depressed arterial PO_2 in salmon exposed sublethally to BKME (Davis, 1973). In the present study, no excess mucus production was noted, although an increase

in thickness of the order described by Ultsch and Gros (1979) could have

occurred in response to DHA exposure.

Additional evidence for DHA-induced hypoxia may be suggested from the

pattern of plasma electrolyte changes in salmon exposed to DHA in fresh water

which followed closely that reported in the literature for hypoxic fish. At

the end of the freshwater exposure period plasma Na^+ was unaltered, Mg^{++} was

elevated and K^+ was significantly lowered. An identical pattern was observed

by Soivio et al. (1975) during in vitro incubation of rainbow trout blood under

falling oxygen tension. A progressive rise in hematocrit was accompanied by

an influx of K^+ into the erythrocytes from the plasma. Platner (1950) found

that a combination of hypoxic stress and lowered temperature resulted in a

marked increase in plasma Mg^{++} levels in the goldfish which he attributed to

a leakage of Mg^{++} from the erythrocytes into a plasma diluted by water uptake.

In the present experiments the lowering of plasma osmolality and elevation of

muscle water concentrations indicate a water retention and/or an increase in

water uptake from the environment by DHA-exposed fish.

Kidney function

As no attempts were made to measure kidney function in the present

investigation, the following discussion is based on an interpretation of the

changes observed in plasma electrolytes in the light of the known role of

the kidney in hydromineral balance.

The kidney plays an indispensable role in osmoregulatory adaptations

which determine euryhalinity in teleost fishes such as the salmon. In

fresh water, in spite of a relatively low overall permeability, there is a

continuous osmotic influx of water into the fish due to the osmotic gradient

between the plasma ($\sim$ 300 mOsM/kg) and the medium (< 5 mOsM/kg). The kidney

counteracts this influx of water by producing an abundant, strongly hypotonic (to plasma) urine and together with the urinary bladder achieves conservation of filtered ions. In sea water the osmotic gradient between the plasma ($\sim$ 300 mOsM/kg) and the medium ($\sim$ 1000 mOsM/kg) is considerably greater and in the reverse direction so that the fish tends to become dehydrated. The functions of the kidney and bladder in sea water are to minimize water loss by re-absorption of water and monovalent ions, to produce a urine isotonic to the plasma, and to excrete divalent ions which enter with swallowed sea water (Smith, 1930).

The implication that DHA exposure of salmon to fresh water results in hypoxia has already been discussed. One of the adaptive mechanisms by which fish respond to hypoxic stress is that of increasing the functional surface area of the gills (Steen and Kruysse, 1964; Holeton and Randall, 1967a). While an increase in the perfusion of gill lamellae facilitates oxygen uptake, it concurrently augments the surface area available for passive osmotic and ionic movements. This leads to an increased uptake of water over the gills in fresh water (Loretz, 1979). In hypoxic trout Lloyd and Swift (1976) found an increased permeability to water which persisted beyond the time when ventilation had returned to normal. This increased permeability was reflected in compensatory increases in urine flow rate as well as a reduction of the active uptake of sodium and chloride by the gills (Swift and Lloyd, 1974). Increased diuresis combined with a reduction of salt reabsorption by the kidney led to the excretion of abnormally high concentrations of Na^+, K^+, Mg^{++} and Cl^- in the urine of rainbow trout after hypoxic stress (Hunn, 1969).

In the present study, arguments have already been presented to support the view that DHA exposure in fresh water led to an increase in water loading possibly via the mechanism discussed above. The rapid influx of water across

the gills and possibly also across the gut may have exceeded the capacity

of the kidney to maintain water balance. At high rates of diuresis, the

efficiency of the renal salt reabsorptive mechanism is reduced due to shortened

urine residence time both in the kidney tubules and in the urinary bladder.

As normally between 80-90% of NaCl is reabsorbed (Lahlou, 1970) this can

result in the excretion of urine containing abnormal concentration of electro-

lytes,especially chloride, such as has been shown to occur after handling

stress and "laboratory diuresis" (Forster, 1953; Forster and Berglund, 1956;

Grafflin, 1931; Holmes, 1961). The characteristic lowering of plasma Cl^-

in DHA-exposed fish during freshwater residence may be due to an unavoidable

increase in the renal excretion of this ion accompanying a compensatory rise

in urine production.

Another possibility, other than a simple overloading of the kidney,

which could contribute to a renal insufficiency may be a direct effect of

high levels of DHA on the kidney function per se. During the sublethal

exposure employed in the present study, DHA levels in the kidneys reached

278.1 µg/g. The effects of such a concentration are unknown but could

possibly disrupt normal cellular functions. Based on the unit surface area

available for salt transport, the cells of the renal tubules are the sites

of intense exchanges (Lahlou, 1970), which have been shown to involve Na^+-K^+

ATP-ase (Jampol and Epstein, 1970, Epstein et al.,1969). Enzyme systems

involved in active transport may be sensitive to high concentrations of DHA.

Trump and Jones (1977) described a fundamental correlation between the

inhibition of active transport and ultrastructural changes in cells of the

teleost nephron. These were related to the alteration in permeability of

the plasma membrane and mitochondria by a variety of toxic agents. Studies

on organ histopathology were not conducted in the present study but Fujiya (1965) reported kidney tubule necrosis in marine fish taken from waters adjacent to a kraft pulp mill.

DHA-induced kidney dysfunction could have equally important consequences for fish moving into sea water as the successful transition of a migrating salmon from a river to the marine environment depends to a large extent on the ability of its kidney to assume a very different osmoregulatory role, often within a matter of hours. The critical adjustment required of the kidney in a fish faced with increased salinity is a prompt reduction in urine flow to limit water loss. This is accomplished by a reduction in glomerular filtration rate (GFR) (Hickman and Trump, 1969). Failure to accomplish this quickly will lead to a rapid dehydration.

After the transfer of DHA-exposed sockeye salmon to sea water, hydration was replaced by a rapid dehydration as demonstrated by a rise in plasma osmolality and all measured ions. This was followed by a gradual regulation back down to normal levels after 120 h in sea water. In DHA-exposed fish in fresh water, urine flows could be expected to be elevated to contend with the toxicant-induced hydration. If such a fish is rapidly transferred to sea water, it is conceivable that there might be a delay in the necessary adaptive reduction in GFR. Initially this would aid in the disposal of excess water but if continued could lead to a rapid dehydration.

It is probable that the changes observed at the 24 h sampling period do not represent the maximum excursions in plasma electrolytes which occurred. Utida (in Fontaine, 1975) observed that during the transfer of smolts of the salmon Oncorhynchus masou from FW to SW, maximum plasma

osmolality was reached within 8 h and was subsequently regulated back down to

normal seawater values. In DHA-exposed sockeye salmon the measurements of

plasma electrolytes in behaviorally abnormal fish revealed that they were not

able to regain control of hydromineral balance after this rapid departure from

normal electrolyte levels. The severity of locomotory difficulty appeared to

be related to the extent of this departure from the plasma ionic levels

measured in affected(but still regulating)fish. In fish which were drifting

(i.e. could not maintain position against the current) or had just lost

equilibrium,plasma electrolyte levels were grossly increased above both the

controls and exposed (but resisting) groups. Of all the ions measured,

plasma Mg^{++} showed the largest relative increase and the slowest regulation.

This key finding provides more direct support for the involvement of the kidney

in DHA-induced hydromineral balance observed in salmon during seawater

adaptation. Beyenbach (1974) found that the tolerance (before loss of

equilibrium) to Mg^{++} in rainbow trout was directly related to the capacity

of the renal secretory system to maintain magnesium homeostasis.

During the adaptation of fish to sea water, the temporally second most

important function of the kidney becomes that of divalent ion secretion;

especially of magnesium and sulfate which are absorbed across the intestine

from ingested sea water. Tubular secretion of Mg^{++} maintained urine/plasma

ratios (U/P) in the 50:1 to 100:1 range in seawater-adapted flounder (Hickman,

1968; Lahlou, 1970) coho salmon (Oncorhynchus kisutch (Miles, 1971) and

rainbow trout (Beyenbach and Kirschner, 1975).

Some DHA-exposed salmon apparently increased water ingestion while

still in freshwater and thus could have had mucosal permeabilities increased

by the direct contact with the lipid-soluble resin acid. Such an increase

in gut permeability could increase the uptake of Mg^{++} from subsequently

swallowed sea water.

Although the intestinal absorption route is generally considered to be the most important, Kirschner et al. (1974) found that the gill epithelium exhibited a low but definite permeability to Mg^{++}. As equilibration between plasma and sea water according to the electrochemical gradient would result in a 10-20 fold increase in plasma Mg^{++} concentration (Beyenbach, 1974), it is imperative that fish maintain a low gill permeability to this ion. In view of the changes in gill permeability to water it may be that prior DHA exposure increased gill permeability to Mg^{++} in sea water.

Even though it is not known whether Mg^{++} uptake via the gills increased in fish previously exposed to DHA, the increased entry through the gut alone would probably suffice to rapidly elevate plasma Mg^{++} concentrations. The dehydration occurring in sockeye salmon which were transferred to sea water after sublethal DHA exposure was shown to be accompanied by a compensatory increase in sea-water ingestion (Table XII). Since a net absorption of 44% of the ingested (SW) Mg^{++} was shown to occur across the intestine of normal trout (Shehadeh and Gordon, 1969) an increase in sea water ingestion beyond normal levels could be expected to rapidly raise plasma Mg^{++} concentrations.

As an increased uptake of Mg^{++} can only be eliminated by the kidney, and because this ion must be tightly regulated, the teleost kidney has developed a powerful tubular Mg^{++} secretory function. Magnesium infusion in a variety of euryhaline fish species brings about a powerful diuretic response which persists until plasma Mg^{++} levels return to normal (Bieter, 1933; Brull and Cuypers, 1955; Forster, 1953; Hickman, 1968). Natochin et al.(1970) found similar results in migrating sockeye salmon smolts which they loaded experimentally with Mg^{++}. As the rate of urine flow in sea water is determined

by the intensity of Mg^{++} excretion, Mg^{++} loading of salmon can be expected

to aggravate dehydration in sea water (Beyenbach, 1974).

Plasma Mg^{++} levels are normally regulated within narrow limits and small

variations are known to block neuromuscular transmission (del Castillo and

Engbaek, 1954) suggesting that the observed changes in locomotor performance

of DHA exposed fish may be related to myoneural blockage by Mg^{++}. Juvenile

pink salmon subjected to the stress of scale loss in sea water became un-

responsive to visual and mechanical stimulation after plasma Mg^{++} levels

increased by 63% (personal communication, Dr. L.S. Smith, University of

Washington). These symptoms were followed by a progressive paralysis. In

the present study identical symptoms were observed in DHA-exposed sockeye

salmon in the first 48 h after sea water transfer. Plasma Mg^{++} levels in

these fish increased by ∿50%. Fish which were drifting or moribund had

plasma Mg^{++} levels 3 to 4 times higher than the exposed (but resisting) fish.

Block et al. (1978) found that plasma Mg^{++} levels underwent the greatest

increase of all electrolytes measured in white perch (Morone americana)

exposed to chlorine in estuarine waters. These changes were attributed to

alterations in branchial permeability due to damage to the gill epithelium

by the toxicant.

In the present study, a comparison of the observed changes in plasma

Mg^{++} levels with those described in the literature on renal function suggests

that DHA exposure of salmon reduces the efficiency of kidney function in sea

water and/or increases gill permeability to Mg^{++} to such an extent that

renal excretory mechanisms may become overloaded. Based on the literature,

elevated plasma Mg^{++} levels may be responsible for many of the behavioral

changes observed in DHA exposed sockeye salmon during seawater adaptation.

Presumably the recovery of fish thus affected involves a successful reduction in GFR and gill permeability, thereby reducing water loss and the necessity for sea water ingestion. This would be followed by a gradual return of plasma Mg^{++} levels to normal.

PART III. ECOLOGICAL IMPLICATIONS

Environmental stress has been defined as "a state produced by any environmental factor which extends the normal adaptive responses of an animal, or which disturbs the normal functioning to such an extent that the chances of survival are significantly reduced" (Brett, 1958). Pursuing this philosophy further, in a review of methods used in the study and interpretation of sublethal effects of toxicants, Sprague (1971) emphasized the need to relate the often subtle toxicant-induced physiological changes measured in the laboratory to successively higher ecological levels of integration. In other words, is the measured change within the normal adaptive range or can it be expected to contribute to a reduction in the survival potential of the entire organism? This decision poses a singularly difficult problem as a determination of the ecological significance of laboratory findings usually has to be made in the face of limited knowledge of what happens in the field and therefore requires a higher than usual level of personal judgement by the investigator.

In the present study, the exposure of juvenile sockeye salmon to DHA for a period of 5 days led to significant departures of plasma electrolytes from "normal" values. Subsequent transfer of these fish into sea water again resulted in marked changes in hydromineral balance in the direction of the osmotic and ionic gradients. The majority of the fish so-affected managed to return plasma electrolyte levels to control values within 120 h after the toxicant exposure had been discontinued, indicating that no permanent reduction in osmoregulatory capacity had occurred. To suggest whether such a temporary loss in regulatory precision is meaningful to the survival of the fish one must consider not only the physiological effects but also the ecological implications.

Special efforts were made during this study to attempt to correlate changes in plasma electrolyte levels, gut water content and muscle water levels with observed changes in general behavior of the salmon. Measurement of these parameters at gradually more severe stages of locomotory dysfunction, i.e. drifting, equilibrium loss, and mortality, confirmed that the more seriously the fish were affected, the greater were the departures of the measured variables from the control values. This was especially true after the transfer of fish to sea water, with plasma Mg^{++} showing the greatest percent change. Although a cause/effect relationship was not established, based on the literature it is suggested that the observed locomotory difficulties may be related to an interference with neuromuscular function due to high Mg^{++} concentrations. In addition the high DHA residues occurring in brain tissue could have led to dysfunction at the integrative level and contributed to locomotor difficulties through poor muscle co-ordination.

One of the first noticeable effects of sublethal DHA toxicity was a gradual break up of the schooling response. Initially, fish-to-fish distances increased and a progressive reduction in cover response developed. Although schooling in fish is known to be under visual control, the lateral line is also involved (Pitcher et al.,1976). In the initial stages of intoxication, salmon which were more widely spaced still manifested a cover response to visual stimulation, indicating that vision was probably not impaired. At a later stage, when cover response was completely reduced, a tap on the tank would trigger an undirected spasmotic swimming behavior suggestive of a hypersensitivity and may indicate a dysfunction of the lateral line system. Gardner (1975) linked behavioral alterations to histopathological changes in marine teleosts exposed to a number of water-borne toxicants. A loss in the ability to maintain schooling patterns was related to lesions in the olfactory

epithelium of Atlantic silverside (Menidia menidia) exposed to crude oil in

the laboratory. In the field, the unnatural behavior and hypersensitivity to

mechanical stimulation in menhaden (Brevoortia tyrannus) collected in the

effluent discharge area of a nuclear generating station were linked to

lesions in the lateral line organs. Gardner (1975) also reported that exposure

of juvenile Atlantic salmon (Salmo salar) to pulp mill waste both in

laboratory and in field studies resulted in lesions in olfactory organs.

The changes in behavior of DHA-exposed sockeye salmon may have arisen, in part,

from the toxicant-induced changes discussed above. On the other hand, the

responses could have originated in dysfunction of the nervous system as DHA

was shown to accumulate to high levels in brain tissue.

Until recently the establishment of water quality criteria has depended

almost exclusively on the results of acute or long-term chronic life cycle

bioassays (Sprague, 1976). Only a few workers have directed their attention

to the ecological significance of subtle, toxicant-induced behavioral changes.

For example, Basch and Truchan (1976) observed gulls feeding on small fish

floundering near the surface during a period of cooling water chlorination at a

Lake Michigan power plant. While the temporary exposure of a prey species to

a physiologically sublethal concentration of a chemical toxicant may directly

cause little more than abnormal behavior, if greater predation is the conse-

quence then a sublethal effect has indirectly become lethal to the prey

(Goodyear, 1972). These observations form a good example of the concept of

"ecological death". Another example was provided by an experiment reported

by Farr (1977) who exposed grass shrimp (Palaemonetes vulgaris) sublethally

to Mirex (an organochlorine insecticide) and found that after 13 days there

was no difference in survival between controls and exposed groups. However,

within 24 h of the introduction of predatory pinfish (Lagodon rhomboides) the

survival of the previously exposed shrimp dropped precipitously. Similar

results have been demonstrated using other simple fish predator/prey systems

(Goodyear, 1972; Kania and O'Hara, 1974; Sullivan and Atchison, 1978).

Schooling behavior is well developed in sockeye salmon smolts and it may

have adaptive value at the estuarine stage of the life cycle (Hoar, 1958) or

in the search for food (Eggers, 1976). Very little is known about the

migratory behavior, routes followed, or physiological and behavioral responses

during entry into the sea (Northcote, 1974; Hanamura, 1966). Ricker (1966)

suggests that Fraser River sockeye . . ." move out into the offshore pelagic

environment rather quickly" based on their absence from seine catches. In

interpreting the selection behavior of salmon in a laboratory salinity gradi-

ent, Williams (1969) suggested that sockeye salmon smolts move rapidly out into

the Strait of Georgia within several hours of entering the Fraser River

Estuary. Williams (1969) also found that smolts from the Cultus Lake run were

capable of surviving a direct transfer into sea water. My results support

these latter findings since in the present experiments control fish at the

smolt stage maintained their blood electrolyte concentrations within very

narrow limits after transfer to 28 $^{o}/oo$ sea water. On the other hand, DHA-

exposed fish could not. If the changes in schooling and swimming behavior

observed in the laboratory were to occur in the field upon entry into sea

water, sockeye smolts could suffer a heavy predation. However, very little is

known at the present time about the extent or importance of natural predation

on normal sockeye salmon smolts on their seaward migration.

Often neglected in the interpretation of studies on electrolyte balance

of salmonids is the consideration of salinity. Many laboratories, the present

one included, utilize coastal sea water (salinity 25-30 ^{o}/oo) in their

investigations. In the wild, migrating sockeye salmon may move into full

strength sea water shortly after leaving the river and would be exposed to

considerably higher salinities ($\sim$ 35 ^{o}/oo). Boeuf et al. (1978) found that

coho salmon smolts which remained in an essentially homoiosmotic state after

transfer into sea water of 30 ^{o}/oo salinity suffered from a significant

hydromineral imbalance when placed in waters of 35 ^{o}/oo salinity. Thus DHA-

exposed sockeye salmon moving into waters of the Strait of Georgia could be

considerably more seriously affected than the present study has shown.

The laboratory exposure experiments established that salmon can rapidly

accumulate DHA in major organs such as the brain, liver and kidney and that

these high residue levels are probably related to the physiological dys-

function reported in this thesis. However, DHA depuration rates were not

measured, so it is not known how long these residues can exert their toxic

action, nor is there any information on the levels of DHA or other resin

acids in sockeye salmon which have moved down the Fraser River past the

pulp mills at Prince George and Quesnel or down the Thompson River past

Kamloops. The presence and the possible interactions of DHA with other

persistent toxicants known to be present in kraft mill waste have not been

studied in these fish nor in the rivers. The question of a potentiation of

KME toxicity by natural fluctuations in temperature and dissolved oxygen in

these rivers must be addressed. Results reported in the present study showed

that DHA toxicity was remarkably increased by a reduction of oxygen to 75%

of saturation, a drop well within natural fluctuations.

In view of the demonstrated persistence, bioaccumulation potential,

and known toxicity of DHA, safe limits of discharge of this, and possibly

other resin acids, would better be determined by establishing an "Ecological

Limit"[4] for these toxicants. This concept takes into account persistence, bioaccumulation, and sublethal thresholds to calculate environmentally safe discharge levels. Although there appears to be no published information to date on sublethal thresholds of any resin acid, sublethal thresholds for a variety of physiological and behavioral functions have been determined for salmonids exposed to whole kraft mill effluent (Davis, 1976). Perhaps these estimates could be used for establishing Ecological Limits while studies on resin acid sublethal thresholds are being conducted.

This study has demonstrated that chronic exposure to sublethal DHA concentrations can result in substantial toxicant accumulation. Regulatory agencies attempting to set water quality criteria utilizing "safe" concentrations should consider the bioconcentration potential of resin acids after their discharge into receiving waters.

When viewed in the light of Brett's (1958) definition of an environmental stress, sublethal DHA exposure was shown to "disturb the normal functioning" of the sockeye salmon hydromineral homeostatic mechanism and if the behavior observed in the laboratory also occurs in the field, then it is highly probably that the "chances of survival are significantly reduced".

The present study, while limited in its scope, underlines the general paucity of information on the physiological ecology of the sockeye salmon especially in relation to the interactions with pulp mill wastes during migration. Furthermore, the study has elucidated sublethal effects of DHA on salmonids and possible mechanisms not previously described. While the magnitude and scope for deleterious effects of such toxicants upon Fraser River stocks has not been directly investigated, the potential for such effects upon a sensitive life stage of migratory salmonids has been demonstrated.

[4] Ecological Limit (EL) concept was proposed in the Netherlands by Canton and Sloof and is discussed in van Esch (1978).

APPENDIX I-1. REMOVAL OF DHA FROM FRESH WATER BY SOCKEYE SALMON

In this experiment, the acute toxicity of DHA was investigated in relation to fish loading density during a static bioassay. In addition, analytical methods were developed which permitted the monitoring of the actual concentration of DHA in the aquarium water.

MATERIALS AND METHODS

The experiment was conducted using underyearling sockeye salmon which had been hatched at PEI from eggs obtained from the Cultus Lake 1973 brood stock. At the time of the experiment the fish were 4 months old and had been transferred to laboratory tanks from outdoor facilities a week earlier. In the laboratory, they were kept in tanks provided with a continuous flow of well water at the same temperature as outside (10.5 ±0.5 $^{\circ}$C). Daily feeding with OMP was discontinued 24 h prior to transfer of the fish to glass aquaria filled to a volume of 30 L with well water kept at 10.5 ±0.5 $^{\circ}$C by means of a water bath. An air-stone maintained dissolved oxygen levels above 90% saturation during the study.

After a 24 h acclimation period, the fish were gently transferred to identical test aquaria containing a theoretical DHA concentration of 1.88 mg/L. One aquarium contained the resin acid but no fish, to determine the extent of normal adsorption and/or degradation of the toxicant, a second tank was stocked with 5 fish (low loading density) and a third with 10 fish (high loading density). A fourth aquarium was used as a control and contained similar dilutions of only the DHA carrier solvents and 10 fish. Times to death (as judged by cessation of opercular movement) were recorded, and dead fish were weighed and measured.

DHA was prepared to a purity of 94% and a concentrated stock solution was made by dissolving 640 mg of DHA in 5 mL ethanol followed by 5 mL 5N NaOH

and made up to 1000 mL with distilled water. The addition of 94 mL of this

solution to the aquarium water yielded a theoretical concentration of 1.88

mg/L DHA.

At 24 and 96 h, water samples (500 mL) were taken from both aquaria for

determination of DHA concentrations. Water samples were acidified with 1

drop of conc. H_2SO_4 and extracted with chloroform (4 x 50 mL) in a 1 L

separatory funnel. The chloroform extracts were evaporated to dryness under

vacuum, re-dissolved in diethyl ether, methylated with diazomethane and dried

under a stream of nitrogen. Methanol dilutions of these extracts were

analyzed by GLC and quantified by comparison of integrated peak areas to a

calibration curve based on an external DHA standard. Analyses were done on a

Hewlett Packard 7620A gas chromatograph equipped with an integrator and

fitted with a 1.2 m x 3 mm stainless steel column packed with 10% Silar 5CP

on Gas Chrom Q (80-100 M). Oven temperature was 240^oC while injection port

and detector (flame ionization) were operated at 250^oC. Nitrogen was the

carrier gas.

<div align="center">RESULTS AND DISCUSSION</div>

At the low loading density, all fish were dead by 54.3 h (3255 min)

whereas at the high loading density only 3 out of 10 fish died in the first

39.3 h (2355 min) and no further mortalities occurred during the remainder

of the 96 h exposure period (Fig.22).   This high rate of survival of

fish exposed to 1.88 mg/L DHA can be attributed to their rapid reduction of

DHA concentration to a sublethal range (0.86 mg/L) after 24 h followed by

a further reduction to 0.29 mg/L after 96 h (Table XVIII).On the other hand,

after 24 h at the lower loading density, DHA levels were still at 1.16 mg/L;

a concentration rapidly lethal to the salmon. In addition, a 32% reduction

in DHA concentration in the tank containing no fish indicates that some
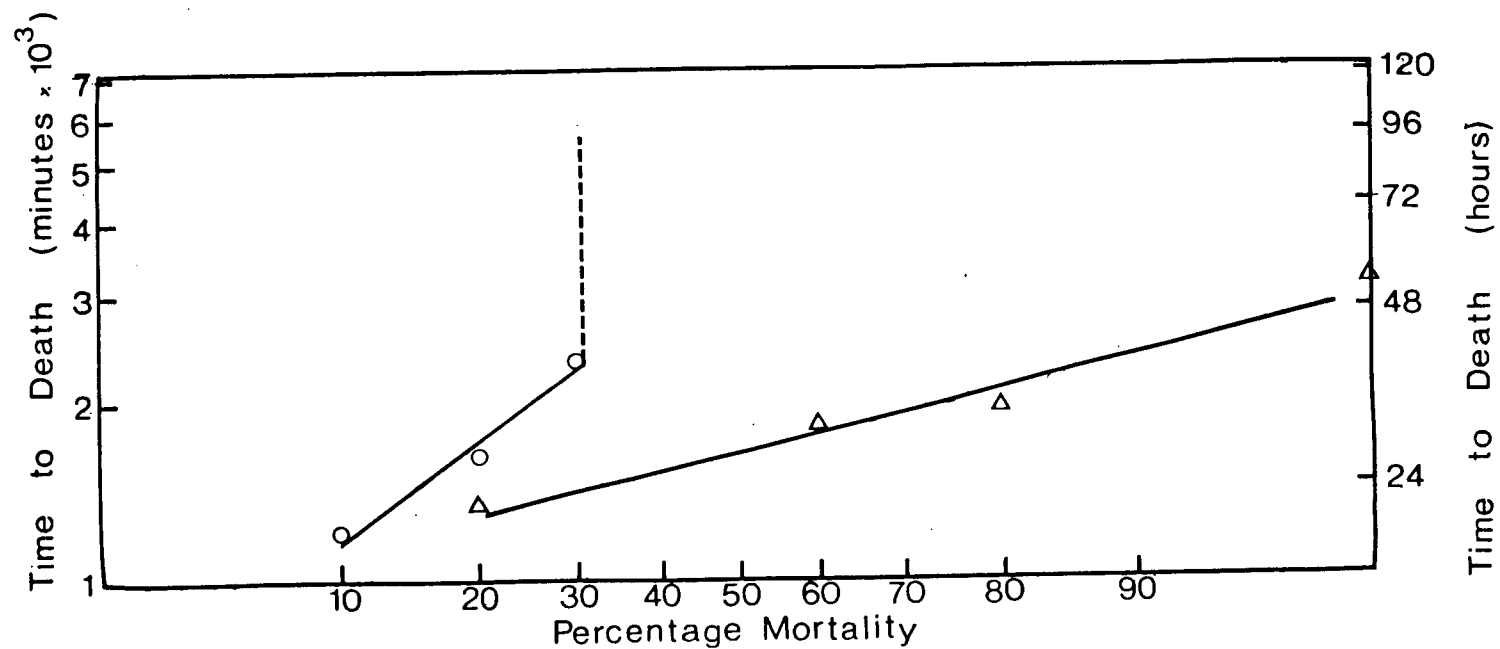
Figure 22. The effects of fish loading density in a static bioassay on the acute toxicity of DHA (1.88 mg/L) to juvenile sockeye salmon.

Table XVIII. The effect of fish loading density on
concentration of DHA in aquarium water[1].

Loading density	DHA concentration	
g/L	mg/L	
	24 h	96 h
0	1.44	0.98
1.1	1.16	0.77
2.2	0.86	0.29

[1]·Initial theoretical concentration 1.88 mg/L.

adsorption or degradation took place.

These results showed that a reduction in DHA levels in the water occurred and that this reduction was greatly enhanced by the presence of fish. However because DHA may not have been actually in solution or may have been removed by simple adsorption to the tank walls or to the surface of the fish, experiments were conducted to answer these questions. These experiments are described in the rest of Appendix I.

APPENDIX I-2. EFFECTS OF FILTRATION ON DHA RECOVERY FROM FRESH WATER

Although DHA recovery experiments confirmed that the resin acid was present in the water at close to the theoretical concentrations, they did not indicate whether the toxicant was in solution. The resin acid could have been adsorbed to particulates present in the water or could simply have been present in suspension and as such might have been bound or not freely available to the fish. As these particulates should be readily filterable, a comparison of DHA recovery from filtered and unfiltered solutions was made.

METHODS

Replicate solutions of DHA were made up using laboratory well water as used in bioassay experiments. A concentrated stock solution (238.5 mg/L DHA) which had been prepared for a bioassay was the source of the resin acid and the same dilution ratio as used in continuous-flow bioassays was utilized (3 mL stock/500 mL water). Four identical solutions were prepared in 1000 mL glass bottles which were stoppered, shaken to ensure mixing and then left for 36 h in a water bath at 10.5 ±0.5°C. Two of the samples were then acidified with 1 drop conc. H_2SO_4 and extracted with chloroform as previously described while the other two were suction filtered through a 0.22 μm Nucleopore filter before extraction. The extracts were then analyzed by GLC as before.

RESULTS AND DISCUSSION

Filtration had no effect on the recovery of DHA from the water (Table XIX). As the original theoretical concentration was 1.35 mg/L DHA, and as the mean percentage recovery was 86.9% some loss did occur during the experiment, probably by adsorption to the glass in the bottles as well as some loss during the extraction process. As "apparent solubility" has been operationally defined by filtration through a 0.45 μm membrane filter (Suffet and Faust,

Table XIX. Comparison of concentrations of DHA recovered from filtered and unfiltered water samples.[1]

	Replicate samples	DHA concentration mg/L	Recovery %
Filtered	A	1.17	86.7
	B	1.18	87.4
Unfiltered	A	1.18	87.4
	B	1.16	85.9

[1] Original concentration 1.35 mg/L.

1972) and in the present experiment a 0.22 µm filter was used, it was
concluded that the DHA was indeed dissolved and as such should be freely
available to the fish.

APPENDIX I-3.   ESTIMATE OF THE DIRECT SOLUBILITY OF DHA IN FRESH WATER

Although DHA may be considered as being water insoluble according to the chemical definition (Grant, 1944), the extent of this solubility may nevertheless be sufficient to be of toxicological and therefore biological significance.   To test this hypothesis, the direct aqueous solubility of DHA was determined by gas chromatographic methods.

METHODS

Dehydroabietic acid (95% purity) crystals were ground to a fine powder using a mortar and pestle and three samples were weighed on an electrobalance (Perkin Elmer Autobalance Model AD-2) to 0.01 mg and then transferred to 500 mL well water (pH 6.76) in 1000 mL glass bottles.   Magnetic stir bars were used to keep the mixture in suspension.   After 24 h, the contents of each bottle, now at room temperature (20°C) were filtered through a 0.45 μm filter. After acidification, the filtrates were extracted and analyzed as previously described.

RESULTS AND DISCUSSION

The recovery of DHA from the water showed that the resin acid was directly soluble in well water to an average of 3.3 mg/L (Table XX). Nyren and Back (1958) determined the total solubility (sum of ionized and unionized DHA) to be 6.6 mg/L while for the unionized acid it was 4.9 mg/L and demonstrated that the solubility was a function of pH.   These authors suggested that the aromatic ring of DHA rendered it more hydrophilic than other resin acids such as abietic.   The results of the present experiment suggest that DHA may be capable under certain conditions, of dissolving directly in water to a level acutely toxic to salmonids.   While chemically "insoluble", dehydroabietic acid can be considered toxicologically "soluble"

Table XX . Concentrations of DHA directly soluble in fresh
water at pH 6.76 and 20°C.

Replicate samples	Concentration of DHA in original mixture mg/L	Concentration of DHA in solution mg/L
A	29.78	3.15
B	31.68	3.20
C	33.26	3.61

as the free acid. As discussed in the Introduction, the precise chemical

status of DHA in receiving waters has not been established although it is

usually discharged as a sodium salt. The exact chemical species will then

be determined by receiving water chemistry. In all bioassay experiments

conducted during the present study, DHA was prepared as the sodium salt

before addition to the aquarium water.

APPENDIX I-4.   UPTAKE OF DHA FROM FRESH WATER AND ITS DISTRIBUTION IN THE
TISSUES OF JUVENILE SOCKEYE SALMON AND A MATURE RAINBOW TROUT

INTRODUCTION

The fish-loading density experiment described in Appendix I-1 showed that DHA was removed from the water by the presence of the salmon, however this removal could be accomplished by simple adsorption to the surface of the fish or by an actual absorption into the fish.

It is known that in fishes the entry of a toxicant across the gill membranes is related to its partition coefficient and its pKa (Hunn and Allen, 1974).   Dehydroabietic acid is a weak organic acid of very low aqueous solubility but which is freely soluble in lipid solvents such as chloroform.   Although no published information on the partition coefficient of DHA could be found in the literature, the partition coefficients of a wide spectrum of compounds are inversely related to their aqueous solubility and in turn determine biomagnification and lipophilic storage (Chiou et al., 1977; Neely et al., 1974; Yang and Sun, 1977).   Based on its solubility properties and at the pH of well water DHA could be expected to pass readily through the gill epithelium (McLeay et al., 1979).

After entering the vascular system, lipid soluble compounds are thought to dissolve in plasma lipoproteins (Fromm, 1970) and are then distributed throughout the body by the blood (Holden, 1962) where they may be deposited in tissues of high lipid content (Branson et al., 1975; Freed et al., 1976).

As a preliminary experiment (unpublished) showed that DHA was indeed taken up and concentrated from the water by sockeye salmon during an acute bioassay, two detailed experiments were performed to measure the extent of DHA accumulation during sublethal exposure and to determine the tissue

distribution of these residues.

MATERIALS AND METHODS

Fish and Test Conditions

Two separate experiments were conducted, one with sockeye salmon and one with a single rainbow trout (Salmo gairdneri). Sockeye salmon smolts were obtained from the Great Central Lake (Vancouver Island) migration and kept in the PEI outdoor holding facility for 2 months prior to use. A single mature female rainbow trout (fork length 47 cm, 1520 g wet weight) was used for the trout experiment and was part of a stock obtained from a commercial supplier in Mission, B.C. These fish were 2+ years old and had been held at PEI for an additional 18 months before the experiment. The fish were held at 11.5 ±0.5^{o}C in well water (see Table I).

In the salmon experiment, 10 fish (15-20 g) were transferred to the laboratory donut tanks receiving well water at the same temperature and were held there for a further 72 h before the toxicant exposure began. The fish were not fed during the acclimation period or during the 120 h exposure period. Ten salmon were exposed to 0.65 mg/L DHA in one tank and another 10 served as controls in a second tank; a third tank contained the single rainbow trout. A continuous current of 15 cm/sec was maintained and the flow of well water at a rate of 500 mL/min provided a 90% replacement time of ∿ 4 h (Sprague, 1969). The fish loading density for the salmon was 3.6 L water/g fish/day and 0.47 L/g/day for the trout.

Toxicant

The DHA toxicant was prepared and metered into the incoming water system by procedures described previously. A GLC check of the DHA concentration actually present in the water showed that ∿ 90% of the theoretical dosage had been attained. The pH of the bioassay water after addition of the

stock solution was 6.96 for the DHA and 6.97 for the control tank. The water temperature was maintained at 11 ±0.5°C and dissolved oxygen > 90% saturation.

Fish tissue preparation

At the conclusion of the exposure period, the salmon were anesthetized with 200 mg/L MS-222, while the trout was stunned by a blow on the head. Blood was collected from severed caudal peduncles of both species. Blood from each salmon was spotted on a microscope slide for the preparation of blood smears and after centrifugation the hematocrit was measured and the plasma was pooled for the determination of osmolality. In the salmon the packed red blood cells were pooled for the determination of DHA residues while for the rainbow trout, DHA residues were determined in a sample of whole blood.

The gall bladder bile was collected from both species using 3 mL B-D Vacutainers. The liver, kidney, spleen, gill, gut and brain were rapidly removed from both species and in the salmon, the remainder was termed the "carcass".

Individual tissues were pooled and freeze-dried to constant weight. For the trout, additional samples of ova and lateral muscle were taken but the carcass was not analyzed. In the salmon, the entire branchial basket was utilized while in the trout only the gill filaments were used. The trout muscle sample was removed from the dorso-lateral muscle mass immediately posterior to the dorsal fin and the skin was removed before weighing. The large mass of the various tissues in the trout permitted accurate wet weights to be determined before freeze-drying so that the % water could be determined. In salmon, where the wet weights of pooled tissues were not measured, wet weights were approximated by using the salmon dry tissue weights which had been measured and applying the % water obtained for the trout data. In the case of salmon bile, erythrocytes, gills and carcass, the relationship

between dry and wet weight was established from control samples.

Chemical Extraction

Dry tissue samples were ground by mortar and pestle with sodium sulfate, acidified with a drop of 10% H_2SO_4, packed on a chromatographic column (1 cm x 20 cm or 2 cm x 30 cm, depending on sample volume) and extracted with 100-200 mL pesticide grade methylene chloride (Burdick & Jackson Laboratories Inc., Michigan, USA). The extracts were evaporated to dryness under vacuum, redissolved in diethyl ether, methylated with diazomethane, and suitable hexane dilutions were then analyzed by GLC. Analysis of two control liver samples spiked with DHA yielded 92.2 and 99.3% recovery for this extraction procedure. Bile in the Vacutainer vials was decanted 5 times with methylene chloride, acidified, evaporated to dryness, methylated and then analyzed by GLC.

Analytical Procedure

The presence of DHA was confirmed in hexane dilutions of the fish extracts by gas chromatography-mass spectrometry (GC-MS), using a Hewlett Packard HP 5992A fitted with a 1.8 m x 6 mm glass column packed with 3% OV-101 on Supelcoport 80-100 mesh. The oven temperature was programmed from 150 to 250°C at 8 C/min after an initial isothermal hold of 2 min. Detector and injection port temperature was 250°C and helium was the carrier gas. An external DHA standard was used for comparison of the mass spectra. Ions were scanned from 40 to 400 mass units with a single ion being monitored at m/e 239 (Enzell and Wahlberg, 1969).

Quantitative determination of DHA was made by GLC on a Hewlett Packard HP 5700 fitted with a flame ionization detector utilizing a split ratio of 100:1 and a 30 m x 0.2 mm column wall-coated with OV-101. The oven

temperature was programmed from 124 to 205°C at 8 C/min after an initial

isothermal hold of 2 min. Detector and injection port temperature was

250°C and helium was the carrier gas. DHA was used as an external standard.

DHA Residue Calculation

Resin acid residues are expressed both as μg DHA/g dry tissue and wet

tissue in order to facilitate the comparison of concentration factors

(tissue : water). The salmon wet tissue weights are estimates derived from

measured dry weights as previously described. The whole body residues for

the sockeye salmon were determined by summing the dry weight of all the

tissues to reconstitute the "original" fish. As the % water for the carcass

was known from control samples, the original total wet weight of the exposed

fish could be back-calculated from the dry weights. The total DHA residue

recovered from the various tissues was summed and divided by the wet weight

of the fish.

For the trout, the total body residues were calculated by summing the

individual tissue DHA residues and dividing by the original total fish wet

weight. Estimates were made for blood and muscle according to proportions

in the literature. Total blood residues were estimated on the basis of a

blood volume of 3% of body weight (Smith, 1966) while total "muscle"

residues were calculated on the basis of a muscle mass of 67% of body

weight (Stevens, 1968). As the trout was gravid and heavily laden with

eggs, "body weight", for the purposes of these calculations, was taken as

the total wet weight minus eggs.

<div align="center">RESULTS</div>

Both fish species accumulated DHA from the water. On a whole body, wet

weight basis, the sockeye salmon DHA residues were 19.2 mg/kg while the

rainbow trout contained 22 mg/kg. The DHA distribution in various tissues

is shown for salmon (Fig.23) and for rainbow trout (Fig.24) expressed on a wet weight and dry weight basis (left and right ordinate scale, respectively). All calculations which follow are based on wet tissue weights.

As shown in Fig. 23, the highest overall concentration of DHA was found in the salmon bile (647.3 µg/g); this yields a bile/water ratio of 996. Of the salmon organs, the brain contained the largest amount (619.8 µg/g) followed by the kidney (278.1 µg/g) and liver (262.5 µg/g) yielding bioconcentration ratios of 954, 428, and 404 respectively. The carcass, which consists of the original body minus the tissues listed to the right in Fig.23 and therefore includes the head, skeleton, muscle, skin etc., contained 7.7 µg/g DHA. On a wet weight basis, the internal organs and tissues represent only 17% of the total fish weight yet contained 29% of the total body residue. The liver and kidney accounted for 40% of the organ DHA burden.

The rainbow trout residue tissue distribution (Fig.24) shows the liver (290.6 µg/g) kidney (182.5 µg/g) and brain (154.3 µg/g) contained the highest residues, yielding bioconcentration factors of 447, 281 and 238 respectively. The bile contained 17.9 µg/g resulting in a bile/water ratio of 27.5. For the trout, the liver and kidney accounted for 56% of the total organ DHA burden.

While the whole body bioconcentration factors for sockeye smolts and rainbow trout amounted to 30 and 34 respectively, it can be seen that in individual organs, for example in the liver, DHA was present at 400 times the 0.65 µg/mL level present in the water during the 120 h exposure period.

Bile samples of both the salmon and trout were found to contain at least three metabolic derivatives of the parent DHA molecule. One of the derivatives was identified as methyl abietatetraenoate. DHA was not detected in any of the tissues taken from control fish.
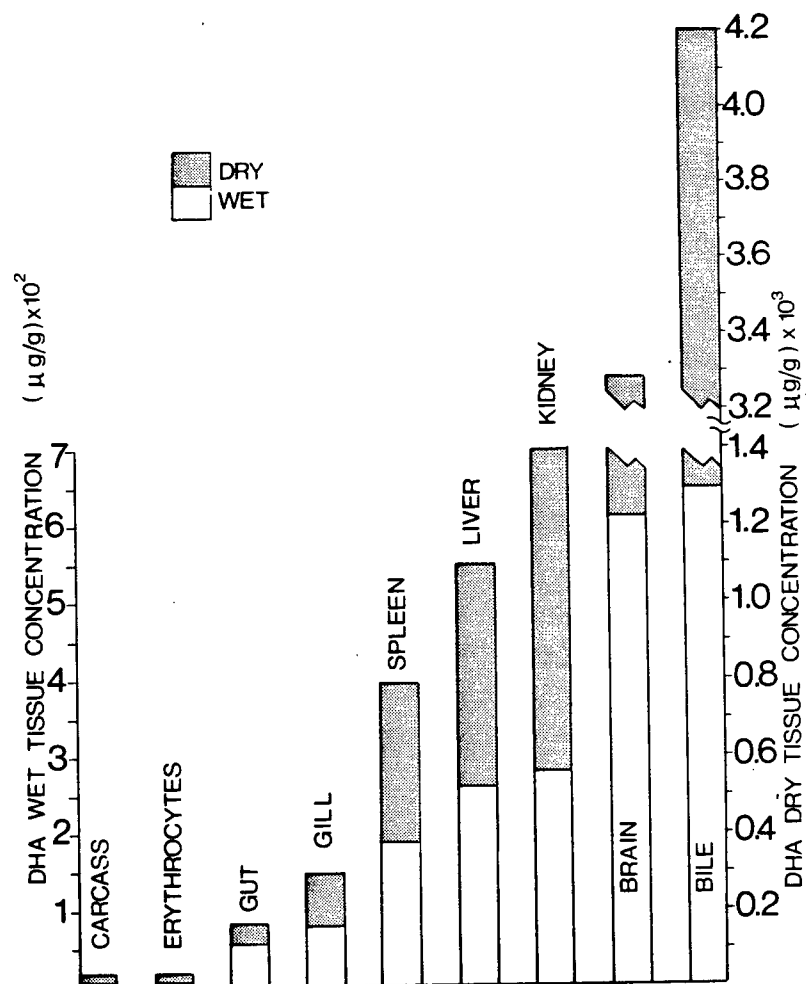
Figure 23. Distribution of dehydroabietic acid in pooled tissues of
sockeye salmon smolts after a 5 day laboratory exposure
to 0.65 mg/L DHA.
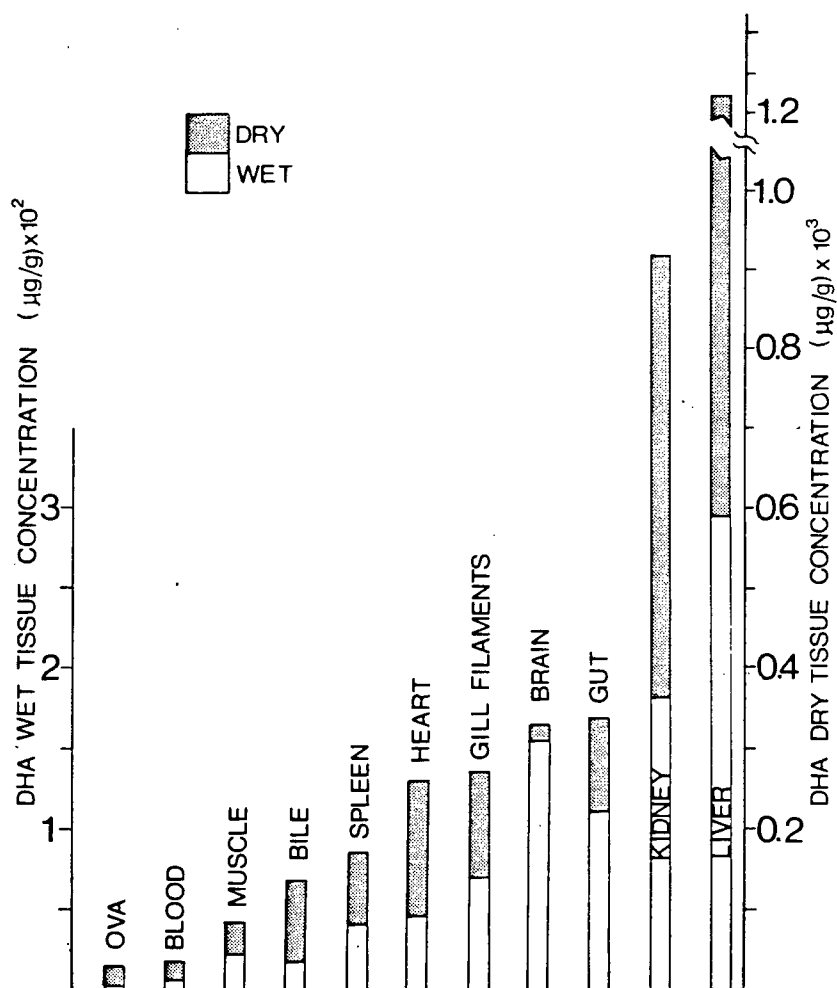
Figure 24. Tissue distribution of dehydroabietic acid in a rainbow trout after a 5 day laboratory exposure to 0.65 mg/L DHA.

DISCUSSION

Dehydroabietic acid is a weak organic acid which, based on its aqueous

solubility (Appendix I-3), can be classified chemically as water "insoluble"

(Grant, 1944). On physicochemical grounds DHA at the pH of well water

would be expected to partition rapidly from the water, across gill epithelia,

into the blood and from there be distributed to the various lipid pools with-

in the body (Hunn and Allen, 1974; McLeay et al., 1979). This is substan-

tiated by the presence of high DHA residues in lipid extracts of tissues

in salmon and trout in the present study.

No published information is available on levels of DHA in individual

organs of fish. Hagman (1936) in analyses of moribund fish collected down-

river from a sulfate pulp mill, found a positive (colorimetric) reaction for

resin acids in extracts of liver, pancreas, kidney and mucus, with an

especially strong reaction for the "liquid surrounding the brain". The

present study has shown that the brain of the sockeye salmon accumulated the

highest DHA residues of any organ. Tomiyama (in Fujiya 1965) suggested that

penetration of resin acids into fish tissues may have led to chronic toxic

effects such as the histopathological changes reported by Fujiya. Fujiya

noted a variety of necrotic changes in the kidney, intestine, pancreas and

gills of fish taken from waters receiving kraft pulp mill waste, with

particularly serious effects on the liver (Fujiya 1961, 1965). In the

present work, the liver contained among the highest DHA residues in the

two salmonid species tested.

The high level of DHA in the bile of salmon as well as the presence of

more polar metabolites in both fish species suggests a hepatobiliary

excretion route for this resin acid. In fishes, this route of xenobiotic

excretion is now well established, with the liver playing a fundamental role in biotransformation of lipid soluble compounds into more polar, water soluble forms which are then excreted via the bile (Adamson and Sieber 1974; Klaassen 1975). A number of pesticides, polychlorinated biphenyls, phenols, and detergents have been isolated from the bile of a variety of fish species (Lech et al., 1973; Gakstatter, 1968; Melancon and Lech, 1976; Statham et al., 1973;Lech, 1973; Tovell et al., 1975). Smith (1971) reported that among the requirements for extensive biliary excretion, a balance between polarity and non-polarity, molecular weight of 300-400, and the presence of an easily ionizable group are important physicochemical characteristics. DHA, with a molecular weight of 300 and a carboxyl group would appear to fit these requirements. Hydroxylation by the liver would increase molecular weight, make the resin acid more polar and thus facilitate its biliary excretion.

Bile flow is also an important determinant of the rate at which many compounds are cleared from the plasma and excreted in the bile (Tuttle and Schottelius, 1969). During the exposure to DHA, the sockeye salmon developed a marked yellowish tinge which was particularly noticeable in the membranes of the pectoral and pelvic fins as well as in the plasma. These symptoms were found to be due to a form of jaundice caused by a DHA-induced rise in plasma bilirubin. These results are discussed in depth in Appendix III.

Jaundice in mammals is known to result when hepatic clearance of bilirubin is reduced by acute liver disease or obstruction of the bile duct (Nosslin,1960). Bile is known to be toxic to human liver parenchyma when released into the tissues during acute obstructive jaundice (Sherlock, 1968); similarly Hendricks et al., (1976) reported that rainbow trout bile was highly caustic to trout liver tissue. In the present experiment, the

failure or overloading of the hepatobiliary system in salmon exposed to DHA may have contributed to the accumulation of DHA residues to toxic levels in various organs. Presumably when uptake exceeds metabolism and excretion, accumulation of DHA and/or bile in the liver may occur and result in localized tissue necrosis.

The rainbow trout did not exhibit jaundice. Its much larger size may have enabled it to store or metabolize more of the DHA before its toxic effects become apparent; the results of preliminary acute bioassays (unpublished) indicated that larger fish may be more resistant to the acute toxic effects of DHA.

The sublethal exposure of fish to a toxicant can lead to its accumulation at high concentrations in various internal organs which subsequently undergo histopathological changes. This mode of toxic action has been well documented for pesticides and related chemicals accumulated by fish in the laboratory (Buhler et al., 1969; Eller, 1971; Kruzynski, 1972; Mathur, 1962; Mount, 1962) or in the wild (Johnson, 1968; Kennedy et al., 1970). In many cases rapid and pronounced changes occur in the liver (Couch, 1975) although frequently most of the organ systems are affected (see Walsh and Ribelin, 1975 for review).

The accumulation of heavy metals such as mercury and cadmium has been shown to result in damage to fish kidney tubules (Trump et al., 1975) as well as in renal lesions and liver and gonad degeneration (Tafanelli and Summerfelt, 1975). These authors concluded that the histopathological changes and subsequent physiological dysfunction were caused directly by the accumulation of large organ residues after sub-lethal exposure.

Although histopathological observations were not attempted in the present study, it would be illuminating to determine whether the high DHA residues can be linked to internal organ histopathology; such as reported by Fujiya (1961) in fish exposed to kraft mill waste in the vicinity of a coastal pulp mill. Renal damage may be one of the main contributing factors to the hydromineral imbalance observed in the DHA-exposed fish.

The calculated whole body wet weight DHA residues obtained in this study compare favorably with those I found in a preliminary (unpublished) study. Using a semi-quantitative technique (Mahood and Rogers, 1975) to achieve the separation of interfering fatty acids from DHA prior to GLC analysis I measured whole body residues of 27.3 mg/g DHA in sockeye salmon which had died during freshwater exposure to 1.1 mg/L DHA. This represents a bio-concentration of $\sim$ 25 x that theoretically present in the water.

Fox et al. (1977) exposed 200 g rainbow trout to dilutions of kraft mill waste (KME) and found whole fish residues of DHA approximately 20 times the DHA concentrations present in the diluted effluent. Based on their figures, a 48 h continuous exposure of trout to KME containing a mean concentration of 0.67 μg/mL DHA resulted in residues of 10 μg/g.

In the present study, an exposure of 120 h to 0.65 μg/mL DHA resulted in residues of 22 μg/g for the rainbow trout and 19.2 μg/g for the sockeye salmon. If the uptake rate of DHA for rainbow trout as reported by Fox et al. (1977) was linear, then after 120 h their fish should have contained approximately 25 μg/g DHA. This estimate compares favorably with the results obtained in the present experiment. If such a comparison is valid, then the time for equilibration of the uptake rate would exceed 5 days. Technically, the synthesis of radio-labelled DHA would greatly simplify the estimation of uptake and depuration rates of this resin acid.

APPENDIX I-5.   THE ACCUMULATION OF DHA IN SALMON VIA THE DIETARY ROUTE-
EVIDENCE OF ACCUMULATION IN THE SALMON FOOD ORGANISM
Anisogammarus confervicolus.

INTRODUCTION

In addition to absorbing DHA directly from the water, salmon may feed
on organisms which have accumulated the resin acid above ambient levels.
The uptake and bioconcentration of some pesticides by members of the various
trophic levels of the aquatic food chain has been well documented (Hatfield,
1969; Johnson et al., 1971; Schoenthal, 1963) and in some cases, this route
is thought to be the major source of contamination for fish in natural waters
(Macek and Korn, 1970).

Field studies on feeding habits have established the general importance
of estuarine amphipods in the diet of juvenile salmon (Goodman and Vroom,
1972; Levings, 1973). Healey (1978) found that amphipods dominated the diet
of sockeye salmon smolts sampled in Georgia Strait, B.C. and the amphipod
Anisogammarus confervicolus was found to be the most important food item in
the diet of juvenile chinook salmon O. tshawytscha within the area of
influence of a coastal pulp mill (Kask and Parker, 1972).

As an increase in the abundance of these food organisms has been
observed in the vicinity of pulp mill outfalls and log storage areas
(Birtwell, 1978; Harger and Nassichuk, 1974; Waldichuk and Bousfield, 1962)
it is conceivable that juvenile salmonids may be "attracted" into waters
containing relatively high concentrations of toxic waste components. In
addition, if food organisms such as amphipods were to accumulate these
toxicants above ambient levels, there would arise the potential for bio-
concentration of the more persistent toxic components by fish utilizing this
contaminated food source.

As DHA is known to be one of the more persistent toxicants present in kraft pulp mill waste, a laboratory study was conducted to determine whether this resin acid was taken up from the water by a representative salmon food organism; the amphipod A. confervicolus.

MATERIALS AND METHODS

Amphipods were collected from troughs draining the outdoor fish holding tanks at PEI. Water in the troughs (salinity ~ 10 ^{o}/oo) supported a thick growth of mussels (Mytilus edulis) among which lived the amphipods and the isopod Gnorimosphaeroma oregonensis. No effort was made to separate the amphipods from the mussel mass and approximately equal amounts of the mixture were placed into two glass cylinders 9.2 x 40.7 cm (2.76 L) with the ends covered by fiberglass mosquito netting. Each cylinder was then laid flat on the bottom of separate donut tanks in which the recirculating pump maintained a continuous water current of ~ 15 cm/sec. Approximately 30 g of the alga Fucus vesiculosus collected from the beach was added to each cylinder to provide additional shelter.

After a 48 h acclimation period, the amphipods were exposed to 0.4 mg/L DHA in brackish water (10 ±1 ^{o}/oo; 10.5 $\pm0.5^o$C; pH 6.97; dissolved oxygen > 90% saturation) under continuous-flow conditions. A flow rate of 740 mL/min provided a 95% replacement time of 4.5 h (Sprague, 1969). The control tank received the diluent solvents without the resin acid.

At the conclusion of the 120 h exposure period, the cylinders were lifted out of the tanks, the contents emptied into a tray and the amphipods were picked out with forceps. During the sorting procedure, the amphipods were placed into a tray containing uncontaminated brackish water. After sorting was complete, they were placed on a screen, rinsed with deionized water, blotted and then frozen rapidly with liquid CO_2. Each amphipod mass

was weighed and then freeze dried to determine the relationship between wet and dry weight.

After grinding with Na_2SO_4, the sample was extracted with methylene chloride and processed in the same manner as previously described for the fish samples. After GLC analysis, the results were expressed on both a wet weight and dry weight basis.

RESULTS

Shortly after their transfer into the laboratory donut tanks, the amphipods appeared to have settled into the various crevices and the mussels were actively feeding. During the course of the experiment, no dead or behaviorally abnormal amphipods were observed and the accumulation of fecal pellets downstream of the glass cylinders indicated that active feeding continued throughout the exposure period.

The results show that the amphipods contained DHA at a level 21 x that theoretically present in the water (Table XXI). On a whole body wet-weight basis, this figure compares favorably with the 29.5-fold concentration of DHA by sockeye salmon exposed to 0.65 mg/L of the resin acid for the same length of time. As both exposures were of relatively short duration the data do not provide information on the shape of the uptake curve so that DHA residues at equilibrium are not known. No DHA was detected in the control samples.

DISCUSSION

This experiment indicates that there exists a potential for bio-accumulation of DHA via the dietary route, however the estimate of its relative importance must await uptake and assimilation studies. As fish-food organisms such as amphipods may be exposed to DHA from their own food supply

Table XXI. DHA residues in the amphipod <u>Anisogammarus</u> <u>confervicolus</u>
exposed to 0.4 mg/L DHA for 120 h in sea water of
salinity 10 %oo.

	Total Weight of sample		N^1	DHA		Concentration Factor[2]
	Wet g	Dry g		Wet μg/g	Dry μg/g	
Exposed	6.8400	1.9760	133	8.39	29.03	21
Control	10.7136	3.0793	208	ND	–	–

1
Estimate based on individual wet weight (51.6 mg) calculated from the
pooled wet weight of 100 amphipods.

2
Wet weight basis relative to maximum theoretical concentration in the
water.

as well as directly from the water, such studies could best be done in a model ecosystem utilizing several members of the aquatic food chain.

Nevertheless, recent studies have shown that the regions around pulp mills may be attractive to juvenile salmonids because of the presence of a more readily available food supply. Under laboratory conditions, the addition of dilute KME to artificial freshwater stream communities led to an enhancement of the biomass of the amphipod Crangonyx (Ellis, 1967) and it appears that similar effects do occur in the field. Kelso (1977) suggested that the intense aggregation of the fish community near a kraft pulp mill outfall in Lake Superior was due to the extreme abundance of benthic invertebrates adjacent to the point of entry of the effluent plume. He indicated that the feeding response to a high benthic biomass may override any avoidance reaction to the effluent. Increased numbers of juvenile chinook salmon in the area adjacent to a coastal pulp mill at Port Alberni B.C. may have been due to such an enhancement of the food supply (Birtwell, 1978). Fish thus "attracted" into regions of increased food supply would be exposed to elevated levels of toxicants in the water as well as in the diet.

Although the consequences of consuming contaminated fish food organisms in the wild are unknown, Tokar (1968) conducted a laboratory growth study on juvenile chinook salmon fed on tubificid worms (oligochaetes). Salmon fed on worms which had previously been exposed to full strength KME for 24 h consumed equal amounts of food but had much lower growth rates than the controls. This was attributed to a striking reduction in the efficiency of food utilization. As the fish were kept in effluent-free water these effects strongly suggest a sublethal toxic action by some component accumulated from the effluent and absorbed by the fish.

Since species such as chinook, coho and chum salmon can spend extended periods of time feeding in estuarine habitats which are also being used for the discharge of pulp mill wastes (Birtwell, 1978; Davis et al., 1978; Sibert and Kask, 1978), the ingestion of DHA-contaminated food organisms could be of considerable significance to these as well as to other salmonid species. If the rate of transit of sockeye salmon smolts through other estuaries is as rapid as that thought to occur in the Fraser River Estuary, (Williams, 1969), it is unlikely that the consumption of DHA-contaminated amphipods would play a significant part in the buildup of DHA residues. On the other hand, while still in the river, sockeye smolts feed primarily on aquatic insects (Goodman, 1964) so that this food source could be a potential source of DHA contamination.

It is not known whether the potential for bio-accumulation of DHA suggested by this preliminary study is realized in the field situation. However circumstantial evidence indicates that this may indeed be the case. Brownlee et al. (1977) found DHA in bottom sediments at a distance of up to 15 km from the outfall of a kraft pulp and paper mill in Lake Superior. Such sediment-bound DHA may be taken up by benthic organisms and subsequently translocated to bottom-feeding fish. Brownlee and Strachan (1977) found DHA in the sucker (Catastomus); a bottom feeder, and in yellow perch (Perca flavescens) which feed on aquatic invertebrates as well as small fishes.

It is possible that these fish accumulated DHA directly from the water in the region of the pulp mill, but based on the amphipod data obtained in the present study, there appears to be little reason to exclude the food chain route from a contributing role in the buildup of DHA in wild fish.

APPENDIX II. BACTERIAL KIDNEY DISEASE(BKD)

Salmonid bacterial kidney disease(BKD) is caused by a genus of

Corynbacterium and affects all five species of Pacific salmon, the Atlantic

salmon, as well as most of the commonly cultured trout species. It may be

present in the chronic stage and progress slowly with no apparent symptoms

for much of the year but frequently reaches epizootic proportions in the

early spring months. Because it is basically asymptomatic in the chronic

stages, it usually passes unnoticed until some of the more severely infected

fish begin developing external symptoms or start dying.

As BKD infections are highly resistant to antimicrobial drugs (Suzomoto

et al., 1977) no treatment has yet been found, so that when the disease

arises in aquaculture operations the entire infected stock is routinely

destroyed. As a result, BKD infections have seriously affected production in

a number of U.S. trout hatcheries (Snieszko et al., 1955) and are currently

one of the most serious problems facing Pacific salmon culture both in

hatcheries and marine net pens.

Although most prevalent in fish culture operations, it is likely that

BKD also affects natural stocks of salmon although the consequences on marine

survival are unknown. The disease has been found in "wild" rainbow trout in

B.C. (Evelyn et al., 1973) and recently in a "wild" chinook salmon in Puget

Sound, Washington (Ellis et al., 1978). In 1977, 11 of 41 coho salmon

sampled returning to the Capilano River (West Vancouver, B.C.) as spawning

adults were found to be infected with BKD (G.E. Hoskins, Pacific Biological

Station, personal communication). Recent evidence suggests that resistance

to BKD in coho salmon may be genetically determined (Suzomoto et al., 1977).

Once the disease is established, its course and severity appear to be affected by a variety of factors such as water temperature and hardness, season, degree of crowding and diet. In a consideration of water chemistry in 37 U.S. salmonid hatcheries, Warren (1963) found that as the constituent load of the water decreased, the severity of corynbacterial kidney disease increased. However an etiologic relationship was not established. Wedemeyer et al. (1976) suggested that increased mortalities in soft waters may be due to increased energy requirements for osmoregulation.

Although BKD is termed kidney disease, it is actually a systemic infection and can affect most of the vital organs, although the hematopoietic tissue of the kidney and spleen are among the first tissues infected (Wood and Yasutake, 1956; Snieszko et al., 1955). In more acute stages, the infection leads to necrosis of the entire kidney and can extend to the gills, liver, spleen, eyes, musculature and anterior gastrointestinal tract (Bell, 1961). Young and Chapman (1978) described ultra-structural changes in the glomerulus and renal tubules of BKD infected brook trout (Salvelinus fontinalis) which were interpreted as signs of irreversible cell injury.

The progressive destruction of kidney tissue can be expected to lead to osmoregulatory dysfunction and it has been postulated that eventual death may be due to renal insufficiency (Bendele and Klontz, 1975). Wood and Yasutake (1956) suggested that the complex and extensive morphological changes observed in other organs were probably not a direct result of impaired renal function as the excretory system was one of the latter tissues to be affected by the disease, but that each organ was affected directly by the bacteria. These authors attributed the often-reported edema to damage to the circulatory system, especially the mesenteric blood vessels. A reduction in hematocrit,

hemoglobin, and plasma protein in infected fish was interpreted as a symptom

of osmoregulatory dysfunction (Hunn, 1964; Suzumoto et al., 1977). While it

is difficult to determine how far along the disease has progressed before the

excretory function of the kidney is seriously affected it is known that its

hematopoietic function is reduced early in the infection. The progression from

chronic subclinical to the manifested stages is therefore accompanied by a lower-

ing of hematocrit (Suzomoto et al., 1977; Iwama, 1977).

The Occurrence of BKD During Electrolyte Balance Experiments

In the present study, indications of the presence of a chronic BKD

infection in the sockeye salmon smolts became apparent during blood sampling

in Expt. 1. Several fish had lowered hematocrits and one showed a swollen

abdomen which is one of the visible symptoms of infection. However no

mortalities were observed in the stock holding tanks containing ∿ 2000 smolts

in the week prior to or during Expt. 1. Hematocrits obtained in Expt. 2,

which followed immediately, indicated that the disease was progressing more

rapidly and a chronic mortality (1-2 fish per day) began in the stock holding

tanks at this time. Shortly after, a confirmation of the presence of BKD in

the salmon was obtained through the Diagnostic Service of the Fish Health

Program, Pacific Biological Station, Nanaimo, B.C. and the entire stock was

destroyed. At this time tank mortalities remained low (2-3 fish per day in a

stock of ∿ 2000), typical of the chronic stage of BKD infection. As it was

impossible to judge the severity of the chronic BKD infection in fish used in

Expts. 1 and 2 until after the blood sampling was completed, fish showing

lowered hematocrit were judged to be in an advanced stage of chronic

infection and were deleted from the statistical consideration of the data.

This procedure was based on two assumptions: A) that as the disease pro-

gressed in severity, hematocrit dropped and B) that the incidence of infection

was normally distributed in the fish used in the experiments. Support for the first assumption was provided above, while the basis for the second assumption was ensured by a random sampling of fish from the stock tanks for use in Expts. 1 and 2.

Hoffman (1963), using the properties of the normal distribution, described a graphical method which separates clinically healthy from diseased persons. By using arithmetic probability paper, a normal distribution can be transformed into a straight line by plotting cumulative frequencies, expressed as percentages of the total frequency against the end points of the class intervals.

When treated in this manner, a composite distribution comprising clinically "normal" and "sick" components can be graphically split into two by eye fitting a straight line to the component which represents the clinically normal data with maximum weight being given to the points around 50%. Normal limits are then arbitrarily defined to be the values which graphically enclose 95% of those obtained by testing clinically "normal" subjects. In the present experiment therefore, fish were defined as "diseased" if their hematocrits fell in the lower 2.5% of the clinically "normal" values. Separate curves were plotted using the hematocrits of all the "control" fish for Expt. 1 and Expt. 2, the lower limits were established and then applied to eliminate fish from both control and experimental groups having hematocrits below the lower limits. In Experiment 1 the lower limit was 26.8% while for Experiment 2 it was 25.2%. The data for the fish which were thus eliminated are presented in Table XXII and XXIII respectively.

Due to the progressive nature of BKD, this screening method was considered to eliminate fish which were in the advanced chronic or severe stages of a disease which may compromise osmoregulatory performance. This screening

Table XXII. Size, hematocrit and plasma ionic composition of fish deleted[a] from data of Expt. 1[b] because of a suspected advanced infection of bacterial kidney disease.

Sampling Time (hours)	Group[c]	Fish size Fork length cm	Wet weight g	Hematocrit %	Osmolality mOsm/kg	Chloride mEq/l	Sodium mEq/l	Potassium mEq/l	Calcium mEq/l	Magnesium mEq/l
24	C	14.5	33.2	26.4	298	126.0	159.7	2.78	5.57	1.82
48	E	15.8	36.4	26.3	298	140.0	152.0	3.23	4.90	3.79
72	E	14.8	31.7	25.9	264	113.0	150.5	3.21	6.31	1.52
96	C	14.2	28.1	20.8	284	129.5	148.8	3.33	5.27	1.59
120	E	14.6	29.2	25.5	298	135.5	161.0	3.41	5.25	1.68

[a] Based on hematocrit < 26.8%

[b] Sockeye salmon smolts were exposed to sea water after a 120 h exposure to 0.65 mg/L DHA in fresh water.

[c] C=control, E=exposed.

Table XXIII. Size, hematocrit and plasma ionic composition of fish deleted[a] from data of Expt.2[b] because of a suspected advanced infection of bacterial kidney disease.

Sampling Time (hours)	Group[c]	Fish size Fork length cm	Wet weight g	Hematocrit %	Muscle Water %	Gut Water %	Osmolality mOsm/kg	Chloride mEq/l	Sodium mEq/l	Potassium mEq/l	Calcium mEq/l	Magnesium mEq/l
0	C	16.4	47.7	21.7	75.69	77.93	290	113.0	148.2	4.47	5.48	1.56
	C	17.2	52.9	17.7	75.38	79.72	274	114.0	144.2	3.66	6.13	1.56
24	C	16.5	45.5	21.3	71.81	73.61	300	130.5	148.1	3.29	5.53	2.65
	C	16.4	40.5	23.4	73.31	76.77	334	131.0	170.9	4.36	7.30	4.11
	C	13.6	28.1	39.0[d]	71.29	72.76	350	162.5	172.6	4.17	3.61	2.09
48	C	14.0	28.5	20.2	73.55	77.27	294	129.5	156.7	4.74	5.49	1.59
	C	15.7	41.2	22.7	75.25	79.07	304	131.5	158.3	3.45	5.10	1.91
	C	13.8	28.6	19.9	74.47	81.17	306	127.5	160.0	4.45	5.51	1.73
72	C	16.4	48.7	20.6	74.95	77.98	306	129.0	150.1	4.26	5.13	1.58
	C	14.9	33.4	19.8	74.50	79.44	304	122.5	161.0	3.99	5.91	1.89
	C	14.7	29.1	22.8	75.92	79.46	292	129.0	154.3	3.97	5.58	1.95
	E	17.7	58.0	24.0	74.90	84.17	302	138.5	157.6	4.03	5.03	1.76
	E	16.6	43.1	23.2	75.03	80.03	232	100.0	122.4	2.59	4.18	2.45
96	C	17.6	53.0	9.3	74.67	77.15	304	132.0	153.7	3.25	5.13	1.76
	C	15.8	41.1	18.6	74.93	80.24	296	133.5	158.8	4.02	5.26	1.51
	E	16.0	36.0	21.4	75.12	79.71	304	134.0	144.3	5.53	4.85	2.20
	E	14.9	37.3	20.6	74.75	81.39	282	131.5	150.2	3.62	5.13	2.43
120	C	14.1	27.7	24.5	75.15	77.15	316	130.0	156.9	4.47	6.74	1.75
	C	16.0	37.8	18.4	75.88	79.43	300	124.5	146.2	4.25	5.95	1.72
	C	15.9	40.8	22.5	74.50	80.31	298	129.5	153.4	4.55	5.29	1.61
	E	15.0	37.3	21.9	81.20	75.41	282	127.5	150.7	4.06	4.43	1.62

[a] Based on hematocrit < 25.2%.

[b] Sockeye salmon smolts were exposed to sea water after a 120 h exposure to 0.65 mg/L DHA in fresh water.

[c] C=control, E=exposed.

[d] Eliminated on basis of BKD symptoms; fluid filled abdomen.

method could not eliminate chronically infected fish so that the results of

Expt. 2 may be viewed as an interaction of disease and the toxicant.

The Interaction of BKD with the Toxicity of DHA

The presence of a chronic BKD infection appeared to increase the suscep-

tibility of sockeye salmon to DHA toxicity (Fig. 25). In Expt. 3 conducted

with healthy fish, only 2 out of the 72 exposed fish died; one near the end of

the freshwater toxicant exposure period and the second after 65.5 h in the

seawater recovery phase. In Expt. 1, in which the BKD infection was first

detected, the disease appeared to contribute to an increase in the latent

toxicity of DHA, that is, mortalities began only after the fish had been in

sea water for 24 h with a total of 6/60 exposed fish dying in this period.

The "mortality" curve shown for Expt. 2 was generated in a slightly

different manner than for Expts. 1 and 3 in which actual times to death were

recorded. In Expt. 2 four fish were collected after death but eight fish

were sampled in a "moribund" condition to permit the determination of % water

in muscle and gut while still alive. Based on past observations, these fish

would have died within 3-4 hours but for the purposes of the construction of

the toxicity curve, sampling time was taken to be time to death. The addition

of several hours would have made a small difference in the location of the

line; shifting it slightly to the right (Fig. 25). Three fish were sampled

at the "inverted" stage (fish which had lost equilibrium) but were not used

to plot the toxicity curve. In Expt. 2, in which the BKD infection

was reaching an advanced chronic stage in some fish, the combined stress

loading due to the disease and toxicant was shown by mortalities occurr-

ing both earlier and in greater numbers, as illustrated in Fig. 25.

That the slope of the mortality curve does not break until 24 h into

the seawater recovery period indicates a persistence in the enhancement
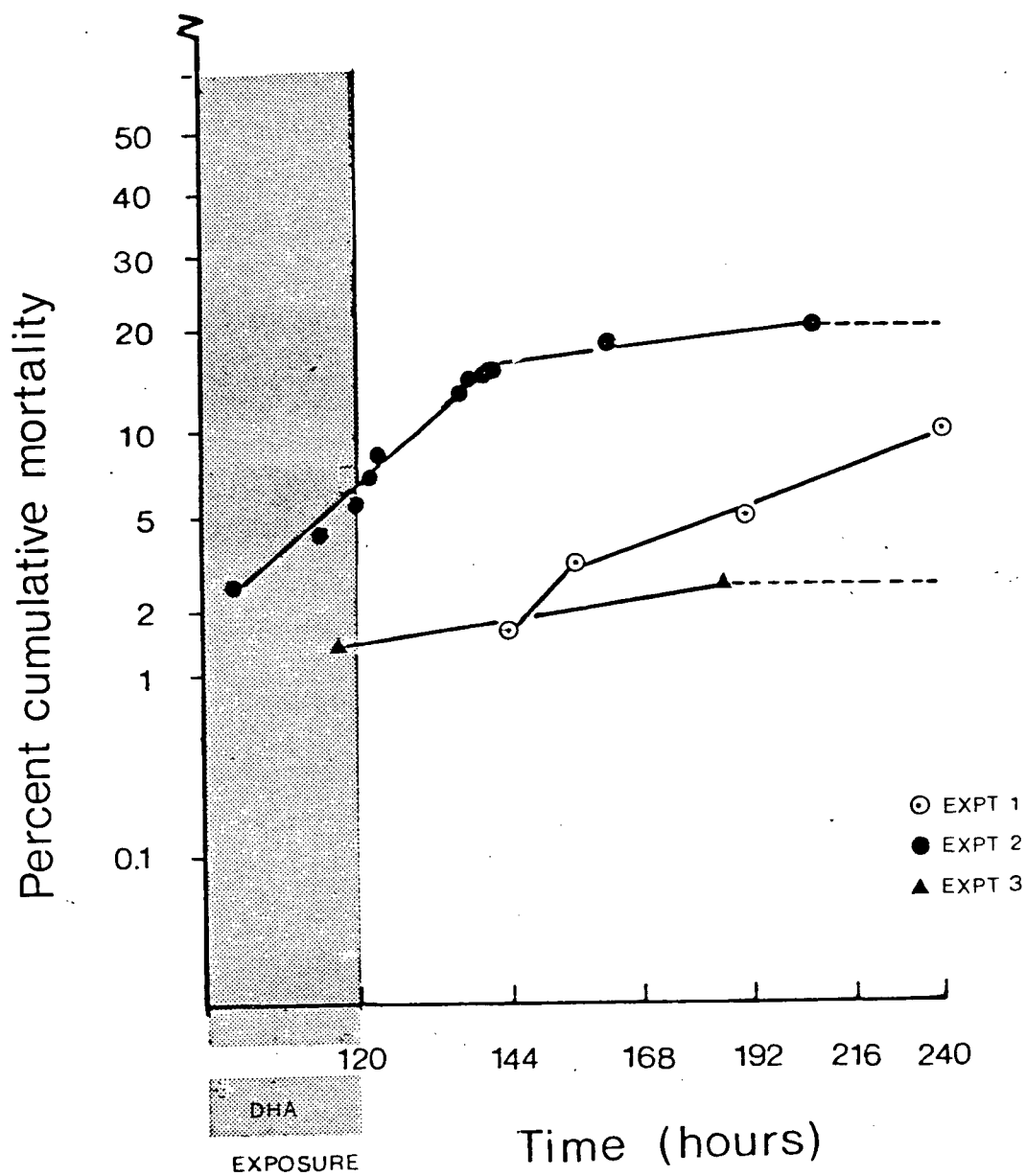
Figure 25. The extent of salmon mortality during the three
electrolyte balance experiments (Expts. 1, 2
and 3) in which sockeye salmon smolts were exposed
to sea water after a 120 h exposure to 0.65 mg/L
DHA in fresh water.

of the sublethal toxicity of DHA. After the break, two more fish died for a total of 15/75. Evidence that the BKD infection per se or in combination with the salinity stress was not lethal is provided by the total absence of control mortality in Expt. 1 and only 1 mortality out of 72 control fish in Expt. 2 in which the disease was more advanced.

In the present context "acute toxicity" is defined as that occurring during the 96-h toxicant exposure period. The "sublethal" exposure used for the electrolyte balance experiments was purposely chosen to cause negligible mortality in an additional 24-h exposure to DHA. The mortality data confirm that this design was achieved and that depending on the severity of the BKD infection, this "sublethal" exposure shifted closer to the "acute" level of exposure and the interaction of stresses of disease/toxicant/sea water contributed to an increase in the latent toxicity of DHA. This would amount to a shift to the left of the acute toxicity curve (Fig. 9) which was the basis for the choice of a concentration of DHA which would be "safe" for 120 h.

APPENDIX III. PLASMA BILIRUBIN

INTRODUCTION

During the course of exposure to DHA, juvenile sockeye salmon were seen
to develop a jaundiced appearance characterized by a yellowish tinge which
became particularly noticeable in the membranes of the paired fins. As
jaundice in mammals is brought about by a buildup of bilirubin in the blood,
plasma bilirubin levels of exposed fish were compared to those of controls.

In vertebrates, bilirubin is a natural end-product of the catabolism of
hemoglobin, formed in the reticuloendothelial system, delivered to the blood
stream and cleared by uptake in the liver cells. Two types of bilirubin are
recognized: unconjugated (UCB) and conjugated (CB) bilirubin. Bilirubin
normally circulating in the blood stream (UCB) is protein bound and in-
soluble in water. In the liver, it undergoes a conjugation and is thus
rendered soluble before excretion into the bile. This differential solubility
forms the basis of the "direct" - soluble (CB) vs. "indirect" - insoluble(UCB)
test for bilirubin and the relative proportions of CB/UCB are used to
characterize the type of jaundice involved. For example, a rise in serum
UCB without a concomitant rise in CB can indicate an increase in blood
destruction or hemolysis but that the hepatobiliary system is still excreting
bilirubin in a normal fashion.

As normal serum contains negligible to very low amounts of CB, its rise
is interpreted as a sign of a hepatobiliary disorder such as acute parenchymal
disease or biliary obstruction (Gray, 1961; Nosslin, 1960; Wintrobe, 1961).

To determine whether the apparent jaundice was related to elevated
bilirubin levels, both CB and UCB concentrations were measured in sockeye
salmon smolts in two experiments (Expt. PB-1 and PB-2).

MATERIALS AND METHODS

In both experiments a 5-day exposure of smolts to 0.65 mg/L DHA in fresh water was followed by terminal blood sampling and a determination of plasma bilirubin levels. In Expt. PB-1 bilirubin was determined in blood pooled from 10 fish which had been exposed during the DHA tissue residue experiment (Appendix I-4). When elevated plasma bilirubin levels were confirmed, the exposure was repeated using 20 fish. In addition to the controls for each experiment, bilirubin was also measured in a sample of plasma pooled from 5 salmon taken from the laboratory stock tank during preliminary work.

The sockeye salmon smolts used in these experiments were from the same stock as used in the DHA tissue residue experiment and holding, acclimation, toxicant exposure and blood sampling protocol was the same as described in the General Methods.

After the measurement of hematocrit (Hct) of each fish, the blood plasma was pooled for measurement of osmolality (mOsM), total (CB+UCB) and direct (CB) bilirubin as well as plasma iron (Fe). Plasma Fe was measured to determine whether the bilirubinemia could be attributed to increased hemoglobin breakdown as occurs in hemolytic jaundice (Smith, 1973).

Plasma bilirubin was measured by the modified diazo procedure developed by Michaelsson (1961) for the determination of serum bilirubin in newborn infants. However, rather than using 1 mL of 1/10 diluted serum, 50 µL of undiluted plasma was added to the reagents halved in volume. Absorbance was measured at 600 nM on a Pye Unicam or a Gilford 2400 Spectrophotometer. To check the accuracy of the method, a calibration curve was constructed using total bilirubin levels given in human serum calibration references (General Diagnostics, Calibrate I, II, III). As this showed

that the absorption law was being followed, the calibration constant of 43

was used as described by Michaelsson (1961).

Plasma iron was measured in pooled samples of blood taken from salmon

in Expts. PB-1 and 2, as well as from a single rainbow trout (1400 g) during

the development of the analytical procedures. Preparative methods followed

those described for human serum (Perkin Elmer, 1971) and plasma iron was

measured by Atomic Absorption Spectrophotometry using an air-acetylene flame

at 248.3 nm UV.

RESULTS

No mortalities were observed in either experiment during the 5-day

exposure period. While blood plasma of control fish was essentially clear,

that of the DHA-exposed fish developed the characteristic yellow tinge. The

results of the blood chemistry analyses are shown in Table XXIV for Expt.

PB-1 and in Table XXV for Expt. PB-2. Both experiments show similar

results. Plasma bilirubin levels are elevated in fish exposed to DHA, with

the direct (CB) accounting for 81.6% and 69.7% of the total bilirubin

(Expts. PB-1 and 2 respectively). There appears to be very little change in

plasma iron, while hematocrit was elevated and osmolality lowered by the

resin acid exposure. In the blood plasma pooled from the 5 salmon taken from

the stock holding tank, conjugated bilirubin was not detectable while total

bilirubin was 0.30 mg%. The plasma of the single rainbow trout contained

30 µg% iron.

DISCUSSION

An increase in plasma bilirubin can be caused by increased blood destruc-

tion or by the reduction in the capacity of the liver to remove the pigment

from the blood and excrete it in the bile. However, the liver is generally

Table XXIV. Blood chemistry of sockeye salmon smolts[a] exposed to
0.65 mg/L DHA for 5 days in Expt. PB-1.

	Pooled Plasma			Blood
	Bilirubin	Iron	Osmolality	Hematocrit[b]
	Total Direct			
	mg%	μg%	mOsm/kg	%
Exposed N=10	2.06 1.68	46	282	49.0 ±1.55 [c]
Controls N=10	0.19 0.11	42	290	41.7 ±0.69

[a] Size range 15-20 g

[b] Mean ±SE

[c] Significantly different from controls p< 0.05 (t-test)

Table XXV. Blood chemistry and size of sockeye salmon smolts exposed to 0.65 mg/L DHA for 5 days in Expt.PB-2.

	Pooled Plasma			Blood	Fish	
	Bilirubin	Iron	Osmolality	Hematocrit	Fork length	Wet weight
	Total Direct mg%	μg%	mOsm/kg	%	$\bar{x}$ ±SE cm	g
Exposed N=20	2.28 1.59	38	288	52.4[b]±0.8	13.7 ±0.3	22.41 ±1.57
Controls N=18	0.47 ND[a]	38	297	43.0 ±0.6	14.0 ±0.2	22.0 ±1.01

[a]Not detectable

[b]Differs significantly from control $p<0.05$ (t-test)

considered to have a large reserve capacity and an increase in plasma

bilirubin can usually be linked to a dysfunction in the hepatic excretion of

the pigment (Wintrobe, 1961). Nevertheless, the levels of plasma bilirubin

have been selected as a measure of increased Hb degradation in rainbow trout

and coho salmon exposed to formalin (Wedemeyer,1971).

Hemoglobin is broken down into globin, iron and bilirubin. In the

rainbow trout, most of the iron is bound to the plasma protein transferrin

(Fromm, 1977), however during increased red blood cell destruction, this

binding capacity may be exceeded and lead to increased plasma Fe levels.

The method used in the present study measured the levels of unbound plasma

Fe and the results indicate no measurable effect of DHA on this parameter in

sockeye salmon. The range of plasma iron values (38-46 μg%) is lower than

the mean of 55 μg% obtained for the rainbow trout (Fromm, 1977).

In the present study it is unlikely that the observed increase in plasma

bilirubin was due to increased red blood cell destruction or hemolysis. The

exposure of salmon to DHA in fresh water invariably led to an elevation, not a

lowering of hematocrit, no change or a slight drop in plasma K^+ and virtually

no change in plasma Fe levels; both of which could be expected to rise as a

result of increased erythrocyte destruction. The observed rise in plasma

bilirubin can therefore be attributed to a reduction in the efficiency of its

excretion.

The reliable measurement of trace amounts of bilirubin in plasma is

difficult and considerable work has been done towards the development of

methodology, mostly because of the importance of accurate determinations in

infants suffering from neonatal jaundice. The method of Michaelsson (1961)

is considered to be the most reliable (With, 1968) and was applied to the

measurement of salmon bilirubin in this study. Due to the use of different methods, widely varying levels of plasma bilirubin have been reported for fish. Sakai and Kawazu (1978) reported 0.025 mg% for the carp (Cyprinus carpio) and 0.032 mg% for the rainbow trout. Satia et al (1974) and Buckley (1976) reported 0.05 mg% for rainbow trout and coho salmon respectively while Wedemeyer (1971) found plasma bilirubin levels of 0.6 mg% for rainbow trout and 1.6 mg% for coho salmon. In the present study, total plasma bilirubin levels in control sockeye salmon averaged 0.33 mg%. The sample of plasma pooled from 5 salmon taken from the stock tank yielded 0.30 mg%. The accompanying very low or non-detectable levels of conjugated bilirubin in control samples are consistent with the mammalian literature (Gray, 1961; Wintrobe, 1961).

When there is increased blood destruction, the conjugated form of bilirubin constitutes less than 15% of the total; higher proportions are usually attributed to the regurgitation of the conjugated bilirubin glucuronide by the liver (Michaelsson, 1961). In salmon exposed to DHA, there was a dramatic rise in the total bilirubin, of which the bulk (70-82%) was attributed to the conjugated form. This constitutes further evidence against increased blood destruction and these symptoms therefore can be interpreted as a result of obstructive jaundice.

Two main types of obstructive jaundice are recognized: A) Mechanical - due to extra-hepatic obstruction of bile flow and B) Parenchymatous - due to intra-hepatic obstruction stemming from the distortion of liver cell architecture by local necrosis of liver cells (Gray, 1961). Extra-hepatic obstruction leads to the forced retention of bile in the liver, distending the biliary passages and eventually rupturing the bile capillaries. This results in the passage of bile into the sinusoids and back into the blood. As the

bile is toxic to human liver parenchymal cells,its leakage out of the bile

canaliculi can lead to local necrosis (Sherlock, 1968). Hendricks et al.

(1976) reported that rainbow trout bile was highly caustic to trout liver

tissue.

The jaundice observed in salmon sublethally exposed to DHA could be of

both types. In fish which swallowed large amounts of fresh water the increased

hydrostatic pressure of a turgid stomach may have reduced bile flow. In fish

in which no excess water intake was observed, intra-hepatic obstruction is

suggested and may be related to liver histopathology.

Residue analyses showed that the liver had among the highest residues

of DHA of all the tissues investigated (Fig.23 and Fig.24). The high

concentrations of DHA in the liver, the jaundice and the suggested importance

of the hepato-biliary route for excretion of the toxicant (p.138) make it

tempting to speculate that an interrelationship exists. Fujiya (1961, 1965)

observed necrotic changes in many of the major organs, especially the liver,

particularly around the biliary ducts, of fish taken from waters receiving

kraft pulp mill waste. Tomiyama (1965) proposed that these histopathological

changes may have been caused by resin acids. Although histopathological

observations were not conducted in the present study, DHA accumulation may

result in necrotic changes in the liver which could account for the observed

obstructive jaundice.

On the other hand, the accumulation of DHA may be secondary to the

obstructive jaundice. Bile flow is an important determinant of the rate at

which many xenobiotics (foreign compounds) are cleared from the plasma and

excreted in the bile (Tuttle and Schottelius, 1969). The failure or over-

loading of the hepatobiliary system in fish exposed to DHA may have contributed

to the accumulation of this toxicant in various organs. This could be especially marked if the bile leaked out into the liver parenchyma leading to necrotic changes and a reduction in physiological function. A reduction in excretion of DHA via the bile could compromise the function of other organs.

The DHA-induced jaundice may have other chronic effects related to the direct influence of free bilirubin on tissue metabolism. At elevated plasma bilirubin levels such as occur in infants during neonatal jaundice, bilirubin crosses the blood/brain barrier and accumulates in brain tissue. At these levels, in vitro studies have demonstrated the uncoupling of oxidative phosphorylation in isolated mitochondria (Gray, 1961). These findings are remarkably close, albeit indirectly, to one of the possible modes of toxic action of kraft mill effluent on fish, as proposed by Warner (1965).

Although these mechanisms remain speculative, hyperbilirubinemia is frequently related to a reduced clearance due to hypoxia and can interfere with the cell volume regulatory mechanism of red blood cells (Wintrobe, 1961). In the present study, increased hematocrit and jaundice have been shown to be characteristic symptoms of sublethal DHA toxicity to sockeye salmon. As one of the suggested mechanisms is one of toxicant-induced hypoxic stress, there may be a relationship between hypoxia, hematocrit, and the accumulation of bilirubin in the blood of salmon exposed to DHA.

APPENDIX IV.   THE EFFECTS OF SUB-LETHAL DHA EXPOSURE IN FRESH WATER ON THE
RED BLOOD CELL DIMENSIONS OF SOCKEYE SALMON SMOLTS

In the present study, the sublethal exposure of juvenile sockeye salmon

to DHA in fresh water invariably resulted in elevated blood hematocrit values.

As a rise in hematocrit may result from an increase in red blood cell size,

measurements were made of the length and width of erythrocytes taken from

exposed and control fish.

MATERIALS AND METHODS

Blood smears were made during blood sampling at the conclusion of the

exposure period in the DHA residue experiment (Appendix I-4). Duplicate

smears were air dried, fixed in methyl alcohol and stained with Giesma

(Hesser, 1960). The length and width of 25 red blood cells was determined on

each slide using an ocular micrometer in a microscope under 400 x magnifi-

cation.

Cell areas were calculated using the formula(length x width x 0.25 π)and

the ratio of width/length was used as a measure of cell roundness (Murray and

Burton, 1979). As preliminary calculations showed no difference between

replicates for each fish, the values were pooled and the means were calculated

on the basis of the measurements of 50 cells per fish. Grand means for cell

area and roundness for exposed and control fish were then compared using

Student's t-test. In addition, the osmolality of pooled plasma samples was

measured after the hematocrits had been determined for individual fish.

RESULTS AND DISCUSSION

The results (Table XXVI) show that DHA-exposed salmon displayed two of the

symptoms characteristic of sublethal exposure to this toxicant in fresh water;

a lowering of plasma osmolality and a rise in hematocrit. Elevated hematocrit

was accompanied by a highly significant increase in the area of the red blood cell (Table XXVI) but this occurred without any change in the degree of cell roundness. As the observed rise in hematocrit (17.4%) was considerably greater than the measured change in cell area (10.9%), the difference is probably due to a concomitant increase in cell thickness (height).

An approximation of the change in cell volume can be calculated by taking the cell height to be $\sim$ 4 μm (Eddy, 1977) and assuming that the increase in height was proportionately similar to that observed for the length and width dimensions. By applying the formula for a prolate sphere (V=4/3 π lwh), where l, w and h refer to the radii of the red blood cell, the mean volume for the control cells is 211 μm^3. If the cell height increased $\sim$10%, the mean volume of the "exposed" cells would be 367 μm^3. This represents an 18% increase in volume and corresponds well to the observed rise of 17.4% in blood hematocrit.

Based on the results of this experiment as well as on a more thorough discussion of other mechanisms which could lead to hemoconcentration (p.98), it can be concluded that the sublethal exposure of sockeye salmon to DHA in fresh water leads to a dilution of the blood plasma and to a swelling of red blood cells which contributes to an increase in blood hematocrit.

Table XXVI. Red blood cell dimensions[1] of juvenile sockeye salmon exposed to 0.65 mg/L DHA for 120 h in fresh water.

	Erythrocyte				Blood	Plasma[2]
	Length μm	Width μm	Area μm^2	Roundness	Hematocrit %	Osmolality mOsM/kg
Exposed	17.02[a]±0.08	9.36[b]±0.06	125.03[a]±0.94	0.552 ±0.005	48.98[a]±1.55	282
Control	16.06 ±0.52	8.94 ±0.10	112.74 ±1.82	0.556 ±0.005	41.72 ±0.69	290

[1]Based on the means of measurements of 50 red blood cells for each of 10 fish.

[2]Pooled plasma

Differs significantly from control [a] $p<0.001$ [b] $p<0.01$ Student's t-test

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