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**Function of Muscle Adipocytes in Atlantic Salmon (*Salmo salar*)**
**Exposed to Hydrocarbons**

by

Shengying Zhou

A Thesis Submitted to the
Faculty of Engineering
in Partial Fulfilment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Food Science and Technology

APPROVED:

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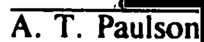

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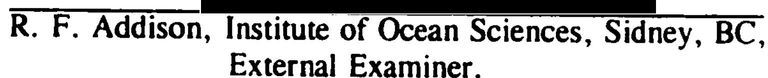

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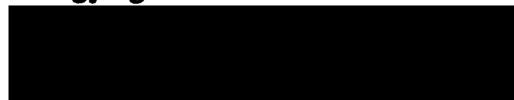
Signature of Author: 

Date: December 12, 1996

## DEDICATION

This manuscript is dedicated to:

**My Parents** - for their love and support over the years

**My Parents in Law** - for their support and devotion in taking care of LuLu during my studying in Canada

**My Wife, Li Yang**, - for her support, love and understanding

**My Daughter, Lulu,** - who has inspired me toward success

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## LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| AA | aggregated adipocytes |
| AD | adipocyte |
| AHH | aryl hydrocarbon hydroxylase |
| AMPL | acetone-mobile polar lipids |
| AM | adipocyte membrane |
| AN | adipocyte nucleus |
| bdl | below detectable limit |
| BF | belly flap |
| BV | blood vessel |
| C(numeric subscript) | straight chain alkane (number: chain length) |
| CDA | connective tissue devoid of adipocytes |
| CHO | free sterol |
| CWA | connective tissue with adipocytes |
| DG | diacylglycerol |
| DM | dark muscle |
| DMF | dark muscle fiber |
| DMWM | dark muscle without myosepta |
| FFA | free fatty acid |
| FID | flame ionization detector |
| GLC-FID | gas-liquid chromatography-flame ionization detector |
| GLC-MS | gas-liquid chromatography-mass spectrometry |
| HPLC | high-performance liquid chromatography |
| ITD | ion trap detector |
| LC-GC | liquid chromatography-gas chromatography |
| LF | lower flank muscle |
| LPC | lyophosphatidyl choline |

| | |
|---|---|
| MA | mesenteric adipose tissue |
| MYO | myoseptum |
| n.d. | not detected |
| PA | phosphatidic acid |
| PAH | polycyclic aromatic hydrocarbon |
| PBB | polybrominated biphenyl |
| PC | phosphatidyl ethanolamine |
| PCB | polychlorinated biphenyl |
| PE | phosphatidyl ethanolamine |
| PM | polar materials |
| ppb | part per billion (ng/g) |
| ppm | part per million ($\mu$g/g) |
| SF | subdermal fat tissue |
| SPH | sphingomyelin |
| TG | triacylglycerol |
| TLC-FID | thin-layer chromatography-flame ionization detector |
| tr | trace amount |
| WF | whole fillet |
| WM | white muscle |
| WMF | white muscle fiber |
| WMT | whole muscle tissue |
| WMWM | white muscle without myosepta |
| WSF | water-soluble fraction |
| WSF-HC | water-soluble fraction-hydrocarbon(s) |

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# ABSTRACT

The role of muscle adipocytes in Atlantic salmon (*Salmo salar*) exposed to the water-soluble fraction-hydrocarbons (WSF-HC) of crude oil was thoroughly investigated by histologically examining the adipocyte and lipid distribution in different parts of muscle tissues, and by analyzing the WSF-HC in tainted adipocytes isolated from the muscle tissue and by analyzing the WSF-HC in different muscle tissues exposed to a series of WSF-HC concentrations.

Belly flaps, subcutaneous fat layer, and myosepta in the muscle tissue were found to be the main sites for adipocytes. In muscle tissue proper, adipocytes were mainly distributed in myosepta and to a lesser extent in connective tissues surrounding bundles of white muscle fibers. Lipid droplets occurred in the connective tissue around bundles and individual dark muscle fibers, but were only occasionally occurred around individual white muscle fiber. Finely dispersed intracellular lipid droplets were present in dark muscle cells but were not observed in dorsal white muscle cells. Small adipocytes (20-40 $\mu$m) predominate in the adipocyte population in tissues of Atlantic salmon. Lipid storage in the myosepta of muscle tissues was quantitatively determined by Chromarod thin-layer chromatography with Iatroscan flame ionization detection (TLC/FID). It was found that this type of connective tissue, not the actual muscle fibers, stores most of the muscle lipids in Atlantic salmon.

Exposures of Atlantic salmon to both high levels of WSF-HC for a short period of time (8 h) and low levels of WSF-HC for a prolonged period (96 h) were carried out by using WSF-HC of Flotta North Sea crude oil rich in alkylated benzenes and by employing WSF-HC of K-D diluent, a commercial petroleum product rich in polycyclic aromatic hydrocarbons (PAHs). Atlantic salmon rapidly took up the WSF-HC in exposure water during both the 8 h and the 96 h exposure periods. The amounts accumulated in various tissues of salmon and their rates of approaching the plateau values were initially independent of tissue lipid content, but gradually became partially

correlated with lipid contents after 6 h of exposure in 0.27 ppm of WSF-HC. Maximum concentrations of WSF-HC were reached after only 12 h of exposure in tissues having relatively lower lipid reserves (dorsal white muscle and dark muscle). It would take much more time (beyond 24 h) for tissues rich in lipids (subdermal fat tissue, mesenteric adipose tissue and belly flap) to attain equilibrium of WSF-HC between the tissue lipids and the exposure water. WSF-HC recovered from various tainted tissues were similar to those in the exposure water with respect to their relative abundance. Atlantic salmon were rapidly freed of most of the tainting WSF-HC (mainly alkylated benzenes of lower molecular weights) during the first 10 days of depuration in clean seawater, but the complete discharge of the remaining WSF-HC (mostly PAHs) from muscle tissue would take months. The depuration rates of accumulated WSF-HC were highly correlated with the lipid content of different muscle tissues and decreased as the molecular weights of individual WSF-HC increased. WSF-HC accumulated in the muscle tissue through the short-term exposure were depurated much faster than those accumulated through the long-term exposure. Prolonged starvation of the exposed Atlantic salmon did not help to accelerate the clearance of the accumulated tainting hydrocarbons from either whole muscle tissue or the subcutaneous fat tissue.

After 96 h exposure of market-sized Atlantic salmon to 0.2 ppm WSF-HC, adipocytes isolated from the muscle tissue accumulated 14.3 times more WSF-HC (59.4 ppm) than the dorsal white muscle (4.2 ppm), while 54% of the tainting WSF-HC in the dorsal white muscle was found to be stored in associated adipocytes. When returned to clean seawater, WSF-HC accumulated in dorsal white muscle were released much faster than those in the adipocytes. After four days of depuration the adipocytes became the principal storage site of residual WSF-HC in white muscle and the depuration of WSF-HC from muscle tissue then reflected the release of WSF-HC from adipocytes in the muscle tissue. It is the adipocytes in the muscle tissue which control the actual accumulation and release of hydrocarbons in the whole muscle tissue of Atlantic salmon. The role of adipocytes in the storage and retention of xenobiotics may be applicable not

only to the WSF-HC but also to various other various organic pollutants due to their similarity in hydrophobicity. This key role of adipocytes would also be expected to occur in various other aquatic organisms, particularly those with high lipid content.

# GENERAL INTRODUCTION AND THESIS OBJECTIVES

Hydrocarbons enter the aquatic environment via three general processes: Biosynthesis, geochemical processes and anthropogenic inputs (Farrington and Meyer 1975, Dudley 1976, Clark et al. 1977). Accordingly, hydrocarbons present in most aquatic organisms can be classified into two types: biogenic and non-biogenic (or abiogenic). The non-biogenic hydrocarbons or pollutants are derived from many sources. These include vessel activities, off-shore crude oil (petroleum) production, oil spills, sewage discharges, direct or indirect precipitation of airborne pollutants, and the leaching of terrestrial organic contaminants into the water column with rainwater from percolating through the soil etc. (Canton et al. 1984, Nevenzel 1989, McCahon and Pascoe 1990, Wiener et al. 1990, Chesterikoff et al. 1991, Napolitano et al. 1992). The impacts of non-biogenic hydrocarbons on the aquatic environment have received increasing attention in recent decades and research concerned with their accumulation and retention in aquatic organisms has intensified because of a substantial commitment towards understanding hydrocarbon pollution problems.

Various aquatic organisms, including fishes and shellfishes, have been investigated in studies of the toxicity, accumulation, depuration and retention of petroleum hydrocarbons. Both complex mixtures of hydrocarbons [e.g., crude oil or the water-soluble fraction (WSF) of crude petroleum] and individual hydrocarbons (mainly aromatic hydrocarbons such as toluene, naphthalene, and alkylnaphthalenes etc.) can be employed as "typical" pollutants (Melancon and Lech 1978, Roubal et al. 1978, Varanasi et al. 1978, Capuzzo et al. 1984, Niimi and Palazzo 1986, Kennish et al. 1988, Purdy 1989, Heras et al. 1992). Extensive investigations have also been made on the wide distribution of relatively stable industrial organic chemicals in the aquatic environment, including pesticides, PCBs (polychlorinated biphenyls), and PBB (polybrominated biphenyls) etc. (Gruger et al. 1977, Ogata et al. 1980, Safe 1984, Miller et al. 1992, Granby and Spliid 1995). It has been found that there are substantial species-specific

differences in the rates of uptake, depuration and levels of retention of organic pollutants by aquatic organisms. It has been suggested that lipid plays an important role in the behaviour of organic pollutants in the tissues of aquatic organisms. The higher the lipid content of the tissue, the more likely the accumulation and retention of organic pollutants in the tissue.

Despite the numerous studies concerned with organic pollutants in aquatic organisms, the role of individual cell compartments, particularly adipocytes present in the tissues in controlling the uptake, depuration and retention of organic pollutants in aquatic organisms, is still not clear. Therefore, fundamental investigations on the distribution of adipocytes and lipid in tissues and the isolation of adipocytes from tissues of aquatic organisms are important steps towards the complete understanding of interactions between organic pollutants and aquatic organisms.

Atlantic salmon (*Salmo salar*) provide one of the most important fish species in fisheries and aquaculture industries in the cold water regions of the world. It is a fatty fish species and normally contains 10-12% total lipid in muscle tissue (wet tissue basis) (Ackman 1989). It is the combination of these two factors that promote its selection in this study as the best candidate for investigation of the role of lipids in the uptake and retention of organic pollutants in aquatic organisms. The water-soluble fraction of crude petroleum oil was used as the target organic pollutant since the most important and wide spread effect of petroleum based accidents is the intimate contact of the WSF hydrocarbons (WSF-HC) with fish and other aquatic organisms rather than the obvious slick or dispersed droplets of crude petroleum (Rice et al. 1977).

Fig. 1 shows a schematic outline of the thesis work. The objectives of these studies are summarised as follows:

(1) To fully understand the distribution of adipocytes and lipid in the muscle tissue of Atlantic salmon.

(2) To quantitatively determine the lipid storage in the myosepta, the main connective tissue of muscle tissue.

(3) To reveal the role of different cell compartments, particularly adipocytes, in controlling the uptake, depuration and retention of WSF-HC in the muscle tissue of Atlantic salmon.

(4) To understand the uptake and depuration of WSF-HC in different types of muscle tissues and to reveal the relationship between lipid content of different muscle types and the uptake and depuration behaviour of WSF-HC in the muscle tissue.

(5) To determine the uptake and depuration rates of total WSF-HC in the muscle tissue of Atlantic salmon under various exposure conditions.

(6) To determine the effect of prolonged starvation on the elimination of accumulated WSF-HC from the muscle tissue.

(7) To explore the uptake pattern of WSF-HC by the muscle tissue and subdermal fat tissue of Atlantic salmon and to determine the minimum exposure time required for reaching an equilibrium of WSF-HC partitioning between the exposure water and the muscle tissue.

(8) To reveal the difference in the uptake, depuration and retention of different types of WSF-HC, such as aromatics, aliphatics and cyclics etc., in the muscle tissue.

(9) To determine the minimum time required for the complete clearance of accumulated WSF-HC in muscle tissue.

(10) To improve the efficiency for recovering tainting WSF-HC from tissue samples by steam distillation.

(11) To understand the behaviour of hydrocarbons in water.

(12) To develop a method for the continuous preparation of WSF-HC stock solution by a Karr reciprocating-plate countercurrent extraction column.

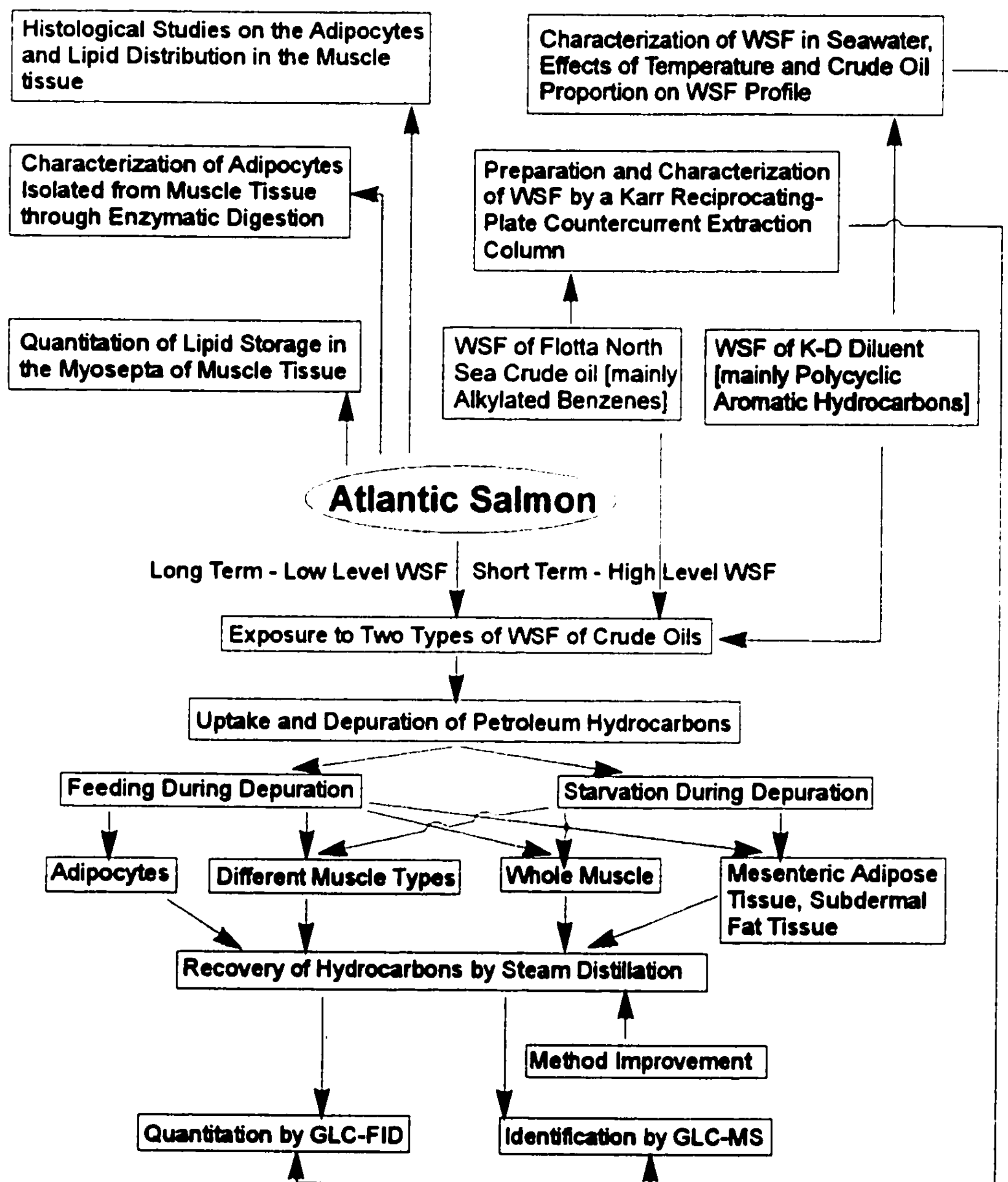


Fig. 1.0. A brief outline of the thesis work.

# SECTION I. ADIPOCYTES AND LIPIDS IN THE MUSCLE TISSUE OF ATLANTIC SALMON (*Salmo salar*)

## 1. ENZYMATIC ISOLATION OF ADIPOCYTES AND HISTOLOGICAL STUDIES ON THE DISTRIBUTION OF ADIPOCYTES AND LIPIDS IN THE MUSCLE TISSUE OF ATLANTIC SALMON

### 1.1 Introduction

Connective tissues are defined as the complex of cells and extracelluar materials that provide the support and framework for all of the other tissues of the body. In fish, the body is supported more by muscle fibres than by the skeleton, which is not as substantial as in terrestrial animals. The numerous muscle fibres are held together through connective tissue. Loose connective tissue is widely distributed in the fish body and is characterized by a relative predominance of resident cells. Adipocytes are commonly found in loose connective tissue. In mammalian tissues adipocytes have been studied for characteristics such as morphology, size distribution and metabolism (DeMartinis and Francendese 1982, Johnson and Greenwood 1983, Sinnett-Smith and Waddington 1992). Lipids in fish have generally been studied with respect to their chemical, biochemical, or biological aspects (Robinson and Mead 1973, Sheridan et al. 1983, Polvi and Ackman 1992). Only a few papers report details of the histological and morphological characteristics of these lipid bodies in fish (Yamada and Nakamura 1964, Yamada 1972, Eastman and DeVries 1982, Fauconneau et al. 1993, Aursand et al. 1994). Farmed Atlantic salmon of commercial size contain up to 12% lipids in the edible muscle, the principal organ and chief lipid storage site in salmonids (Ackman 1989). Understanding the distribution of adipocytes within the muscle tissue is essential to further studies on their biological function, their chemical and biochemical aspects, and even their influence on the textural quality of muscle tissue.

## 1.2 Literature Review

### 1.2.1 Enzymatic Isolation of Adipocytes

Isolates of fat cells were first prepared and studied by Rodbell (1964) who found that free fat cells could be isolated from white adipose tissue by incubation of the tissue with bacterial collagenase. Since then, many studies have been conducted to reveal the metabolic behaviour of adipocytes released from rat adipose tissue, and all were based on the Rodbell's isolation procedures.

In Rodbell's method, adipose tissue was added to a collagenase solution in a siliconized flask containing albumin-bicarbonate buffer, collagenase and glucose. Incubation was carried out for 1 hour at 37°C in a metabolic shaker, and fat cells were liberated from the tissue fragments by gentle stirring with a rod. Fragments of tissue remaining after this treatment were removed with forceps. The resulting suspensions of cells were centrifuged in polyethylenic centrifuge tubes. The fat cells floated to the surface, and the stromal-vascular cells were sedimented. The stromal-vascular cells were removed by aspiration, and the fat cells were washed three time in the same buffer. Fat droplets, which may have been formed from the breakage of the fat cells, floated more rapidly to the surface than the fat cells after gently stirring the cell suspension and were aspirated from the surface.

Since the development of Rodbell's isolation method, several slight modifications were made by other researchers. Fain et al. (1965) excluded glucose from the incubation buffer and the solution which was used to wash the cells. Christiansen et al. (1985) isolated the adipocytes from rainbow trout (*Salmo gairdnerii*) using a high concentration of collagenase (3 mg/mL buffer), which is still considered harmless for the cells. Fain (1975) reported a low concentration of collagenase (0.2-0.5 g/mL) and long incubation time (45 min) for the isolation of white fat cells and found that it had the same result as the original method. Recently, Meignen et al. (1990) reported a simple device for the

isolation of fat cells which shows appreciable lowering of the degree of cell damage and the extent to which larger cells are ruptured during the isolation process.

The roles of collagenases and other proteolytic enzymes in the dispersal of animal tissues were extensively investigated by Kono (1969). The author indicated that crude collagenase is a mixture of a number of hydrolytic enzymes, and any purified collagenases or mixture of purified collagenases, unlike the crude preparation, did not disperse all of the rat tissues tested; however a combination of the actions of trypsin and the purified collagenases could disperse fresh rat tail tendon, adipose tissue, and cardiac muscle. Accordingly, it may be postulated that the cells in these tissues are doubly bound by two types of intercalated proteins, one digestible with trypsin and the other with a mixture of the purified collagenases A-$\alpha$ and B-$\alpha$. Studies employing bicarbonate buffer must be done with 5% $CO_2$ in the gas phase since the final pH will be around 8.2 to 8.4 in its absence. A Krebs-Ringer phosphate buffer with air as the gas phase instead of the bicarbonate buffer had been used by Fain (1975) for the isolation of fat cells and it was found that the response of fat cells to hormones was generally the same as for cells incubated in bicarbonate buffer. Unfortunately all of the reported isolation procedures were only suitable for the isolation of small quantities of adipocytes suitable for studying the metabolic activities of adipocytes. The amount of the isolated adipocytes was not enough for quantitative analysis of WSF-HC accumulated in those cells. Moreover, the adipocytes released from adipose tissue employing the above methods were normally a mixture of intact adipocytes and free fat from ruptured adipocytes.

The isolation of adipocytes from aquatic organisms has rarely been studied. One study by Christiansen et al. (1985) reported that adipocytes isolated from the mesenteric adipose tissue of rainbow trout were fragile and withstood incubation rather poorly. Besides rainbow trout, another marine organism, female mussels *Mytilus edulis* have been subjected to the isolation and purification of adipogranular cells for examination of their biochemical characteristics (Peek and Gabbott 1989, Peek et al. 1989).

### 1.2.2 Size Distribution of Adipocytes in Aquatic Organisms

Compared with the morphological studies of adipocytes in mammalian adipose tissues, much less attention has been paid to the size distribution of adipocytes in aquatic organisms. Most studies related to adipocytes in aquatic organisms were conducted through histological procedures, rather than through the isolation of adipocytes. Fauconneau et al. (1993) investigated the size distribution of adipocytes from abdominal subcutaneous adipose tissue of rainbow trout and found that very small adipocytes (20-30 $\mu$m) predominated, although larger cells were also present. The study also indicated that the size of adipocytes increased with the lipid content of the flesh. Yao et al (1994) studied the effect of feeding frequency on lipid accumulation in ayu *Plecoglossus altivelis* using scanning electronic microscopy (SEM). They found that increases in feeding frequency resulted in a reduction in the mean diameter of fat cells. A histological examination of salmonid adipose fin through both paraffin and frozen sectioning procedures revealed that small lipid droplets from 10 - 50 $\mu$m in diameter were the main population of adipocytes (Weisel 1968). The mean adipocyte volume in fin whale *Balaenoptera physalus* and the morphology of adipocytes in female *Euchaeta marina* and *Pleuromamma xiphias* (Copepoda: Calanoida) have also been studied (Pond and Mattacks 1988, Blades-Eckelbarger 1991). Based on all the information available on the size distribution of adipocytes in aquatic organisms, domination of adipocytes by a small cell population (20-50 $\mu$m) is rather common in the adipose tissues, except for the notothenioid fishes from McMurdo Sound, Antarctica (*Pleuragramma antarcticum* and *Aethotaxis mitoptery*) in which larger size populations of adipocytes, 60 - 120 $\mu$m, were reported (Eastman and DeVries 1982).

In human and other mammalian animals, the size distribution of adipocyte population is mainly centered around 80 - 100 $\mu$m in diameter, while the population of small adipocytes accounts for only a small fraction of the total population (Björntorp and Karlsson 1970, Smith et al. 1972, Meignen et al. 1990, Sinnett-Smith and Waddington

1992).

## 1.3 Materials and Methods

### 1.3.1 Atlantic Salmon Samples

Atlantic salmon, *Salmo salar*, were held in aerated seawater (6-8°C) at the Dalhousie University Aquatron and fed up to sacrifice on a diet of Fundy Choice feed (Corey Mills Ltd, Fredericton, New Brunswick). Two fish were used for histological studies, one male (2950 g, 61 cm) and the other a maturing female (3180 g, 65 cm). For the enzymatic isolation of adipocytes, one moderate-sized male (2218 g, 58 cm) and one large male (6084 g, 75 cm) were used. Another well fed smaller salmon (955 g, 41 cm, female) was also used for the enzymatic isolation of adipocytes, but was not included in the calculation of morphological size distribution. All the fish were transported in chilled seawater to the Canadian Institute of Fisheries Technology at the Technical University of Nova Scotia. They were then killed by a blow on the head, gutted and the gut cavities were washed thoroughly with water immediately before dissection. Total lipid content of muscle tissue was determined following the procedures of Bligh and Dyer (1959).

### 1.3.2 Histological Observations

Cubes of 1 $cm^3$ or less were dissected from different areas of the muscle tissue, adipose fin, and mesenteric tissue. The samples were immediately fixed in 4% formaldehyde and 1% glutaraldehyde in phosphate buffer at pH 7.2 for at least three days at 3°C.

Fixed samples were rinsed with running tap water and cut into small pieces (about one fourth of the original fixed tissue size). The sample blocks were immersed in O.C.T. compound (Tissue-TEK) on a sample block holder; and sectioned in a Harris

Cryostat (IEC Ltd., Needham Heights, Mass). The temperature of the Cryostat chamber was set at -20°C for white muscle and -30°C for other tissue blocks. Sections were then stained with Oil-Red-O as described by Humason (1979). Thick sections had a tendency to float off the slides. In this case, the sections were held in a small basket for the staining process and picked up again by subbed slides (Humason 1979) at the final stage of washing. The sections were mounted in glycerin jelly (Fisher Scientific Company, Fairlawn, New Jersey) and observed using a Zeiss Photo Microscope.

### 1.3.3 Isolation of Adipocytes

Adipocytes were isolated from belly flaps, mesenteric tissue and white muscle locations as illustrated in Fig. 1.1. Belly flap pieces were cut about 1.5 cm in width from a side of the half fillet as a source of the connective fatty adipose tissue. White muscle was taken from a dorsal portion starting about 2 cm behind the head and a concentrated myoseptum material was further dissected. The dissected tissues were immediately placed in a Petri dish containing Krebs-Ringer phosphate buffer with 1% albumin (bovine, fraction V, Sigma Chemical Co. St. Louis, MO). The Krebs-Ringer phosphate buffer contained 120 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 17.2 mM $HPO_4^{2-}/H_2PO_4^{1-}$ (Na salts) and the pH was adjusted to 7.4. The tissues were cut into small pieces in the Petri dish and added to 4 oz Nalgene plastic bottles containing 12 mL of 1% albumin in phosphate buffer plus 40 mg collagenase (type II, Sigma). The bottle was flushed with oxygen and stoppered. The tissues were digested for 1 h at 30°C in a shaking water bath.

The suspension of adipocytes was strained through a mesh (350 $\mu$m, metal) and centrifuged at 100 x g for 30 s in a polyethylene centrifuge tube. The proteinaceous digest solution in the bottom of the centrifuge tube was removed carefully with a long needle syringe. The adipocytes were resuspended in buffer, centrifuged, and the bottom layer again removed by syringe. A section of dialysis tubing (Spectra/Por, Los Angeles,

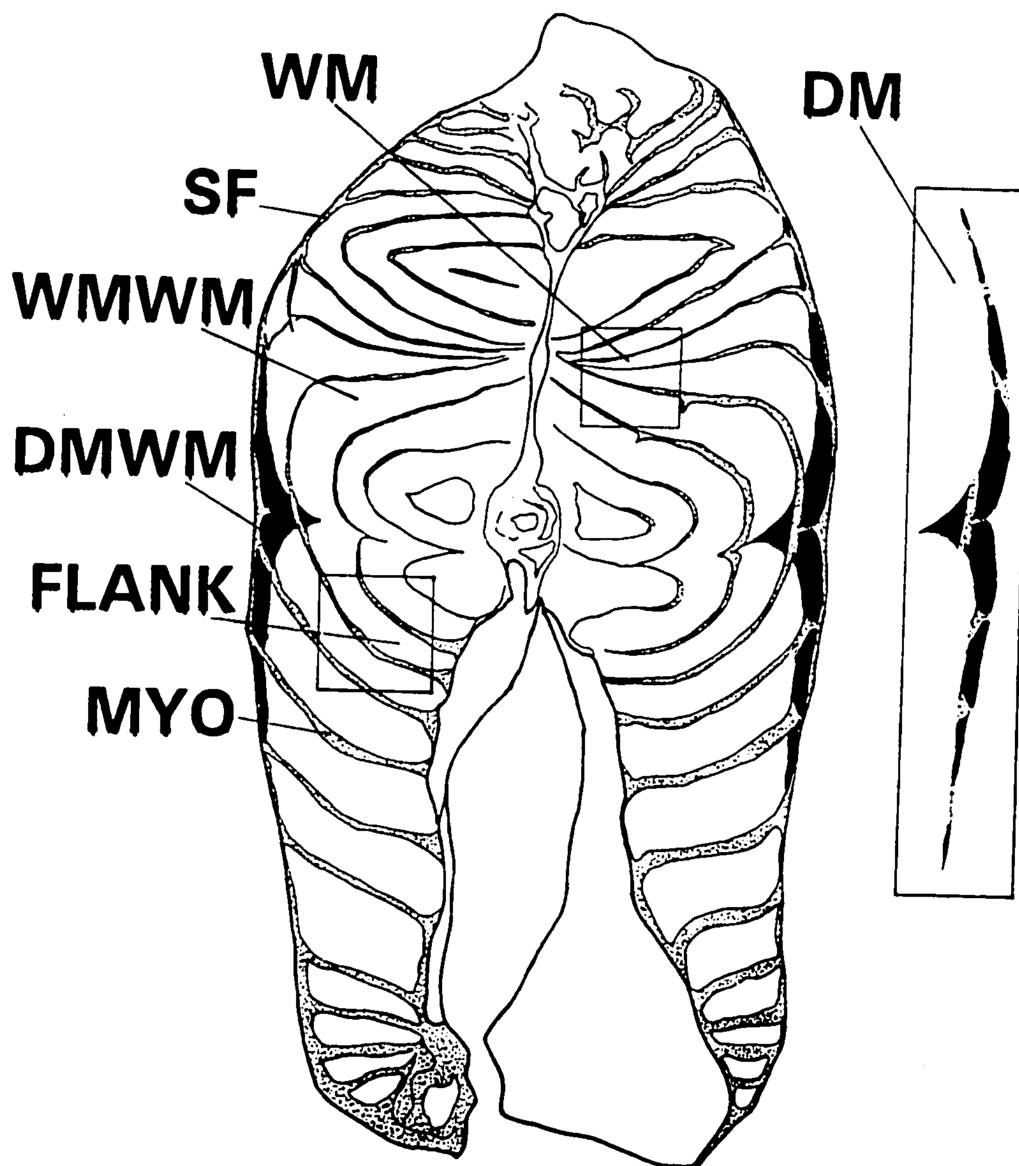


Fig. 1.1. Cross section of Atlantic salmon body showing the distribution of myosepta and the dissection areas of dorsal white muscle and dark muscle. WM - dorsal white muscle, DM - dark muscle, WMWM - dorsal white muscle without myosepta, DMWM - dark muscle without myosepta, MYO - myosepta, SF - subdermal fat tissue.

CA), of 80 cm length and 1.46 cm diameter, was used to separate free fat from adipocytes. Fig. 1.2 is a diagram illustrating the separation of adipocytes from free fat. The tubing was filled with the phosphate albumin buffer. A clamp was applied about 15 cm from the bottom. The adipocyte suspension was loaded into this empty tubing section, which was sealed by applying a clamp at the bottom end of the tubing. The tubing was suspended vertically for a few seconds; then the clamp on top of the loaded cell suspension was released. As soon as most of the rapidly rising droplets of free fat floated to the top of the tubing, another clamp was applied just below that free fat layer. The adipocytes continued to rise until they collected below the top clamp and could be removed for microscopic observation.

Each isolated cell suspension (5 $\mu$L) was applied from underneath a slide into a rectangular framed (1 x 1 cm) cell. This was made by cutting adhesive tape and gluing it to a subbed slide. Both the subbed slide and the cover glass were freshly siliconized before observation. The adipocyte suspension was observed under a Nikon Biological Microscope (Nippon Kogaku K.K., Japan) equipped with a photomicrographic attachment (Microflex, Nikon Model HFX, Japan). Photomicrographs were immediately taken at different locations of the slide.

The diameters of both isolated and histologically revealed (*in situ*) adipocytes were measured by projecting slides and reading the adipocyte images. Adipocyte size distribution revealed through histological sections was obtained by measuring five sections per tissue. The plots of diameters of adipocyte populations were achieved by measuring at least 500 cells from belly flaps and mesenteric adipose tissue, except for adipocytes isolated from the myosepta of white muscle, of which only 180 cells were counted. Due to the polygonal characteristics of adipocytes on the histological sections, the measurement of apparent diameter of each adipocyte was achieved by averaging two widths of cytoplasmic rim to rim taken at 90 °.

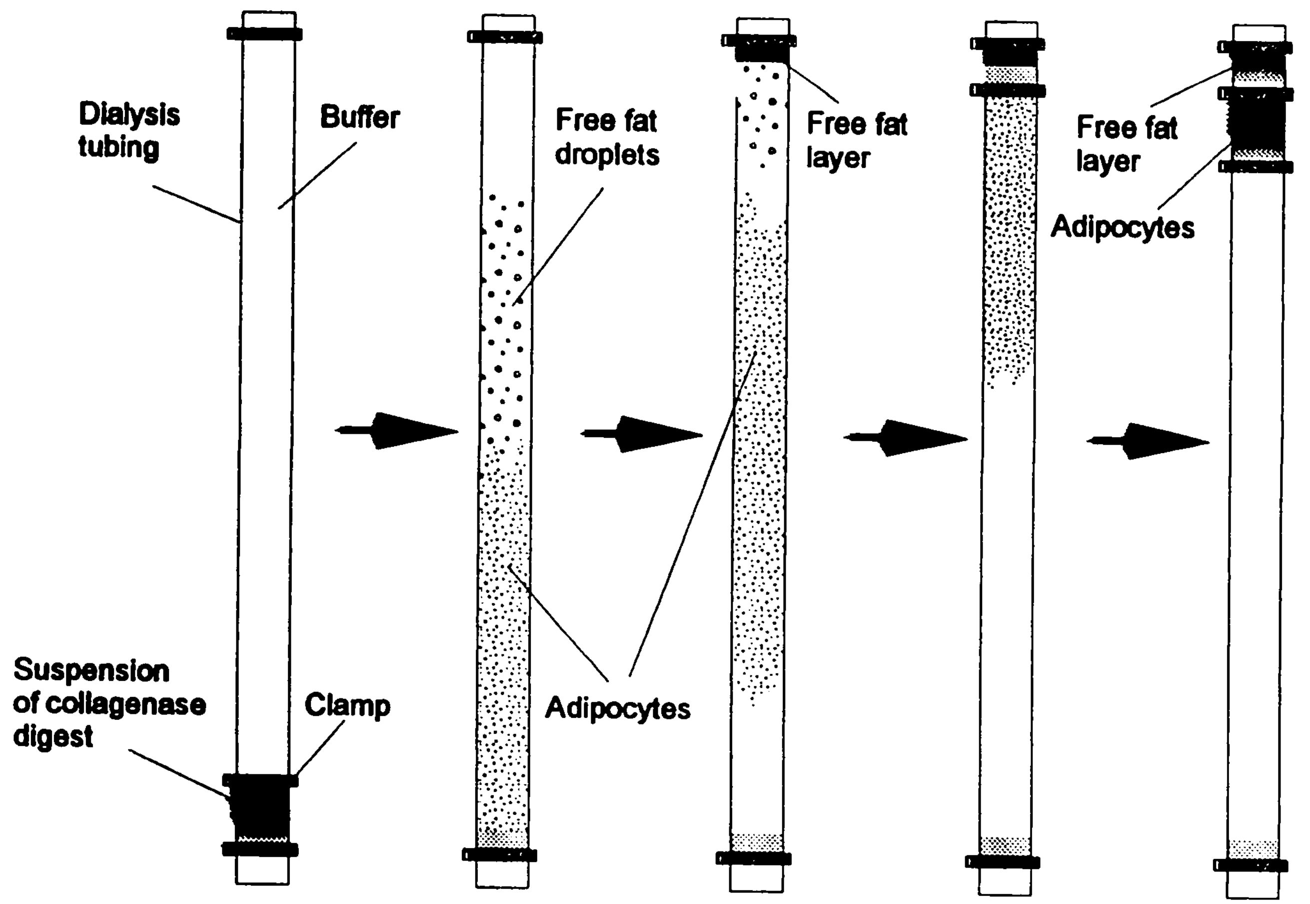


**Fig. 1.2. An outline of the floatation procedures for the separation of free fat droplets from isolated adipocytes**

## 1.4 Results

The lipid content of whole muscle tissue used for histological studies was 10.82±0.34% and 9.01±0.45% (wet basis, triplicates) for the male and female fish respectively. The slightly lower lipid content of the female salmon was possibly due to the mobilization of lipids from the muscle tissue to the maturing eggs.

Adipocytes were found in all connective tissues but their size and density varied according to the type of tissue and its location. The connective tissues of the mesentery and belly flaps were characterized by numerous adipocytes, which displayed the same characteristics of cell morphology, size range and density in both tissues (Figs. 1.3A and 1.3B); blood vessels (BV) were also clearly visible.

High densities of adipocytes were also present in the myosepta of the lower flank region, but more intercellular fibres or matrix were observed in this type of connective tissue than in the belly flaps (Fig. 1.4). Adipocytes are polygonal when contiguous to other cells, and their peripheral nuclei were often clearly seen in the sections of belly flaps (Fig. 1.3B), mesenteric tissue, subcutaneous fat layer (Fig. 1.5A), myosepta and adipose fin (Fig. 1.5B). Most of the adipocytes were found *in situ* in frozen sections, but in a small number of adipocytes the lipids had moved during sectioning because they were not fixed. However, the cytoplasmic membrane of these adipocytes was fixed and clearly visible.

The muscle tissues were surrounded by a layer of subcutaneous fat, which was more concentrated along the lateral line of the dark muscle, and became much thicker towards the belly flap regions. Adipocytes constituted the major cell type in this layer of subdermal tissue (Fig. 1.5A). Almost all of the myosepta from the white muscle penetrated the dark muscle layer and passed through to the subdermal fat layer (Fig. 1.1). Very few myosepta were isolated solely within the muscle tissue.

Adipocytes in the adipose fin were present mainly in the loose connective tissue of the core area (about 1/3 to 1/4 of the fin thickness) at a slightly lower density (Fig.

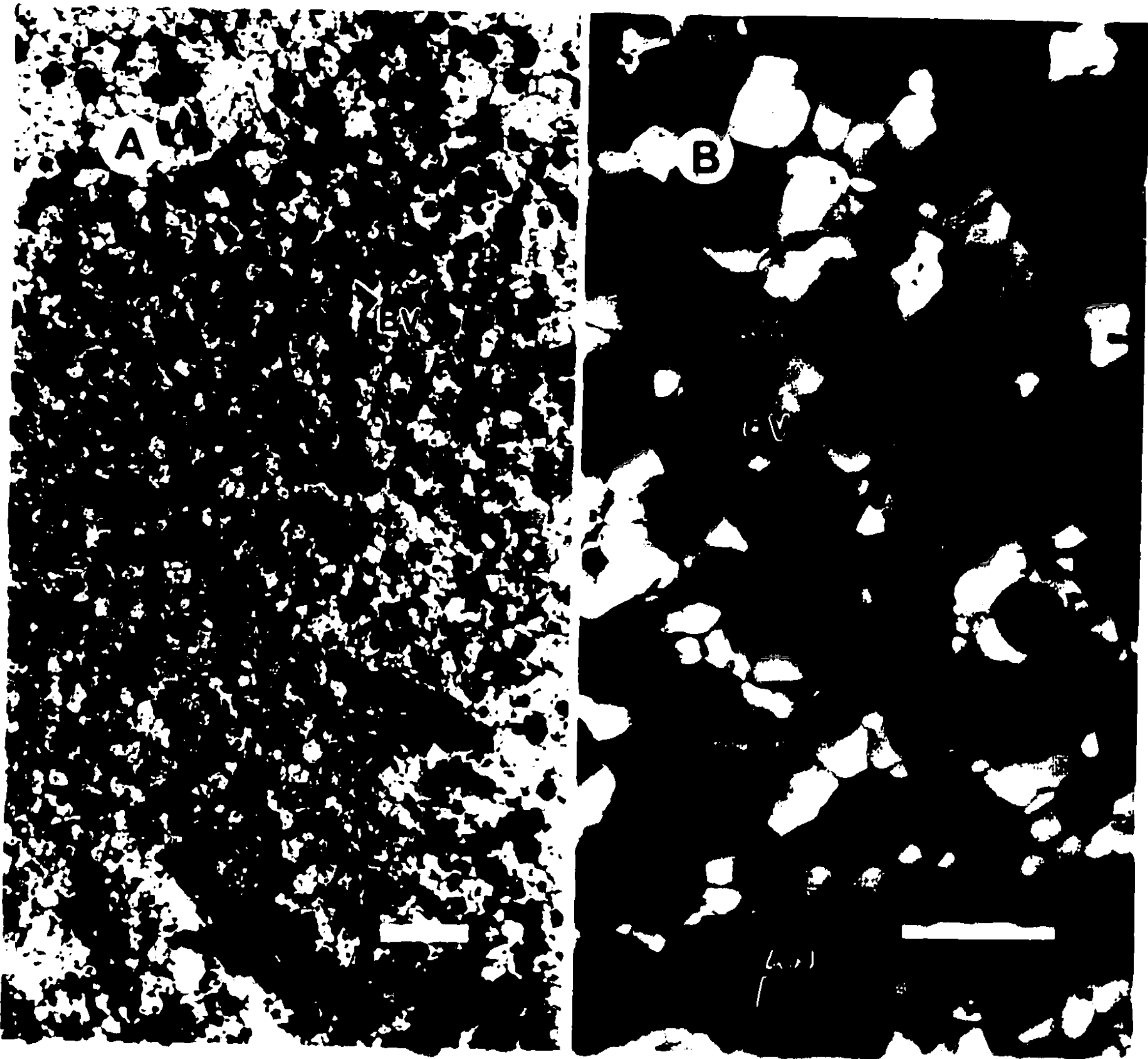


Fig. 1.3. Light micrographs of adipocyte distribution in (A) mesenteric tissue and (B) belly flaps of Atlantic salmon (frozen sections, oil red O staining) showing a high density of adipocytes, the adipocyte membrane (AM) and nucleus (AN), as well as numerous blood vessels (BV). The light micrographs for both belly flaps and mesenteric tissue were similar in terms of adipocyte distribution. (A) Scale bar = 250 $\mu$m. (B) Scale bar = 100 $\mu$m.

Fig. 1.4. Light micrograph of myoseptum in lower flank region of Atlantic salmon (frozen section, oil red O staining) showing adipocytes (AD) and bands of white muscle fibers (WMF) attached to the myoseptum. Scale bar = 250 $\mu$m.

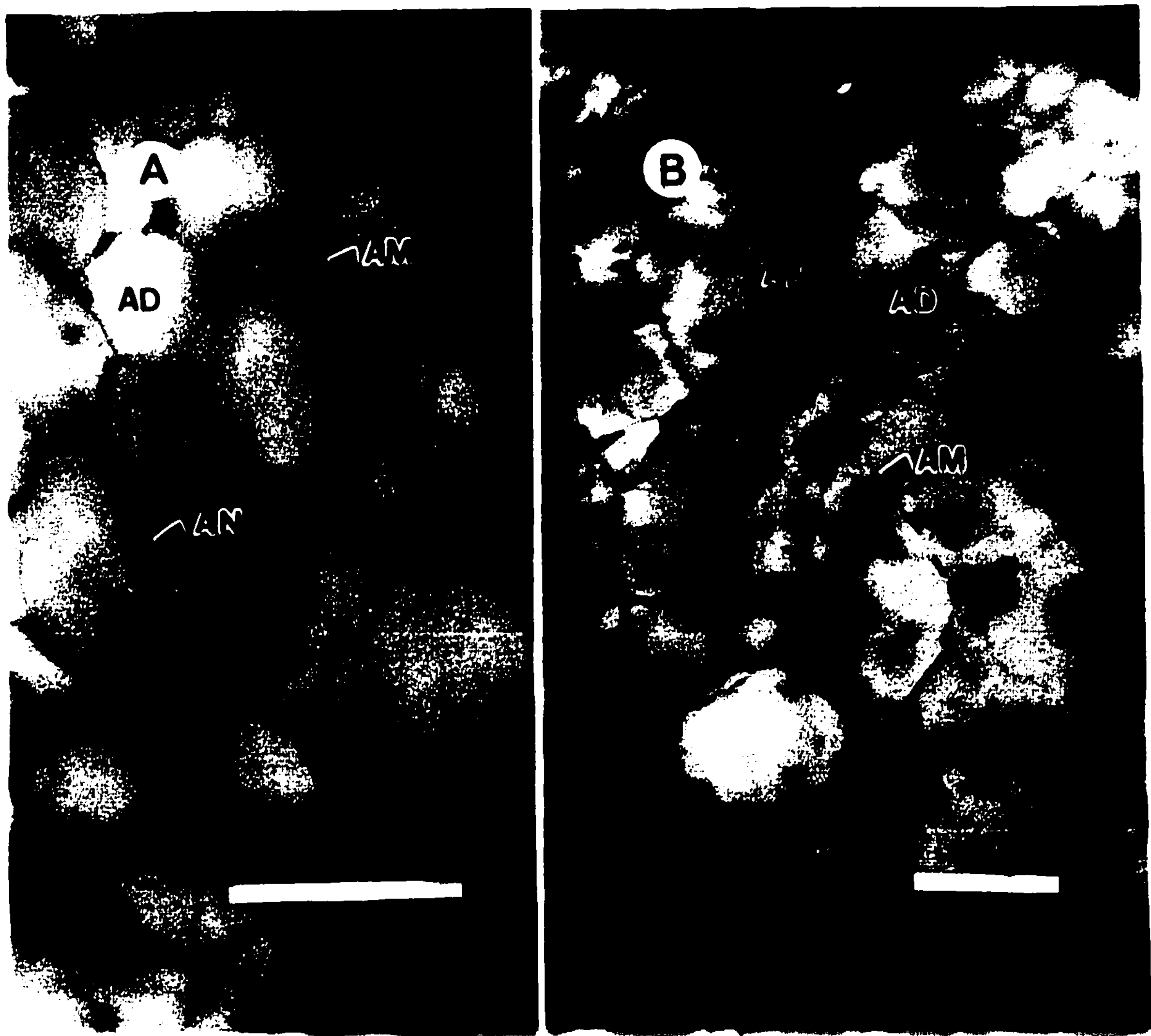


Fig. 1.5. Light micrographs showing adipocyte distribution in (A) subcutaneous fat tissue and (B) core area of adipose fin of Atlantic salmon (frozen sections, oil red O staining). AD, adipocyte; AM, adipocyte membrane; AN, adipocyte nucleus. Scale bars = 100 $\mu$m.

1.5B) than those in the belly flaps. Single adipocytes were also observed in the dermal layer near the core area of the adipose fin.

Adipocytes were a common cell type in the myosepta, which consist mainly of tough collagenous connective tissue. The myoseptum is one of the major connective tissues present in muscle tissue and the volume proportions increased sharply from the dorsal region to the belly flaps. There were high proportions of intercellular fibre, ground substances (glycosaminoglycan, Wheater et al. 1979), capillaries and non-adipocyte cells in the myosepta of the dorsal region (Fig. 1.6), especially in the leaner fish. Adipocytes were densely packed in the myosepta although there were differences in the size range and density of the adipocytes in different regions of the muscle. The adipocytes were more densely packed and larger closer to the belly flaps. The volume of myosepta in all areas of the muscle decreased with the depletion of lipids, showing more differences at different locations. As a result, adipocytes in the myosepta of the leanest portions of the muscle had a smaller size and showed a slightly lower adipocyte or lipid droplet population than those of the adipocytes in the myosepta of belly region. Sometimes it was very difficult to distinguish adipocytes from lipid droplets as the depletion of lipid caused a sharp reduction of the size of adipocytes. Adipocytes were mainly found in the myosepta of the muscle, but both adipocytes and lipid droplets were observed in the connective tissue and in the vicinity of the myosepta although they were in a much lower population.

Adipocytes were also present in the perimysium surrounding bundles of muscle fibres and the endomysium surrounding each individual muscle fibre, although their densities were much lower than those in the myosepta. Adipocytes were rarely observed in the endomysium of dorsal white muscle (Fig. 1.6), but they were easily observed in the perimysium and endomysium closer to the belly flaps. Lipid droplets were present in the endomysium surrounding each individual dark muscle cell (Fig. 1.7A) and were finely dispersed throughout the dark muscle cells (Fig. 1.7A). Most of the connective tissue at the junction between dark muscle and white muscle fibres contained a high

Fig. 1.6. Light micrograph of dorsal white muscle showing the cross section of white muscle fibers and myoseptum across the muscle tissue. AD, adipocyte; WMF, dorsal white muscle fiber; MYO = myoseptum. Scale bar = 100 $\mu$m.

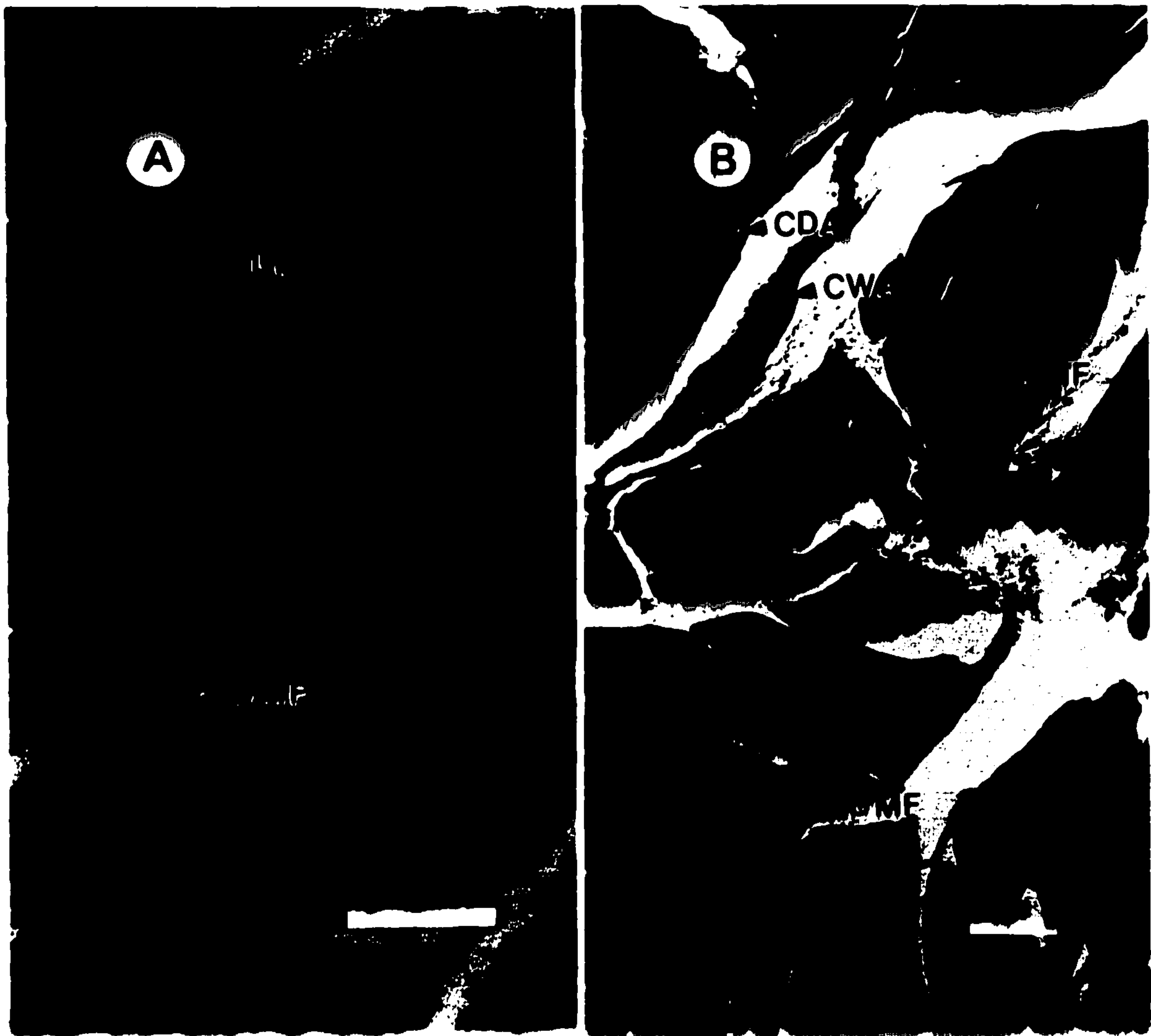


Fig. 1.7. Light micrographs of dark muscle illustrating (A) cross section of dark muscle fibers and (B) of muscle fibers in the junction area between dark muscle and white muscle (frozen sections, oil red O staining). DMF, dark muscle fiber; LD, lipid droplet; WMF, white muscle fiber; CWA, connective tissue with adipocytes; CDA, connective tissue devoid of adipocytes. (A) Scale bar = 100 $\mu$m. (B) Scale bar = 250 $\mu$m.

concentration of lipids; however, some regions were totally devoid of lipids (Fig. 1.7B).

The adipocytes isolated from belly flaps and mesenteric tissue were spherical (Figs. 1.8A and 1.8B). The freed adipocytes had a strong tendency to form aggregations, especially those from belly flaps. The presence of bi- or multilocular adipocytes could not be excluded as we were not able to completely distinguish the aggregations from multilocular adipocytes. A portion of the freed adipocytes was broken during the incubation of tissue with collagenase, and thus free lipid droplets were present in every collagenase digest. However, the volume from this damage was usually small in proportion to the amount of isolated adipocytes. Examination of the isolated adipocytes under the microscope showed that flotation of the cell suspension in buffer removed most of the free lipid droplets (Fig. 1.9).

A population of relatively small adipocytes dominated both the adipocytes observed *in situ* by histology and those isolated from collagenase treatment (Fig. 1.10). Both methods of size examination were in full agreement that the major population of adipocytes in both belly flaps and mesenteric tissue was centered around 20-40 μm. The average diameters of adipocytes in belly flaps and mesenteric tissue were 38.9 μm and 38.4 μm respectively from the *in situ* examination, and 40.5 μm and 41.2 μm respectively from the examination of the isolate. There was no significant difference in the average size of adipocytes between belly flaps and mesenteric adipose tissue. However, the enzymatically freed adipocytes showed a wider size range of 5-140 μm, while the histological sections only displayed an adipocyte size range of 10-90 μm. The population of adipocytes isolated from the myosepta of white muscle was centered in a smaller size range, with those of diameter 10-30 μm constituting the major cell fraction (Fig. 1.10). This agrees with the histological observations that adipocytes in the myosepta of the dorsal white muscle are smaller than those in the belly flap region. No significant difference in adipocyte morphology and size distribution was observed in the same tissues from fish of different sizes.

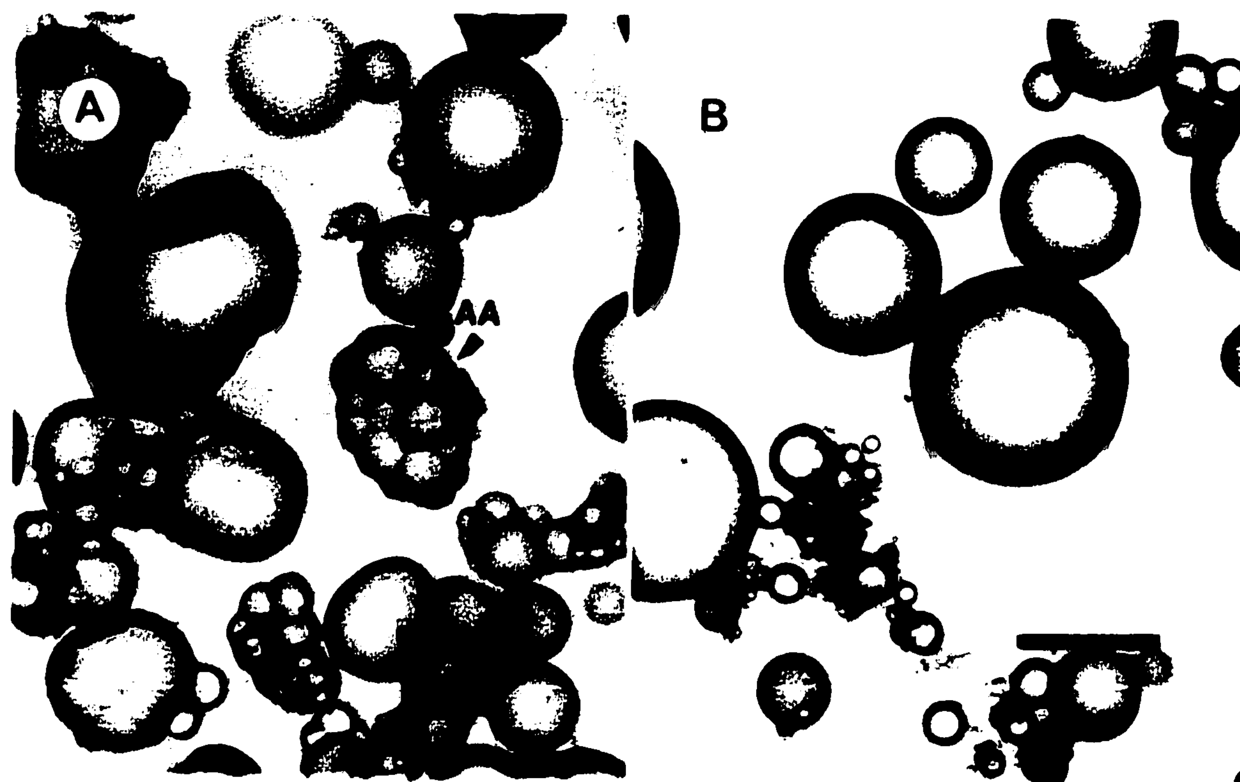


Fig. 1.8. Light micrographs of adipocytes enzymatically isolated from (A) belly flaps and (B) mesenteric tissue. AA, aggregated adipocytes. Scale bar = 100 $\mu$m.

Fig. 1.9. A picture showing the free fat and the isolated adipocytes in a dialysis tubing separated through the floatation procedures.

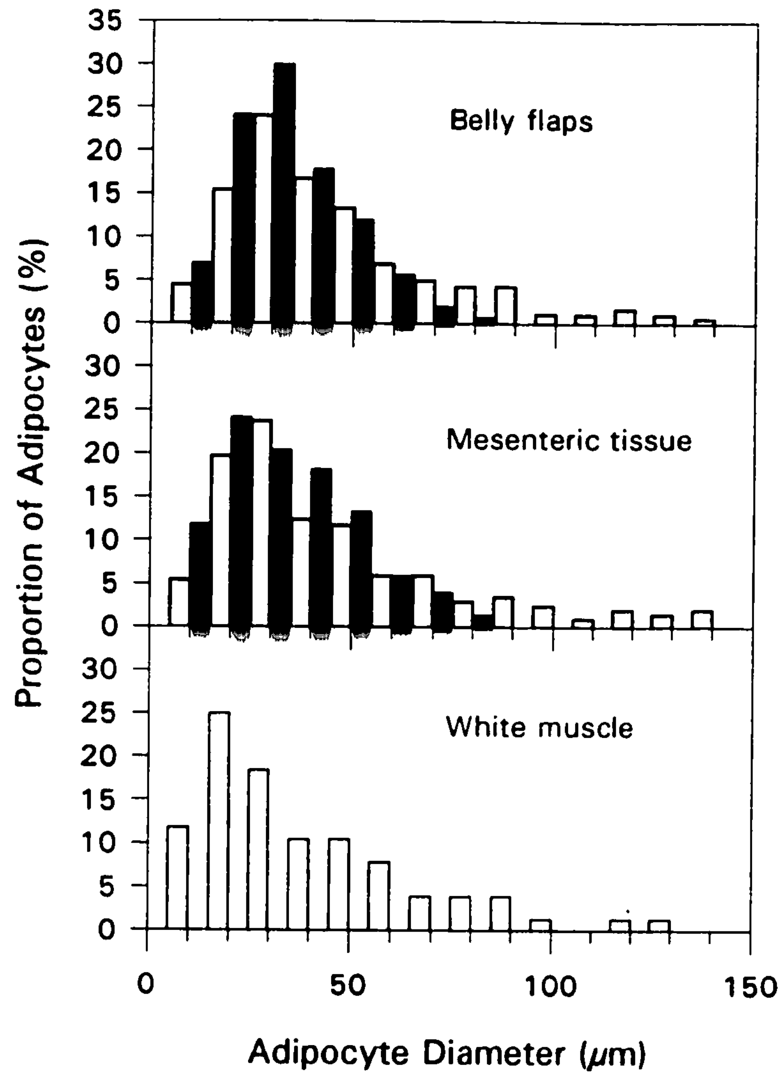


Fig. 1.10. Size distribution (10 μm classes) of adipocytes measured in situ in histological sections (solid bars) and those isolated through collagenase digestion (open bars) from Atlantic salmon belly flap, mesenteric tissue, and myosepta of white muscle (average from two fish for each procedure). Adipocyte size distributions in myosepta of white muscle were measured only through the enzymatic isolation procedures.

## 1.5 Discussion

Belly flap tissue is the richest lipid depot of the muscle tissue in Atlantic salmon, especially next to the abdominal skin (Fig. 1.1). It is reported that the lipid content of belly flaps is up to 10 times higher than that of dorsal white muscle generally (Ackman et al. 1993). The concentrated packing of adipocytes in myosepta, especially in the belly flaps, supports the observation that this region is the major energy storage site of the fish. Conversely these white muscle connective tissues became very thin in fish depleted in lipids through starvation (unpublished observations), This suggests that they are the major sites for lipid deposition and depletion in Atlantic salmon.

Connective tissues are able to interconnect and nourish other tissues. They carry blood vessels and mediate the exchange of metabolites between tissues and the circulatory system. The blood vessels and comparable lymphatic capillaries are ensheathed by the amorphous ground substance in loose connective tissue. Blood vessels in or adjacent to adipose tissue are abundant because they are the only route for the transportation of lipids towards or from the adipocytes (Cormack 1984).

The difference in the deposition and depletion of lipids in different areas of the muscle tissue might be responsible for the size variation of adipocytes between the dorsal white muscle and the belly flaps. During periods of fasting or active movement of fish, the hormone-sensitive lipase within the adipocytes is activated and fatty acids are hydrolysed from triacylglycerols (Bloom and Fawcett 1975). The free fatty acids are then released from the adipocytes, pass through the ground substance by diffusion, and are carried away by capillaries. The diffusion of free fatty acids through the ground substance of connective tissue is probably one of the key steps in controlling the mobilization of lipids in adipocytes, and thus affects the size and density of adipocyte population. In the first instance, the presence of a lower density of adipocytes, and therefore the relatively higher vascularity around the adipocytes in the myosepta of the dorsal region, favours the absorption of released free fatty acids and thus increases the

gradient density of free fatty acids between the blood capillaries and the adipocytes. In the second instance, myosepta in the dorsal white muscle are surrounded by abundant muscle fibres, and the adipocytes in this region are probably used as the localized source for supplying energy to the adjacent muscle fibres. Both factors lead to the smaller size and lower density of adipocytes present in the dorsal white muscle region.

The lack of lipid inclusions inside white muscle fibres and the presence of few adipocytes in their surrounding connective tissue may also indicate that the main energy source in this muscle tissue comes from glycogen, not from lipids. This agrees with the finding of Nag (1972) that the dark muscle fibres in rainbow trout are rich in lipid and relatively low in glycogen whereas white muscle fibres have little lipid and much glycogen.

Besides Atlantic salmon, intracellular lipid droplets in dark muscle fibres have also been observed in Japanese char (*Salvelinus pluvius*), rainbow trout (*Oncorhynchus mykiss*), horse mackerel (*Trachurus japonicus*) and mackerel (*Scomber japonicus*) (Shindo et al. 1986). These observations support the hypothesis that dark muscle is used for long and sustained swimming movement. Lipids would provide the major energy source for this activity because the proportion of mitochondria in dark muscle is much higher than that in white muscle (Nag 1972) and these droplets are most probably surrounded by mitochondria where energy is produced from lipid oxidation. Fauconneau et al. (1993) also remarked on the depot fats associated with dark muscle fibres of rainbow trout. Electron microscopy observations on dark muscle fibres of Antarctic notothenioid fish revealed that large numbers of lipid droplets were intimately surrounded by mitochondria (Lin et al. 1974). The observations of a rich lipid presence in the endomysium of dark muscle cells ensures a continuous supply of intracellular lipids for release and oxidation during long and sustained swimming. The depleted lipid store in the endomysium would, in turn, be supplied from the adipocytes of the nearest myosepta. The presence of both intra- and inter-cellular lipids, and the high proportion of myosepta, are the reasons for the lipid content of dark muscle being higher than that of white muscle (Ackman et al.

1993).

There are few reported morphological studies on size distribution of adipocytes in fish. The dominant presence of small (<50 $\mu$m) adipocytes in Atlantic salmon differs from the normal uniform adipocyte population found in human and other mammalian animals, which are dominated by much larger adipocytes (80-100 $\mu$m). Very small adipocytes (8-35 $\mu$m) have been observed in some mammals, but in a very low proportion (DeMartinis and Francendese 1982, DeMartinis 1985). However, adipocytes isolated from the chick embryo were mainly composed of very small adipocytes and the mean adipocyte diameter increased from 6 to 35 $\mu$m between days 12 and 19 of the embryonic period (Farkas et al. 1996).

The presence of small adipocytes in Atlantic salmon may well be a characteristic feature of adipocyte populations in this family. According to our unpublished data, the daily diet intakes of Atlantic salmon were totally dependent on the water temperature. This is, to some extent, similar to the hibernating animals which completely stop eating during their hibernating period and where brown adipocytes play an important role in heat production during the process of arousal from hibernation (Joel 1965). The small adipocytes in Atlantic salmon might significantly contribute to the maintenance of an energy supply at low temperatures, as they have relatively more mitochondria associated with the main lipid body. For the same reason, they may be the most active sites for lipid deposition and depletion when excess diet energy must be stored or when they are fasting. Fauconneau et al. (1993) suggested that the small adipocytes in fish are the result of a developmental stage when additional fat is being deposited.

Aggregation of isolated adipocytes was not observed for the adipocytes isolated from humans and other mammals (Lorch and Rentsch 1969, Smith et al. 1972). The isolation of adipocytes from belly flaps and myosepta of white muscle is different from mesenteric tissue because of the absence of muscle fibres in the latter tissue. The aggregation of adipocytes isolated from belly flaps and myosepta of white muscle was postulated to be due to the presence of phospholipids released from the membranes of

muscle fibres, or of protein fragments produced during the digestion period. These compounds possess amphoteric characteristics and have a strong affinity with the membranes of adipocytes. The aggregated adipocytes were not split up through the subsequent washing and flotation procedures. The lower incidence of adipocyte aggregation from mesenteric tissue was apparently due to the low concentration of protein fragments and phospholipids in the digest.

# 2. QUANTITATIVE DETERMINATION OF LIPID STORAGE IN THE MYOSEPTA OF ATLANTIC SALMON

## 2.1 Introduction

Fish muscle is composed of parallel muscle fibres separated by layers of connective tissue termed endomysium, perimysium and epimysium. At the ends of the muscle fibres, the connective tissue elements blend with strong connective tissue sheets called the myosepta (Harder 1975), or sometimes myocommata, separating the layers of muscle fibres into myotomes. The partition of lipids between the muscle fibres and the connective tissues has not been recorded. In our histological studies of Atlantic salmon tissues, dense distributions of adipocytes in the myosepta were observed. Lipids present as adipocytes in the connective tissue may be functionally different from those inside muscle fibres, which would be primarily cellular membranes in white muscle, and it is necessary to consider them separately.

Fish lipid content is generally based on the extraction of lipids from the whole of a part of a body or of a specific tissue, and is expressed as the weight percentage in that part or tissue. Therefore, the literature does not show anatomical discrimination and lipids from different compartments of fish muscle have always been considered as one unit when studies such as depletion, deposition or mobilization of lipid were carried out. As it is virtually impossible to completely separate the myosepta from the other muscle units without cross contamination, the commonly used solvent extraction method for the determination of lipid content is not applicable. The objective of this study was to develop a method for the quantification of lipids stored in the myosepta of Atlantic salmon through the analysis of lipid classes recovered from different muscle tissues.

## 2.2 Literature Review

Lipid content and distribution in fish vary markedly from species to species (Sidwell et al. 1974, Henderson and Tocher 1987, Ackman and McLeod 1988, Sheridan 1988). The major lipid storage sites in fish are also variable with species but include mesenteric tissue, muscle, liver, and the often overlooked subdermal fat layers (Ackman 1980). In the cod, *Gadus morhua*, there is relatively little lipid (~0.7%) in the muscle, occurring chiefly as cellular phospholipids. In contrast, the liver contains a high proportion (30%-50%) of oil (Jangaard et al. 1967, O'Keefe and Ackman 1987). The Atlantic salmon, *Salmo salar*, on the other hand, stores most of its reserve of lipids in the muscle instead of the liver (Ackman 1989, Polvi and Ackman 1992). As much as 18.6% muscle lipid has been reported by Sigurgisladóttir and Pálmadóttir (1993), presumably for farmed salmon (Ackman 1989). Fauconneau et al. (1993) investigated the lipid storage in the flesh of rainbow trout of market size and found a low lipid content (< 10%), which was mainly located in abdominal and dorsal subcutaneous adipose tissues.

Two basic types of fish muscle, white and dark, are easily distinguished in most fish (Shindo et al. 1986). The difference between these two types of muscle has been discussed by Burt and Hardy (1992). It is accepted that the white muscle is responsible for bursts of activity such as fast swimming, while the dark muscle is for slow, steady swimming and for maintaining the horizontal balance of the body (Hudson 1973, Walker and Pull 1973). Dark muscle has usually been reported to contain more lipid than white muscle (Polvi 1989, Burt and Hardy 1992), sometimes by a factor of 10 (Ackman and Eaton 1971, Porter et al. 1992). This opinion is based on reliable methods for the efficient extraction of total lipid from lean fish muscle. In the case of *G. morhua* it refers to the cellular membrane lipids, mostly polar lipids such as phospholipids (Bligh and Scott 1966) which comprise 0.6% of the white muscle and 1.6% of the dark muscle (Burt and Hardy 1992). The mackerel (*Scomber scombrus*) also exhibits similar

phospholipid concentrations with 0.5% polar lipid in the white muscle and 1.6% in the dark muscle, even though total lipid by weight percent in fat autumn fish is roughly equal between white (10%) and dark (14%) muscle (Ackman and Eaton 1971). Polvi (1989) and Polvi and Ackman (1992) showed an increase in proportions of muscle triacylglycerol with growth for well-fed Atlantic salmon. Researchers working with small juvenile fish of any species may not notice the uneven distribution of lipid in fish muscle simply because younger fish have relatively little total lipid compared to adults.

## 2.3 Materials and Methods

### 2.3.1 Sample Preparation

Atlantic salmon, *Salmo salar*, of commercial origin, were held in aerated seawater at the Dalhousie University Aquatron Laboratory and fed on a diet of Fundy Choice feed (Corey Mills Ltd, Fredericton, New Brunswick). Three live fish (average weight 1907 g, average length 56 cm, male) were taken from the fish tank at the Aquatron Laboratory, and transported in chilled seawater to the Canadian Institute of Fisheries Technology at the Technical University of Nova Scotia. Fish were then killed by a blow on the head, gutted and the gut cavities washed thoroughly with water. Dissection was carried out immediately and carefully to avoid any cross contamination of lipids from different locations. The skin was separated without carrying away any adherent dark muscle. The subdermal fat layer external to the dark muscle was then scraped off as completely as possible. A wedge-shaped cut was made along the entire length of the lateral line and the dark muscle was carefully separated from the white muscle. A cylindrically-shaped portion of dorsal white muscle (1.5 x 1.5 x 10 cm) was excised adjacent to the vertebrae and starting about 2 cm behind the head. The white muscle and dark muscle sections were cut into pieces of about 1 cm length each. The same muscle types from the three fish were pooled and ten pieces of each pooled muscle type were

randomly selected for dissection of white muscle and dark muscle from myosepta. The resulting pooled white muscle and dark muscle portions without myosepta are denoted WMWM and DMWM respectively. About 1 g of WMWM and 100 mg of DMWM were analysed for lipids. About 70 mg of myosepta were also taken in the flank region (Fig. 1.1) where there was a high density of adipocytes and the same myosepta went through both the white and dark muscle (Fig. 1.1). All dissection work was done in a cold room (3°C) and the samples were frozen at -35°C until analysis.

### 2.3.2 Lipid Extraction

Lipid extraction from portions of the whole white muscle (2 x 60 g) and dark muscle (2 x 35 g) was conducted following the method of Bligh and Dyer (1959). Owing to the small samples of WMWM, DMWM, and myosepta available, the Bligh and Dyer method was modified for the extraction of lipid from these samples. The tissues were cut as finely as possible and each sample was put into a 15 mL screw-cap centrifuge tube flushed with nitrogen. The tissue sample was extracted with about 10 mL of a mixed solvent of chloroform:methanol:water (2:2:1.5, v:v:v). The centrifuge tube was vortexed vigorously for 5 min, left in a domestic refrigerator overnight and then vortexed again for another five min. The centrifuge tube was centrifuged at 2000 rpm for 10 min. Two clearly separated solvent layers were observed, with the solids of extracted tissue residues staying in the top layer. The clear bottom layer of chloroform was then removed by syringe and its concentration was adjusted under nitrogen for lipid class analysis.

### 2.3.3 Lipid Classes

Lipid classes were separated by thin layer chromatography on silica gel (Chromarods-SIII) with quantification by flame ionization detection (Iatroscan Model TH-

10, Iatron Laboratories Inc., Tokyo, Japan; Canadian distributor, Scientific Products & Equipment, Concord, ON), broadly as described by Parrish (1987). The chloroform extract was adjusted to an appropriate concentration. After spotting, the Chromarods were conditioned in a constant humidity chamber for 5 min. The actual separation was conducted with a sequence of three different solvent systems. The first development was carried out for 55 min in hexane:chloroform:isopropanol:formic acid (80:14:1:0.2, v:v:v:v). The Chromarods were then dried at 100°C for 1.5 min and partially scanned from the top to a point just below the diacylglycerol (DG) peak. The Chromarods were then developed in acetone for 15 min, dried at 100°C for 1.5 min and partially scanned to below the acetone-mobile polar lipid peak. Finally, the Chromarods were developed in chloroform:methanol:water (70:30:3, v:v:v) for 60 min, dried at 100°C for 3 min and completely scanned to reveal the different phospholipids. Only the first solvent development was needed for the determination of lipid classes of myosepta, but all the other lipid samples were developed with the three solvent systems. The calibration of the system was conducted under the same conditions using authentic standards.

### 2.3.4 Lipids in Myosepta

In this study, both white muscle (WM) and dark muscle (DM) are considered to be composed of only two basic tissue components, respectively myosepta and fibrous muscle tissue without myosepta (WMWM or DMWM). The calculation of the lipid proportion distributed in the myosepta is based on two assumptions: One is that the total lipid amount in white muscle or dark muscle is equal to the lipid contribution from both myosepta and muscle without myosepta. The other is that triacylglycerol (TG) is always the major component in total lipid of all specific tissue samples; its partition between both tissue components is then used for a second calculation, i.e., the amount of TG in WM or DM is equal to the amount distributed in both myosepta and muscle without myosepta. The amount of TG is the multiplication of lipid content in specific tissue

sample and the TG percentage of total lipid in that tissue sample. These basic two equations are shown as follows:

$$T = M + m_{myo} \quad \text{.......................................} \quad (1)$$

$$T \times T_t = M \times M_t + m_{myo} \times m_t \quad \text{.......................} \quad (2)$$

where T is the total lipid percentage in the muscle; M is the amount of lipids (g) stored in either WMWM or DMWM of 100 g of the specific muscle type analyzed. $m_{myo}$ is the amount of lipids (g) stored in myosepta of 100 g of the specific muscle type analyzed; $T_t$ and $m_t$ are the TG percentages in total lipids of the specific muscle type and myosepta respectively; $M_t$ represents the TG percentage in lipids of WMWM or DMWM. The calculation formula for lipid storage in myosepta derived from the above two equations is shown as follows:

$$\frac{m_{myo}}{T} = \frac{T_t - M_t}{m_t - M_t} \quad \text{.......................................} \quad (3)$$

## 2.4 Results

Lipids extracted from Atlantic salmon muscle are effectively separated into different lipid classes and individual phospholipid by the Iatroscan-Chromarods under the three solvent systems. Fig. 2.1 shows the Iatroscan chromatogram of lipids from the sample of white muscle without myosepta (WMWM).

The lipid classes of the samples are shown in Table 2.1. Lipids from dark muscle were found to contain a higher proportion of TG than lipids of white muscle. Upon subtraction of myoseptum lipid, the percentage of TG in total lipids decreased from 83.6% to 73.8% for white muscle and from 91.9% to 80.6% for dark muscle, while polar lipids increased from 13.9% to 21.4% and from 6.6% to 15.4% respectively. TG

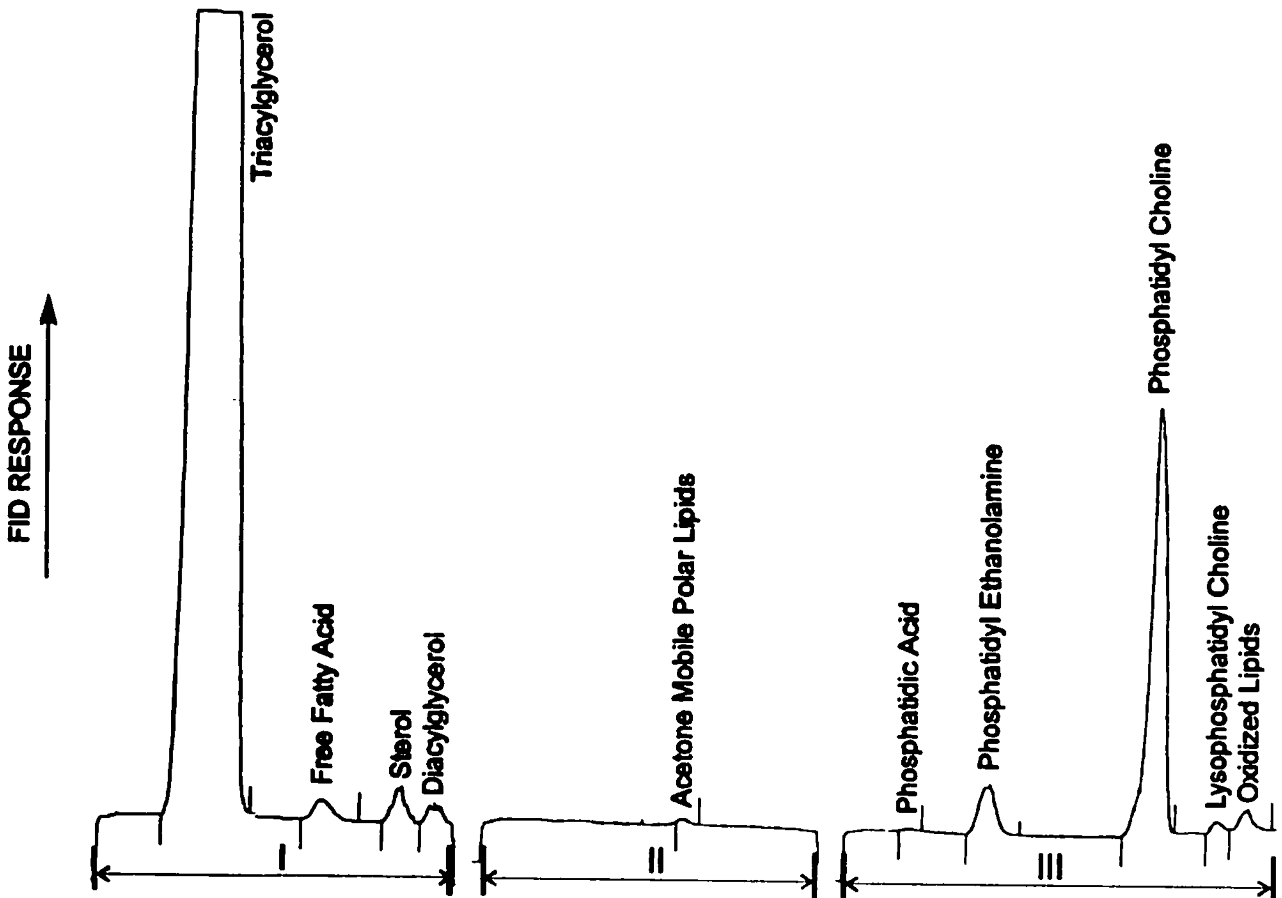


**Fig. 2.1 Sequential Iatroscan TLC-FID profiles of the lipid classes extracted from the dorsal white muscle without myosepta (WMWM) of Atlantic salmon. Symbols of I, II and III represent partial chromatograms from the three-stage development sequence of total lipids on a Chromarod-SIII.**

Table 2.1. Lipid classes as percentages in total lipids (w/w)[a,b] of white muscle, dark muscle, muscles without myosepta, and myosepta (pooled from three fish).

| | Tissue examined[b,c] | | | | |
|---|---|---|---|---|---|
| Lipid classes | WM | WMWM | DM | DMWM | MYO |
| TG | 83.56 | 73.81 | 91.92 | 80.63 | 98.73 |
| FFA | 0.65 | 1.86 | 0.39 | 1.33 | bdl |
| CHO | 1.36 | 1.59 | 0.71 | 1.04 | bdl |
| DG | 0.55 | 1.39 | 0.39 | 1.11 | bdl |
| ΣNeutral Lipids | 86.12 | 78.65 | 93.41 | 84.61 | 98.73 |
| AMPL | n.d. | 0.24 | n.d. | 0.13 | bdl |
| PA | n.d.[c] | 0.47 | 0.55 | 1.50 | bdl |
| PE | 2.55 | 3.94 | 1.57 | 4.14 | bdl |
| PC | 10.03 | 14.85 | 3.45 | 8.67 | bdl |
| SPH | n.d. | 0.29 | n.d. | 0.24 | bdl |
| LPC | 0.87 | 0.92 | 0.52 | 0.76 | bdl |
| PM | 0.42 | 0.64 | 0.49 | 0.45 | bdl |
| ΣPolar Lipids | 13.87 | 21.35 | 6.58 | 15.39 | 1.27 |

[a] Average of two determinations.

[b] Abbreviations: WM - white muscle, WMWM - white muscle without myosepta, DMWM - dark muscle without myosepta, DM - dark muscle, MYO - myosepta, TG - triacylglycerol, FFA - free fatty acid, CHO - free sterol, DG - diacylglycerol, AMPL - acetone-mobile polar lipids, PA - phosphatidic acid, PE - phosphatidyl ethanolamine, PC - phosphatidyl choline, SPH - sphingomyelin, LPC - lysophosphatidyl choline, PM - polar materials.

[c] n.d. = not detected, bdl = below detectable limited.

was, as expected from histological observations, predominant in lipids from myosepta and only trace amounts of polar lipids (1.3%) were detected. The major phospholipids present in both muscle types were phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE), with PC > PE as is commonly reported for fish muscle (Ackman 1980). The ratio of PC to PE in white muscle was about twice that in dark muscle. However, removal of myoseptum lipid had no effect on the ratio of these two major phospholipids present in both muscle types. The lipid content of dark muscle (20.2%) was much higher than that (3.8%) of white muscle (Table 2.2). Of the 3.85 g total lipids from 100 g white muscle, 1.51 g was present in myosepta, representing 39.1% of total lipids. In the dark muscle, even more lipids were located in the myosepta (62.4% of total lipids extracted from whole dark muscle). There was initially 5.25 times more lipid present in dark muscle than in white muscle, while after removal of myosepta from both muscles types, the ratio was only 3.66.

## 2.5 Discussion

Fish muscle lipids exist mainly in three forms: neutral lipids (mainly TG) in adipocytes for storage of energy, polar lipids serving as major components of the cell membrane, and intracellular lipid droplets distributed in cell cytoplasm as local energy stores. The latter are illustrated by Greenwood and Johnson (1983). When recovered lipids of fish white muscle exceed approximately 1% the excess is normally found to be stored in adipocytes (Sheridan 1994). Membrane lipids (e.g. those of cod white muscle) are generally less than 1% of fish muscle weight (Ackman 1980).

Myosepta in Atlantic salmon are prominent and easily recognized in both white and dark muscle since they appear as bands of a white colour against a pink background (Fig. 1.1). The density of myosepta throughout the flesh is uneven, but they are closer together in the dorsal region; individually they become thicker around the region of the lower belly flaps. Myosepta become the main tissue component in the belly flaps and

Table 2.2. Lipid content of white muscle, dark muscle and amount of lipids stored in myosepta and in muscles without myosepta (pooled from three fish).

| | White Muscle | | | Dark Muscle | | |
|---|---|---|---|---|---|---|
| | WM | WMWM | MYO | DM | DMWM | MYO |
| Lipid content (%)[a] | 3.85 | - | - | 20.20 | - | - |
| Percentages for lipid distribution in total WM and DM lipids[b, c] | - | 60.9 | 39.1 | - | 37.6 | 62.4 |
| Lipids(g) from WMWM,DMWM, and MYO in 100 g muscle[c] | - | 2.34 | 1.51 | - | 7.60 | 12.6 |
| lipid content of WMWM and DMWM (%)[c, d] | - | 2.38 | - | - | 8.70 | - |

[a] Average of two determinations by solvent extraction.

[b] Percentage of total muscle lipids distributed in myosepta calculated according to equation (3)

[c] Calculated value.

[d] For this calculation it is assumed that the myosepta is composed of 100% lipid; thus the lipid content in WMWM or DMWM equal to:

$$\frac{m_m}{100 - m_{myo}} \times 100$$

where $m_m$ is the lipid (g) from WMWM or DMWM in 100 g of the specific muscle type analyzed.

their volume proportion decreases sharply from the belly flaps, through the flank region, to the area where the white muscle sample was taken for this study. Most of the myosepta are connected with each other through subdermal fat layers and only a few are separately located in the white muscle. It was therefore expected that there would be no differences in the characteristics of myosepta in white muscle and dark muscle as the same myosepta obviously go through both muscle types.

In the farmed Atlantic salmon used in our study, the pigments included in the diet was astaxanthin (and sometimes canthaxanthin), which is protein-bound rather than associated with lipid (Sigurgisladóttir et al. 1994). The muscle was therefore selectively pigmented, leaving "white" streaks along the myosepta because the adipocytes clustered in these regions were essentially devoid of colour. This might not be the case in wild Atlantic salmon and it should be emphasized that our salmon are "farmed" fish.

The high content of lipid stored in the muscle of farmed Atlantic salmon indicates that the major form present must be non-membrane lipids. The two possibilities for this are storage inside muscle cells, or in the connective tissue as adipocytes. Both sites have been confirmed with our histological study, which showed fine droplets of lipid inside the dark muscle cells between the myofibrils. As TG is the only major component stored in adipocytes (Fawcett 1981, Body 1988), the presence of adipocytes in the myosepta was further supported by the sharp decrease of the TG percentage in total lipid of WMWM and DMWM, and the presence of nearly pure TG (98.7%) in myosepta. Although the proportion of polar lipids remaining after removal of the myosepta increased, the ratio of PC to PE remained constant in both muscle types, confirming that neutral lipids account for most of the lipids in the myosepta (Table 2.1).

Although the volume proportion of myosepta in the white muscle region was so low that it was almost negligible compared with the total volume of the muscle, 39.1% of white muscle lipids were stored in myosepta. Thus these adipocytes were actually packed very densely in the connective tissue. The sample used for this study was taken from the leanest portion of the muscle. Other parts of the muscle should have a higher

proportion of lipid storage in the myosepta, which appeared to be denser in the flank and belly region, and also in the dorsal region (Fig. 1.1).

Porter et al. (1992) reported 19.42% lipids in dark muscle of sockeye salmon (*Oncorhynchus nerka*), while only 2.37% lipids were present in white muscle. In our study of *Salmo salar*, dark muscle was rich in storage lipid (20.2%). As there was a layer of subdermal fat on top of the dark muscle and the subdermal fat layer was actually connected physically with the myoseptum, it was very difficult to completely avoid the contamination of dark muscle lipids from inclusion of subdermal fat. However, the presence of 62.4% lipids in the myosepta was largely responsible for the high lipid content detected in dark muscle. When the factor of dense lipid packing in the myoseptum was removed, the difference between the lipid content of dark and white muscle tissues decreased markedly from 5.25 times to 3.66 times. This is a more realistic representation of the actual cellular lipid distribution in both types of fibrous muscle, since our histology studies had shown the presence of lipid droplets inside dark muscle fibres, and also surrounding each individual dark muscle fibre.

Lipids stored in the adipocytes of the connective tissue and the majority of the lipids found inside muscle cells were apparently different in their functionality. It is believed that muscle connective tissue, especially myosepta in the case of Atlantic salmon, is often the main reservoir for lipid deposition and depletion. The extent of lipid storage in fish is dependant on species, geographical locations, diet availability, and sexual maturation. Stansby (1981) showed a large variation in fatty acid patterns of fish oils even for one species of fish. The usually accepted reason for such variation is probably the cyclic deposition and mobilization of lipid (mainly TG) in connective tissue under various nutritional conditions, while the phospholipid content of the muscle cell is always fixed at a certain level. Due to the difference in fatty acid patterns between TG and phospholipids present in the same fish muscle (Polvi and Ackman 1992), the extent of lipid storage in the myosepta eventually modulates the total fatty acid composition of the muscle of that fish. Sheridan et al. (1983) reported that the total lipid

content of white and dark muscle of steelhead trout (*Salmo gairdneri*; now *Oncorhynchus mykiss*) was significantly depleted during smolt transformation and TG concentrations were reduced more than those of any other lipid classes. In another study of lipid absorption and deposition in rainbow trout (*S. gairdneri*; now *O. mykiss*), Robinson and Mead (1973) found that starvation for five weeks caused a 63% loss of TG in white muscle, and a 300% increase in TG incorporation was observed in dark muscle after eight hours of force-feeding in the starved groups. Unfortunately, the actual locations of these lipids in the muscle for both studies are unknown.

Considering the overall possible lipid storage sites in Atlantic salmon, it is concluded that if subdermal and mesentery storage are excluded, the primary triacylglycerol storage site is in the adipocytes of the connective tissue of the muscle, especially in the myosepta, and not within the muscle fibres.

The investigations mentioned above on the adipocyte and lipid distributions in the muscle tissue of Atlantic salmon clearly demonstrated that adipocytes are the major form for the storage of lipids and are mainly concentrated in the connective tissue of myosepta. However, the main objective of this thesis work is to reveal the role of adipocytes in controlling the WSF-HC uptake, release and retention in the muscle tissue of Atlantic salmon, which is the key to understanding the results of WSF-HC uptake and depuration in the whole muscle tissue. Studies described in the following sections are therefore continuously focused in this objective by first improving the method for hydrocarbon recovery, and then conducting a series of exposures of Atlantic salmon to the WSF of crude petroleum to understand the WSF-HC behaviour in different types of tissues of Atlantic salmon, and particularly in the adipocytes isolated from the muscle tissue.

# SECTION II. METHOD MODIFICATION FOR RECOVERING HYDROCARBONS FROM TISSUES OF AQUATIC ORGANISMS

## 3. DETERMINATION OF TAINTING WSF-HC OF CRUDE PETROLEUM IN AQUATIC ORGANISMS BY STEAM DISTILLATION

### 3.1 Introduction

The water-soluble fraction of petroleum hydrocarbons encompasses a wide range of both volatile and less volatile hydrocarbons, and is particularly rich in aromatic hydrocarbons from benzene to alkylated naphthalenes (Ernst et al. 1987, Ernst et al. 1989, Heras et al. 1992). This is the major fraction of hydrocarbons which dissolve in the water column and subsequently can be accumulated in aquatic organisms when an oil spill occurs (Burris and MacIntyre 1984, Zbanyszek and Smith 1984, Siron et al. 1987). Fish and fish products tainted with WSF-HC would certainly be rejected by fish consumers, and can be spurned even by association with an oil spill.

The high volatility of WSF-HC poses many difficulties for their quantitative recovery from water or aquatic organisms and the commonly used procedures of recovering higher molecular weight hydrocarbons through extraction, saponification, silica gel chromatography etc. prior to identification and measurement by gas-liquid chromatography (GLC) are not applicable to this hydrocarbon category (Farrington and Meyer 1975, Howard et al. 1978, Saito et al. 1979, Wong and Williams 1980, Vassilaros et al. 1982, Lawrence and Weber 1984, Lebo et al. 1991, Perfetti et al. 1992). A method for recovery of volatile hydrocarbons from homogenized fish tissues by steam distillation was first developed by Ackman and Noble (1973). The characteristic features of this method could be summarised as its being simple, fast and low in cost. Since then, slight modifications of the developed method have been made and the modified method has successfully been used for studying the tainting of fish and shellfish by the

WSF of petroleum hydrocarbons (Ernst et al. 1987, Ernst et al. 1989, Heras et al. 1992). However, limitations in several aspects of the method were occasionally observed in recovering the tainting WSF-HC from the white muscle of Atlantic salmon, lean fish, and shellfish, and the method needed to be modified to further improve accuracy and precision in the determination of tainting WSF-HC from tissues of various aquatic organisms. The problems included the risk of foaming in the distillation of lean fish tissues and shellfish tissues, and low recovery and reproducibility for WSF-HC, particularly for the most volatile compounds (Ernst et al. 1987, Ernst et al. 1989).

### 3.2 Literature Review

Many analytical methods have been developed for the analysis of hydrocarbons, particularly polycyclic aromatic hydrocarbons (PAHs). However, despite the variety of these analytical methods, no standard methods are apparently available for the analysis of petroleum off-flavour contamination in fish, particularly through the WSF of petroleum hydrocarbons. The WSF-HC are predominantly composed volatile aromatic hydrocarbons, ranged from benzene to methylnaphthalenes. However, heterocyclic compounds are notorious adverse flavour compounds and are also probable (Ogata et al. 1980). Large variations in WSF-HC concentrations are expected to occur in the marine environment. High concentrations of WSF-HC could initially be found near the areas of submarine or coastal oil seeps or near an oil spill site. The concentrations of WSF-HC in both water and aquatic organisms then decrease continuously and reach background levels after a certain period of time except in the case of continuous oil release from seeps (Clark et al. 1977). The determination of low-level background petrogenic hydrocarbons in aquatic organisms is subject to strong interference from biogenic hydrocarbons (Farrington et al. 1976, Brassell et al. 1977) and commonly results in lower accuracy of determination.

Steam distillation is one of the oldest methods of separating chemicals on the basis

of differences in vapour pressures over water. For example, it has been widely used in the analyses of volatile fatty acids (AOAC 1995), and in the manufacture of flavours. Steam distillation procedures have been employed for the recovery of halogenated fumigants from cereal products and polychlorinated biphenyls from sediments, and the hexane extracts were analyzed by capillary gas chromatography with electron capture detection (Page et al. 1987, Dunnivant and Elzerman 1988). Steam distillation has also been used for the recovery of pesticides and industrial chemicals from water, sediments and tissue samples, and an exhaustive steam distillation and solvent extraction apparatus has been reported by Veith and Kiwus (1977). Through this apparatus, the trace chemicals in the samples were simultaneously recovered into a small volume of organic solvent described as suitable for direct gas-liquid chromatographic analysis. However, the shortcoming of this apparatus is the time consumed. Boiling of the sample alone requires up to 7 hours.

Application of steam distillation for recovering hydrocarbons from aquatic organisms was first reported by Ackman and Noble (1973), and hydrocarbons were successfully isolated from contaminated whitefish. Since then, the basic method has been slightly modified and has been used for the isolation of WSF-HC from sea scallops (Ernst et al. 1989) and cod muscle (Ernst et al. 1987). There are a few other reported applications of steam distillation in the recovery of hydrocarbon contaminants from aquatic organisms. Farrington and Meyer (1975) described the extraction of fish samples with diethyl ether followed by steam distillation of the ether extract to obtain a volatile fraction. Newton et al. (1991) isolated diesel fuel contaminants from various fish products by steam distillation. The distillate was extracted with *n*-hexane which was then directly injected into gas liquid chromatography (GLC).

Recently an on-line, steam distillation/purge and trap chromatographic procedure was described for the determination of halogenated, non-polar, volatile contaminants in foods (Page and Lacroix 1995). The use of purge and trap techniques for the isolation of volatile contaminants has been increasing during the last decade since coupled

capillary gas chromatography/mass spectrometry makes it possible for the simultaneous separation and identification of numerous volatile compounds. Detailed applications of the purge/trap and headspace methods for the quantitative analyses of volatile organic compounds include bulk edible oils (Thompson 1994), human blood (Ashley et al. 1992), foods and packaging materials (Johnston et al. 1994), vinegars and alcoholic beverages (Williams 1976), and cheese and butter etc. (Page and Charbonneau 1983).

Steam distillation is best used when volatile contaminants are to be determined. However, most of the trace background hydrocarbons in aquatic organisms collected in non-polluted or less-polluted areas are mainly composed of non-volatile hydrocarbons [mainly polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs)], other than the volatile WSF-HC (Brassell et al. 1977, Clark et al. 1977, Dunn and Fee 1979, Risebrough et al. 1980, Lawrence and Weber 1984, Murray et al. 1991, Perfetti et al. 1992, Fayad et al. 1996). The isolation of these non-volatile hydrocarbons is based on the principles of saponification for breaking down the saponifiable lipids, followed by the partitioning of all unsaponifiable lipids into a non-polar solvent, and finally a cleanup by silica absorbent for the complete separation of hydrocarbons from other lipid classes (Farrington and Meyer 1975). The first step is carried out by either saponifying the total lipids extracted from tissues (Farrington et al. 1976) or directly digesting the tissue samples under alkali conditions (Vassilaros et al. 1982, Lawrence and Weber 1984, Lebo et al. 1991, Perfetti et al. 1992, Granby and Spliid 1995). After the breakdown of the saponifiable lipids, the extracted unsaponifiables, which account for a very small fraction of the total lipids, are applied to silica gel absorbent and a pure hydrocarbon fraction usually can be eluted with non-polar solvents such as hexane. Wise et al. (1980) reported a method for the determination of both volatile and non-volatile hydrocarbons by utilizing dynamic headspace sampling of an aqueous caustic tissue homogenate and by solvent extraction of the tissue homogenate. The two hydrocarbon fractions were subsequently analyzed by GLC/MS and high performance liquid chromatography (HPLC).

On-line LC-GC (liquid chromatography-gas chromatography) has also been described by Grob et al. (1991) for the determination of food contamination in mineral oil from jute sacks. The method involves complete transfer of the LC fraction to GC to obtain the required sensitivity.

Gas-liquid chromatography is by far the most frequently used technique for the final separation and quantitation of compounds of interest, and the introduction of the capillary column coupled with mass spectrometry becomes a milestone for the wider application of GLC in analyzing various organic compounds (Vassilaros et al. 1982, Lawrence and Weber 1984, Johnston et al. 1994, Liu et al. 1994, Thompson 1994, Ho et al. 1995). The major advantages for using GLC rely on its excellent resolution capacity, versatility to all organic contaminants, and high sensitivity. However, GLC is incapable of analyzing organic compounds with very high molecular weights and those unstable at high temperature. On the other hand, these particular types of organic compounds can be more effectively analyzed by HPLC. The applications of HPLC in analyzing organic pollutants include PAHs and their metabolites (Zander 1980). HPLC can be performed with high selectivity not only by choosing suitable stationary phases, but also by the application of detection methods selective for PAHs, such as fluorescence (Krone et al. 1992, Perfetti et al. 1992). The two methods of GLC and HPLC are thus complementary.

## 3.3 Materials and Methods

### 3.3.1 Tissue Samples

Tissues from different marine organisms were used for studies on the recovery of tainting WSF-HC by steam distillation. Live Atlantic salmon of market size were obtained from the Aquatron Laboratory of Dalhousie University. The fish were fed on a diet of Fundy Choice feed and held in aerated seawater at the Dalhousie University

Aquatron Laboratory for more than two years. Two live fish (average weight 1791 g) were taken from the fish tank. Fish were then killed by a blow on the head, gutted and skin removed. Whole muscle tissue (WMT), belly flap (BF), dorsal white muscle (WM), dark muscle (DM), lower flank muscle (LF) and mesenteric adipose tissue (MA) were dissected immediately as illustrated in Fig. 1.1. Fresh scallop muscle, cod fillets and live lobster were obtained from a local fisherman's market. Other fish were available from experiments in progress in the CIFT. Lobster were killed by a blow on the head and muscle tissue was dissected. All tissue samples were stored in a -35°C cold room before use.

### 3.3.2 Authentic Hydrocarbon Standards

Twenty one authentic hydrocarbon standards were used to study the efficiency of recovering hydrocarbons from tissue samples by steam distillation. Those hydrocarbon standards encompass the wide spectrum of hydrocarbons present in the WSF of crude petroleum, ranging from benzene to methylnaphthalene. Each hydrocarbon standard, dissolved in and diluted with dichloromethane (Omni-Solv grade, VWR Scientific of Canada, Ltd.), was first examined by GLC for purity (see below for GLC conditions). Fourteen of the standards had purities over 99%, but two others had respective purities of 98.0% and 97.5% (by FID response). The purity of each standard was calculated from its peak area percentage. The amounts of authentic standards spiked to the tissue samples were corrected according to the appropriate purities.

A stock solution of hydrocarbon standards was made by dissolving appropriate weights of the 21 standards in dichloromethane. The stock solution was further diluted to obtain a working solution. Both the stock and working solutions were stored in a freezer at -20°C. The hydrocarbon levels spiked in the tissue samples (200 $\mu$l of working solution in 20 g sample) were equivalent to those in the tainted Atlantic salmon immediately after the exposure of short-term vs. high doses of WSF-HC concentrations

(Table A1, appendix).

### 3.3.3 Efficiency of Recovering Spiked Hydrocarbons from Homogenized Tissue Samples by Steam Distillation

Investigations of the recovery of spiked hydrocarbon standards from tissue samples were carried out by extending the basic procedures of Ackman & Noble (1973). The steam distillation apparatus consisted of a 250 mL round-bottomed flask with a magnetic stirring bar, a 20 mL Barrett-type distilling receiver (Teflon stopcock), a water-cooled condenser, a heating mantle, and a magnetic stirrer (Fig. 3.1).

Several different experiment conditions were tested for recovering hydrocarbons from tissue samples and are summarised in Table 3.1. Procedures for recovering hydrocarbons from tissue samples under experimental conditions IV can be described as follows: Distilled water (80 mL) and pre-purified (baked at 600 °C) reagent grade sodium sulphate (25 g) were placed in a 250 mL flask and were heated to boiling using an electric heating mantle. The distillation was conducted at a rate of 1 mL $min^{-1}$ and was terminated after 20 mL of condensate was collected. The water condensate in the receiver was discarded and the flask cooled. Minced tissue sample (20 g) and the residual sodium sulphate solution in the flask were placed in a Sorvall OMNI-MIXER and the minced tissue was finely dispersed for 15 s. The whole of the dispersion was transferred back to the distillation flask, followed by addition of 1 mL of dichloromethane. The graduated collection receiver was then immersed in an ice/water bath and distillation resumed until 20 mL were collected. After the termination of the distillation, 1 mL of dichloromethane was added immediately to the receiver through the top of the condenser to rinse the wall of the condenser and to reflux briefly within the condenser. This step was intended to recover any of the hydrocarbon distillate which was adhered to the wall of condenser or was floating on top of the aqueous distillate. After cooling, the distillate was drained into a glass centrifuge tube (50 mL) and

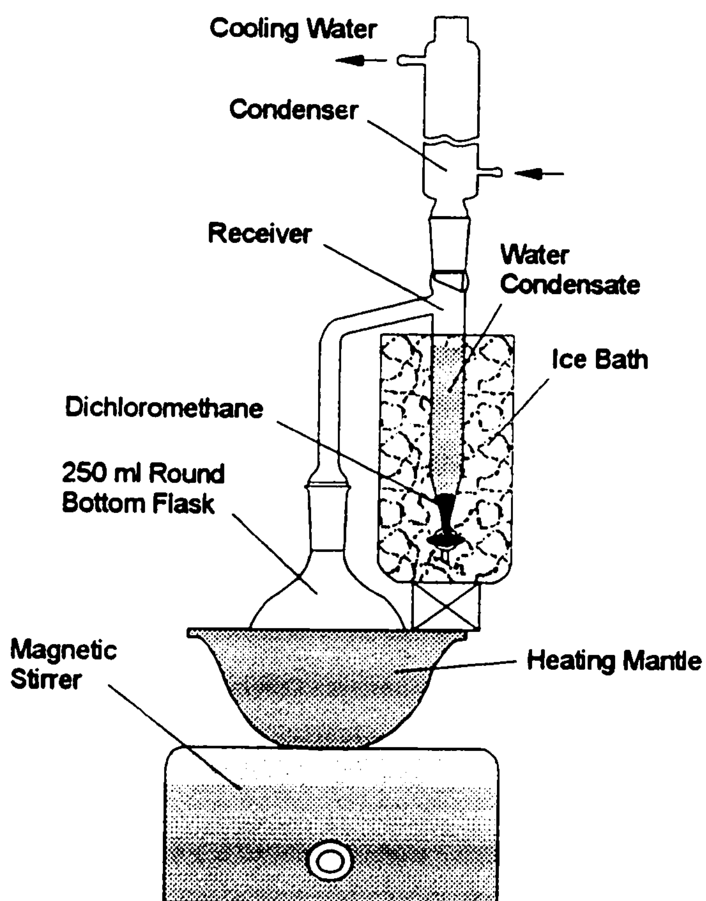


**Fig. 3.1. Steam distillation apparatus for the recovery of hydrocarbons from tissues of aquatic organisms.**

Table 3.1. Experimental designs for the recovery of spiked hydrocarbons from tissue samples of marine organisms.

| | Experiments | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| Addition of $CH_2Cl_2$ (1 mL) before distillation | no | yes | yes | yes |
| Rinse of condenser wall with 1 mL $CH_2Cl_2$ at the end of distillation | no | no | yes | yes |
| Dispersion of tissues in $Na_2SO_4$ solution before distillation | no | no | no | yes |

dichloromethane (100 $\mu$L) containing *n*-heneicosane as internal standard was added. The centrifuge tube was vortexed for 1 min and centrifuged at 1500 rpm for 6 min. The bottom dichloromethane layer was transferred to a 5 mL graduated centrifuge tube and concentrated to 500 $\mu$L under nitrogen. The concentrated solution (4 $\mu$L) was injected into a GLC for hydrocarbon analysis.

### 3.3.4 Gas-Liquid Chromatography Analysis of Hydrocarbons

The hydrocarbon analyses were conducted with a Perkin-Elmer 8420 capillary gas chromatograph equipped with a flame ionization detector (FID) and split injection system (split ratio: 1:32). The analysis was conducted on a DB-1 (methyl silicone) fused silica capillary column (60 m, 0.25 mm ID, 0.25 $\mu$m film thickness). Conditions and temperature for WSF-HC extracted from seawater were: FID, 350 °C; injector, 350 °C; The column temperature was programmed as follows: initial temperature of 45°C held for 15 min; then increased at a rate of 13°C/min; final temperature 280°C, held for 25

min. The carrier gas was helium at a pressure of 138 kPa and hydrogen and air pressures were 90 and 159 kPa, respectively.

The percent recovery efficiency was calculated according to the following formula:

$$R = \frac{\frac{A_1}{A_2} \times M}{M_{sp}} \times RF \times 100$$

where: $R$ = percent recovery efficiency of individual hydrocarbon

$A_1$ = peak area of spiked individual hydrocarbon

$A_2$ = peak area of *n*-heneicosane added to the distillate

$M$ = amount of added internal standard ($\mu g$)

$M_{sp}$ = amount of spiked individual hydrocarbon ($\mu g$)

$RF$ = GLC system response factor of individual hydrocarbon related to *n*-heneicosane

The amounts of tainting hydrocarbons in real tissue samples were calculated according to the internal standard added, the recovery efficiency and the GLC response factors. The recovery efficiency of any tainting WSF-HC not included in the 21 spiked standards was assumed to be the same value as the nearest spiked hydrocarbon on the same chromatogram. The amounts of hydrocarbons in the control fish were used as background levels and were subtracted from those in the spiked samples. Recovery of hydrocarbons from the spiked tissues was carried out in triplicate.

### 3.3.5 Statistical Analysis

Statistical differences on recovery efficiency under different experimental conditions were evaluated by one way analysis of variance, with the aid of Minitab software (Minitab Release 7.2, Minitab, Inc.).

## 3.4 Results and Discussion

### 3.4.1 Effect of Adding Dichloromethane to Tissue-Water Dispersion before Steam Distillation on Recovery Efficiency

The GLC-FID responses and response factors of the 21 selected hydrocarbon standards are shown in Table A2 (appendix). All hydrocarbon standards displayed similar GLC-FID responses which are slightly higher than that of *n*-heneicosane. Among the spiked hydrocarbon standards, the aliphatics displayed slightly lower GLC-FID responses than those of the aromatics, except for *n*-nonane and *n*-decane. The higher GLC-FID responses of spiked hydrocarbons compared to the $C_{21}$ could probably be attributed to the high boiling point of *n*-heneicosane (356.5°C) and therefore some of the injected $C_{21}$ might form fine droplets that pass through the GLC column without being absorbed and appear under the solvent peak. The slightly higher GLC-FID responses of aromatics compared to the aliphatics are due to the higher weight percentages of active carbons in the molecules of aromatics.

The efficiency of recovering spiked hydrocarbons from the whole muscle tissue of Atlantic salmon by steam distillation under the four experimental designs (Table 3.1) is shown in Table 3.2. These recovery efficiency results were obtained under the spiking levels shown in Table A1 (appendix). The results show that the addition of 1 mL of dichloromethane to the tissue-water dispersion before distillation (conditions II) substantially improved the recovery efficiency of spiked hydrocarbons of the lower molecular weights, particularly the aromatic hydrocarbons (from benzene to xylenes) (compared with conditions I). The lower the molecular weights of the aromatics, the larger the increment of recovery efficiency after the addition of dichloromethane. For example, the recovery efficiency of benzene and toluene was originally only 25% and 39%, but increased to 83% and 86% respectively after the addition of dichloromethane before distillation. There is no improvement on the recovery efficiency of aromatic

Table 3.2. Recoveries of spiked hydrocarbon standards from the whole muscle tissue of Atlantic salmon by steam distillation under four experimental designs (refer to Table 3.1). Values are the average of triplicate analyses ± standard deviations. Different transverse lettering superscripts associated with the percent recoveries of each hydrocarbon indicate significant differences ($p<0.05$) between the means of recovery efficiency (only those with superscript letters were tested for statistical significance).

| | Recoveries (%) | | | |
|---|---|---|---|---|
| Spiked Hydrocarbons | I | II | III | IV |
| Benzene | $24.76\pm5.67^{a}$ | $82.59\pm5.49^{b}$ | $90.23\pm2.11^{b}$ | $85.85\pm3.50^{b}$ |
| Cyclohexane | $22.08\pm4.84$ | $49.06\pm5.85$ | $66.84\pm3.68$ | $80.53\pm1.47$ |
| *n*-Heptane | $19.00\pm2.42$ | $45.84\pm6.44$ | $58.96\pm2.48$ | $79.12\pm3.22$ |
| Methylcyclohexane | $28.48\pm5.02$ | $61.15\pm4.18$ | $68.62\pm1.47$ | $76.08\pm3.76$ |
| Toluene | $39.19\pm7.25^{a}$ | $85.91\pm2.60^{b}$ | $76.18\pm3.09^{c}$ | $89.40\pm2.90^{b}$ |
| *n*-Octane | $27.10\pm11.44$ | $35.09\pm3.44$ | $42.19\pm5.49$ | $76.09\pm3.09$ |
| Ethylbenzene | $53.92\pm2.17$ | $83.33\pm6.09$ | $83.63\pm0.95$ | $82.72\pm4.68$ |
| *m+p* Xylene | $59.28\pm3.80$ | $84.75\pm3.89$ | $80.50\pm1.22$ | $78.95\pm1.99$ |
| *o*-Xylene | $51.35\pm4.81$ | $76.34\pm5.11$ | $83.81\pm2.53$ | $76.49\pm4.85$ |
| *n*-Nonane | $33.38\pm3.31$ | $30.96\pm5.33$ | $40.86\pm1.85$ | $70.67\pm4.88$ |
| Isopropylbenzene | $69.03\pm3.09$ | $74.28\pm5.08$ | $76.92\pm3.08$ | $89.01\pm4.93$ |
| Propylbenzene | $72.37\pm4.20^{a}$ | $81.33\pm2.64^{b}$ | $69.14\pm4.72^{a}$ | $75.12\pm1.77^{a}$ |
| 1,3,5-Trimethylbenzene | $78.07\pm7.44$ | $98.21\pm3.58$ | $73.31\pm3.65$ | $90.92\pm4.84$ |
| 1,2,4-Trimethylbenzene | $65.63\pm2.01$ | $77.52\pm1.99$ | $92.08\pm4.70$ | $74.18\pm4.54$ |
| *n*-Decane | $24.43\pm8.00$ | $37.45\pm5.54$ | $40.25\pm2.44$ | $65.25\pm2.21$ |
| *n*-Undecane | $24.18\pm3.02^{a}$ | $19.27\pm6.01^{a}$ | $21.88\pm4.54^{a}$ | $62.54\pm3.24^{b}$ |
| 1,2,4,5-Tetramethylbenzene | $37.90\pm5.13$ | $40.49\pm4.98$ | $69.57\pm4.70$ | $63.80\pm2.10$ |
| Naphthalene | $42.91\pm7.87$ | $53.00\pm2.91$ | $74.36\pm3.66$ | $76.34\pm3.24$ |
| *n*-Dodecane | $28.94\pm5.02$ | $15.22\pm5.43$ | - | - |
| 2-Methylnaphthalene | $34.77\pm6.04$ | $41.64\pm7.32$ | $63.55\pm3.18$ | $67.46\pm3.05$ |
| 1-Methylnaphthalene | $39.36\pm7.93^{a}$ | $36.95\pm4.77^{a}$ | $62.88\pm3.92^{b}$ | $67.79\pm5.55^{b}$ |

hydrocarbons of the higher molecular weights such as methylnaphthalenes with the addition of dichloromethane to the tissue-water dispersion. The recovery efficiency of cyclohexane, methylcyclohexane and *n*-heptane was also improved after the addition of dichloromethane, but to a much less extent comparing to those of their aromatic counterparts having similar molecular weights.

The WSF of crude petroleum are normally dominated by volatile aromatics, mainly benzene and alkylated benzenes (Heras et al. 1992). Most of these hydrocarbons are evaporated from the tissue-water dispersion in the flask during the early stage of steam distillation owing to their relatively higher vapour pressures (CRC Handbook of Chemistry and Physics 1973-1974). The vapour pressure of benzene is even higher than that of water. Therefore, a large fraction of the spiked benzene would have been lost before the collection of any distillate in the receiver if dichloromethane were not added to the tissue-water dispersion prior to distillation. Other volatile aromatics such as toluene and xylenes are similar to benzene and are also easily evaporated from the tissue-water suspension at the early stage of steam distillation. Initially in simple steam distillation, most of these early distilled aromatic hydrocarbons adhered to the wall of the condenser since their amounts in the tissue samples were not high enough to become discrete droplets in the collection receiver. This part of these volatile hydrocarbons may then have gradually evaporated and been lost in the air. The other part was subsequently brought down to the receiver by the distilled water condensate and dissolved in the water condensate. Under the circumstance of high WSF-HC load in the tissue samples, the water condensate would be saturated by the recovered hydrocarbons, and the non-dissolved fraction would float on top of the collected water condensate and being gradually lost in the air. The addition of dichloromethane into the tissue-water dispersion substantially increased the amount of hydrocarbons being recovered at the early stage of steam distillation. The condensed dichloromethane vapour immediately became droplets and ran down to the bottom of the collection receiver. Therefore a large fraction of the most volatile hydrocarbons could be kept dissolved in this dichloromethane layer and

their losses were substantially reduced.

The boiling points of cyclohexane (80.7°C), methylcyclohexane (100.9°C) and *n*-heptane (98.4°C) are between those of benzene (80.1°C) and toluene (110.6°C), but their recovery efficiency was consistently lower than their aromatic counterparts (Table 3.2) even though the addition of dichloromethane did improve their recoveries. This could most probably be attributed to the their lower solubilities in water compared to those of the aromatic hydrocarbons. A fraction of the vapour of these non-aromatic hydrocarbons was condensed but presumably unable to be rinse down by the initial dichloromethane vapour into the collection receiver.

### 3.4.2 Effect on Efficiency of Recovering Hydrocarbons by Rinsing the Condenser Wall of the Steam Distillation Apparatus at the End of Steam Distillation

The percent recoveries of the less volatile aromatic hydrocarbon spikes such as tetramethylalkylated benzenes, naphthalene and methylnaphthalenes were around 40% with and without the addition of dichloromethane before steam distillation (Table 3.1, conditions I and II) (Table 3.2). These hydrocarbons are much less soluble in water than benzene and xylenes, and their boiling points are over 200°C (CRC Handbook of Chemistry and Physics 1973-1974). They were presumably slowly distilled along with water vapour from the tissue-water dispersion during the entire distillation processes. Therefore, collection of fixed volume of steam distillate is important in order to maintain their constant recoveries from the samples. The percent recoveries of these less volatile aromatic hydrocarbons were however significantly improved by rinsing the condenser wall of the distillation apparatus with dichloromethane (Table 3.1, conditions III) immediately after 20 mL of water distillate was collected (Table 3.2). Without rinsing the condenser at the end of distillation, the recovered less volatile aromatic hydrocarbons tended to stick to the condenser wall and/or float on top of the water distillate due to their relatively lower solubilities and their higher boiling/melting points (e.g., m.p. of

2-methylnaphthalene, 34.6°C ). These portions of hydrocarbons were normally lost when the distillate was simply drained from the receiver into a centrifuge tube and led to a low recovery efficiency. The rinse process utilized the residual heat from both the distillation receiver and the vapour of the distillate in the receiver to help rinse down and/or dissolve both portions of hydrocarbons. Table 3.2 shows that this rinse process increases the percent recoveries of methylnaphthalenes from about 40% to about 63%.

### 3.4.3 Suppression of Foaming by the Incorporation of Sodium Sulphate Salt into the Water Phase of the Tissue-Water Suspension

Foaming of tissue-water suspension in the distillation apparatus during steam distillation severely interferes with the recovery of hydrocarbons from tissue samples and generally can result in failure of the whole steam distillation processes. This is a commonly observed problem during the steam distillation of tissue samples from certain marine organisms, particularly those with low lipid content. Examination of foaming by distilling various tissue samples revealed that lipid content of the tissues plays an important role in foam production and foam stability (Fig. 3.2). The dividing line for lipid content of the tissue samples to initiate foaming interference with the distillation process is about 4 % on a wet tissue basis. Serious foaming would definitely occur if the tissue lipid content is less than 3 % and the foaming would not interfere with the steam distillation processes if the tissue lipid content is higher than 5%. The lipid content of dorsal white muscle of of some experimental Atlantic salmon was just around the borderline for foaming (4%) and therefore both foaming and no foaming were observed for the steam distillation of this type of tissue.

Investigations have been carried out to overcome this problem. Physical disruption of actual foam by the rapid stirring of the tissue-water dispersion through a magnetic stirring bar is helpful in the suppression of foaming, but only to a limited extent. Ernst et al. (1989) reported that preventing foam from entering the distillation receiver could

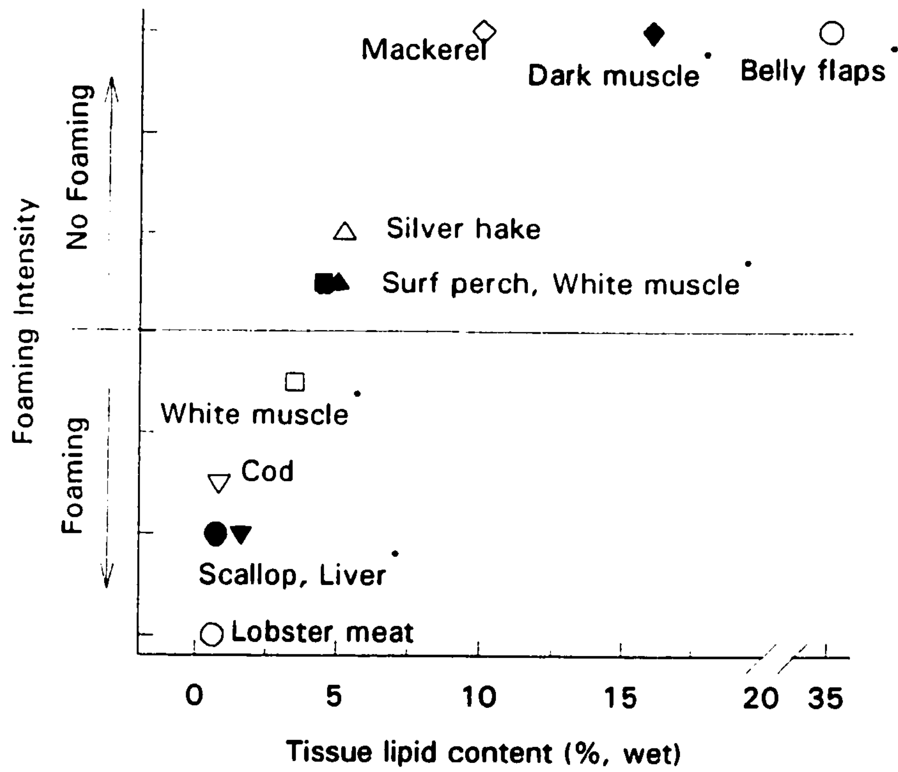


Fig. 3.2. Relationship between foaming and tissue lipid content of marine organisms. *, Atlantic salmon tissue.

be achieved by placing a glass wool plug at the inside joint of the gooseneck tube and the round-bottomed flask used in their steam distillation. However, this would lead to the substantial reduction in hydrocarbon recoveries since the glass wool plug provides an ideal site for the preferential condensation of most of the distilled hydrocarbons in the vapour phase. Addition of pure lipids, such as refined vegetable oil, into the tissue samples to raise the total lipid content is another effective way for suppression of foaming. However, a few extra peaks then appeared in the background profile of volatile organic compounds recovered from the non-spiked fish tissues. Therefore, this foaming suppression technique is not applicable in recovering trace organic pollutants from the tissues of aquatic organisms. Commercial antifoam reagent is specifically used for suppression of foaming. The use of antifoam for foam suppression in analyses of volatile organic compounds has been reported and cited by Ashley et al. (1992) and Page & Lacroix (1995) respectively. The capability of antifoam in foaming suppression under the current conditions of steam distillation was also examined and it was found to have only limited benifits. The addition of antifoam could not effectively prevent foaming when very lean tissue samples (e.g. cod and scallop muscle) were subjected to steam distillation. It was also observed that a few extra peaks could be found in the background chromatogram of the control tissue samples even though the antifoam reagent was pre-cleaned by non-polar organic solvents.

Dispersion of minced tissue into saturated solutions of salts was found to have remarkable effect in suppression of foaming. Among the saturated salt solutions tested, sodium sulphate was the most effective. The percent recoveries of the spiked hydrocarbons using saturated sodium sulphate solution (conditions IV, Table 3.1) are shown in Table 3.2. The recovery data show that distillation of minced tissue in saturated sodium sulphate solution had similar efficiencies for recovering most aromatic hydrocarbons although significant difference ($p<0.05$) in recovery efficiency was observed for a few aromatics compared to those obtained under improved distillation conditions III (Table 3.1). It is interesting to note that the percent recoveries of *n*-

alkanes were significantly improved when the minced tissue was dispersed in the saturated sodium sulphate solution.

The incorporation of saturated sodium sulphate solution in tissue-water dispersion for recovering volatile compounds in aquatic organisms has the following advantages over the classic steam distillation processes: (1) Tissues from most aquatic organisms can be steam-distilled smoothly without foaming interference. For example, lobster meat and scallop muscle are two of the most difficult tissue samples for steam distillation due to their extremely low lipid content. When these tissues were dispersed in the saturated sodium sulphate solution, steam distillation could be carried out without foaming. (2) Sodium sulphate is easy to preclean and would not introduce any background interference to the recovered volatile organic compounds from tissues. (3) The recovery efficiency of *n*-alkanes increased significantly ($p<0.05$) from the levels without the incorporation of this salt. Figs. 3.3 and 3.4 show the correlation between percent recoveries of the spiked hydrocarbons and their boiling points with and without the addition of sodium sulphate. It is clearly demonstrated that the percent recoveries of hydrocarbons decrease as their boiling points or molecular weights increase for steam distillation conducted under both conditions III and IV. Aromatic hydrocarbons and *n*-alkanes could be classified into two separate groups with respect to their percent recoveries in which constantly lower levels of recoveries for *n*-alkanes were observed than for their aromatic counterparts unless sodium sulphate was added to the tissue-water dispersion (Fig. 3.4). The presence of sodium sulphate in the tissue-water dispersion would inevitably raise the boiling point of salt solution. However, only a small increment in the boiling point of tissue-water dispersion was observed ($\sim 2$ C°, not accurately determined). This would not have significant effects in recovering hydrocarbons from tissue samples.

Volatile organic compounds and water are proportionally distilled from the tissue-water dispersion according to their relative proportions of vapour pressures if there is sufficient interfacial area between the organic compounds and water. Sufficient interfacial area could be achieved by either partially dissolving the organic phase into the

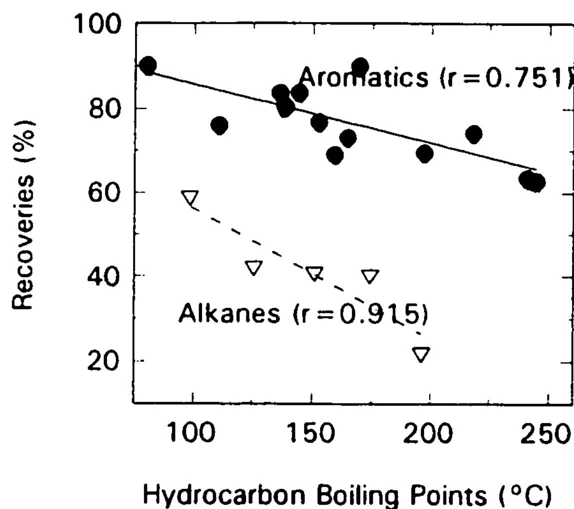


Fig. 3.3 Correlation between boiling points of spiked hydrocarbons and their recovery efficiency from whole muscle tissue of Atlantic salmon by steam distillation (conditions III, i.e., without sodium sulphate). The solid and dotted regression lines represent aromatic and straight chain aliphatic hydrocarbons respectively.

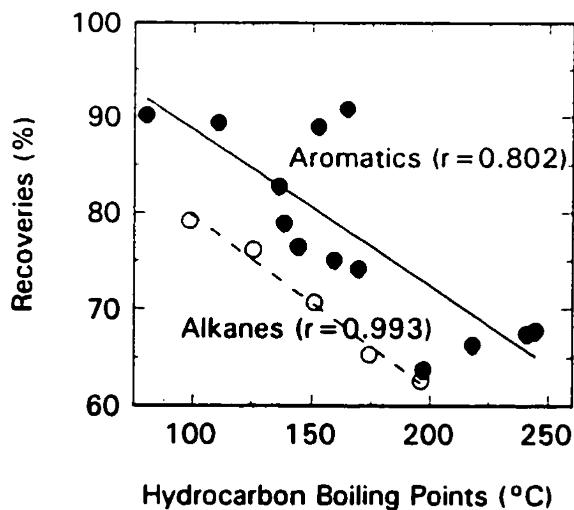


Fig. 3.4 Correlation between boiling points of spiked hydrocarbons and their recovery efficiency from whole muscle tissue of Atlantic salmon by steam distillation (conditions IV, i.e., with sodium sulphate). The solid and dotted regression lines represent aromatic and straight chain aliphatic hydrocarbons respectively.

water phase or by finely dispersing the organic phase in the water phase. The consistent higher recovery efficiency of aromatic hydrocarbons obtained without the incorporation of salt could be attributed to their higher solubilities in water than those of the *n*-alkanes. The increase in percent recoveries of *n*-alkanes in the presence of sodium sulphate is probably contributed from the effect of salt which enhances the physical interaction between water and *n*-alkanes.

Foams are an agglomeration of gas bubbles separated from each other by thin liquid films, walls or lamellae. Foams are stabilized by the incorporation of foaming agents into the thin liquid films, walls or lamellae. The foaming agents generally include molecular solutions (inorganic salts, organic acids and salts, alcohols and sugars), colloidal solutions (proteins, gums, saponins, tannin) and finely divided particles. The major foaming agents present in marine organisms include proteins, phospholipids and finely divided tissue particles. It is a general rule that differences in concentration of solutes in surface layers and bulk solution are essential for the production of stabilized foams. In the dispersed system of tissue-saturated sodium sulphate solution, salt is the predominant solute and therefore the concentration of solutes in the surface layer of the dispersion is the same as the concentration of solutes in bulk liquid. This explains the remarkable effect of saturated sodium sulphate solution on foaming suppression. A likely explanation for the effects of high tissue lipid content on suppression of foaming is that phospholipids, one of the major forming agents, are trapped by large amount of neutral lipids (triacylglycerols). Therefore the amount of phospholipids incorporated into the thin lipid films of foam for the stabilization of foam was substantially reduced.

### 3.4.4 Recoveries of Spiked Hydrocarbon Standards from Different Types of Tissues of Atlantic Salmon, from the Muscle Tissue of Cod and Scallop, and from Lobster Meat

The efficiency of recovering hydrocarbons from dorsal white muscle, dark muscle, belly flap and mesentery tissue of Atlantic salmon under experimental conditions IV (Table 3.1) is shown in Table A3. The major compositional differences among these four type of tissues are their lipid content determined by following the Bligh and Dyer (1959) procedures: white muscle, 3.6%; dark muscle, 15.3%; belly flap, 34.7%; mesenteric adipose tissue, 82.3%. The overall recovery data of spiked hydrocarbons (Table A3) demonstrate that higher tissue lipid content is generally accompanied by lower efficiency of recovering hydrocarbons from the tissue. The general trend in recovery efficiency for the four tissue samples is: dorsal white muscle > dark muscle > belly flap > mesenteric adipose tissue. However, this difference in recovery efficiency is primarily noted in the less volatile hydrocarbons such as naphthalene and methylnaphthalenes, and there is little difference on the efficiency in recovering the most volatile hydrocarbons among the four different types of tissues of Atlantic salmon. The inverse correlation between the percent recoveries of hydrocarbons and the lipid content in the tissues could obviously be attributed to the high solubility of hydrocarbons in droplets of the lipid fraction (oil) which would substantially reduce the chance of their being distilled from the tissue-water dispersion.

Efficiency of recovery of the spiked hydrocarbon standards from lobster meat and muscle tissues of cod and scallop (Table A4) was also examined by steam distillation under the improved experimental conditions IV (Table 3.1). The overall recovery efficiency of hydrocarbons from these three tissue samples and from the dorsal white muscle and dark muscle of Atlantic salmon is more or less in the same range.

Several articles have been published on the determination of organic pollutants in various materials by steam distillation and on-line steam distillation/purge and trap

analysis. Veith and Kiwus (1977) proposed an exhaustive steam distillation and solvent extraction unit for the determination of pesticides and industrial chemicals in water, sediments and fish tissues. Newton et al. (1991) recovered diesel contaminants in various fish products by steam distillation. Although these published methods are effective in recovering volatile organic compounds, the distillation processes take a much longer time than does our proposed method.

# SECTION III. BEHAVIOUR OF WATER-SOLUBLE FRACTION OF PETROLEUM HYDROCARBONS IN ATLANTIC SALMON MUSCLE TISSUE

## 4. UPTAKE, DEPURATION AND RETENTION OF WSF-HC OF CRUDE PETROLEUM IN THE MUSCLE TISSUES AND ADIPOCYTES OF ATLANTIC SALMON

### 4.1 Introduction

Hydrocarbon contaminants found in aquatic animals have usually included aromatics and aliphatics. Both could be detected in very low concentrations in aquatic animals and are concentrated in the animal tissues via polluted water or food sources during the periods of continuous and intermittent exposures (Handy 1994)

Salmon in wild conditions are known to avoid parts of the water column contaminated with the WSF-HC of crude petroleum, but farmed, pen-reared salmon cannot avoid this type of oil pollutant. Natural situations that could be encountered by this species are high doses of WSF-HC for short periods of time or low doses for prolonged periods. The first possibility was in fact the case in the Shetland Islands in January of 1993 where salmon stocks were exposed for several days to the oil spill from the Braer tanker (Ritchie and O'Sullivan 1994), and could certainly occur in Canadian salmon farms located on shores where heavy tanker traffic increase the chances of oil spills. Low level exposure can also be found along shorelines where normal vessel activity can contribute continuously to low-level pollution, or where hydrocarbons are spilled into natural drainage systems or leached from sand beaches. To understand the uptake and depuration of hydrocarbons in salmon under these two naturally occurring situations it is necessary to perform a series of exposures of salmon to the WSF-HC of crude oil and to reveal the functions of key elements or cell compartments in the muscle tissue which control the WSF-HC behaviour. The objectives of the current studies are

outlined as follows:

(1) To determine the uptake and depuration rates of total WSF-HC, different types of WSF-HC and individual WSF-HC of the same type in the muscle tissue of Atlantic salmon under various exposure conditions.

(2) to explore the patterns of uptake of WSF-HC by the muscle tissue of Atlantic salmon and to determine the minimum exposure time required to reach an equilibrium between WSF-HC in the exposure water and WSF-HC in the muscle tissue.

(3) to understand the uptake and depuration of WSF-HC in different types of muscle tissues and to reveal the relationship between lipid content of different muscle types and the behaviour of WSF-HC in these muscle types.

(4) to reveal the role of adipocytes in the uptake, depuration and retention of WSF-HC in the muscle tissue.

(5) to determine the effect of prolonged-starvation of Atlantic salmon on the depuration of accumulated WSF-HC from the muscle tissue.

(6) to determine the minimum time required for the depuration of accumulated WSF-HC in the muscle tissue of Atlantic salmon.

## 4.2 Literature Review

### 4.2.1 The Most Recent Example of Impact of the Wreck of an Oil Tanker on the Nearby Salmon Farming Industry

On January 5, 1993, a serious oil spill took place near Shetland Islands where salmon farming is an important aquaculture industry (Ritchie and O'Sullivan 1994). An estimated 84,700 tonnes of light Norwegian Gullfaks crude oil were released over a period of a few days. The oil, which was of a type particularly apt to disperse, was thoroughly mixed into the turbulent sea and was finally deposited in a small area of fine sediments on the sea bed. Around inshore areas, levels of oil in seawater immediately

after the incident rose to 50 ppm, 20,000 times background concentrations. High concentrations of polycyclic aromatic hydrocarbons (PAHs) (up to 14 ppm) were detected in the salmon flesh near the sites of spill. The accumulated PAHs in salmon were then gradually depurated with time and salmon from all affected sites were in fact approaching background values for PAHs content after about 7 months. The oil spill however led to a ban on harvesting salmon affected by the spill for market, and a decision to dispose of the whole of the 1991 and 1992 salmon year-classes. The effect of the oil spill on wild fish was reported to be less severe (George et al. 1995), and they recovered more quickly than captive salmon from taint and contamination because of their ability to avoid the oil by simply swimming away.

### 4.2.2 Lipids in Aquatic Organisms and Their Role in Hydrocarbon Uptake, Depuration and Retention

The gross effects on aquatic organisms resulting from exposure to petroleum hydrocarbons have been thoroughly investigated in many ways. There are important variables which must be taken into consideration in experimentally evaluating the effects of petroleum hydrocarbons on aquatic organisms: (a) the levels to which petroleum hydrocarbon are accumulated; (b) the duration of hydrocarbon residence in the organisms; and (c) the composition of the hydrocarbon mixture in the water and, subsequently, in the organisms. With aquatic organisms one biological factor, i.e., lipid content, plays a major role in the above variables. Studies have shown that the main entry route of organic pollutants into aquatic organisms is through the partitioning of organic contaminants between water and membrane lipids (Boryslawskyj et al. 1988). The epithelial membrane of the gill surface is reported to be the major exit route of naphthalene elimination from the tissues of *Hemigrapsus nudus* (Crustacea: Decapoda) (Laurén and Rice 1985). Membrane composition may therefore be an important factor in this partitioning. This would imply that the temperature history of the organisms

should be carefully examined since many organisms adjust their lipid bilayer composition in order to retain fluidity at low temperatures.

Both laboratory and field studies on bivalves suggest that hydrocarbons and other lipophilic compounds are accumulated by simple equilibration between seawater and body lipids (Fossato and Canzonier 1976, Burns and Smith 1981, Pruell et al. 1986, Murray et al. 1991). Stegeman (1974) studied the exposure of oysters differing in lipid content to low levels of petroleum hydrocarbons for extended periods, and found that high tissue concentrations of hydrocarbons could be achieved even when the concentration in the water was low. The studies showed that the extent of accumulation was apparently linked to the neutral lipid content of the organisms. As the concentration of hydrocarbons accumulated in tissue exceeded 50 ppm, the levels of accumulation in lipid-rich oysters were much higher than in those with lower lipid content. This relationship between lipid content and hydrocarbon accumulation by oysters has also been discussed by Stegeman and Teal (1973) as being indicative of some degree of equilibration between hydrocarbons in the water and hydrocarbons which become associated with oyster lipid. It was observed that the quantity of hydrocarbons accumulated by oysters during an exposure period of 7 weeks was greater in animals that contained a higher proportion of body lipid. Conceivably, the small quantities of hydrocarbons retained by the animals over long periods are concentrated in the lipid fraction from which they can be released only very slowly. Neff et al. (1976) investigated the accumulation of hydrocarbons in the tissues of marine animals and pointed out that in all species tested, accumulation of aromatic hydrocarbons appeared to be dependent primarily on the partitioning of the hydrocarbons between the exposure water and the tissue lipids. Holden and Marsden (1967) reported that while the concentration of the fat-soluble chlorinated hydrocarbons in the whole tissue varied widely, those expressed in term of extractable fat were in much closer agreement. Examination of the organic contaminants in five fish species and their eggs from Lakes Ontario and Erie also indicated that the percent lipid in the fish and eggs significantly influenced the contaminant transfer (Niimi 1983). Teal (1977)

believed that no accumulation process is involved on the hydrocarbon accumulation and release, other than the lipid/water partitioning apparently dominant in animals.

Significantly positive correlations between naphthalene retention and copepod size measured as total lipid content were also found by Harris et al. (1977) in studying the retention of an aromatic hydrocarbon by marine planktonic copepods. It was found that the lipid content of the animals might be more important than metabolic effects in determining naphthalene retention. Prolonged starvation caused a fall in lipid level in the animal, which would thus have less chance of retaining a lipid-soluble substance such as aromatic hydrocarbons. Comparing the hydrocarbon retention by males and females of this species, it was found that although females and males retained similar amounts of naphthalene (86.60 and 87.45 $\mu$g/animal respectively), the males had roughly thrice the amount of lipid (33.12 $\mu$g/animal compared with 12.12). This implies that there might be differences in hydrocarbon behaviour between male and female aquatic organisms. There have been no other publications confirming this effect.

Species-specific differences in the uptake and depuration of organic pollutants could also be attributed to the differences in percent lipids of each species. Cod (*Gadus morhua*) and scallop (*Placopecten magellanicus*), typical of lean fish and shellfish (< 1% lipids) (Dambergs 1964, Ackman 1980), took up small amounts of hydrocarbons from water and the tainted muscles were rapidly freed of the accumulated hydrocarbons by holding them for a few days in clean seawater (Ernst et al. 1987, Ernst et al. 1989). On the other hand, farmed salmon, a fatty fish species, accumulated higher concentrations of hydrocarbon pollutants (Heras et al. 1992). The accumulated pollutants in fatty species were depurated very slowly into the water column and could stay in the muscle tissue for months (Ritchie and O'Sullivan 1994). The species-specific characteristics have also been observed by Hektonen et al. (1992) and Hebert and Haffner (1991) for cod, rainbow trout and cyprinids. The role of lipids in the uptake and depuration of organic compounds was found to be important in a freshwater species (channel catfish) with differences in muscle lipid content (Johnsen and Lloyd 1992). The fatter channel

catfish (>2.5% muscle fat) accumulated nearly three times more 2-methylisoborneol than lean fish (<2%). In clean water, the accumulated contaminants in leaner fish were depurated much faster (8 h) than those in fatter fish (48 h).

The major site of lipid deposition in cod is the liver which may contain some 60% fat and occupy 7% of the body weight. This marked centralization of fat deposition in the liver may well account for the lack of any apparent effect of hydrocarbon-feeding in the muscle tissue of cod compared with the marked changed noted in the muscular tissue of other fatter species (Whittle et al. 1974). The plaice, which stores significant amounts of lipid in the muscle under the skin, took up hydrocarbons which were identified in the flesh within 5 days after hydrocarbon-feeding. The partitioning of an organic contaminant (Mirex) between adipose tissue and serum in human was studied by Burse et al. (1989) and Mirex was found to be much more concentrated in adipose tissue than serum.

Discrimination in the uptake and retention of hydrocarbons certainly occurs. It is believed that the reason for the difference in the extent of uptake and retention of different aromatic hydrocarbons is their different lipid/water partition coefficient (Anderson et al. 1974, Whittle et al. 1974, Neff et al. 1976). Hydrocarbons with higher lipid/water partition coefficient favour a more rapid uptake and longer retention in the tissue of marine animals. The aromatic hydrocarbons are accumulated to a great extent and are retained longer than the alkanes (Stegeman and Teal 1973, Anderson et al. 1974, Neff et al. 1976).

The model for the uptake of organic pollutants from water by aquatic organisms at equilibrium has been defined as the octanol-water partition coefficient (Chiou et al. 1977, Boese 1984). If pollutant uptake from water is dependent upon lipid and aqueous phase partitioning, then uptake efficiency should be related to the octanol-water partition coefficient.

Concentrations of lipophilic contaminants are often adjusted for variation in tissue lipid content, which is called lipid normalization (Hebert and Keenleyside 1995). Lipid

normalized data are used in a variety of applications, including developing biota-sediment accumulation factors (Lake et al. 1990, Ankley et al. 1992, Thomann, et al. 1992, Macdonald et al. 1993, Pruell et al. 1993, Schell et al. 1993) and modelling biomagnification in foodwebs (Thomann and Connolly 1984, Connolly and Pederson 1988).

A contrary effect of lipids on the accumulation of organic pollutants in fish has also been reported. Miller et al. (1992) investigated the patterns of organochlorine contamination in lake trout and claimed that lipid may not be an important factor influencing PCB bioaccumulation in this species, within the range of lipid concentrations observed.

### 4.2.3 General Aspects of Hydrocarbon Uptake, Depuration and Retention in Aquatic Organisms

The accumulation of hydrocarbons by aquatic organisms is quite rapid and so is depuration, although a long period is needed for the complete discharge (Stegeman and Teal 1973, Anderson et al. 1974, Fossato and Canzonier 1976, Harris et al. 1977, Stainken 1977). Heras et al. (1992) conducted a short-term (6 h) exposure of Atlantic salmon (*Salmo salar*) with low lipid content (3.6%) to WSF-HC. The fish were heavily tainted by the WSF-HC and the sensory panel evaluation indicated that the short-term exposed fish were significantly different from the control fish. The accumulation of WSF-HC by coho salmon (*Oncorhynchus kisutch*) and starry flounder (*Platichthys stellatus*) were studied by Roubal et al. (1978). Both species accumulated a complex spectrum of low molecular weight aromatic hydrocarbons, but bioconcentration factors for most hydrocarbons in starry flounder muscle were substantially higher than those for coho salmon muscle. Accumulated hydrocarbons in coho salmon exposed for six weeks fell below detection limits within a week when fish were transferred to clean water. Starry flounder exposed for two weeks retained substantial concentrations (7-26 ppm) of

accumulated hydrocarbons in muscle two weeks after the termination of exposure.

Selective uptake occurs between different parts of the same fish. Many experimental results have revealed that the *n*-alkanes in muscles tended to have a uniform carbon number distribution while those in the liver show a high odd carbon predominance (Mackie et al. 1974, Whittle et al. 1974). Alkanes in the water column were similar to those in the muscle; sediment alkanes to those in the livers. This agrees with the hypothesis that liver hydrocarbons came mainly from the food, carried by the blood directly from the gut via the hepatic portal circulation to the liver. There they are absorbed before having the chance to enter the other tissues. The muscle hydrocarbons instead are probably absorbed through the gills from the water. Blood from the gills passes through the muscles before reaching the gut and liver. Therefore, hydrocarbons absorbed from the water tend to be localized in muscle.

Roubal (1974) applied the method of spin-labelling of living tissue, and indicated that "insoluble" hydrocarbons are transported by carriers in the blood in which the hydrocarbon pollutant is able to intercalate into a region of low viscosity (such as lipid).

Studies on the ultrastructure of *Cyclotella cryptica* have shown that alkanes affect the thickness of the cell wall (Karydis and Fogg 1980). The interaction of hydrocarbons with cells and their constituents in coho salmon was investigated by Roubal (1974) using very low levels of spin-labelled hydrocarbons. It was reported that alkanes tend to occur in membrane interiors and aromatics on the surface. In Roubal's studies alkanes were found to be much less subject to disposal than any aromatics.

A large proportion of current publications have focused on the distribution of organic pollutants in aquatic organisms collected from various polluted locations (Risebrough et al. 1980, Rowland and Volkman 1982, Granby and Spliid 1995, Khan et al. 1995, Meador et al. 1995, Payne et al. 1995, Widdows et al. 1995), particularly from the areas where oil spills occurred previously (Squire 1992, Fayad et al. 1996).

### 4.2.4 Health, Growth and Physiological Performance of Aquatic Organisms during and after Exposure to Organic Contaminants and Ingestion of Contaminated Foods

WSF-HC are more toxic to aquatic organisms than their parent crude oil because they are mainly composed of aromatic hydrocarbons and are relatively soluble in water (Morrow et al. 1975). The toxicity of WSF-HC has received less attention than the studies in their accumulation in aquatic life. In a critical review, Malins (1982) documented the fact that individual petroleum components, model mixtures of hydrocarbons, fractions of petroleum, and whole oil alter cellular and subcellular structures. Purdy (1989) studied the effects of brief exposure to seven aromatic hydrocarbons on feeding and avoidance behaviour in coho salmon and found that exposure to aromatic hydrocarbons could limit a fish's ability to survive. Moles and Rice (1983) studied exposure of juvenile pink salmon for 40 days to stable, sublethal concentrations of naphthalene and WSF-HC and reported that chronic marine oil pollution at a concentration as low as 0.4 ppm total aromatic hydrocarbons could reduce the growth of juvenile pink salmon. Rainbow trout were exposed to a model mixture of water-soluble aromatic hydrocarbons at concentrations of 0.3-15.2 ppm to study their effects on some haematological parameters (Zbanyszek and Smith 1984). All parameters, except blood glucose, changed at the 7.2 ppm level and all fish died within 1 h at 15.2 ppm. However, in the normal case of hydrocarbon pollution or oil spills, the WSF-HC concentrations are rarely raised to such a high level and unusual fish mortalities have not been observed (Ritchie and O'Sullivan 1994). Besides aromatic hydrocarbons, other water-soluble constituents, such as phenolic compounds, could also be found in the WSF-HC of crude oil. Korn and Rice (1985) investigated the toxicity of phenol and *p*-cresol to pink salmon and kelp shrimp and concluded that most of the toxicity of the WSF-HC remains unaccounted for since phenols do not contribute substantially to the toxicity of the WSF-HC of crude oil.

Studies have also focused on the direct exposure to petroleum hydrocarbons such

as crude oil instead of WSF-HC to aquatic organisms. American lobster (*Homarus americanus*) at larval stages were exposed to a sublethal concentration of seawater-crude oil dispersion through a continuous flow system (Capuzzo et al. 1984). It was found that the normal patterns of lipid storage, utilization and synthesis during larval development and metamorphosis were altered with oil exposure. Increased rates of protein catabolism, delayed molting and reduced growth were also evident among lobster larvae exposed to oil, which suggests either deficiency or immobilization of lipid reserves. Atlantic salmon parr were investigated for growth and food conversion during exposures to two sublethal flow-through concentrations of crude oil (Vignier et al. 1992). The results suggest that medium-term exposure to concentrations of oil may have transitory effects on Atlantic salmon parr, but that chronic oiling may impair growth and may influence the timing of length-dependent smoltification. Another study on farmed Atlantic salmon by Aabel and Järvi (1990) showed that exposure to both crude oil and handling increased mortality significantly. Mortality was also observed when rainbow trout (*Oncorhynchus mykiss*) were exposed for 21 days to sublethal levels of crude oil (Steadman et al. 1991). However, following this exposure period the ability of pre-exposed trout to survive exposure to acutely lethal levels of crude oil was observed.

Ingestion of organic pollutants or foods contaminated with organic pollutants also affects various aspects of the performance of aquatic organisms. Adult rainbow trout were exposed to a single oral dose containing a mixture of eight PAHs and it was found that, unlike the exposure to PAHs in water column, PAHs are poorly absorbed by trout (Niimi and Palazzo 1986). Polar cod (*Boreogadus saida*) were examined for the effects of feeding foods contaminated with 200 or 400 ppm crude oil on food selection patterns and appetite-growth relationships (Christiansen and George 1995). The results showed that the ingestion of oil-contaminated food led to a significant depression in growth performance in both male and female fish. Schwartz (1985) studied the effect of oil contaminated prey on the feeding and growth rate of pink salmon fry. The growth rate of pink salmon fry exposed to oil contaminated prey for 50 days decreased with increased

dose levels of oil contaminated prey. Reduction in the growth of fry exposed to the WSF-HC of crude oil was greater than the reductions in growth of fry fed oil contaminated prey.

The influence of chronic ingestion of saturated hydrocarbons on the performance of rainbow trout has been studied by Cravedi and Tulliez (1982) and Luquet et al. (1983). Pristane, dodecylcyclohexane and a mixture of common *n*-alkanes were incorporated separately into the diets of rainbow trout. A general depressive effect of hydrocarbons was observed on both appetite and growth. Total lipids in the whole carcass and muscle were lower in the test groups than in the control group. An interesting observation was noted in that cyclohexyldodecanoic acid was detected in the fish receiving dodecylcylohexane.

## 4.3 Uptake and Depuration of WSF-HC of Flotta North Sea Crude Oil in Different Muscle Types of Atlantic Salmon during and after a High Dose and Short Term (8 h) Exposure

### 4.3.1 Introduction

Fish muscle contains two basic muscle fibres, white and dark, which differ in their biological, physiological and biochemical functions. White muscle can be further subdivided into dorsal white muscle, flank muscle and belly flap according to their compositional differences, primarily the proportion of muscle fibres vs. connective tissue. It has been generally recognized that the dark muscle works for slow swimming and for keeping and horizontal balance of the body, while the white muscle works for fast swimming (Hudson 1973, Walker and Pull 1973). Lipids are the main energy source for dark muscle, while the energy of white muscle is derived mainly from the aerobic breakdown of glycogen (Bone 1966).

The correlations between lipid content and hydrocarbon uptake and depuration are well documented (Johnsen and Lloyd 1992, Hebert and Keenleyside 1995). These correlations are the characteristics of both inter-species (Ernst et al. 1987, Ernst et al. 1989, Heras et al. 1992) and intra-species comparisons of aquatic organisms having different lipid content (Niimi 1982, Johnsen and Lloyd 1992). However, the uptake, depuration and retention of hydrocarbon pollutants in different muscle types of each species have not been studied and remain unknown. In this study, Atlantic salmon were exposed to the WSF-HC for 8 h and subsequently depurated in clean seawater for one month. The objectives are to fully understand the differences in the uptake, depuration and deposition of WSF-HC in dorsal white muscle, dark muscle, flank muscle and belly flap of Atlantic salmon.

### 4.3.2 Materials and Methods

**Atlantic Salmon**

Atlantic salmon, *Salmo salar*, were held in aerated seawater at the Dalhousie University Aquatron Laboratory and fed on a diet of Fundy Choice feed (Corey Mills Ltd, Fredericton, New Brunswick) (Fig. 4.1). The fish used for the exposure study (26 fish) had the average weight 1470 ± 235 g, length 47.8 ± 2.7 cm, and were composed of 52% male salmon. The fish were starved for 24 h before exposure.

**Preparation of WSF-HC Stock Solution**

Flotta North Sea crude oil, obtained from the Dartmouth, Nova Scotia refinery of Esso Petroleum Canada, was used to prepare the water-soluble fraction. The crude oil was stirred with cold seawater in a 450 L stainless steel mixing vessel equipped with a powerful mechanical stirrer, a bottom drain, and a jacket cooling-water system. Crude petroleum and seawater of salinity 31.6‰, pumped from the Northwest Arm, an inlet near Halifax, and sand-filtered in the Dalhousie University Aquatron (Parrish et al. 1992), in a ratio of 1:99 (v/v), were stirred for 24 h and allowed to settle for an additional 48 h. The contents were kept cool by circulating tap water through the outer jacket of the mixing vessel. The prepared WSF-HC stock solution was removed through the bottom drain. The preparation of WSF-HC stock solution was started immediately prior to the exposure study and the prepared WSF-HC stock solution was stored in two layers of 70 L capacity bags (polyethylene, Fig. 4.2) (Heras et al. 1995).

**Exposure and Depuration of Atlantic Salmon**

Every effort was made to avoid stress caused by handling fish during the exposure

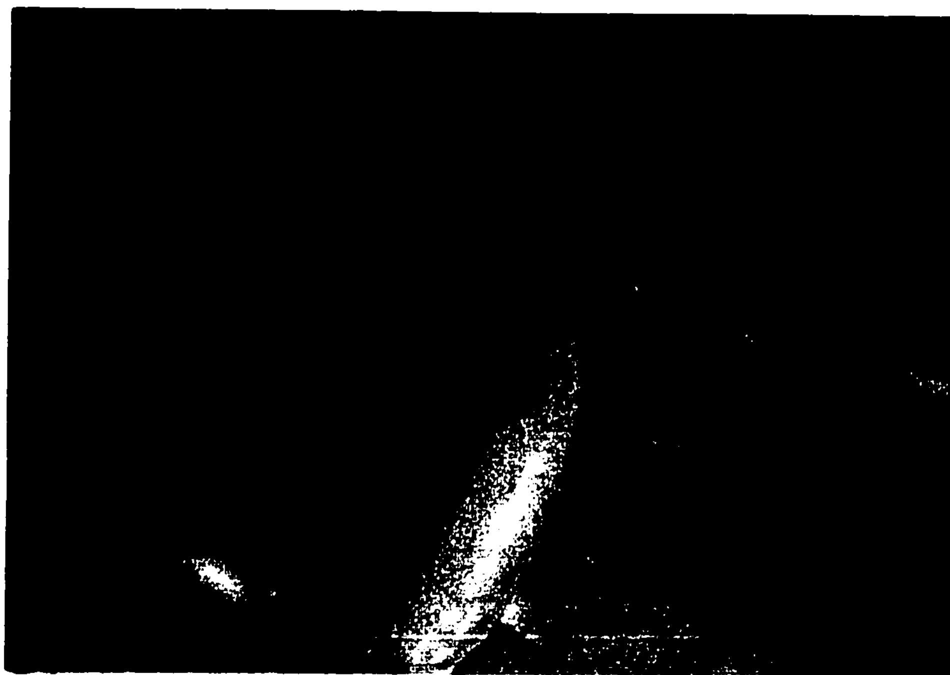

Fig. 4.1. Stock Atlantic salmon held in aerated seawater at the Dalhousie University Aquatron Laboratory

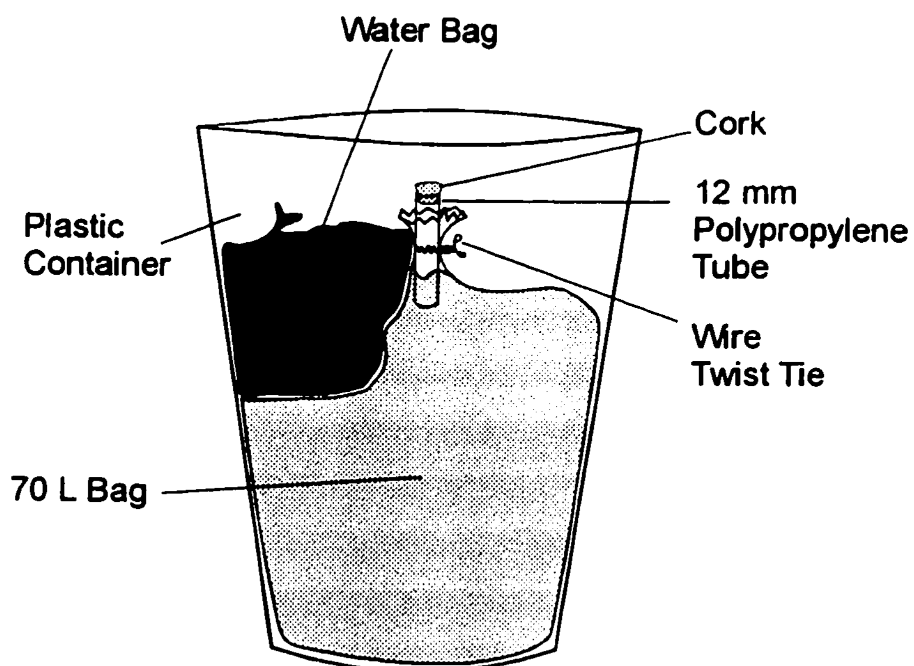


Fig. 4.2. Schematic diagram of the polyethylene bag used for the storage of WSF stock solution.

period and transferring fish from tank to tank. The exposure tank (2 m in diameter and 0.8 m in depth, 2500 L water holding capacity) with an overflow system was pre-filled with diluted WSF-HC stock solution (0.3 m in depth) and Atlantic salmon (18 fish) were then carefully placed into the tank. The tank was immediately covered with polyethylene film and sealed by taping the plastic film along the body of the tank. The tank was kept sealed during the entire exposure period in order to maintain a stable hydrocarbon profile in the exposure water. Constant concentration of WSF-HC and oxygen level in the exposure water were achieved by continuously and proportionally pumping oxygenated seawater (3600 mL/min) from a header tank and WSF-HC stock solution (470 mL/min) from the sealed plastic bags to the exposure tank (80-110% saturation). Two Masterflex pumps (Cole-Parmer Instrument Co., Niles, IL) with Tygon tubing were used to supply seawater and WSF-HC stock solution respectively. All tubing was pre-cleaned by pumping seawater through it for several hours. The WSF-HC stock solution was sampled at the beginning and end of exposure and analyzed immediately for WSF-HC concentration. During the exposure period, the flow rates of both seawater and WSF-HC stock solution were measured each hour and an appropriate adjustment was made if necessary. The exposure water temperatures ranged from 8.5 to 8.9 °C.

At the end of exposure, three exposed fish from the exposure tank were removed immediately for the recovery of tainting WSF-HC taken up during exposure. In addition, three control salmon were taken from the reserve fish holding tank at the same time. The remaining exposed fish were immediately transferred to a clean seawater aquarium for depuration. The photoperiod was set for 12 h light and 12 h dark. The tainted fish were continuously fed on Fundy Choice feed during the depuration period. Salmon were depurated for 31 days at seawater temperatures ranging from 5 - 9°C and the fish (three each time) were randomly taken at day 1, day 4, day 10, day 17 and day 31 for analyses of WSF-HC in different muscle types. A second batch of three control fish was taken at day 10 of the depuration period.

**Sampling of Muscle Tissues**

The live Atlantic salmon removed for study were immediately transported in chilled seawater to the Canadian Institute of Fisheries Technology at the Technical University of Nova Scotia. The fish were killed with a blow on the head and the gut cavities were cut open along the middle of the abdomen. The viscera were then removed and the fish were thoroughly washed with cold water. The tainted and control fish were kept separate from each other at all times. The mesenteric adipose tissue was carefully collected. The dorsal white muscle, dark muscle, lower flank muscle and belly flap were dissected from each fish as shown in Fig. 4.3. The dissected samples from each of three fish were pooled and immediately frozen at -35°C until analysis.

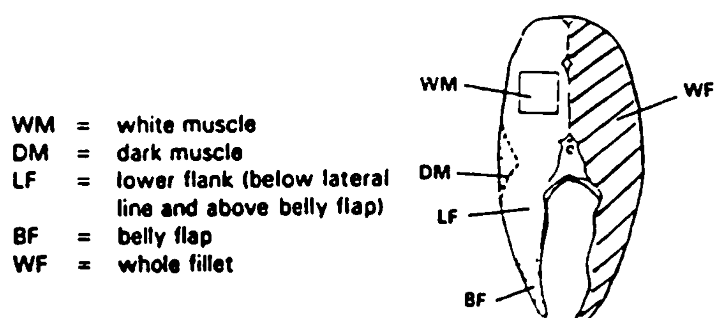


Fig. 4.3. Cross section of Atlantic salmon muscle showing the different muscle types used for hydrocarbon analyses

**Extraction of WSF-HC from Seawater**

The stock WSF-HC water samples in the plastic bags were pumped through the Masterflex tubing into 1 L brown screw cap bottles. The bottles were sealed with

Parafilm and the hydrocarbons in water were extracted immediately according to the Murray microextraction procedures (Murray 1979, Murray and Lockhart 1981, Murray et al. 1984). In brief, an extraction flask of 1 L capacity was constructed with a side arm and a capillary tube at the top-centre of the flask. The WSF-HC solution (980 mL) was placed in the flask. The flask was stoppered, and the mixture was shaken with hexane (1 mL, Omnisolv grade, VWR Scientific of Canada, Ltd.) for 1 min. The mixture was allowed to settle for a minimum of 15 min in a domestic refrigerator (5 °C) to permit the hexane droplets dispersed throughout the aqueous phase to collect on the upper surface. The hexane layer was directed to the centre capillary tube by adding chilled distilled water through the side arm, while tilting the flask at an angle of 45 °. Two $\mu$L of the hexane layer from the capillary tube were taken into a Hamilton GC syringe containing 0.2 $\mu$L of clean hexane, followed by 1 $\mu$L of a hexane standard solution of *n*-heneicosane. Both were injected simultaneously into the GLC. Quantitative analyses were based on the use of $n$-$C_{21}$ as the internal standard, and the concentrations of WSF-HC in the samples were calculated according to correction factors from a published report (Ernst et al. 1989).

**Recovery of Hydrocarbons from the Muscle and Mesentery Adipose Tissues by Steam Distillation**

Hydrocarbons were recovered from the muscle tissues and mesentery adipose tissue by steam distillation as described in Section 3.3.3 (experimental conditions III).

**GLC Analysis of Hydrocarbons**

The hydrocarbon analyses were conducted with a Perkin-Elmer 8420 capillary gas chromatograph equipped with a flame ionization detector (FID) and split injection system (split ratio: 1:32). The analysis was conducted on a DB-1 (methyl silicone) fused silica

capillary column (60 m, 0.25 mm ID, 0.25 μm film thickness). Conditions and temperature for WSF-HC extracted from seawater were: FID, 280 °C; injector, 280 °C; and for hydrocarbons extracted from tissues: FID, 350°C; injector, 350°C. The column temperature was programmed as follows: initial temperature of 45°C held for 15 min; then increased at a rate of 13°C/min; final temperature 280°C, held for 25 min. The carrier gas was helium at a pressure of 138 kPa and hydrogen and air pressures were 90 and 159 kPa, respectively.

**Calculation of Tainting WSF-HC in the Muscle Tissues and Mesentery Adipose Tissue**

$$T(ppm) = \frac{A_S - A_C}{A_{IS} \times R \times m} \times M \times RF$$

where: T = amount of tainting WSF-HC in tissue sample (ppm, wet tissue basis)

$A_S$ = GLC peak area of hydrocarbon in tainted sample

$A_C$ = GLC peak area of the same hydrocarbon present in the control Atlantic salmon sample.

$A_{IS}$ = GLC peak area of *n*-heneicosane internal standard

R = hydrocarbon percent recovery by steam distillation

m = amount of tainted wet tissue used for steam distillation (g)

M = amount of added *n*-heneicosane internal standard (μg)

RF = GLC-FID response factor

**Determination of Lipid Content and Analyses of Lipid Classes of Total Lipids Extracted from Atlantic Salmon Muscle Tissues**

The lipid content of different muscle tissues and mesenteric adipose tissue was determined in duplicate according the procedure of Bligh and Dyer (1959). In this

procedure, about 100 g of muscle tissue was weighed accurately and transferred to a covered glass Waring Blendor jar. Chloroform (100 mL) and methanol (200 mL) were added and the mixture was blended for 2 min. A further 100 mL chloroform was added and the mixture again blended for 30 s and then 100 mL of water was added to the blend and mixed for another 30 s. The blend was then filtered through a Buchner funnel under water aspirator vacuum suction. The Blender jar was rinsed with chloroform (2 x 25 mL) and the rinses were poured through the Buchner funnel cake. When filtering was nearly complete, the cake was squeezed dry by pressing with the bottom of a small beaker in order to obtain complete recovery of lipid. The cake was rinsed with chloroform (2 x 15 mL) and pressed dry again. The filtrate was transferred to a 1 litre separatory funnel and was allowed to stand to completely separate the organic and aqueous layers. After the layers had separated, the chloroform layer containing the extracted lipid was collected in an Erlenmeyer flask and dried with anhydrous sodium sulphate. The chloroform solution was then filtered into a pre-weighed round bottom flask and the solvent stripped from the lipid on a rotary-evaporator under vacuum. The recovered lipid was weighed and the percent lipid calculated. In the case of insufficient tissue available for lipid content determination (<200 g wet tissue), the volume of solvents was proportionally reduced according to the sample weight.

The separation and quantitation of lipid classes were accomplished by thin layer chromatography on silica gel (Chromarods-SIII) with quantitation by flame ionization detection (Iatroscan-TLC-FID). The detailed procedures have been described in Section 2.3.3.

### 4.3.3 Results and Discussion

#### 4.3.3.1 Lipid Content and Lipid Classes of Different Muscle Types and Mesentery Adipose Tissue

Table 4.1 shows the gravimetrically determined lipid content and the chromatographically measured percentages of lipid classes in total lipid extracted from the different muscle types and mesenteric adipose tissue of Atlantic salmon. Mesenteric adipose tissue contained up to 79.07% of total lipids on wet tissue basis and triacylglycerol accounted for 99% of the total lipids. Lipids were not evenly distributed along the cross section of the muscle tissue (Fig. 1.1) and the lipid content was dependent on the types of muscle analyzed. As much as 33.23% of lipids was found in belly flap, but white dorsal white muscle contained only 3.45% of tissue lipids. The lipid content of low flank muscle was 12.47%, which is slightly lower than that of dark muscle (15.67%). Analyses of lipid classes by Iatroscan TLC-FID showed that triacylglycerol was the major lipid class (>80%) in lipids extracted from all muscle types. However, the percentage of triacylglycerol in total lipids decreased as the lipid content declined from belly flap to dorsal white muscle. The decreases in triacylglycerol percentage in total lipids extracted from different muscle types of Atlantic salmon was usually accompanied by the increases in the proportion of phospholipids.

#### 4.3.3.2 WSF-HC Profile in Seawater

Fig. 4.4 is a typical GLC chromatogram of WSF-HC prepared from Flotta North Sea crude oil. Aromatics ranging from benzene to dimethylnaphthalenes were the predominant hydrocarbons present in the WSF of the crude oil and exhibited a pattern similar to those previously reported (Heras et al. 1992). This WSF-HC profile is completely different from that of the parent crude oil, in which *n*-alkanes from *n*-heptane

Table 4.1. Lipid contents and lipid classes of different muscle types and mesenteric adipose tissue of Atlantic salmon. Values of lipid contents and lipid classes are the average ± standard deviations of duplicate analyses (pooled samples from three fish).

| Tissue and Muscle Types | Lipid Classes (% w/w of total lipids) | | | | | Lipid Contents (%, wet tissue basis) |
|---|---|---|---|---|---|---|
| | TG[a] | FFA | CHO | DG | PL | |
| Dorsal White Muscle | 81.39±2.45 | 0.62±0.20 | 1.14±0.54 | 0.61±0.35 | 16.25±1.24 | 3.45±0.38 |
| Dark Muscle | 90.34±3.09 | 0.60±0.24 | 0.73±0.32 | 0.56±0.14 | 7.76±1.41 | 15.67±0.98 |
| Lower Flank | 94.69±2.67 | 0.26±0.24 | 0.54±0.11 | 0.36±0.26 | 4.15±0.55 | 12.47±0.27 |
| Belly Flap | 98.13±1.33 | tr[b] | 0.26±0.19 | nd[c] | 1.61±1.42 | 32.33±1.09 |
| Mesenteric Tissue | 99.30±0.85 | tr | 0.21±0.18 | nd | 0.47±0.39 | 79.07±1.86 |

[a] TG - triglyceride, FFA - free fatty acid, CHO - free sterol, DG - diglyceride, PL - phospholipid.

[b] tr - trace (<0.1%).

[c] nd - not detected.

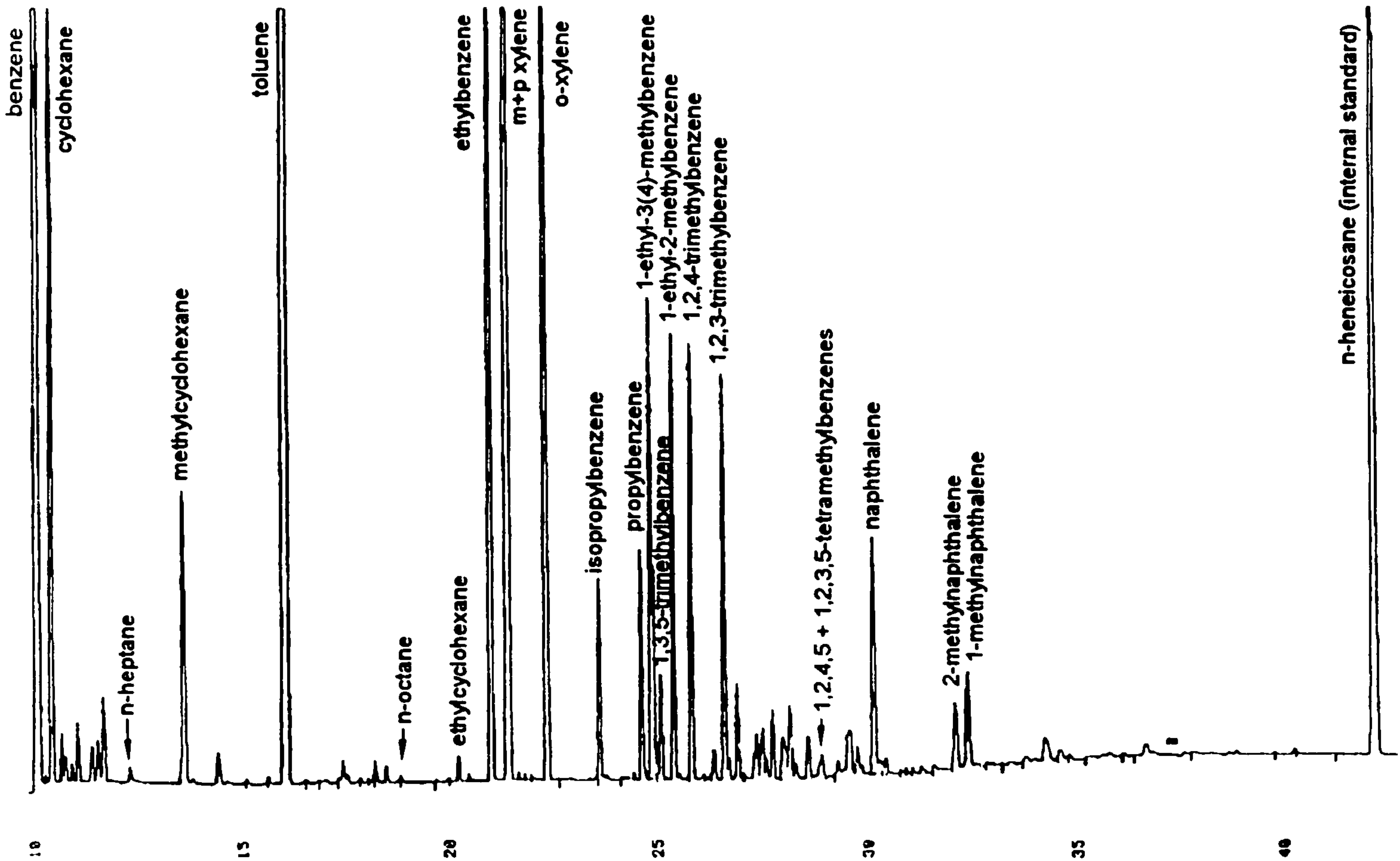


Fig. 4.4. GLC chromatogram of WSF stock solution of Flotta North Sea crude oil used for the short term (8 h) exposure

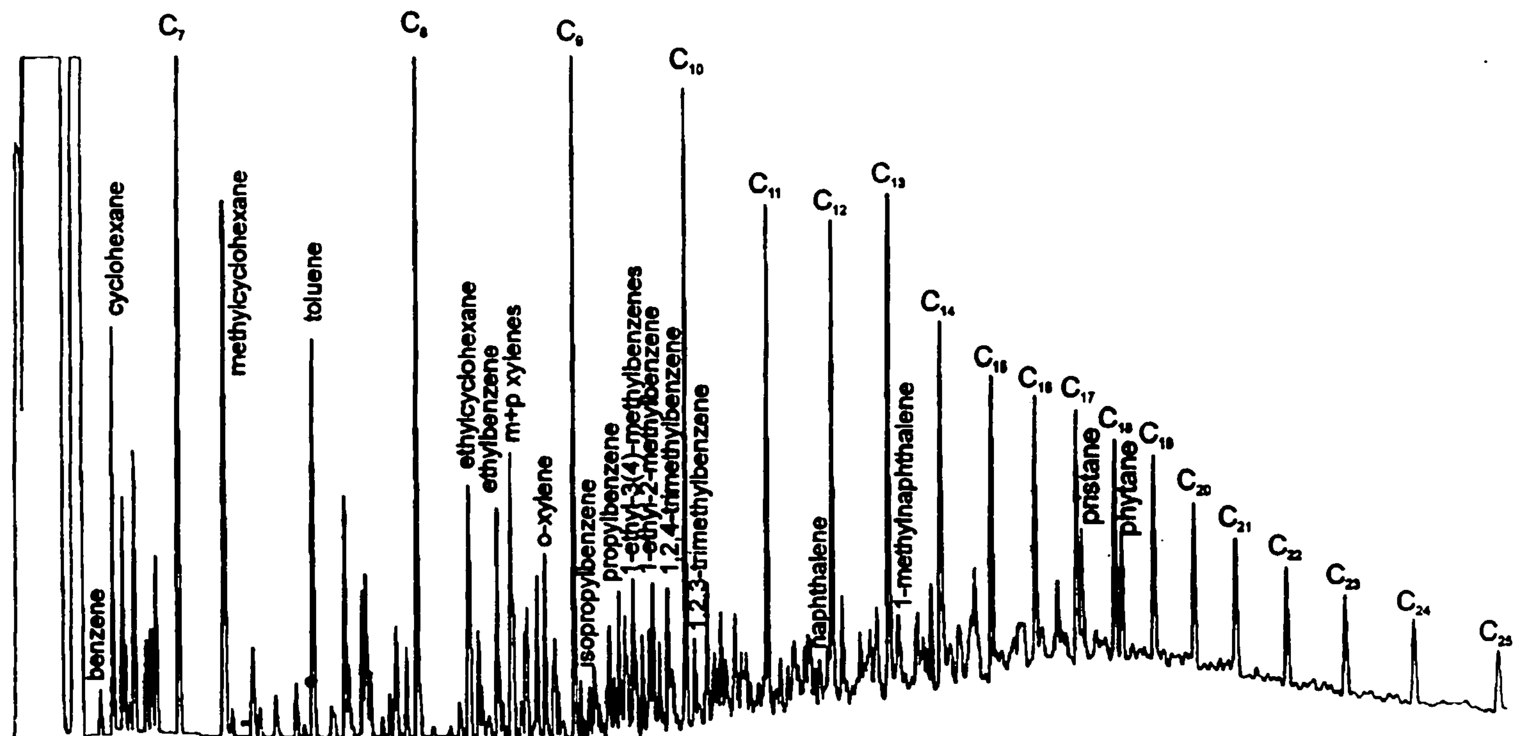


Fig. 4.5. GLC-FID chromatogram of Flotta North Sea crude oil

to *n*-dotriacontane account for a high percentage of the total crude oil hydrocarbons (Fig. 4.5). Table 4.2 shows the average concentrations of WSF-HC in the stock solution used for the 8 h exposure. The average concentration of total WSF-HC of the stock solution was 11.51 ppm. After the proportional dilution with seawater the actual average concentration of total WSF-HC in the exposure water was 1.5 ppm. Among the WSF-HC in the water column, benzene and alkylated benzenes dominated the entire aromatics, while PAHs, such as naphthalene and alkylated naphthalenes, accounted only for a small fraction of the total aromatics. *n*-Alkanes above *n*-nonane were not detected or present merely in trace amount in the exposure water. Non-aromatic cyclic hydrocarbons such as cyclohexane, methylcyclohexane and dimethylcyclohexanes were another group of WSF-HC with detectable concentrations in the WSF-HC solution and their peaks were clearly visible in the GLC chromatogram of WSF-HC stock solution (Fig. 4.4). A nearly horizontal baseline of the GLC chromatogram was observed for the WSF-HC of Flotta North Sea crude oil. However, for most parent crude oils and their WSF-HC an unresolved GLC baseline envelope is normally expected (Murray et al. 1984, Siron et al. 1987, Grob et al. 1991, Tan and Kuntom 1994, Draper et al. 1996). The observation of unresolved baseline envelopes reflects numerous hydrocarbon components in a narrow area of the GLC chromatogram which could not be completely separated.

#### 4.3.3.3 Uptake of WSF-HC by the Muscle Tissue of Atlantic Salmon

The results of the uptake and depuration of tainting WSF-HC by different muscle types are shown in Tables A5-1 to A9-2. Fig. 4.6 is a summary of the changes in total tainting WSF-HC in the different muscle tissues and mesenteric adipose tissue during the 31 days of depuration period. It was found that the higher the lipid content of the tissue, the more the WSF-HC were accumulated at the end of the 8 h exposure. This was not only observed in different muscle types but also in other tissues, hence mesenteric adipose tissue is included in this study. The bioaccumulation of WSF-HC in

Table 4.2. Concentrations of hydrocarbon components in the stock WSF-HC of Flotta North Sea crude oil prepared for the short-term exposure (8 h) of Atlantic salmon by the batch extraction method.

| Hydrocarbon Components | Concentration (ppm) | Percentage (wt%) |
|---|---|---|
| Total Hydrocarbons (ppm) | 11.51 ± 1.90 | 100 |
| **Aromatics** | | |
| benzene | 2.10 | 18.23 |
| toluene | 3.51 | 30.50 |
| ethylbenzene | 0.61 | 5.34 |
| *m*+*p*-xylenes | 0.93 | 8.08 |
| *o*-xylene | 0.59 | 5.12 |
| isopropylbenzene | 0.10 | 0.88 |
| propylbenzene | 0.12 | 1.03 |
| 1-ethyl-3-methylbenzene | 0.21 | 1.86 |
| 1-ethyl-4-methylbenzene | 0.06 | 0.51 |
| 1,3,5-trimethylbenzene | 0.05 | 0.43 |
| 1-ethyl-2-methylbenzene | 0.18 | 1.54 |
| 1,2,4-trimethylbenzene | 0.17 | 1.47 |
| 1,2,3-trimethylbenzene | 0.15 | 1.32 |
| tetramethylbenzenes | 0.01 | 0.11 |
| $C_4$-benzenes[a] | 0.20 | 1.77 |
| naphthalene | 0.16 | 1.41 |
| 2-methylnaphthalene | 0.07 | 0.61 |
| 1-methylnaphthalene | 0.07 | 0.65 |
| other PAHs[b] | 0.07 | 0.56 |
| Total Aromatics | 9.37 | 81.42 |

Table 4.2 (continued)

| Hydrocarbon Components | Concentration (ppm) | Percentage (wt%) |
|---|---|---|
| **Aliphatics** | | |
| cyclohexane | 1.07 | 9.33 |
| 2-methylhexane | 0.03 | 0.24 |
| isooctane | 0.06 | 0.54 |
| *n*-heptane | 0.02 | 0.20 |
| methylcyclohexane | 0.20 | 1.78 |
| dimethylcyclohexanes | 0.13 | 1.09 |
| *n*-octane | 0.01 | 0.12 |
| ethylcyclohexane | 0.03 | 0.30 |
| *n*-nonane | tr[c] | tr |
| *n*-decane | tr | tr |
| Total Aliphatics | 1.57 | 13.6 |
| **Unknown** | **0.57** | **4.98** |

[a] excludes tetramethylnaphthalenes

[b] other polycyclic aromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

[c] trace amount (< 0.01 ppm or <0.01%)

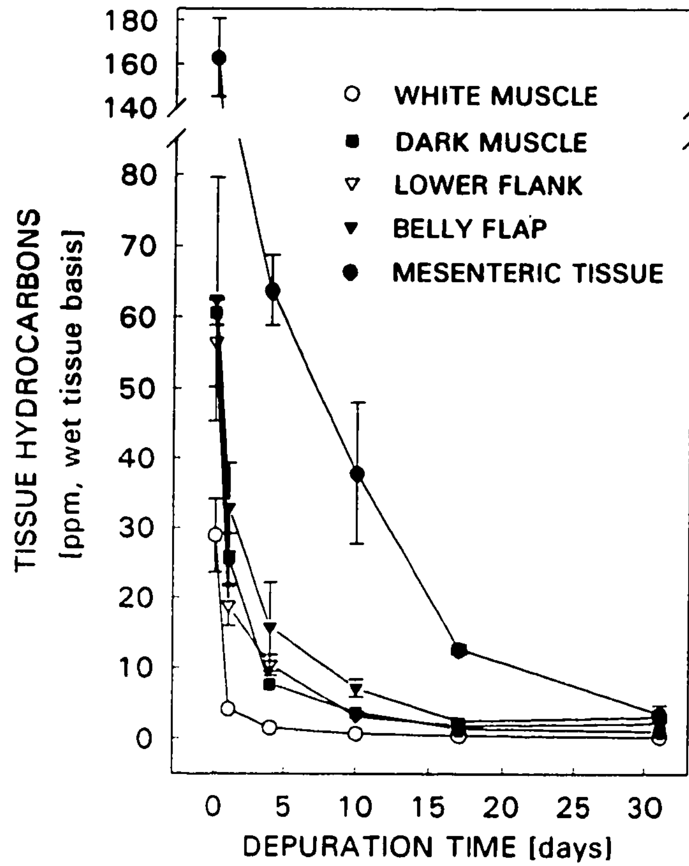


Fig. 4.6. Depuration of accumulated hydrocarbons from different muscle types of Atlantic salmon for 31 days following 8 h exposure to 1.5 ppm WSF of Flotta North Sea crude oil.

the muscle tissues increased as the following order: belly flap > dark muscle > lower flank muscle > dorsal white muscle. However, the concentrations of accumulated WSF-HC in different muscle types and mesenteric adipose tissue were not directly proportional to their lipid content. The mesenteric adipose tissue, which was predominantly composed of triacylglycerols (79.07% lipid), took up 163.0 ppm of WSF-HC. On the other hand, the dorsal white muscle, which contained only 3.45% of lipids, was tainted by 29.7 ppm of WSF-HC at the end of exposure. This was one half the accumulated level of WSF-HC in dark muscle (60.6 ppm) and lower flank muscle (56.2 ppm), although its lipid content was approximately one fourth the lipid content of both tissues (15.67% and 12.47% respectively). The difference in the uptake of WSF-HC between belly flap and dark muscle is quite small (62.4 ppm compared with 60.6 ppm of dark muscle), although the lipid content of belly flap is two times higher than that of dark and lower flank muscle. This may suggest that the accumulation of WSF-HC into the salmon tissues on this short term exposure had not reached a saturation plateau, and there is also the possibility that the vascularization delivering WSF-HC to the belly flap may be less than in other muscle tissues. This last hypothesis is further sustained by looking at the WSF-HC depurated after one day; more than half of the tissue WSF-HC were released from the different parts of the muscle, except for the belly flap which needed 1.3 days.

#### 4.3.3.4 Depuration of Accumulated WSF-HC from Different Muscle Tissues of Atlantic Salmon

The accumulated WSF-HC were rapidly depurated when the exposed fish were returned to clean seawater (Fig. 4.6). After 4 days of depuration, only 1.5 ppm of the total 29.7 ppm WSF-HC was left in the dorsal white muscle tissue (Table A8-1), while 15.8 ppm (of 62.4 ppm) of WSF-HC could still be detected in the belly flap (Table A5-1). WSF-HC in the dark muscle and the lower flank muscle were depurated in a similar rate, which showed that 7.7 ppm and 10.3 ppm of WSF-HC were present in those two

muscle types at the end of 4 days of depuration (Tables A6-1 and A7-1). The discharge of WSF-HC from the muscle tissues slowed down thereafter. After 10 days of depuration, WSF-HC concentrations in the dorsal white muscle were very close to the background levels of the non-tainted Atlantic salmon (Table A8-2), while in other muscle types at least 3.2 ppm of tainting WSF-HC could be detected. These remaining WSF-HC were exclusively composed of $C_4$-benzenes, naphthalene and alkylated naphthalenes (Tables A5-2, A6-2 and A7-2). At the end of 31 days of depuration, all of the muscle types were freed of the tainting WSF-HC except for the belly flap in which 1.8 ppm of WSF-HC (mostly PAHs) could still be found. The complete depuration of those remaining PAHs may well take months.

Fig. 4.7A illustrates the correlation between the lipid content of the muscle types and the time needed to depurate 80% of the WSF-HC in different muscle types. It is clearly demonstrated that the depuration rates of WSF-HC from different muscle types are highly correlated with lipid content in the tissues (Fig. 4.7B). Tissues with a higher lipid content display a lower depuration rate than tissues with a lower lipid content. The belly flap needs 6.2 days, while white muscle needs only 0.94 days to reach the same extent of WSF-HC removal. Muscle with intermediate lipid values (i.e. dark muscle and lower flank) of similar lipid levels accumulated similar amounts of WSF-HC (60.6 ppm for dark muscle and 56.2 ppm for lower flank). They also depurated to 80 % at a similar rate (3.3 days for dark muscle and 3.8 days for lower flank muscle).

The depuration rates of individual WSF-HC in a specific muscle type are mainly dependent on their molecular structures such as the number of aromatic rings, the alkylation on the ring and the degree of unsaturation of the ring. This point is clearly illustrated in Fig. 4.8. Among the aromatics, the depuration rate decreased as the number of aromatic ring and the alkylation on the ring increased. For example, in belly flap benzene, toluene, xylenes and $C_3$-benzenes were depurated in 1 day, 4 days, 10 days and 17 days respectively, while significant amount of methylnaphthalenes (0.22 ppm) could still be detected in the belly flap after 31 days of depuration in clean water (Fig.

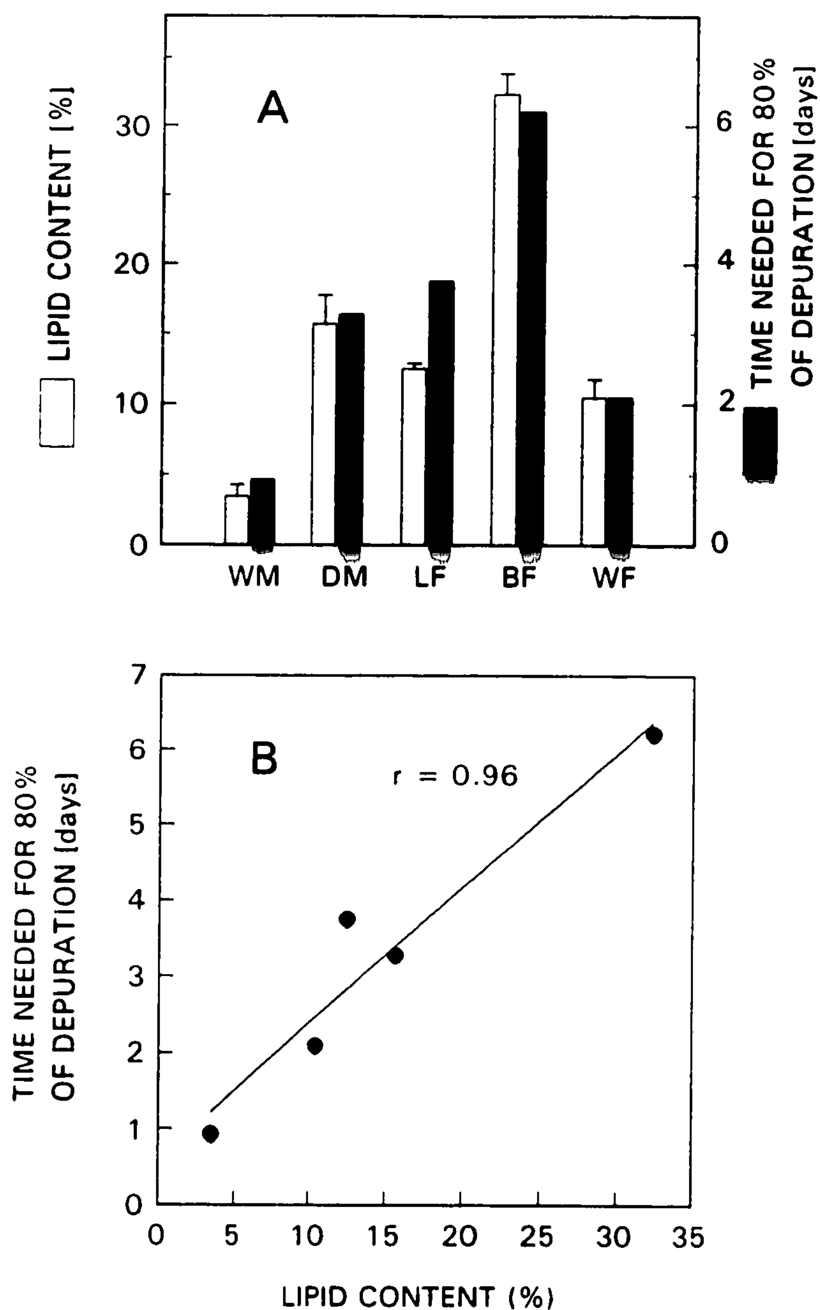


Fig. 4.7. Relationship between lipid content of different muscle types and the time needed to depurate 80% of the accumulated WSF hydrocarbons. This relationship is illustrated by a bar chart (A) and a chart with regression analysis (B). WM = dorsal white muscle, DM = dark muscle, LF = lower flank muscle, BF = belly flap, WF = whole fillet.

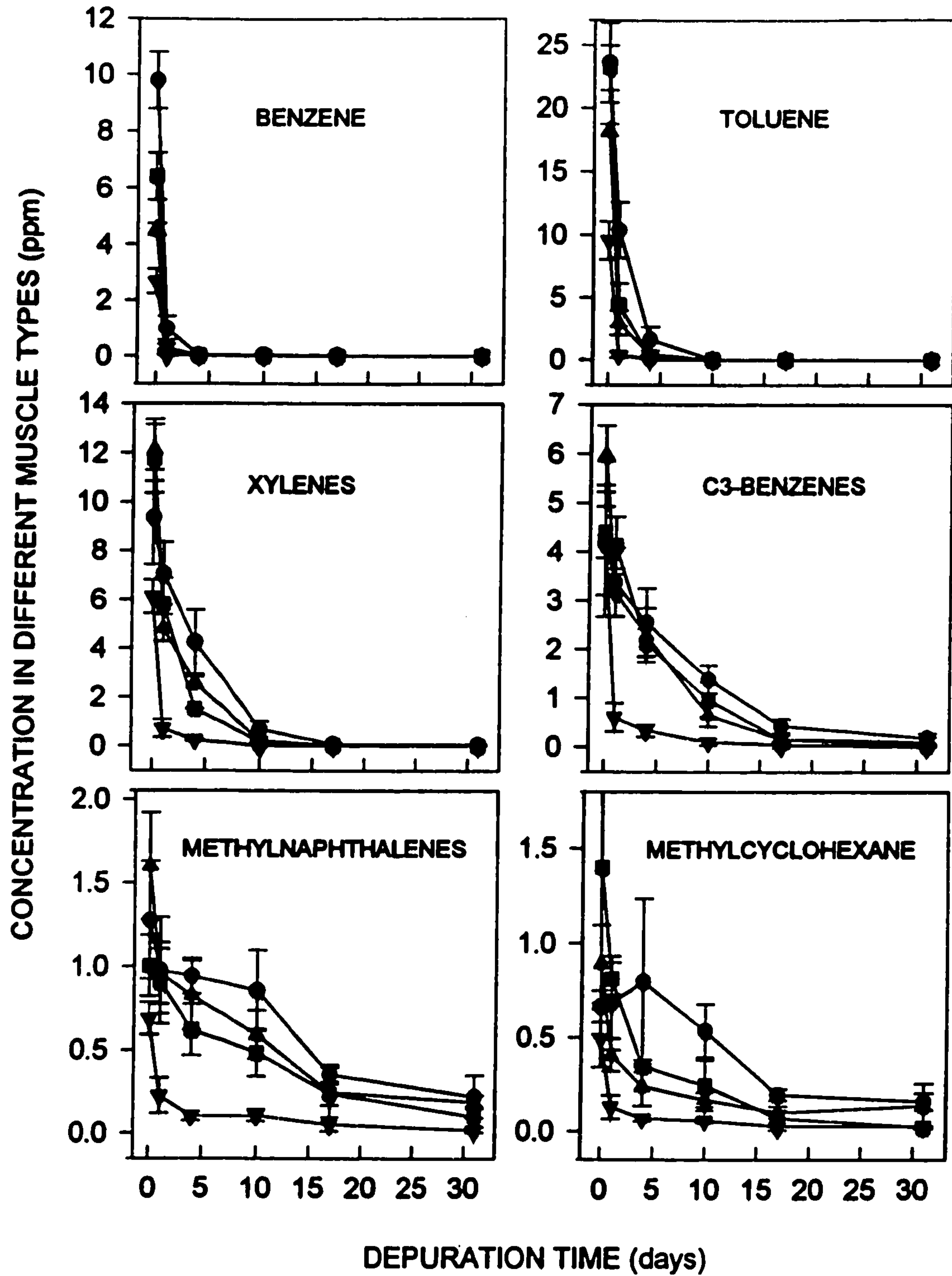


Fig. 4.8. Depuration of individual hydrocarbons in different muscle types of Atlantic salmon during the 31 days of depuration period in clean seawater. The Atlantic salmon were exposed to 1.5 ppm WSF of Flotta North Sea crude oil for 8 hours. —●— Belly Flap, —■— Dark Muscle, —▲— Lower Flank Muscle, —▼— Dorsal White Muscle.

4.8, Table A5-1 to A8-2). The higher solubility in body fluids of monoaromatics and also their faster enzymatic metabolization (Thomas and Rice 1981, Kennish et al. 1988) may be responsible for their relatively swift depuration compared to the higher molecular weight hydrocarbons that could be sequestered in lipid tissues. The depuration of methylcyclohexane was much slower than that of its aromatic counterpart of toluene and even $C_3$-benzenes (Fig. 4.8). This slower depuration behaviour of the non-aromatic cyclic hydrocarbons is probably due to their low water solubility and resistance of being metabolized by the liver of Atlantic salmon although evidence had showed that dodecylcyclohexane could be metabolized in rainbow trout (Cravedi and Tulliez 1981).

The depuration and retention of individual WSF-HC in different muscle types were similar to those of the total WSF-HC (Fig. 4.8). Up to 88% of xylenes and 86% of $C_3$-benzenes were released from the dorsal white muscle after the initial day of depuration, while only 24% and 19% of the same WSF-HC were released from belly flap during the same depuration period. The fast release of lower molecular weight hydrocarbons led to only the detection of naphthalene, methylnaphthalenes and highly alkylated benzenes in the late depuration stage.

Hydrocarbons retained in the tissues of aquatic organisms are expected to be mainly associated with lipids (Burns and Smith 1981, Johnsen and Lloyd 1992, Hebert and Keenleyside 1995). This association could primarily be attributed to the similarity in hydrophobicity between hydrocarbons and lipids.

Differences in the hydrophobicity of different lipid classes may probably also have some effects on the uptake and depuration of WSF-HC in different muscle types. Lower solubility or smaller attractive forces between tainting WSF-HC and phospholipids, the principal fraction of polar lipids, are expected due to the higher polarity of phospholipids compared to other lipid classes. Therefore, phospholipids would generally have lower retaining power for hydrocarbons compared with neutral lipids (mainly triacylglycerols). Thus at similar level of tissue lipids, the higher the proportion of polar lipids in tissues, the faster the depuration rate of hydrocarbons would be. Lipids extracted from the dorsal

white muscle contained up to 16.25% of phospholipids, while those from belly flap had only 1.61% phospholipids. This difference probably contributed in part to the rapid release of accumulated WSF-HC from the dorsal white muscle and to the slow depuration of WSF-HC from belly flap.

WSF-HC in the exposure water enter aquatic organisms mainly via gills and through partitioning with membrane lipids (Lee et al. 1972) and the tainting WSF-HC are subsequently associated with blood fluid. The lipoproteins and chylomicra in the blood then carry the WSF-HC through the circulatory system to the whole fish body including muscle tissue. When returned to the clean water, the aquatic organisms were rapidly freed of most of the accumulated hydrocarbons through the gills again, by the reverse processes of uptake partitioning between water and tissue lipids (Thomas and Rice 1981). However, metabolism of the accumulated hydrocarbons would also take place in the tissues, primarily in the liver (Pederson et al. 1974, Gruger et al. 1977, Gerhart and Carlson 1978, Melancon et al. 1978, Statham et al. 1978, Kennish et al. 1988). This may be important for the depuration of PAHs because of their lower water solubility and thus slower depuration rate via gills. Metabolism would also occur directly in the muscle tissues although this could be rather slow compared to the metabolic activity of liver. White muscle and dark muscle are two functionally different muscle types in fish (Bone 1966, Hudson 1973, Walker and Pull 1973). The functional and metabolic differences in those two muscle types of Atlantic salmon might not be a significant factor affecting the depuration of WSF-HC in this study since similar depuration rates were observed for lower flank muscle and dark muscle (Fig. 4.6) which both contained a similar level of lipid content (Table 4.1).

Our findings are different from those of Miller et al. (1992) who compared the PCB content of relatively lean (5.5-17.1% lipid) lake trout (*Salvelinus namaycush namaycush*) with that of the much fatter (21-27% lipid) siscowet (*S. namaycush siscowet*). In their opinion there were similar rates of PCB bioaccumulation on a basis of mg/kg of PCBs in skin-on edible portion fillets. At this time we simply draw attention

to our results for depuration of different tissues. Although PCBs are commonly held to be readily stored in marine animal body depot fats, this view is not always based on sound data, methods, samples, and understanding of fats (Addison 1989). Intestinal absorption of organochlorines has also been split into two possible routes, lipid co-transport and chemical diffusion (Gobas et al. 1993). Long-term, low-level exposure to PCBs may not be the same as our "pulse" exposure to WSF-HC. Similarly exposure of cod *Gadus morhua* to petroleum oils in production areas on a presumed long term basis showed little evidence of tainting by sensory evaluation but uptake and retention of naphthalene and similar high molecular weight molecules could be detected (Rasmussen et al. 1992).

In previous studies, lipids in white muscle were found to be mainly stored in adipocytes (see Section I). Since the depuration and retention of WSF-HC are highly correlated with the lipid content of different muscle types, it is evident that adipocytes in the muscle tissue of Atlantic salmon might play an important role in the uptake, depuration and retention of WSF-HC.

## 4.4 Role of Adipocytes in the Muscle Tissue of Atlantic Salmon (*Salmo salar*) in the Uptake, Retention and Release of Water-Soluble Fraction of Crude Petroleum Hydrocarbons

### 4.4.1 Introduction

Fish tainted with WSF of petroleum hydrocarbons are unmarketable and must be held in clean water for a period of time until the objectional flavours disappear (depuration). Various investigations have been conducted to study the accumulation and release of hydrocarbons in aquatic organisms, but they have usually been based on the whole tissue as a unit (Stegeman and Teal 1973, Fossato and Canzonier 1976, Neff et al. 1976, Heras et al. 1992). Much of the work emphasized only the fate and metabolism of hydrocarbons, especially aromatic hydrocarbons, in aquatic organisms (Pedersen et al. 1974, Lee et al. 1976, Thomas and Rice 1981, Cravedi and Tulliez 1986). The mechanisms of hydrocarbon uptake and storage behaviour in individual cell compartments have rarely been studied and remain largely unknown.

Any species-specific difference in the accumulation and release of hydrocarbons is reported to be related to tissue lipids (Stegeman and Teal 1973, Neff et al. 1976, Boryslawskyj et al. 1988, Hebert and Keenleyside 1995). Lean fish and shellfish, such as cod and scallops, took up small amounts of WSF-HC in their muscle and were freed of the tainting WSF-HC very rapidly when returned to clean water (Ernst et al. 1987, Ernst et al. 1989). We have recently shown that Atlantic salmon, a fatty fish, accumulated much more WSF-HC at similar exposure conditions than the lean cod, and that the tainting aromatic hydrocarbons of high molecular weights could still be detected in muscle tissue after one month of depuration (See Section 4.3). It has been proposed that the accumulated hydrocarbons are associated with lipids and that this is probably a process of partitioning between the exposure water and the tissue lipids (Stegeman and Teal 1973, Neff et al. 1976, Johnsen and Lloyd 1992). In fact, octanol-water partition

coefficients have been used in defining the probable uptake of organic pollutants from water by aquatic organisms at equilibrium (Chiou et al. 1977, Boese 1984). Normalization to a lipid basis has been used as an approach to compare variations in the concentrations of organic pollutants for differences in tissue lipid content (Hebert and Keenleyside 1995); this was also the basis for modelling bioaccumulation of organic pollutants in foodwebs (Thomann and Connolly 1984, Connolly and Pederson 1988).

The muscle tissue of aquatic organisms is mainly composed of muscle cells and to a much smaller extent of adipocytes as well as other tissue components. It is important to understand the roles of different cell compartments in the uptake and release of hydrocarbons in order to see the actual role of lipid containing compartments and to clarify the species-specific phenomena of hydrocarbon behaviour observed in different aquatic organisms. The main objective of this work is to reveal the role of adipocytes in the muscle tissue of Atlantic salmon in relation to the uptake, release and retention of WSF-HC by the whole of the muscle tissue.

### 4.4.2 Materials and Methods

#### 4.4.2.1 Exposure of Atlantic Salmon to WSF-HC

Preparation of WSF-HC stock solution was conducted mainly by following the same procedures as in Section 4.3.2. with only a few changes. The preparation of WSF-HC was conducted in a cylindrical vessel with a water holding capacity of 800 L. The concentration of total WSF-HC in the tank was controlled at approximately 0.2 ppm for 96 h. Samples from both plastic storage bags (stock solution) and exposure tank water were taken twice daily for the analysis of WSF-HC concentration. The fluctuations of WSF-HC in the exposure tank were kept to a minimum by immediately adjusting the flow rate of WSF-HC stock solution based on the WSF-HC levels in exposure water as analyzed by gas-liquid chromatography.

Eighteen market-sized Atlantic salmon, *Salmo salar*, were used for the exposure experiment (average weight: 2434 ± 520 grams, length: 59 ± 3 cm). Twelve salmon were female and contained mature eggs (4-5 mm in diameter). The fish had been previously held in aerated sand-filtered seawater for three months at the Dalhousie University Aquatron and fed on a diet of Fundy Choice feed (Corey Mills Ltd, Fredericton, New Brunswick). The fish were starved for 24 h before the exposure. The temperature of the exposure water was 4.5-6.5°C. The photoperiod was set for 12 h light and 12 h dark. Meanwhile, six control fish were kept in clean seawater under the same conditions. At the end of the exposure period, three fish were killed for immediate dissection of muscle tissue and for the isolation of adipocytes, and the remaining fish were transferred to a clean seawater tank for depuration. The salmon were depurated for 20 days at seawater temperatures ranging from 6.5 to 8.0°C and the fish (three each time) were taken at day 1, day 4, day 10 and day 20 for analyses of WSF-HC in adipocytes and white muscle. Three of the six control fish were killed at day 0 and day 10 respectively.

#### 4.4.2.2 Isolation of Adipocytes and Sampling of White Muscle

The live Atlantic salmon removed for study were immediately transported in chilled seawater to the Canadian Institute of Fisheries Technology at the Technical University of Nova Scotia. The fish were anaesthetized with 2-phenoxyethanol and blood was taken. The fish were then killed with a blow on the head and the gut cavities were cut open along the middle of abdomen. The viscera were then removed and the fish were thoroughly washed with cold water. The subdermal fat sample was obtained by taking the skin off the fish body and then scraping the top fat tissue off the muscle tissue. A cylinder-shaped portion of dorsal white muscle (about 2 x 2 x 10 cm) was excised adjacent to the vertebrae, 2 cm from the head. The dorsal white muscle portions thus dissected from each of the three fish were cut into several pieces, pooled, and separated

into two portions. One portion was used for WSF-HC analysis and the other portion for the determination of total lipids and lipid storage in the myosepta of the dorsal white muscle tissue. Strips of belly flaps, about 2 cm in width, were cut along one side of the half fillet from the pectoral fin to the pelvic fin. The skins of the cut belly flaps and bundles of muscle fibres were then removed, leaving belly flaps enriched in connective tissue from the three fish which were pooled. All dissection work was performed in a cold room at 5 °C.

About 9 g of the dissected belly flaps were immediately placed in a Petri dish containing Krebs-Ringer phosphate buffer with 1% albumin (bovine, fraction V, Sigma Chemical Co., St. Louis, MO). The tissues were cut into small pieces in the Petri dish and added to a 4 oz Nalgene plastic bottle containing 22 mL of 1% albumin in phosphate buffer plus 180 mg collagenase (type II, Sigma). The digestion processes applied to tissue from the belly flaps and the technique for discrimination between isolated adipocytes and free fat have been described in Section 1.3.3. The resulting dialysis tubing containing sections of the isolated adipocytes, the free fat layer and the buffer solution was immediately frozen at -35 °C. The adipocyte block was then cut off and stored at -35 °C until analysis. Isolation of adipocytes from belly flaps was performed in duplicate.

#### 4.4.2.3 WSF-HC Analysis

The WSF-HC in the dorsal white muscle and the isolated adipocyte block/subdermal fat tissue were recovered by modification of the procedures of Ackman and Noble (1973), which is shown in Section 3.3.3 (experimental conditions III). The steam distillation apparatus consisted of a 250 mL (for dorsal white muscle) or 125 mL (for adipocyte block and subdermal fat tissue) round-bottomed flask with a magnetic stirring bar, a 20 mL Barrett-type distilling receiver (Teflon stopcock) and a water-cooled condenser. The flask was filled with 80 mL (for dorsal white muscle) or 50 mL (for

adipocyte block and subdermal fat tissue) distilled water and heated to boiling using an electric heating mantle. The distillation was terminated when 20 mL of water condensate was collected. The flask was then cooled to room temperature and the water condensate discarded.

The dorsal white muscle was thawed in a domestic refrigerator and immediately upon softening was minced in a Sorvall Omni-Mixer. The minced dorsal white muscle (20 g) was added to the remaining water in the 250 mL flask for distillation. The subdermal fat tissue (~5 g) was directly added to the flask. The frozen adipocyte block was left at room temperature for only a few minutes until the frozen block could be slipped out of the dialysis tubing. The frozen adipocyte block was immediately transferred intact to the 125 mL flask with pre-distilled water. Methylene chloride (1 mL) was added to both flasks. The distillation resumed until 15 mL of condensate had been collected. Dichloromethane (1 mL) was then immediately added through top of the condenser. After distillation the condensate was drained to an ice-cooled 50 mL graduated centrifuge tube. Methylene chloride (200 $\mu$L) containing *n*-heneicosane as internal standard was added to the centrifuge tube. The water and methylene chloride in the centrifuge tube were vortexed for 1 min and centrifuged. The methylene chloride layer was removed by syringe and used for GLC (gas-liquid chromatography) analysis. Distillations of similar control fish dorsal white muscle, subdermal fat tissue and their non-tainted adipocytes were performed by exactly the same procedures as the tainted samples. All samples were distilled in duplicate and each WSF-HC extract was injected twice into the GLC unit.

The recovery efficiency for hydrocarbons by steam distillation was evaluated by spiking 21 hydrocarbon standards ranging from benzene to methylnaphthalenes into both the control adipocyte block and the control dorsal white muscle and by performing the same recovery procedures described previously (Section 3.3.3). The amounts of tainting WSF-HC in the samples were calculated according to the internal standard added, the recovery efficiency and the GLC response factors of individual hydrocarbons (Table A2)

(Section 4.3.2). Table 4.3 shows the recovery efficiency of some of the 21 hydrocarbon standards spiked to the dorsal white muscle and the adipocyte block by steam distillation. All of the percent recoveries of spiked hydrocarbons were higher than 50% of the spiked amounts with the recovery of methylnaphthalenes being the lowest. The recovery efficiency of distilling hydrocarbons from the adipocyte block was used for the calculation of tainting WSF-HC in the subdermal fat tissue.

The procedures for the determination of WSF-HC in seawater and the conditions of hydrocarbon analysis by GLC have been described in Section 3.3.4.

#### 4.4.2.4 Determination of Lipid Content

The lipid content of dorsal white muscle portions and the subdermal fat tissue was determined in duplicate by following the method of Bligh and Dyer (1959). The analyses of lipid classes and of lipid stored in myosepta of dorsal white muscle were carried out as described Section 2.3.

The total lipid in each collected adipocyte block was recovered immediately after the termination of the steam distillation. Upon removal of the distillation receiver, the flask was cooled and rinsed with 5 mL of chloroform. The water and chloroform in the flask were then pipetted into a 50 mL centrifuge tube. The flask was again rinsed three times with 5 mL of chloroform and all portions of chloroform was combined in the centrifuge tube. Methanol (5 mL) and saturated sodium chloride solution (2 mL) were subsequently added to the centrifuge tube. The centrifuge tube was flushed with nitrogen, vortexed and centrifuged. The bottom chloroform layer was quantitatively transferred to a weighed round bottom flask. The lipids in the flask were stripped of solvent on a rotary evaporator and finally on a mechanical vacuum pump. The effect of distillation on the recovery of lipids from the adipocyte block was evaluated by spiking 2 g of belly flap lipids into the flask and by performing the same distillation procedures. The amount of lipids recovered from the adipocyte block was considered as the total

Table 4.3. Recovery efficiency for spiked hydrocarbons from the non-tainted dorsal white muscle and adipocyte block by steam distillation. The concentration of each spiked hydrocarbon is ~0.7 ppm (wet tissue basis). Values are the average of triplicate (white muscle) or duplicate (adipocytes) analyses ± standard deviations.

| | Recovery Efficiency (%) | |
|---|---|---|
| Spiked Hydrocarbons | Dorsal White Muscle | Adipocyte Block |
| benzene | 84.43±4.31 | 90.49±5.26 |
| methylcyclohexane | 56.04±6.66 | 60.28±3.17 |
| toluene | 78.88±2.07 | 85.35±2.95 |
| ethylbenzene | 75.09±4.85 | 77.35±2.60 |
| *m*+*p*-xylenes | 70.93±1.27 | 64.32±5.33 |
| *o*-xylene | 70.32±5.24 | 67.79±2.01 |
| propylbenzene | 74.38±3.44 | 76.07±2.17 |
| 1,3,5-trimethylbenzene | 67.22±2.17 | 62.84±4.36 |
| 1,2,4-trimethylbenzene | 83.76±6.05 | 77.21±3.61 |
| 1,2,4,5-tetramethylbenzene | 63.70±2.54 | 59.87±3.47 |
| naphthalene | 60.19±3.42 | 54.65±1.75 |
| 2-methylnaphthalene | 55.49±2.37 | 51.94±3.46 |
| 1-methylnaphthalene | 57.71±3.75 | 50.77±4.87 |

weight of adipocytes used for distillation since lipids are the predominant component of adipocytes (~90%). The percentages of WSF-HC stored in adipocytes of dorsal white muscle were calculated from two assumptions: (1) Adipocytes in the white muscle and adipocytes in the belly flap regions have the same characteristics with respect to the accumulation and release of WSF-HC; (2) Cell membrane lipids are composed of only phospholipids and sterols and all of the neutral lipid components are associated with adipocytes. Histological studies on the adipocyte and lipid distribution in dorsal white muscle showed that both adipocytes and lipid droplets (or sacs) were present in the intercellular connective tissue (Section 1.4). However, since those lipid droplets were present in smaller amount compared with adipocytes they were classified as part of adipocytes in this study.

### 4.4.3 Results

Adipocytes were successfully isolated from the belly flaps of the muscle tissue through enzymatic digestion and further purified through the flotation procedures. The distribution of adipocytes in the muscle tissue of Atlantic salmon and the characterization of isolated adipocytes have been described in Section 1.4. Steam distillation of the isolated adipocytes had no significant effect on the gravimetric recovery of the amount of isolated adipocytes since 99% of the similar spiked belly flap lipid was recovered after stripping of WSF-HC by steam distillation.

Fig. 4.9 is a typical GLC chromatogram of the stock WSF-HC solution sampled at 50 h of exposure. The WSF-HC profile of the stock solution displays a pattern similar to that as described in Section 4.3.3.2 with respect to the total number of hydrocarbon components and their relative concentrations except for concentrations of naphthalene and methylnaphthalenes which show lower concentrations. Alkylated benzenes, ranging from benzene to $C_4$-benzenes, dominate the WSF-HC in the exposure tank and only traces of n-alkanes are detected. The concentrations of two-ring PAHs in the WSF-HC stock

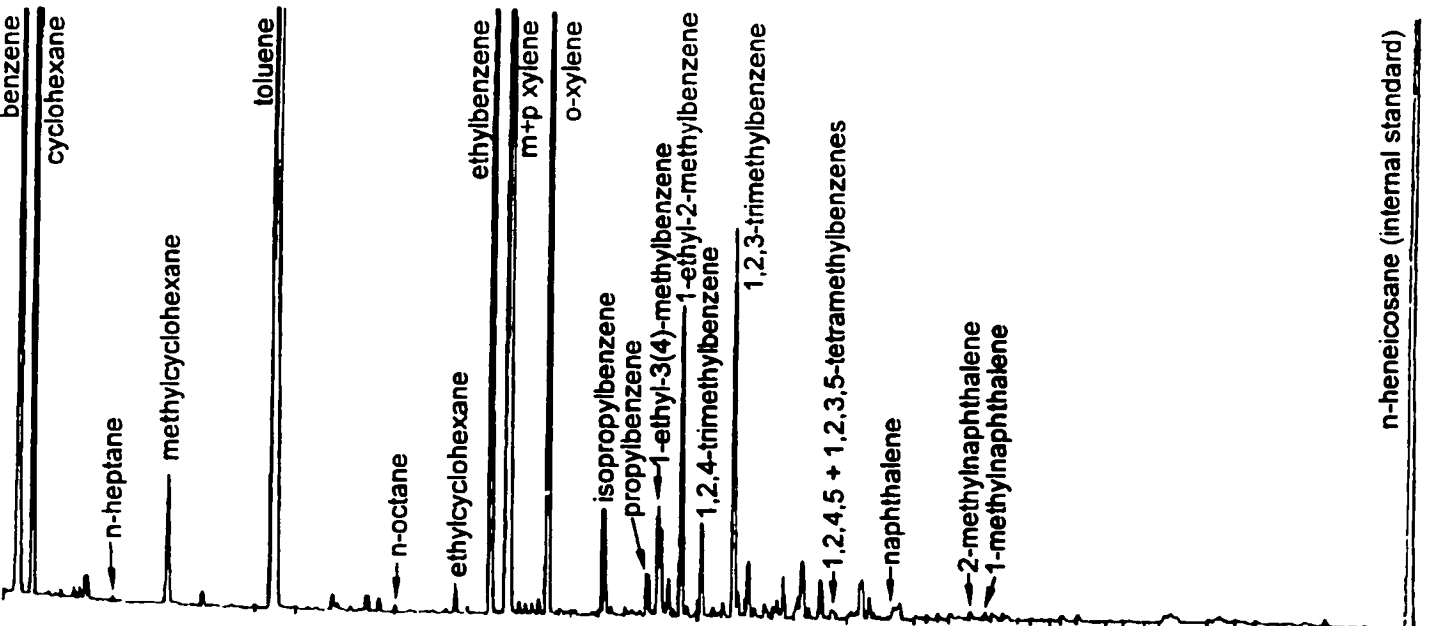


Fig. 4.9. A typical GLC chromatogram of the WSF stock solution of Flotta North Sea crude oil sampled at 50 h of the 96 h exposure period.

solution are relatively low compared with those of alkylated benzenes (Fig. 4.9). The total hydrocarbon concentrations of the stock WSF-HC solution used during the entire 96 h exposure period ranged from 3.87 to 6.06 ppm. However, the effect of this fluctuation on the actual WSF-HC concentrations in the exposure tank was largely minimized by the immediate adjustment of flow rates according to the GLC analysis of WSF-HC concentrations in the stock solution and the exposure water. The average concentration of WSF-HC in the exposure water was 0.2 ppm.

Variations in lipid content of dorsal white muscle ranging from 3.11-4.81% were observed among Atlantic salmon sampled at different depuration stages (Table 4.4). Examination of the lipid distribution revealed that up to ~40% of the dorsal white muscle lipid (35.3-42.0%) was stored in the myosepta of the dorsal white muscle tissue (Table 4.4). The average membrane lipid content in dorsal white muscle was calculated to be approximately 0.6% (wet tissue) (Table 4.4), a figure close to that reported for the very lean muscle of Atlantic cod *Gadus morhua* (Addison et al. 1968, Bligh and Dyer 1959, Jangaard et al. 1967). The lipid content of the pooled subdermal fat tissue was 90.37% (Table 4.4).

Both dorsal white muscle and adipocytes isolated from the belly flap region of the muscle tissue accumulated high levels of WSF-HC after the 96 h long-term exposure, but the difference in the concentrations of tainting WSF-HC between the dorsal muscle tissue and the adipocytes was substantial. The isolated adipocytes bioaccumulated 59.4 ppm of WSF-HC which is 300 times higher than that in the exposure water, while the dorsal white muscle took up only 4.2 ppm of WSF-HC (Fig. 4.10). Moreover, the dorsal white muscle *per se* contained 4.48% (w/w) lipid, of which 86% was in the form of adipocytes or lipid sacs (considered as adipocytes thereafter) in the muscle connective tissue and 42% (of the 4.48%) was specifically in the adipocytes associated with myosepta (Table 4.4). At the end of 96 h exposure, the total adipocyte lipids in dorsal white muscle accounted for the storage of 54% of the total tainting WSF-HC (Fig. 4.11). After subtracting the WSF-HC stored in adipocytes from the total tainting WSF-HC

Table 4.4. Total lipid content of pooled subdermal fat tissue, and total lipid and membrane lipid contents of pooled dorsal white muscle (wet tissue basis) from three fish, and percentage of lipid stored in myosepta of dorsal white muscle of Atlantic salmon sampled during a 20 day period of depuration after 96 h exposure to WSF-HC. The lipid contents of the subdermal fat tissues were obtained by pooling all samples from the tainted and the control Atlantic salmon. All values are the average of triplicate analyses with standard deviations except for the total lipid contents of dorsal white muscle and subdermal fat tissue which were determined in duplicate.

| Depuration Times (days) | 0 | 1 | 4 | 10 | 20 | Average |
|---|---|---|---|---|---|---|
| Dorsal White Muscle | | | | | | |
| total lipid content (%) | 4.48±0.11 | 3.11±0.20 | 4.81±0.08 | 4.55±0.06 | 3.25±0.14 | 4.04±0.71 |
| membrane lipid content (%)[a] | 0.62±0.04 | 0.57±0.03 | 0.62±0.05 | 0.58±0.03 | 0.58±0.02 | 0.60±0.02 |
| lipids in myosepta (%)[b] | 42.0±5.1 | 35.3±3.5 | 41.8±5.0 | 38.1±4.8 | 37.1±6.4 | 38.9±2.64 |
| Subdermal Fat Tissue | - | - | - | - | - | 90.37±0.62 |

[a] Calculated values from lipid class analysis, assuming that lipids in the cell membranes are primarily composed of only phospholipids and sterols.

[b] Percentage of the amount of lipids stored in the myosepta of dorsal white muscle compared to the total lipid of the same muscle tissue.

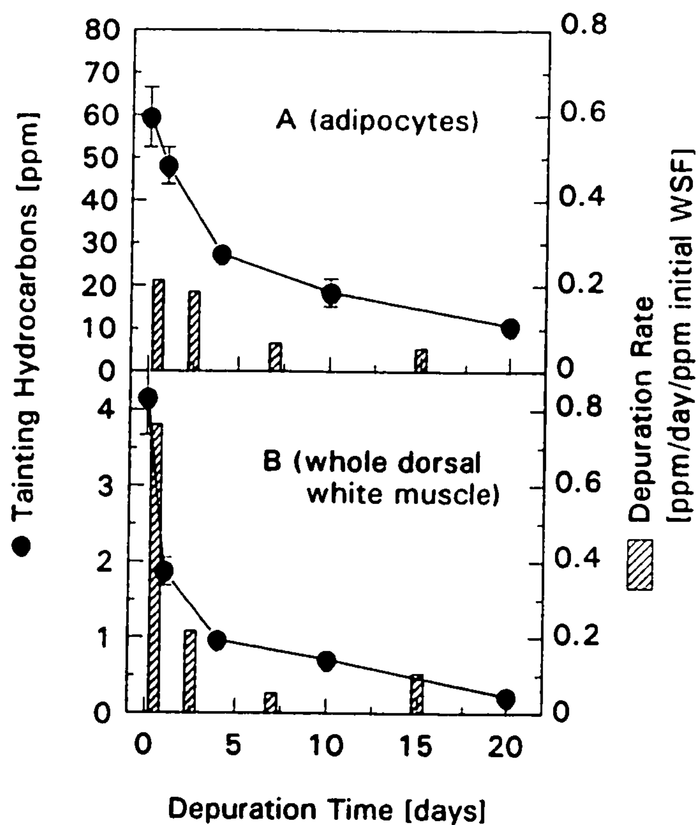


Fig. 4.10. Release of tainting WSF from adipocytes and dorsal white muscle during 20 day period of depuration. Depuration rate: average concentration of WSF released per day and per unit of initial WSF retained in the dorsal white muscle and the adipocytes during the time intervals of day 0 to day 1, day 1 to day 4, day 4 to day 10, and day 10 to day 20.

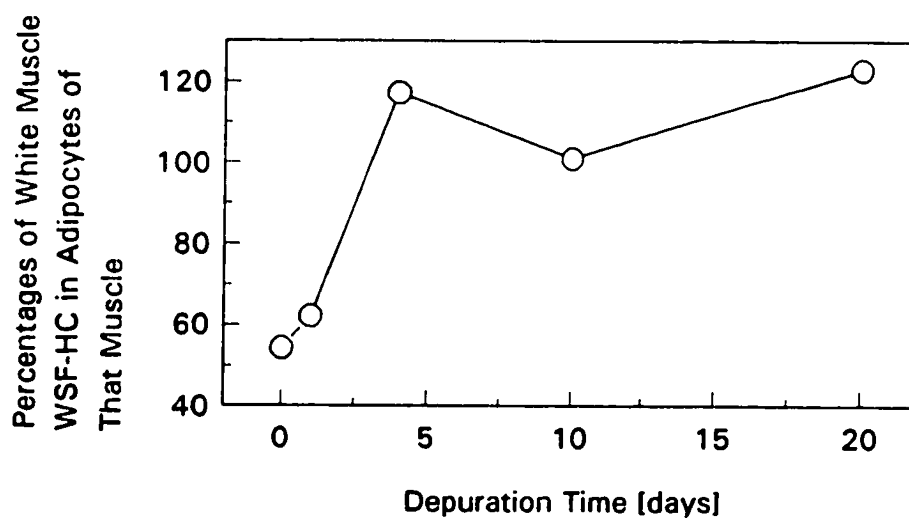


Fig. 4.11. Changes in calculated percentages of tainting WSF-HC in adipocytes of the dorsal white muscle relative to the total tainting WSF-HC in the same muscle tissue.

accumulated in the dorsal white muscle, the non-adipocyte portion of the dorsal white muscle, predominantly composed of white muscle cells, took up only 2.0 ppm of WSF-HC and this is only 10 times higher than the WSF-HC concentration in the exposure water.

The depuration rates of WSF-HC from adipocytes and dorsal white muscle also showed large differences (Fig. 4.10). The characteristic depuration of WSF-HC from dorsal white muscle was distinguished from that of adipocytes by showing a sharp release of WSF-HC on the first day of depuration. The WSF-HC in the dorsal white muscle dropped from 4.2 ppm to 1.9 ppm, accounting for 55% of the total accumulated WSF-HC, while only 19% of the accumulated WSF-HC in adipocytes was released during the same first day of depuration. The release of WSF-HC from dorsal white muscle then slowed down and showed a pattern similar to that from the isolated adipocytes. This characteristic is clearly demonstrated when the depuration rates are expressed as the amount of WSF-HC released per day and per unit of initial WSF-HC in the samples at different time intervals of depuration (Fig. 4.10). From day 0 to day 1, the adipocytes showed a depuration rate (Fig. 4.10) of only 0.21 (ppm/day/ppm initial WSF-HC), while a depuration rate of 0.76 was observed for the dorsal white muscle. Afterwards, the depuration rate of WSF-HC in dorsal white muscle from day 1 to day 4 decreased to 0.21, slightly higher than the 0.18 rate of the isolated adipocytes. A similar depuration rate of about 0.05 was then maintained to the end of the depuration study for both dorsal white muscle and adipocytes. After 20 days of depuration, the dorsal white muscle was almost free of the accumulated WSF-HC and only 0.2 ppm of WSF-HC was detected; however 10.7 ppm of WSF-HC were still stored in the adipocytes.

The uptake and depuration of tainting WSF-HC in the subdermal fat tissue are shown in Fig. 4.12. It is clear that the isolated adipocytes and the subdermal fat tissue rich in these cells displayed the same pattern with respect to the uptake and depuration of WSF-HC.

Fig. 4.11 further illustrates the role of adipocytes in the retention of tainting WSF-

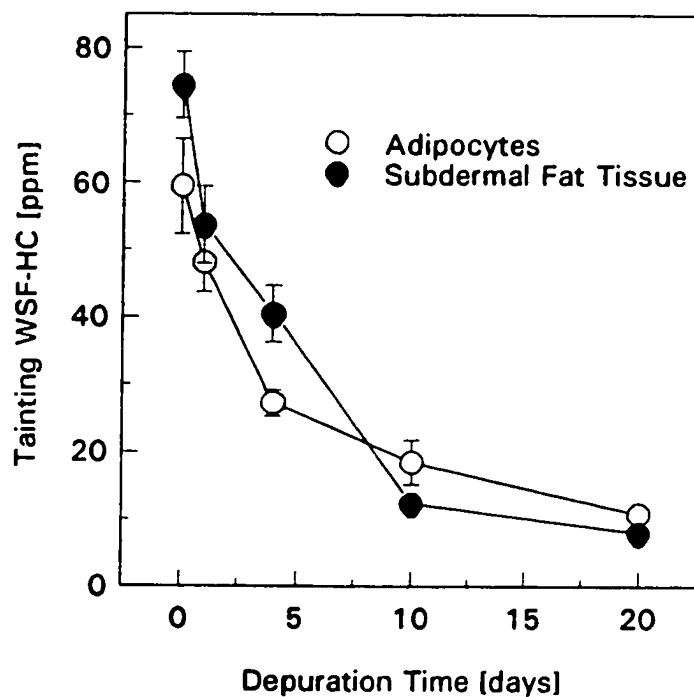


Fig. 4.12. Depuration of tainting WSF-HC of Flotta North Sea crude oil from the isolated adipocytes and the subdermal fat tissue of market size Atlantic salmon. The fish were exposed to 0.2 ppm WSF-HC for 96 h.

HC in dorsal white muscle at different stages of depuration. The percentages are expressed as the amount of WSF-HC stored in the adipocytes of the dorsal white muscle as a percentage of the total amount of WSF-HC in the same white muscle. At the end of the exposure period, 54% of the tainting WSF-HC in the dorsal white muscle was found to be in the adipocytes. Tainting WSF-HC in the dorsal white muscle cells were discharged almost completely and the adipocytes became the principal storage site of WSF-HC in dorsal white muscle after four days of depuration. The release of tainting WSF-HC from dorsal white muscle thereafter was exclusively the depuration of WSF-HC from the adipocytes in the muscle tissue.

Bioaccumulation factors of individual WSF-HC in the adipocytes were much higher than those in the dorsal white muscle (Table 4.5). It was found that the bioaccumulation of individual WSF-HC increased along with the number of aromatic rings and the extent of alkylation on the aromatic ring. The bioaccumulation factor of methylnaphthalenes is almost 23 times higher than that of a single ring counterpart (toluene) in the dorsal white muscle and about 13 times higher in adipocytes. Methylnaphthalenes in the exposure water were concentrated by a factor of 2970 by adipocytes and were the most concentrated components of the tainting WSF-HC in specific cell compartments of the white muscle. That the bioaccumulation factors were also more or less related to the number of substituent alkyl-groups on the ring is noteworthy. Generally, the greater the number of alkyl substitutions, the higher the bioaccumulation factor. This is clearly illustrated for the alkylated benzenes (Table 4.5). An exception in both dorsal white muscle and adipocytes was that ethylbenzene and xylenes were slightly more concentrated than isopropylbenzene and propylbenzene. The bioaccumulation factor of methylcyclohexane was observed to be unusually high in both dorsal white muscle and adipocytes (31 and 480 respectively) relative to that for aromatic hydrocarbons having similar molecular weights, e.g., the bioaccumulation factors of toluene were only 11 and 230 for dorsal white muscle and adipocytes respectively.

The discrimination factors in Table 4.5 reflect the ratios of relative abundance of

Table 4.5. Bioaccumulation factor[a] and discrimination factor[b] of hydrocarbons in dorsal white muscle and adipocytes isolated from the belly flap region of muscle tissue of Atlantic salmon after 96-h exposure to 0.2 ppm WSF-HC.

| | Bioaccumulation Factor | | Discrimination Factor | |
|---|---|---|---|---|
| Hydrocarbons | White Muscle | Adipocytes | White Muscle | Adipocytes |
| Total WSF-HC | 21 | 300 | - | - |
| Benzene | 4 | 100 | 0.2 | 0.3 |
| Methylcyclohexane | 31 | 480 | 1.4 | 1.6 |
| Toluene | 11 | 230 | 0.5 | 0.8 |
| Ethylbenzene | 26 | 430 | 1.2 | 1.4 |
| Xylenes | 47 | 570 | 2.2 | 1.9 |
| Isopropylbenzene | 20 | 270 | 0.9 | 0.9 |
| Propylbenzene | 36 | 430 | 1.7 | 1.4 |
| Ethyl-methylbenzenes[c] | 51 | 520 | 2.4 | 1.7 |
| Trimethylbenzenes[c] | 74 | 740 | 3.4 | 2.4 |
| Methylnaphthalenes[c] | 230 | 2970 | 10.7 | 9.7 |

[a] Calculated as the ratio of hydrocarbon concentration in dorsal white muscle or adipocytes divided by hydrocarbon concentration in exposure water.

[b] Calculated as the ratio of individual hydrocarbon concentration to the total hydrocarbon concentration in the dorsal white muscle or adipocytes divided by the equivalent ratio in the exposure water.

[c] Ethyl-methylbenzenes include 1-ethyl-2(3,4)-methylbenzenes; Trimethylbenzenes include 1,2,3-, 1,2,4-, and 1,3,5-trimethylbenzenes; Methylnaphthalenes include 1-, and 2-methylnaphthalenes.

individual WSF-HC between the dorsal white muscle or the adipocytes and the exposure water. The ratios of relative abundance of benzene were the lowest in both the dorsal white muscle and the adipocytes (0.2 and 0.3 respectively). The percentage of toluene in the accumulated WSF-HC of adipocytes was 80% of that in the exposure water, while it was only 50% in the dorsal white muscle tissue. The discrimination factors of tainting aromatics in dorsal white muscle and adipocytes exceeded those in the exposure water when two carbons or more alkylations were present on the ring, except for isopropylbenzene which had a discrimination factor of 0.9 for both the dorsal white muscle and the adipocytes. The relative abundance of methylnaphthalenes in the dorsal white muscle and the adipocytes was ten times higher than that present in the exposure water.

The depuration of individual WSF-HC was dependent on their alkyl substitutions, number of aromatic rings, and to a large extent their storage sites in the muscle tissue (Fig. 4.13). All the WSF-HC in white muscle were released faster than these in the adipocytes. Benzene in dorsal white muscle was completely depurated within only one day (Fig. 4.13a'), while it took 4 days to be depurated from the adipocytes (Fig. 4.13a). One methyl alkylation on the benzene ring (toluene) required 4 days and 10 days respectively for the complete depuration from the dorsal white muscle (Fig. 4.13b') and the adipocytes (Fig. 4.13b). The rate of release of WSF-HC slowed down with increasing alkyl substitutions (Figs. 4.13c, c', d, d', e, e'). The depuration of methylnaphthalenes was so slow that they would take months to be completely released from the adipocytes (Fig. 4.13e). These characteristics are reflected through the expression of biological half lives (Table 4.6).

An important observation was that all the individual WSF-HC, including those highly alkyl substituted, were rapidly discharged from the dorsal white muscle at the beginning of depuration with a biological half life of less than 4 days, while in adipocytes only benzene and toluene showed this characteristic. The benzenes alkylated with three or more carbons were released so slowly that it took at least 15 days to depurate these

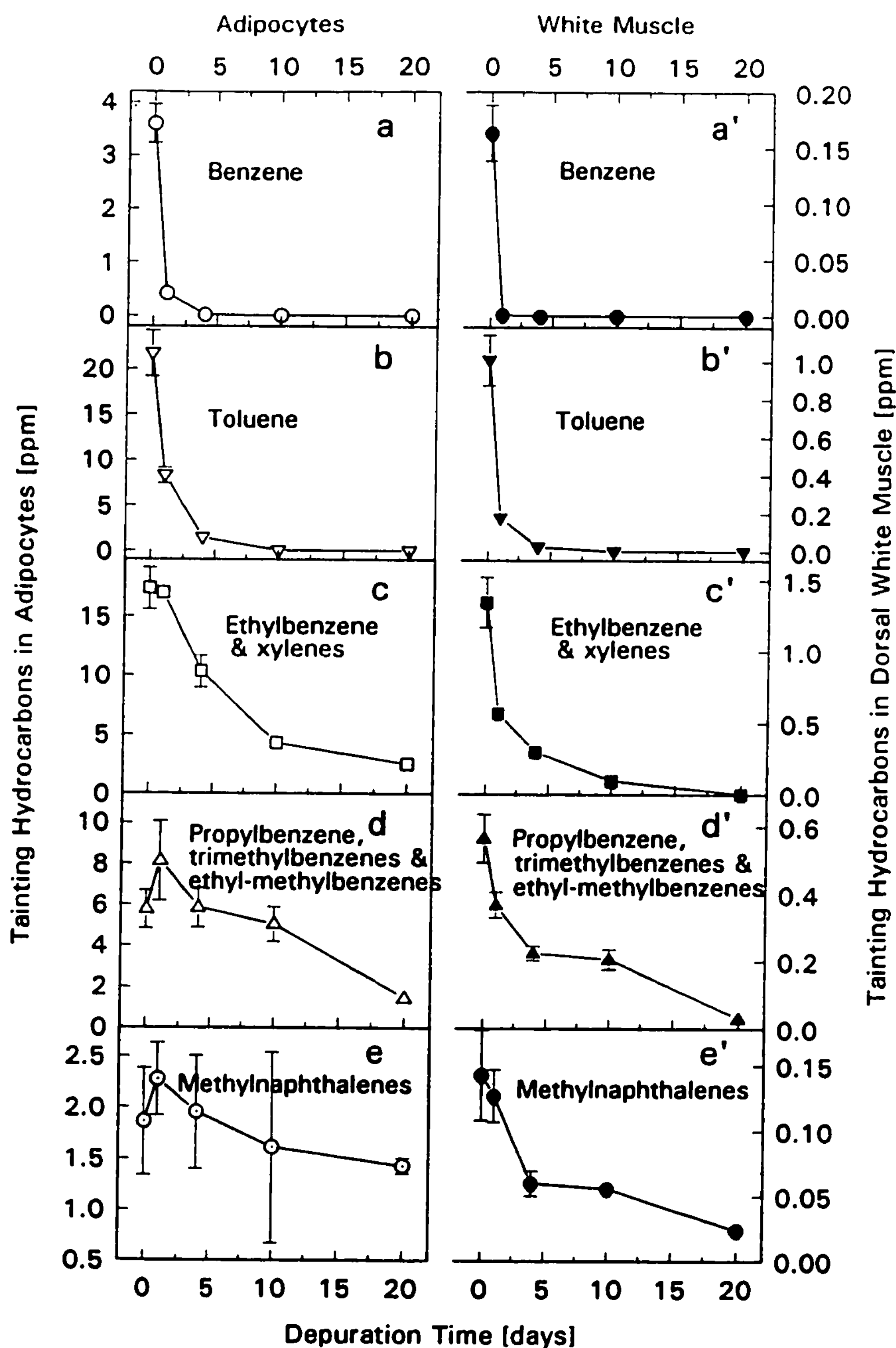


Fig. 4.13. Release of individual or groups of WSF-HC from adipocytes and dorsal white muscle during 20 days of depuration.

Table 4.6. Biological half lives[a] (days) of individual WSF-HC accumulated in adipocytes and dorsal white muscle when depurated in clean seawater.

| Hydrocarbons | White muscle | Adipocytes |
|---|---|---|
| Benzene | 0.5 | 0.6 |
| Methylcyclohexane | 2.5 | 12.0 |
| Toluene | 0.6 | 0.8 |
| Ethylbenzene | 0.8 | 3.9 |
| Xylenes | 0.9 | 6.8 |
| Isopropylbenzene | 3.3 | 17.0 |
| Propylbenzene | 3.8 | 18.0 |
| Ethyl-methylbenzenes | 2.5 | 15.0 |
| Trimethylbenzenes | 2.9 | 16.0 |
| Methylnaphthalenes | 3.5 | >20.0 |

[a] Biological half life: time required for the depuration of half of the accumulated individual hydrocarbon in dorsal white muscle and adipocytes.

to the 50% level in adipocytes. The depuration of polycyclic aromatic hydrocarbons from adipocytes, e.g., methylnaphthalenes, was the slowest among all of the tainting aromatics and the biological half life exceeded the experimental 20 days of depuration in clean water. It is interesting to note that the biological half life of methylcyclohexane was unexpectedly much longer than that of toluene, and even of xylenes and ethylbenzene in both dorsal white muscle and adipocytes.

### 4.4.4 Discussion

The muscle tissue of Atlantic salmon is mainly composed of muscle cells which are held together through connective tissue. Adipocytes in the white muscle tissue of Atlantic salmon are concentrated in part of this connective tissue, specifically in the myosepta, and are specialized for the storage of triacylglycerols. It is the myosepta, the principal connective tissue of white muscle, that stores about 40% of the total white muscle lipids although the volumetric proportion of myosepta in dorsal white muscle is negligible. In farmed Atlantic salmon the muscle fat content ranges from 10-12% (Ackman 1989), or even higher depending on the size and nutritional status of the fish. We have observed, through both lipid class analysis (Table 4.4) and histological examination (Section 1.4), that most of the lipid in white muscle of our relatively lean fish is found in adipocytes or lipid droplets trapped in connective tissue, except for the membrane lipid. On histology no microscopically visible lipid droplets were observed within the section of each individual white muscle cell, whereas finely dispersed lipid droplets were a characteristic of dark muscle cells. Therefore, the accumulation and release of WSF-HC in dark muscle is expected to be more complicated than in white muscle. The belly flap is well-known for a very high fat content which we have found to be due to adipocytes (Section 1.4).

The exposed salmon exhibited signs of physiological stress, e.g., reduction in daily diet intake, due to the long-term WSF-HC exposure and to their transfer from tank to

tank at the beginning and end of exposure. However, examination of the lipid content of dorsal white muscle at different depuration stages (Table 4.4) did not show an obvious trend of lipid depletion caused by the less food intake. The variations in the lipid content of dorsal white muscle among Atlantic salmon sampled at different depuration stages can mainly be attributed to the physical and nutritional differences among individual fish.

The large difference found in the accumulation and release of WSF-HC between adipocytes and white muscle cells confirms that the species-dependent characteristics with respect to the accumulation and retention of hydrocarbons are actually dependent on both the tissue lipid content and the lipid storage format. Our present study on the separation of adipocytes from Atlantic salmon muscle tissue and the subsequent analysis of the tainting WSF-HC in this specific compartment of the tissue have revealed that adipocytes in salmon muscle tissue do play an important role in controlling the uptake and release of hydrocarbons. The WSF-HC accumulated in the tissue were primarily stored in the adipocytes during long-term exposure and were also retained by the adipocytes during the depuration in clean seawater.

Our previous histological studies have shown that the subdermal fat tissue of Atlantic salmon is predominantly composed of adipocytes. This is further supported (Table 4.4) by its very high lipid content (90.37%). Therefore, it is expected that both the isolated adipocytes and the subdermal fat tissue would present the same characteristics in the uptake of WSF-HC from seawater and their subsequent depuration from those two tainted samples. There is no reason to believe that WSF-HC cross the skin barrier, but myosepta passing into this layer are well vascularized.

Neely et al. (1974) suggested that the accumulation and release of hydrocarbons in the tissue is most probably a passive process of partitioning of the hydrocarbons between the exposure water and the tissue lipids. The actual accumulation and release processes of WSF-HC into and from the muscle tissue are probably composed of several partitioning and diffusion steps before the WSF-HC finally reaches the lipid droplets in the adipocytes. Studies have shown that the primary route for the uptake of

hydrocarbons from water and the excretion of the accumulated hydrocarbons was via the gills (Lee et al. 1972, Thomas and Rice 1981). WSF-HC in the exposure water are first partitioned through gills and are subsequently associated with the lipoproteins and chylomicra in the blood fluid, which carry the WSF-HC through the circulatory system to the whole fish body including muscle tissue. The lipoproteins and chylomicra then become adherent to the luminal surface of the capillaries (Porter and Bonneville 1973). The WSF-HC are released from the lipoproteins or the chylomicra and diffuse to the endothelial cells of the capillaries. Due to the concentration gradient of WSF-HC between the bulk lipid droplets of adipocytes and the endothelial cells, the WSF-HC continuously diffuse and pass through the capillary endothelium into the connective tissue ground substance (Wheater et al. 1979). Tissue fluid is loosely bound to the ground substance, thereby forming the medium for passage of WSF-HC throughout connective tissue. The WSF-HC travel through the ground substance and reach the membrane surrounding the individual adipocyte. The WSF-HC then dissolve in and easily pass through the bi-phospholipid layer membrane, diffuse through the thin cytoplasm layer and finally enter the single large lipid droplet in the adipocyte. The accumulation of WSF-HC continues until an equilibrium is reached. Our unpublished results show that high levels of tainting WSF-HC are detected in the muscle tissue of Atlantic salmon after only one hour of exposure. Rapid accumulations of hydrocarbons in the tissues of other fish or shellfish are also reported within hours or even minutes (Lee et al. 1972, Stegeman and Teal 1973, Johnsen and Lloyd 1992). Unfortunately these examinations were only for the whole tissue and there is no report on the accumulation rates of hydrocarbons in specific tissue compartments. It is believed that most of the hydrocarbons detected initially are probably associated only with tissue fluids and it would take a longer time to show their predominant accumulation in the adipocytes.

The non-adipocyte portion of white muscle is mainly composed of water, but the accumulated WSF-HC are most probably associated with the cell membrane polar lipids or the hydrophobic groups of proteins and glycolipids etc. Boryslawskyj et al. (1988)

indicated that lipids in membranes are a key factor in the partitioning of hydrophobic xenobiotics in the freshwater mussel, but changes in the membrane lipid composition did not show a significant effect on the uptake of hydrocarbons. The association of WSF-HC with these polar components is expected to be looser than their association with triacylglycerol lipid in the adipocytes where they are in true solution. Some of the WSF-HC in the non-adipocyte portions are presumably free to move around if they are merely associated with tissue fluids such as plasma. This compartment of WSF-HC can be quickly transported to the gills or to the liver. Thus they will be more readily released into the clean water column or metabolized by the liver (Thomas and Rice 1981). The loose association of WSF-HC with the components in the muscle cells and its presence in tissue fluids are probably the causes for the sharp release of tainting WSF-HC, mostly lower molecular weight aromatics, from white muscle at the early stage of depuration (day 0 to day 4).

Most of the lipids in the muscles of cod and scallop are membrane lipids (<1% of tissue) (Jangaard et al. 1967, Ackman and McLeod 1988) and the presence of adipocytes will obviously be negligible. Scallop adductor muscle exposed to 2.66 ppm WSF-HC for 24 h took up only 15.59 ppm WSF-HC (bioaccumulation factor of 5.9), of which 14.34 ppm was released during the first 24 h of depuration in clean seawater (Ernst et al. 1989). These WSF-HC uptake and depuration behaviours were also observed for muscle of Atlantic cod (Ernst et al. 1987). Thus the low bioaccumulation characteristics of WSF-HC and their subsequently rapid depuration from the lean cod and scallop muscles is other evidence of the fast release of WSF-HC from the non-adipocyte portion of aquatic organisms. After the rapid discharge of WSF-HC from the non-adipocyte portion of the white muscle, i.e. 4 days of depuration in clean seawater, the adipocytes in the salmon muscle tissue became the principal site for WSF-HC storage. This view is supported by the similarity in depuration rates of the tainting WSF-HC from the dorsal white muscle and from the isolated adipocytes at day 4 and thereafter (Fig. 4.10), and also by the changes in the calculated WSF-HC stored in the adipocytes

of the dorsal white muscle as percentages of the total during the 20 day period of depuration (Fig. 4.11).

When the tainted fish return to hydrocarbon-free seawater, the lipid/water partition coefficients for hydrocarbons favour the release of WSF-HC from tissue to water. Rapid depuration rates of WSF-HC from adipocytes were also observed at the early depuration periods (Fig. 4.10), but these were not as fast as the depuration rates from dorsal white muscle and apparently can be attributed to the release from the adipocytes of the more water-soluble hydrocarbons such as benzene and toluene (Table 4.6). The highly alkylated benzenes and polycyclic aromatics such as methylnaphthalenes did not show any sign of rapid release at the early stage of depuration. Generally, depuration is the reverse of the accumulation process since the metabolic rate of hydrocarbons in the fish was negligible during the exposure and the initial depuration period, but this does not necessarily mean an identical rate for the accumulation and the depuration of WSF-HC from adipocytes. Both the accumulation and the release of WSF-HC are controlled by the hydrophobicity of components in the cell compartments and the degree of vascularization surrounding the compartments. It is the affinity of the triacylglycerols in adipocytes for the WSF-HC which leads to the slow release of the accumulated WSF-HC from adipocytes. The persistent retention of polycyclic aromatic hydrocarbons in adipocytes of the exposed fish must be emphasized because of their carcinogenic properties and the potential health hazard they may pose to consumers of marine foods (Heidelberger 1964).

Individual hydrocarbons of WSF accumulated in the dorsal white muscle and adipocytes displayed differences in their abundances in the total tainting WSF-HC when compared with those in the WSF-HC of the exposure water. The selective accumulation of WSF-HC is probably due to the combined effects of differences in their water solubility and in the metabolic processes of Atlantic salmon (Gerhart and Carlson 1978, Melancon et al. 1978, Cravedi and Tulliez 1981). The livers of fish, including salmonids, contain aryl hydrocarbon hydroxylase (AHH), an enzyme system capable of

metabolizing petroleum hydrocarbons (Pederson et al. 1974, Gruger et al. 1977, Statham et al. 1978). Kennish et al. (1988) investigated the metabolic conversion of toluene and ethylbenzene and found that both can be metabolized by Pacific salmon microsomal preparations. Coho salmon were also capable of biotransforming hydrocarbons and the retention and accumulation of parent compounds and metabolites were found to increase along with the increases in aromatic rings (Roubal et al. 1977). Thomas and Rice (1981) compared the metabolism of C-14 labelled toluene and naphthalene by Dolly Varden char (*Salvelinus malma*) and reported that toluene was more easily metabolized than naphthalene. The metabolic conversion of circulating WSF-HC in Atlantic salmon must have begun shortly after they were exposed to the WSF-HC and the process would continue for the 96 h of exposure. Concurrently, the reverse diffusion of WSF-HC from tissue compartments to tissue fluids would have continuously released relatively more of the hydrocarbons with higher water solubility.

The relatively slower depuration of the non-aromatic cyclic methylcyclohexane from adipocytes and dorsal white muscle is probably caused by its lower water solubility and a greater difficulty in its being metabolized by the livers of fish than would be the case for the aromatic counterparts (Cravedi and Tulliez 1986).

In Fig. 4.11 the calculated percentage of tainting WSF-HC stored in adipocytes were higher than 100% of the total tainting WSF-HC in the dorsal white muscle (120%). This could probably be attributed to a number of error sources such as in the hydrocarbon recoveries by steam distillation, in the lipid content determination by $CHCl_3$/MeOH and in the membrane lipid analysis by TLC-FID. Moreover, the assumption that all lipids were stored in adipocytes except for membrane lipids would probably also have contributed to the excess of 100% WSF-HC from calculation since a small fraction of non-membrane lipids would be expected to be present as finely dispersed lipid droplets in the non-adipocyte cells as an energy reserve. However, in spite of the additive errors in the calculated values of percent WSF-HC storage in adipocyte of the dorsal white muscle, there is an obvious trend that adipocytes became

the main storage site for the accumulated WSF-HC after the first 4 days of depuration in clean seawater. Variations in the biological, physiological and nutritional status of individual fish may led to differences in the uptake and depuration of WSF-HC between individual fish. This is probably the reason for the occasional observation of higher concentrations of tainting WSF-HC at depuration day 1 than those before depuration (Figs. 4.13 d,e) although this type of variation was minimized by the random selection of three tainting fish as a group for WSF-HC analysis.

In conclusion, adipocytes in the white muscle tissue of Atlantic salmon were found to be the principal cell compartments for the storage and retention of WSF-HC, particularly after the initial rapid discharge of the accumulated WSF-HC from the muscle cells during the depuration periods. The role of adipocytes in the storage and retention of other xenobiotics may also be applicable to various other organic pollutants due to their similarity in hydrophobicity. This key role of adipocytes would also be expected to occur in various other aquatic organisms, particularly those with high lipid content. The presence of a high lipid reserve in the tissues implies that adipocytes would be the major form for lipid storage and would be abundant in the tissues, while in lean tissues most of the lipids are present in the membranes.

## 4.5 Accumulation of WSF Hydrocarbons in the Muscle Tissues, Subdermal Fat Tissue and Mesenteric Adipose Tissue of Atlantic Salmon during 24 h Exposure to the WSF-HC of Flotta North Sea Crude Oil

### 4.5.1 Introduction

Organic pollutants are concentrated in the tissues of aquatic organisms when their concentrations in the water column exceed critical levels. As the exposure to the pollutants proceeds, their accumulation rates in aquatic organisms will decrease continuously until an equilibrium is finally reached on the partitioning of organic pollutants between the exposure water and the tissue compartments after a certain time. The concentrations of organic pollutants in aquatic organisms will become constant thereafter as long as a constant level of organic pollutants is maintained in the water column. In our previous studies on the 8 h exposure of Atlantic salmon to WSF-HC, the concentrations of accumulated WSF-HC at the end of exposure were not as highly correlated with the tissue lipid contents as those during the depuration stages (Section 4.3). The belly flap, which contains twice as much lipid content as the dark muscle, took up a level of WSF-HC similar to the dark muscle at the end of 8 h exposure. This could probably be attributed to the following two factors: (1) the short term (8 h) exposure is far from reaching an equilibrium of WSF-HC partitioning between the exposure water and the tissue compartments; (2) the belly flap area has a poorer blood vessel supply compared to that of the metabolically active dark muscle.

A lot of studies have been conducted on the accumulation of organic pollutants from exposure to the pollutants or their subsequent depuration in clean water (e.g., Roubal et al. 1978, Niimi 1983, Ernst et al. 1987, Ernst et al. 1989, Johnsen and Lloyd 1992). However, there are only a few investigations exploring the actual uptake course of organic pollutants into aquatic organisms during the exposure period (Stegeman and Teal 1973, Neff et al. 1976, Riley et al. 1981, Laurén and Rice 1985). There has been

no report or correlation between the uptake pattern of hydrocarbons into the tissues of aquatic organisms and their lipid contents.

In this study, Atlantic salmon were exposed to the WSF-HC of Flotta North Sea crude oil for different time periods (maximum 24 h) and the WSF-HC accumulated in different muscle tissues, subdermal fat tissue and mesenteric adipose tissue were subsequently analyzed for the tainting WSF-HC by GLC-FID. The objectives were to understand the actual course of WSF-HC uptake in different muscle types and in subdermal fat tissue, and to explain the fact that lipid appeared to play a less important role in the uptake of WSF-HC during a short term exposure than that of the long term exposure.

### 4.5.2 Materials and Methods

#### Atlantic Salmon

Atlantic salmon, *Salmo salar*, were held in aerated seawater at the Dalhousie University Aquatron Laboratory and fed on a diet of Fundy Choice feed (Corey Mills Ltd, Fredericton, New Brunswick). Twenty one fish were exposed to the WSF-HC and four fish were used as the control sample. These fish had a mean weight of 1852 $\pm$ 420 g, length 53.1 $\pm$ 2.9 cm. The fish were starved for 24 h before exposure.

#### Exposure of Atlantic Salmon to WSF-HC

Preparation of WSF-HC stock solution was carried out by following the same procedures as in Section 4.4.2.1. The procedures for the exposure of Atlantic salmon has been described in Section 4.3.2. Constant concentration of WSF-HC and oxygen level in the exposure water were achieved by continuously and proportionally pumping oxygenated seawater (3000 mL/min) from a header tank and WSF-HC stock solution

(280 mL/min) from the sealed plastic bags to the exposure tanks (80-110% saturation). The concentration of total WSF-HC in the exposure tank was controlled at ~0.27 ppm for 24 h. The temperature of the exposure water was 6.0-7.0°C during the exposure period. Three Atlantic salmon were sampled from the exposure tank after 1 h, 3 h, 6 h, 12 h and 24 h exposure to the WSF-HC. Dorsal white muscle, dark muscle, belly flap, subdermal fat tissue and mesenteric adipose tissue were immediately dissected from the three exposed fish. The sampling procedures for dorsal white muscle, dark muscle and belly flap have been described in Section 4.3.2. The subdermal fat tissue was obtained by taking the skin off the fish body and by subsequently scraping the top layer of fat tissue off the muscle tissue. The dissected samples from each of the three fish were pooled and immediately frozen at -35°C until analysis.

**Analysis and Calculation of WSF-HC in Seawater and Tissue Samples**

Procedures described in Section 4.3.2 were followed for the recovery of WSF-HC from seawater and tissue samples, for the GLC-FID analysis of the recovered hydrocarbons, and for the calculation of WSF-HC concentrations in seawater and tissue samples.

**Determination of Lipid Content**

The lipid contents of different muscle tissues, subdermal fat tissue and mesenteric adipose tissue were determined in duplicate according the procedures of Bligh and Dyer (1959).

### 4.5.3 Results and Discussion

The average concentration of WSF-HC in the exposure tank was 0.27 ppm. The

WSF-HC profile prepared in the cylindrical tank was similar to that described in Section 4.4.3 (Fig. 4.9).

Fig. 4.14 shows changes in the concentrations of accumulated total WSF-HC in the dorsal white muscle, the dark muscle, the belly flap, the subdermal fat tissue and the mesenteric adipose tissue during the 24 h exposure period. The detailed WSF-HC profiles in these tissues at different sampling periods are presented in Table A10-1 to A 14-2. WSF-HC were rapidly taken up by all tissues upon exposure to the WSF-HC solution, but the amounts accumulated in the tissues and the rates at which they approached the plateau values were dependent on both the tissue types and their lipid contents (Table 4.7, Fig. 4.14). However, during the initial exposure period, the WSF-HC uptake in the five tissues of Atlantic salmon was not dependent on their lipid contents. After 1 h exposure, the concentrations of tainting WSF-HC in the five tissues were subdermal fat tissue (17.5 ppm) > dark muscle (7.5 ppm) > mesenteric adipose tissue (5.6 ppm) > belly flap (3.9 ppm) > dorsal white muscle (1.9 ppm), while their lipid contents were ranked in a different order: Subdermal fat tissue (88.42%) > mesenteric adipose tissue (85.09%) > belly flap (34.75%) > dark muscle (16.27%) > dorsal white muscle (3.43%). As the exposure proceeded, the WSF-HC were continuously taken up by the tissues and the role of lipid became more and more evident in the uptake of WSF-HC. After 12 h exposure, the amounts of tainting WSF-HC in the five tissues switched to the same ranking order as their lipid contents.

The uptake rates approaching an equilibrium in WSF-HC partitioning between the tissue and the exposure water were different among the five tissues and were dependent on the lipid contents of the tissues. Dorsal white muscle and dark muscle have the lowest lipid percentages among the five tissues; but their initial 1 h uptake of WSF-HC accounts for 61% and 47% respectively of the accumulated total WSF-HC during the entire 24 h exposure (Tables A10-1 to A14-2). On the other hand, in the lipid-rich tissues such as belly flap, subdermal fat tissue and mesenteric adipose tissue, the same initial 1 h uptake accounts for only 15.6%, 20.5% and 9.1% of the accumulated total

Table 4.7. Gravimetrically determined lipid contents (%, wet tissue basis) of dorsal white muscle, dark muscle, belly flap, subdermal fat tissue and mesenteric adipose tissue pooled from all fish used for the 24 h WSF-HC uptake experiment. Values are the average of duplicate analyses with standard deviations.

| | WM[a] | DM | BF | SF | MA |
|---|---|---|---|---|---|
| Lipid Content | 3.43±0.09 | 16.27±0.12 | 34.75±0.27 | 88.42±0.38 | 85.09±0.52 |

[a] WM - dorsal white muscle, DM - dark muscle, BF - belly flap, SF - subdermal fat tissue, MA - mesenteric adipose tissue.

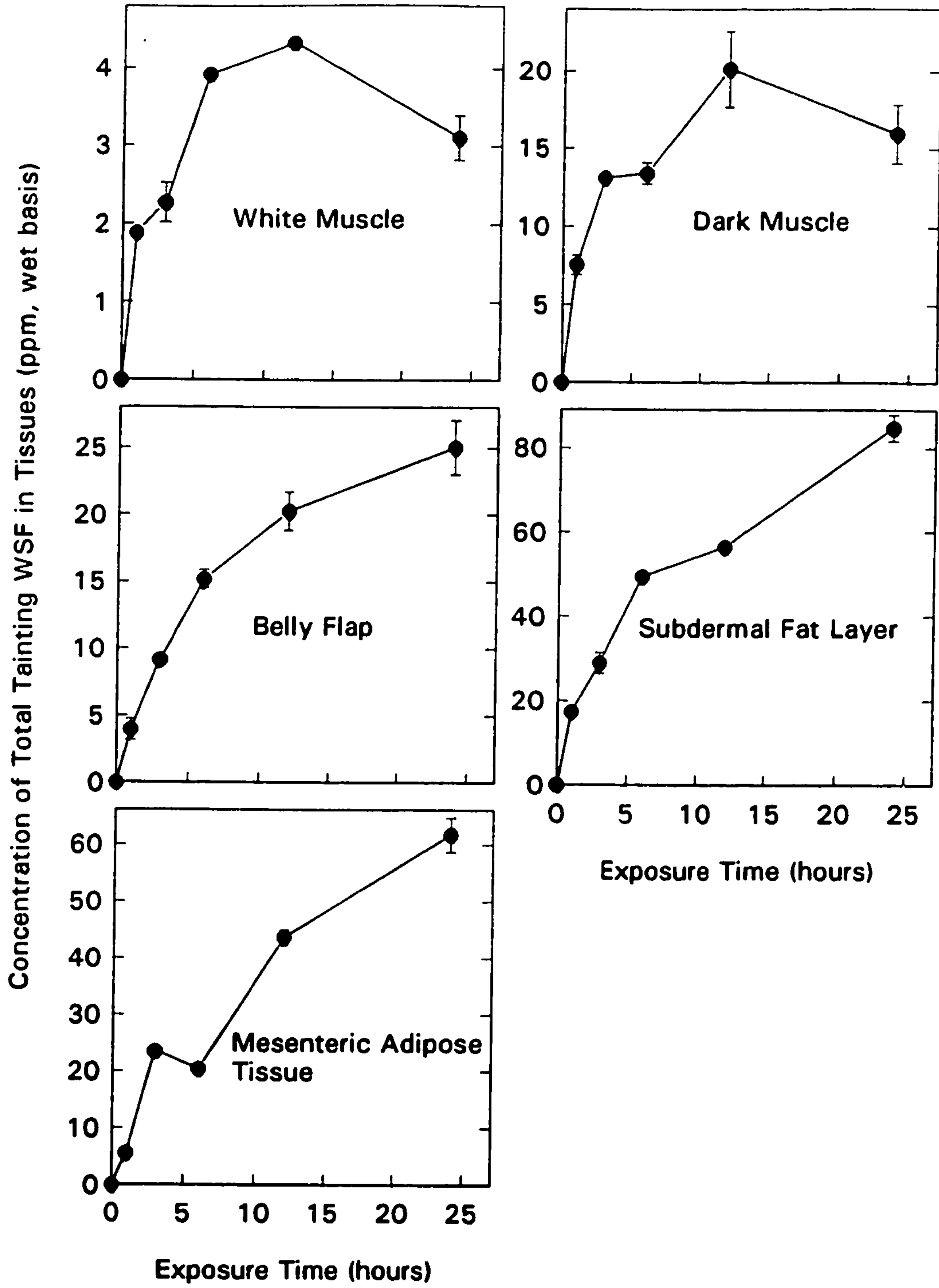


Fig. 4.14. Changes in total tainting WSF-HC concentrations in white muscle, dark muscle, belly flap, subdermal fat tissue and mesenteric adipose tissue of Atlantic salmon sampled during the 24 h exposure period.

WSF-HC in the 24 h exposure. As a result, the plateaus of WSF-HC uptake in dorsal white and dark muscle was rapidly approached after only 12 h exposure to the WSF-HC in water (Fig. 4.14). The WSF-HC concentrations in these two types of muscle tissues in fish exposed for 24 h were slightly lower than those exposed for 12 h, probably reflecting the effects of both the variation in WSF-HC uptake among individual fish and the fluctuation of WSF-HC concentration in the exposure tank. On the other hand, the WSF-HC concentrations in the three lipid-rich tissues continuously showed a steady, almost linear trend during the 24 h exposure in the WSF-HC solution and would probably have taken a much longer time before reaching an uptake plateau (Fig. 4.14). The rapid attainment of a plateau in the uptake of 2-methylisoborneol has been observed for channel catfish, in which the maximum accumulation was reached after 24 h of exposure (Johnsen and Lloyd 1992).

Changes in concentrations of selected individual or groups of WSF-HC in the five tissues during the 24 h exposure period are shown in Fig. 4.15. These WSF-HC span the entire WSF-HC profile ranging from benzene to methylnaphthalenes accumulated in Atlantic salmon. All six types of WSF-HC displayed the same trend as that of the accumulated total tainting WSF-HC with respect to their changes in concentrations during the 24 h exposure period (Fig. 4.14). It is clear that among the three lipid-rich tissues the changes in all individual or groups of WSF-HC in belly flap during the later exposure stages were slightly slower than those in the subdermal fat tissue and in the mesenteric adipose tissue. Therefore, in the belly flap the concentrations of accumulated WSF-HC would probably be much closer to its uptake plateau than the other two lipid-rich tissues at the end of 24 h exposure.

The relative proportions of individual WSF-HC in the five tissues were similar to those in the parent WSF-HC solution and were relatively stable during the 24 h exposure period (Tables A10-1 to A14-2). This indicates that all WSF-HC in the exposure water were taken up by Atlantic salmon in the same proportions as those in the water column and the selective metabolic processes of WSF-HC in Atlantic salmon were

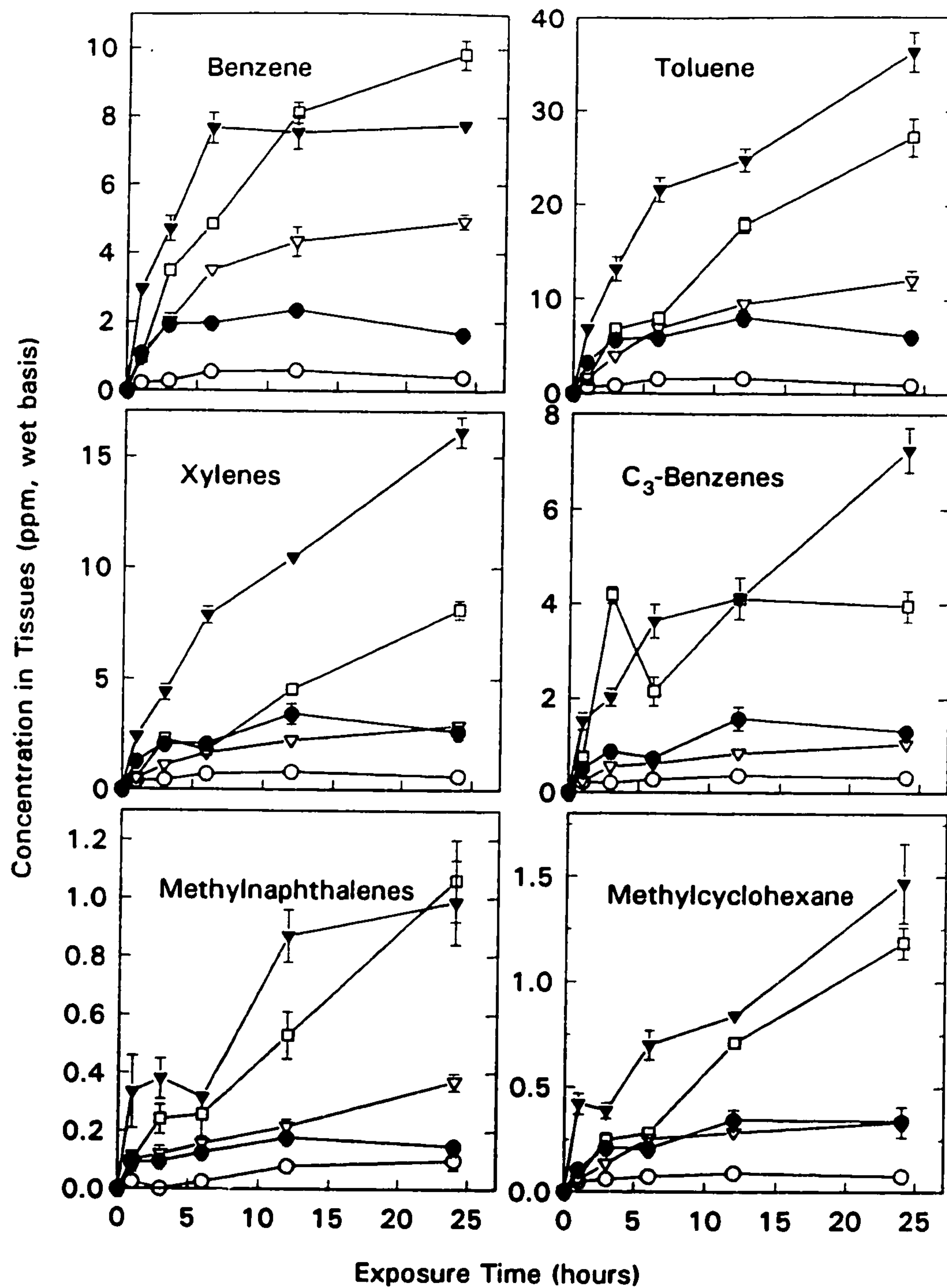


Fig. 4.15. Changes in concentrations of individual or groups of WSF-HC in tissues of Atlantic salmon during the 24 h exposure period. ○ white muscle, ● dark muscle, ▽ belly flap, ▼ subdermal fat tissue, □ mesenteric adipose tissue.

probably not a significant factor in the preferential removal of particular WSF-HC compared with the WSF-HC uptake from the water column.

The accumulation rate of WSF-HC into tissues were probably, to a large extent, dependent on the vascularity or the circulatory system of the tissues. Subdermal fat tissue and mesenteric adipose tissue had a similar level of lipid content, but the subdermal fat tissue took up 3 times more tainting WSF-HC than the mesenteric adipose tissue (17.4 ppm compared to 5.6 ppm) during the initial 1 h exposure (Fig. 4.14). This is probably due to the lower vascularity or the lower surface area of the mesenteric adipose tissue in contact with tissue fluid. The subdermal fat tissue covers the fish body as a thin layer under the skin and has much larger surface area in contact with tissue fluid. As the exposure proceeded, the proportional differences in the total WSF-HC concentrations between these two types of adipose tissues decreased continuously, but a 23 ppm difference in total WSF-HC was still observed at the end of 24 h of exposure (Table A11-1 to A12-2). The uptake of WSF-HC in dark muscle and belly flap was to some extent similar to the above two adipose tissues. Upon the initial 1 h exposure, the concentration of tainting WSF-HC in dark muscle was nearly twice as high as that in the belly flap (7.5 ppm compared to 3.9 ppm), although their lipid contents were exactly the opposite (Table 4.7). However, as the WSF-HC concentrations in dark muscle approached its maximum level rapidly, the accumulation rate of WSF-HC in the dark muscle slowed down compared to that in the belly flap. After 6 h exposure, the concentration of tainting WSF-HC in the belly flap exceeded that in the dark muscle (Table A11-1 to A12-2). At the end of 24 h exposure, an extra of 9 ppm more tainting of WSF-HC was still detectable in the belly flap compared to the increase in the dark muscle and the difference would certainly increase as the exposure continued. This explains our previous observation that similar WSF-HC concentrations were found in those two types of muscle tissues after an 8 h short-term exposure (Section 4.3.3).

Besides the difference in the circulatory systems for tissue fluid, the differences in the distribution and storage of lipids between those two types of muscle tissues would

probably also have contributed to the initially faster WSF-HC uptake into the dark muscle. Histological studies on the lipid distribution in the muscle tissue of Atlantic salmon have revealed that lipids in the belly flap were mainly found in densely packed adipocytes (Section 1.4). In the dark muscle, lipids were not only stored in adipocytes but smaller droplets were also found surrounding individual dark muscle cells, and more importantly, finely dispersed within each dark muscle cell. This would certainly contribute to the faster rate in approaching the uptake plateau in dark muscle than the WSF-HC uptake in belly flap since finely dispersed lipid droplets have a much better chance of contacting tissue fluid and this would increase the partitioning rate of WSF-HC between the tissue fluid and the lipid cell or fat storage body.

## 4.6 Effects of Prolonged Starvation on the Depuration of Tainting WSF-HC from the Whole Muscle Tissue and the Subdermal Fat Tissue of Atlantic Salmon

### 4.6.1 Introduction

When an oil spill occurs near fish farms, those fish in the cages should become heavily tainted by petroleum hydrocarbons, particularly WSF-HC (Ritchie and O'Sullivan 1994). The usual practice for the removal of the accumulated contaminants from aquatic organisms is to keep the fish passively in the cages until the level of contaminants in the fish has fallen to acceptable background levels. Any attempt to move the incompletely depurated fish into markets would result in damage to the reputation of seafood industries. On the other hand, complete depuration of the tainting hydrocarbons from aquatic organisms is a long-term processes, especially for the elimination of PAHs in a fatty fish species such as Atlantic salmon. As a result, the prolonged holding of tainted fish in cages would cause serious economic losses to fish farmer.

High correlations between lipid content and levels of tainting organic pollutants in tissues have been observed in various aquatic organisms (Niimi 1983, Hebert and Haffner 1991, Hektonen et al. 1992, Johnsen and Lloyd 1992). Our previous studies have shown that tainting WSF-HC in the muscle tissue of Atlantic salmon are mainly stored in, and retained by, adipocytes, particularly during the later depuration stage. Therefore, depletion of the lipid reserve in the tainted fish might help to speed up the depuration process for the accumulated hydrocarbons from fish tissues.

Farmed fish obtain their essential nutrients and energy supply mainly from the diet, and a substantial reduction in dietary intake would generally lower body growth rate and decrease lipid reserves in their tissues. It was not known whether depletion in lipid content through prolonged starvation of the tainted fish would indirectly help the clearance of the accumulated hydrocarbons retained by adipocytes in the fish tissues. In this study, Atlantic salmon were exposed to the WSF-HC of Flotta North Sea crude oil

for 8 h and the exposed fish were immediately separated into two groups for their respective depuration in clean seawater. One group was fed intensively and the other group starved. The main objective is to clarify the effects of prolonged starvation of the tainting Atlantic salmon on the depuration of accumulated WSF-HC.

### 4.6.2 Materials and Methods

#### Atlantic Salmon

Atlantic salmon, *Salmo salar*, were held in aerated seawater at the Dalhousie University Aquatron Laboratory and fed on a diet of Fundy Choice feed (Corey Mills Ltd, Fredericton, New Brunswick). Thirty-three fish were exposed to the WSF-HC solution and twelve fish were used as control samples. The control fish came from the same stock as the fish used for exposure and were kept in clean seawater. The fish used for this study had an average weight of 1069 ± 432 g, and an average length of 45.7 ± 7.8 cm. The fish were starved for two days before exposure.

#### Exposure and Depuration of Atlantic Salmon

Preparation of WSF-HC stock solution was conducted by following the same procedures as in Section 4.4.2.1. The procedures for the exposure of Atlantic salmon has been described in Section 4.3.2. Constant concentration of WSF-HC and oxygen level in the exposure water were achieved by continuously and proportionally pumping oxygenated seawater (3000 mL/min) from a header tank and WSF-HC stock solution (500 mL/min) from the sealed plastic bags to the exposure tanks (80-110% saturation). The concentration of total WSF-HC in the exposure tank was controlled at ~0.89 ppm and the fish were exposed to this WSF-HC for 8 h. At the end of exposure, the fish were randomly separated into two groups and transferred to two aquaria for depuration.

One group of fish was fed on Fundy Choice feed three times a day during the initial 30 days of depuration period and fed once a day during the last 30 days of depuration. No food was given to the other group of fish during the entire period of depuration. Every effort was made to avoid causing stress to the fish during the exposure period and during the transferring and handling of the exposed fish. The temperature of the exposure water was 7.0°C during the exposure period and ranged from 9.0 to 16.0 during the depuration period. The photoperiod was set for 12 h light and 12 h dark. Three Atlantic salmon were randomly sampled from both the fed and the starved groups after 4 days, 10 days, 17 days, 30 days and 60 days of depuration in clean seawater. The control fish were also separated into two batches, and control fish from each batch were taken at the beginning of depuration and at the end of 30 days of depuration respectively.

**Tissue Sampling**

The live Atlantic salmon removed for study were immediately transported in chilled seawater to the Canadian Institute of Fisheries Technology at the Technical University of Nova Scotia. The fish were then killed with a blow on the head and the gut cavities were cut open along the middle of abdomen. The viscera were then removed and the fish were thoroughly washed with cold water. The subdermal fat tissue was obtained by taking the skin off the fish body and by subsequently scraping the top fat tissue off the muscle tissue. Whole muscle tissue including dorsal white muscle, dark muscle and belly flap was taken from the middle area of one fillet of each fish. The other entire fillet remaining from each fish was used for the sensory panel evaluation of tainting WSF-HC in the muscle tissue. The dissected samples from each of the three fish were pooled and immediately frozen at -35°C until analysis.

**Analysis for and Calculation of WSF-HC in Seawater and Tissue Samples**

Procedures described in Section 4.3.2 were followed for the recovery of WSF-HC from seawater and tissue samples, for the GLC-FID analysis of the recovered hydrocarbons, and for the calculation of WSF-HC concentrations in seawater and tissue samples. The tainting WSF-HC in pooled tissue sample were analysed in triplicate.

**Determination of Lipid Content**

The lipid contents of whole muscle tissue and subdermal fat tissue were determined in duplicate according the procedure of Bligh and Dyer (1959).

**Statistical Analysis**

Statistical analysis was performed by one way analysis of variance, with the aid of Minitab software (Minitab Release 7.2, Minitab, Inc.).

### 4.6.3 Results and Discussion

The fish showed signs of stress (e.g., refusing to take diet) after being exposed to the WSF-HC solution for 8 h and being transferred from tank to tank. However, the fed fish started to eat after 12 h of depuration in clean water and their dietary intake gradually increased as depuration continued. After six days of being held in clean water the fed fish returned to a normal state of dietary intake . Fig. 4.16 shows the changes in lipid content of both the fed and the starved Atlantic salmon during the 60 days of the depuration period. The whole muscle tissue of Atlantic salmon contained up to 11% of lipids prior to their depuration in clean seawater. Fluctuations in lipid content were observed ranging from 9.61% to 11.79% for the fed group and from 9.73% to 11.02%

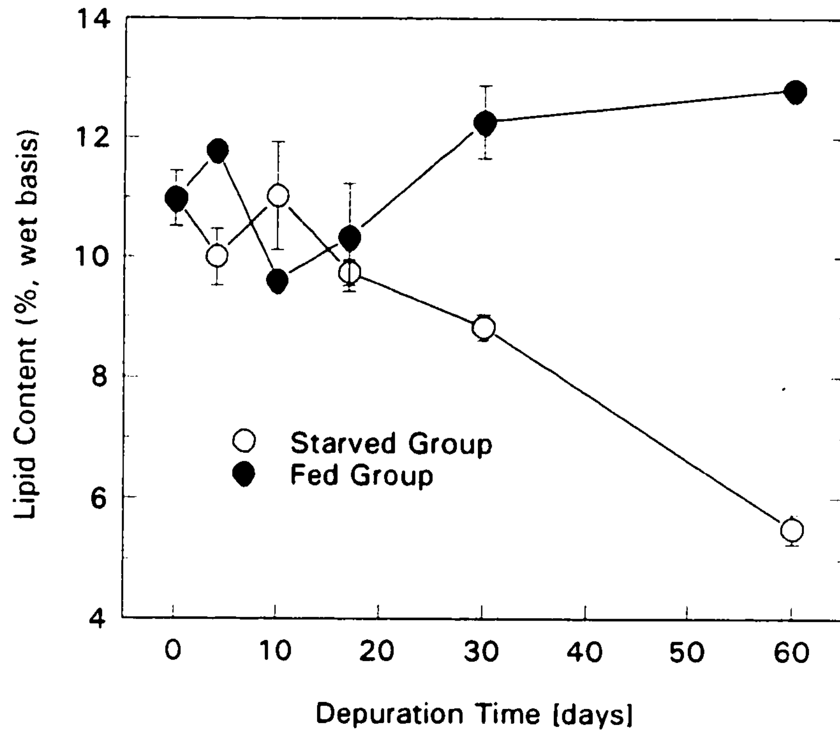


Fig. 4.16. Changes of lipid content in whole muscle tissue of both the fed and the starved Atlantic salmon during 60 days of depuration period.

for the starved group during the initial 17 days of depuration period. However, there was no obvious sign of lipid depletion in this early depuration period, although at day 1 the lipid content in the fed group was significantly higher (1.8%) than that in the starved group ($p<0.01$). These variations in lipid content would mainly reflect the nutritional and biological differences among individual fish and less probably an immediate effect of starvation. However, after 30 and 60 days of depuration in clean seawater, the lipid reserve in the starved Atlantic salmon fell significantly ($p<0.01$) comparing to the level of the non-depurated fish (8.82% and 5.50% compared to 11%), while the lipid contents of the fed group actually increased to 12.27% and 12.82% respectively at the same time period of intensive feeding. The lipid content of the pooled subdermal fat tissue was 84.38%.

Fig. 4.17 shows the GLC-FID profiles of WSF-HC extracted from the exposure water (Fig. 4.17A), and recovered from the whole muscle tissue of the tainted and the control Atlantic salmon (Figs. 4.17B, 4.17C). The composition of the tainting WSF-HC recovered from the whole muscle tissue is similar to that from the exposure water after subtracting the background hydrocarbons in the control fish from the total hydrocarbons in the tainted fish. The WSF-HC were predominantly composed of aromatics, particularly alkylated benzenes ranging from benzene to methylnaphthalenes. There were only trace amounts of both straight-chain and branched-chain alkanes. Cyclohexane and two alkylated cyclohexanes were another group of WSF-HC which were present in the GLC chromatograms besides the aromatics.

At the end of 8 h exposure, both the whole muscle tissue and the subdermal fat tissue were heavily tainted by the WSF-HC, but the WSF-HC concentration in the subdermal fat tissue (139 ppm) was six times higher than that in the whole muscle tissue (23.4 ppm) (Table 4.8). After the exposed fish were transferred into the clean seawater, the tainting WSF-HC were rapidly discharged from the whole muscle tissue of both the fed and the starved groups of Atlantic salmon. At depuration day 4, most of the accumulated WSF-HC was eliminated from the tissue and only 2 ppm of the tainting

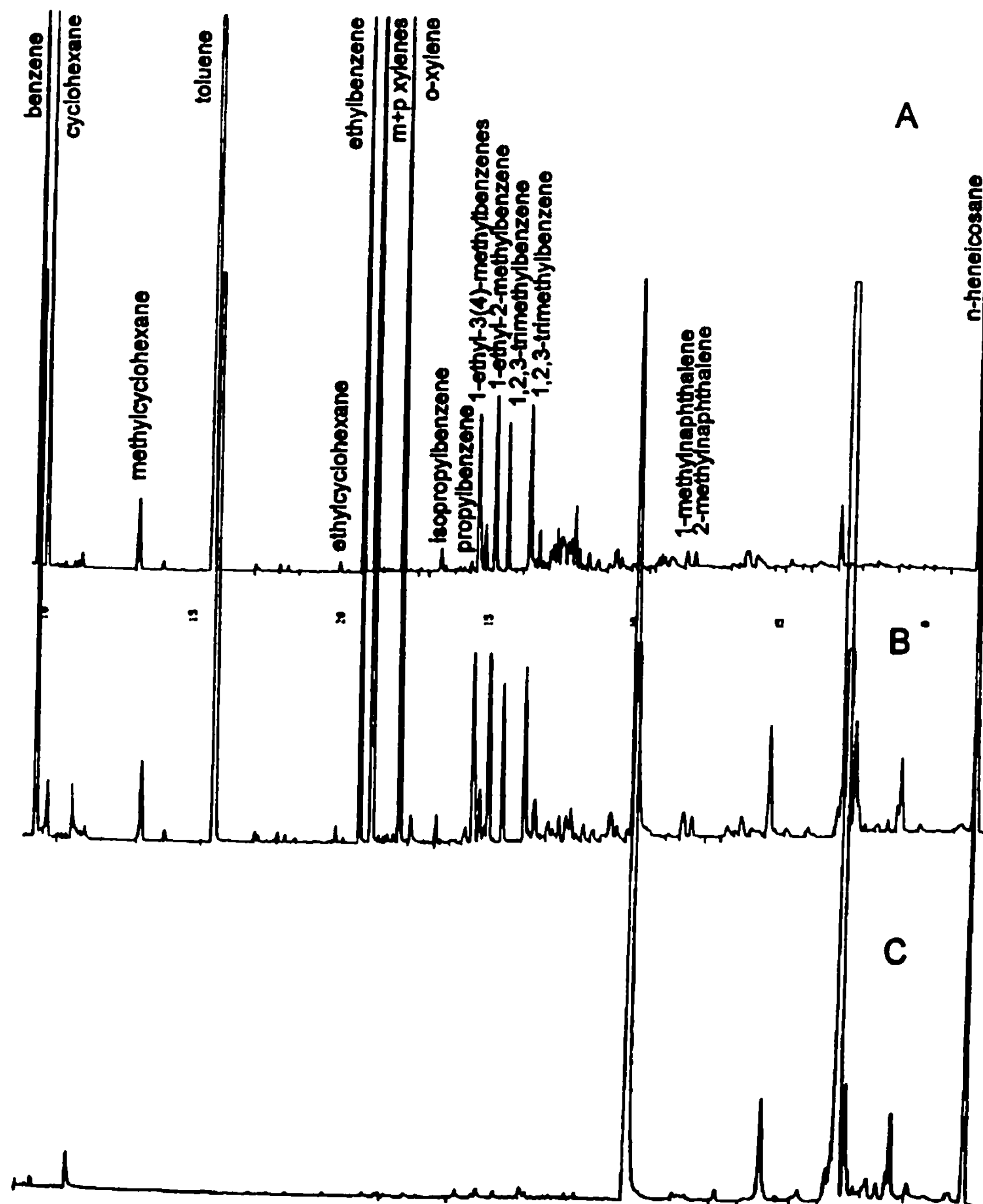


Fig. 4.17. GLC-FID chromatograms of hydrocarbons extracted from (A) the WSF-HC stock solution, and recovered from the whole muscle tissue of Atlantic salmon of (B) the tainted (at the end of 8 h exposure) and (C) the control fish.

Table 4.8. Concentrations of tainting WSF-HC retained by the whole muscle tissue and the subdermal fat tissue of both the fed and the starved Atlantic salmon during 30 days of depuration in clean seawater (ppm, wet basis). The fish were exposed to 0.89 ppm of WSF-HC for 8 h. Samples from three fish were pooled for analyses. Values are the average of triplicate analyses ± standard deviations.

| Depuration Time (days) | Whole Muscle Tissue | | Subdermal Fat Tissue | |
|---|---|---|---|---|
| | Fed | Starved | Fed | Starved |
| 0 | 23.38±0.22 | | 139.38±3.06 | |
| 4 | 2.14±0.58 | 2.03±0.09 | 29.39±1.95 | 25.09±0.11 |
| 10 | 0.79±0.03**[a] | 0.46±0.02 | 12.55±1.45 | 13.91±0.82 |
| 17 | 0.14±0.11 | 0.23±0.00 | 8.51±0.10* | 4.79±0.51 |
| 30 | 0.00 | 0.00 | 0.00 | 0.45±0.66 |

[a] Means of fed and starved groups significantly different at $p<0.05$ (*) and $p<0.01$ (**).

WSF-HC was retained by the whole muscle tissue from both groups of Atlantic salmon. These remaining WSF-HC were depurated very slowly thereafter. At depuration day 17, there was only trace amount of tainting WSF-HC left in the whole muscle tissue of both the fed and the starved Atlantic salmon. The levels of tainting WSF-HC in the muscle tissue from both groups were below the recovery detection limit after 30 days of depuration in clean seawater. The results of this study demonstrated that the accumulated WSF-HC in the whole muscle tissue of both the fed and the starved fish were depurated at the same rate. Although a significant difference ($p<0.01$) in the tainting WSF-HC concentrations (0.79 ppm vs. 0.46 ppm) was observed between the two treated groups at depuration day 17 (Table 4.8), both the total amounts of tainting WSF-HC and their difference were so small that it could be attributed not only to the effect of starvation but also to the variation among individual fish.

The depuration of tainting WSF-HC from the subdermal fat tissue displayed a similar pattern, but more slowly than that from the whole muscle tissue (Table 4.8). The accumulated WSF-HC were rapidly discharged from the subdermal fat tissue of both the fed and the starved Atlantic salmon during the initial 4 days of depuration period. The effect of starvation on accelerating the clearance of the tainting WSF-HC from the subdermal fat tissue was not observed during the first 10 days of depuration, but did occur after holding the fish for 17 days in clean water. At day 17, the amount of tainting WSF-HC in the subdermal fat tissue of the fed fish (8.51 ppm) was significantly higher ($p<0.05$) than that in the starved fish (4.79 ppm). At depuration day 30, the tainting WSF-HC concentration was below the recovery detection limit for the fed fish and was detected only in trace amount for the starved group. In practice, the faster depuration of tainting WSF-HC from the subdermal fat tissue observed at day 17 would not have a significant effect on the elimination of tainting WSF-HC from the whole muscle tissue since the subdermal fat tissue comprises only a very small fraction of the whole muscle tissue.

Tainting hydrocarbons (or background hydrocarbons) present in lipids of aquatic

organisms originate mainly from two sources: Partitioning from water column and absorption from foodwebs (Rowland and Volkman 1982, Niimi and Palazzo 1986, Meador et al. 1995, Zhou et al. 1996). If the organisms are held in clean water, the background hydrocarbons would mainly come from the dietary source, which is usually the case for wild fish (Sasaki et al. 1991). Any hydrocarbons absorbed from WSF-HC exposure would be found mainly in the lipid compartments of the tissues (Section 4.4). Fig. 4.18 illustrates the changes in total background hydrocarbons (from benzene to naphthalene, including trace amounts of retained WSF-HC) in the whole muscle tissue of the fed and the starved Atlantic salmon sampled at depuration day 17, day 30 and day 60. The data in Fig. 4.18 indicate that prolonged starvation of Atlantic salmon did not significantly reduce the levels of background hydrocarbons in the whole muscle tissue until the fish had been starved for 30 days ($p<0.05$). At starvation day 60, the concentration of background hydrocarbons in the whole muscle tissue of the starved fish decreased substantially from the day 30 level and was less than half of the background hydrocarbons (0.48 ppm) was detected in the tissue compared to that in the fed Atlantic salmon (1.13 ppm).

WSF-HC accumulated in the subdermal fat tissue through the current short-term exposure (8 h) are depurated much faster than those through a long-term exposure (96 h). This characteristic is clearly demonstrated in Fig. 4.19 and could be attributed to the fact that the initial step of clearance is faster in the short term exposed fish than that in the long term exposed fish. Both exposures were conducted by using the same type of WSF-HC solution and therefore the same profile of tainting WSF-HC was observed in the Atlantic salmon of the two exposure studies. Although the initial concentration of tainting WSF-HC in the 8 h exposed salmon was almost twice as high as that in the 96 h exposed fish, the faster discharge of the tainting WSF-HC from the 8 h exposed salmon led to much lower WSF-HC concentrations in the subdermal fat tissue than that in the 96 h exposed salmon after initial 4 days of depuration in the clean water (29 ppm and 25 ppm vs. 40 ppm) (Fig. 4.19). During the remaining depuration periods (day 10, day

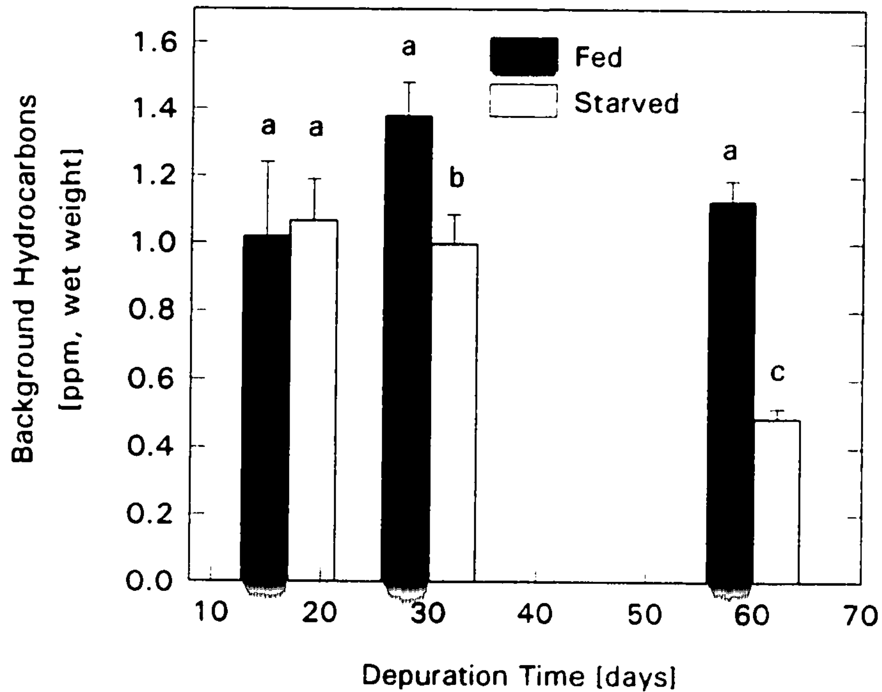


Fig. 4.18. Changes in the levels of volatile background hydrocarbons in the whole muscle tissue of both the fed and the starved Atlantic salmon during the 60 days of depuration period. Different letters above the bars on the same depuration day indicate significant differences between the two means at $p < 0.05$ (a, b) and $p < 0.01$ (a, c). Total background hydrocarbons are the sum of recovered hydrocarbons between benzene and naphthalene on the GLC chromatogram.

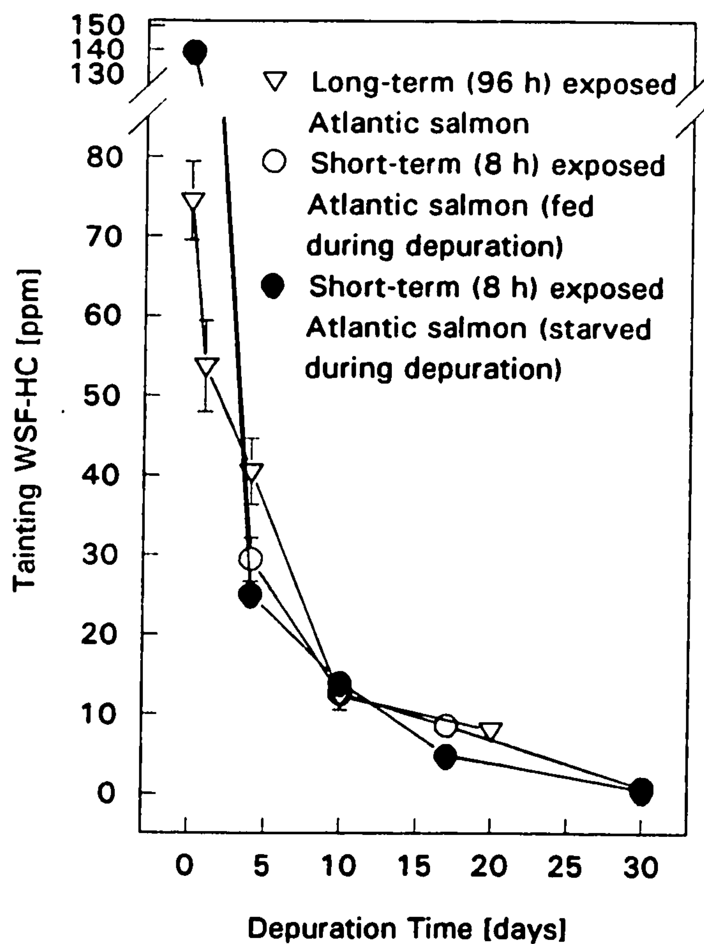


Fig. 4.19. Release of tainting WSF-HC from the subdermal fat tissue of long-term (96 h) and short-term (8 h) exposed Atlantic salmon. One group of the short-term exposed salmon were starved during the entire depuration period.

17 and day 20), similar concentrations of the tainting WSF-HC in the subdermal fat tissue were observed in both the 8 h and the 96 h exposed salmon. The faster discharge of the tainting WSF-HC from the subdermal fat tissue of the 8 h exposed Atlantic salmon suggests that the 8 h exposure is probably far from reaching an equilibrium in WSF-HC uptake from water and consequently a large fraction of accumulated WSF-HC would still be associated with tissue fluid instead of the lipid body at the end of this short term exposure.

The results of this study show that prolonged starvation of the tainted Atlantic salmon did not help to speed up the clearance of the accumulated tainting WSF-HC from the muscle tissue. This could probably be explained by the following two reasons: (a) Most of the tainting WSF-HC were rapidly eliminated from the tissue by holding the tainted fish in clean water only for a few days, while the depletion of lipid reserve from the muscle tissue of Atlantic salmon through starvation is a much slower process. In this study it took one month for the observation of significant reduction in lipid content in the starved salmon group. (2) As soon as the tainting WSF-HC reach the lipid compartments in the muscle tissue of Atlantic salmon, these WSF-HC become strongly associated with the lipid droplets in adipocytes. Prolonged starvation would only increase the consumption of lipids for body energy by retrieving triacylglycerols from some lipid compartments and would have little effect on the clearance of those WSF-HC being stored in the lipid compartments.

## 4.7 A Water-Soluble Fraction of a Crude Petroleum Preparation (K-D Diluent) in Seawater and the Uptake into and Depuration from the Muscle of Atlantic Salmon of That Processed Hydrocarbon Material

### 4.7.1 Introduction

Each year, millions of tons of petrogenic hydrocarbons enter the marine environment through vessel activities, off-shore crude oil production, oil spills, sewage discharge etc. (Farrington and Meyer 1975, Nevenzel 1989). The least understood effect of oil pollution occurrences is the intimate contact in the water column of the invisible water-soluble fraction of petroleum hydrocarbons with marine organisms. The toxicity of petroleum-based oils is largely due to the WSF-HC rather than to films or dispersed droplets of crude petroleum or petroleum products (Rice et al. 1977). Atlantic salmon (*Salmo salar*), a fatty fish species, stores most of its reserve of lipids in the muscle tissue (Ackman 1989, Polvi and Ackman 1992). The uptake and depuration of WSF-HC in this lipid-rich species has been studied previously with 8 h and 96 h exposures to the WSF-HC of Flotta North Sea crude oil (Section 4.3 and Section 4.4). These studies have shown that the presence of a high lipid content in the muscle tissue leads to a heavy accumulation of WSF-HC, and in clean seawater, to slow depuration of the accumulated WSF-HC from the muscle tissue. Unfortunately, in these studies hydrocarbons in the exposure media, the WSF-HC of Flotta North Sea crude oil, were predominantly composed of alkylated benzenes, while the concentration of polycyclic aromatic hydrocarbons is very low. Therefore it became necessary to conduct further studies on the behaviour of PAH-rich WSF-HC in the muscle tissue of Atlantic salmon since numerous studies have shown that PAHs are one of the most probable hydrocarbon contaminants detected in most actual samples of petroleum-tainted aquatic organisms (Neff 1979, Mix 1984, Murray et al. 1991, Misharina and Golovnya 1992, Ritchie and O'Sullivan 1994, George et al. 1995, Meador et al. 1995). The presence of PAHs in

foods has always brought serous concerns over the health issues (Heidelberger 1964).

During a WSF-HC exposure, hydrocarbons transferred through the gills into the blood are accumulated both in the lipid pool (mainly adipocytes) and in the intercellular water pool, including inside muscle cells, of the muscle tissue. The effect of exposure time on the partitioning of accumulated hydrocarbons into the two compartments is not clear. The WSF-HC are transferred first from the blood to the muscle fluids and thence to the adipocytes. It is therefore hypothesized that chronic exposure of marine organisms to petrogenic hydrocarbons should incorporate a higher proportion of WSF-HC into the adipocyte lipid pool than would a short term exposure. This, in turn, would lead to slower depuration of the WSF-HC accumulated during long term exposure. These behaviour characteristics have partially been observed in previous studies on the uptake and depuration of WSF-HC in the subdermal fat tissue of Atlantic salmon (Section 4.6).

The compositional profiles of petroleum products vary largely according to type of crude oil and subsequent fractionation and refining. Our previous exposure studies on Atlantic salmon were mainly focused on the accumulation and depuration of WSF-HC from crude petroleum, dominated by single ring aromatics such as alkylated benzenes and low in PAHs. We have now studied the behaviour of a specific industrial petroleum product, K-D diluent, in seawater, and conducted both short term (8 h) and long term (96 h) exposure of Atlantic salmon to examine the more complicated WSF-HC of K-D diluent rich in PAHs.

### 4.7.2 Materials and Methods

#### Atlantic Salmon and K-D Diluent

Atlantic salmon, *Salmo salar*, were from stocks held in aerated seawater in the Aquatron Laboratory facilities of Dalhousie University and fed on a diet of Fundy Choice feed (Corey Mills Ltd., Fredericton, NB). The fish used for the exposure experiment

had an average weight and length of 559 g and 37 cm respectively. The fish consisted of 62% males and 38 % females, but this was not known at the time of random selection. The petroleum product K-D diluent, an aid to recovery, was obtained from the California Department of Fish and Game, Petroleum Chemistry Laboratory, Rancho Cordova, CA. The gas-liquid chromatographic profile of this material is shown in Fig. 4.20.

**Preparation of the WSF-HC of K-D Diluent**

Sand-filtered seawater at 3°C, 8.5 °C and 23 °C was used to study the characteristics of the WSF-HC prepared from K-D diluent. Pilot studies on the mixing of seawater and K-D diluent were conducted in 4 L brown bottles equipped with magnetic stirring devices. The seawater and diluent were mixed for 24 h and subsequently settled for 48 h after transfer to separatory funnels. The brown bottles were capped and the separatory funnel stoppered during the mixing and settling periods. The seawater and diluent were mixed in ratios of 100:1, 1000:1 and 4000:1. Based on these trials, the ratio of 100:1, which had already been used for our exposure studies with Flotta North Sea crude oil, was selected. The preparation of large quantities of WSF-HC stock solution for the exposure of Atlantic salmon was conducted in a stainless steel mixing tank (450 L) equipped with a glass condenser, a mechanical stirrer and a cooling water jacket. The preparation procedures were the same as mentioned above and the transfer of prepared WSF-HC from the Technical University of Nova Scotia to the Aquatron was effected in sealed plastic bags as described by Heras et al. (1995).

**Exposure of Atlantic Salmon to the WSF-HC of K-D Diluent**

The exposure of Atlantic salmon to the WSF-HC of K-D diluent consisted of two trials: a short term (8 h) with a high WSF-HC concentration (0.50 ppm), and a long term (96 h) with a low WSF-HC concentration (0.08 ppm). Six and twelve fish were used for

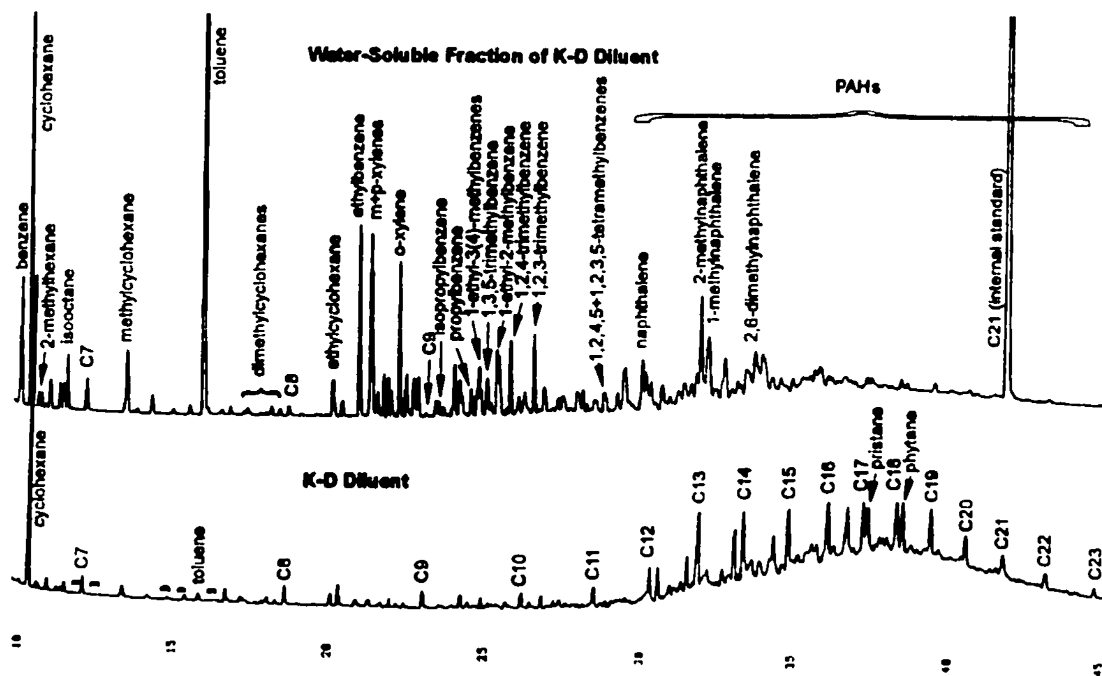


Fig. 4.20. Gas-liquid chromatograms of K-D diluent and its WSF-HC. The WSF-HC was prepared at 3°C with a ratio of diluent : water = 1 : 100..

the short term and long term exposures respectively. The fish had been continuously fed Fundy Choice feed once a day but were starved for 2 days prior to exposures. The exposures were separately conducted in a specially designed circular tank (225 L water-holding capacity) covered with a transparent plastic lid. Diluted WSF-HC concentration in the exposure tank was achieved by continuously and proportionally pumping seawater from a header tank and WSF-HC stock solution from the sealed plastic bag (Heras et al. 1995) to the exposure tank. Two Masterflex pumps (Cole-Parmer Instrument Co., Niles, IL) with Masterflex PHARMED and TYGON tubing were used to supply the seawater and WSF-HC stock solution respectively. The seawater pumped to the exposure tank was refrigerated in order to maintain a below ambient temperature in the exposure tank and was also aerated with oxygen. The WSF-HC stock solution was sampled twice daily; the samples were analyzed immediately for WSF-HC concentration by the procedure of Murray et al. (1984). The seawater flow rate was adjusted accordingly to compensate for the loss of WSF-HC during the storage of the stock solution. The temperature in the exposure tank for both exposures (8 h and 96 h) was in the range of 8-11 °C.

**Depuration of Tainting WSF-HC from Atlantic Salmon**

At the end of exposure periods, three fish were removed from each of the short term and the long term exposure tanks, and also from the control fish tank. The remaining three fish from the short term exposure tank and nine fish from the long term exposure tank were immediately transferred to clean seawater aquaria (2500 L water-holding capacity) for respective depurations. The temperature variations of seawater during the depuration periods ranged from 11.0 to 12.5 °C for the short term exposed fish, from 12.5 to 10.5 °C for the long term exposed fish during the first two weeks of depuration and from 10.5 to 6.0 °C during the last week of depuration. The flow of seawater into the aquaria was set at 25 L/min. The light cycle was set for 12 h of light

and 12 h of darkness. The fish were fed once daily during the entire depuration period. The short term exposure fish were sampled after 7 days of depuration. The long term exposure fish were sampled at day 7, day 14, and day 21 of the depuration period (three fish each time).

**Sampling of Whole Muscle Tissue and Determination of Lipid Content**

All live fish were placed in chilled seawater and transported to the Canadian Institute of Fisheries Technology at the Technical University of Nova Scotia as rapidly as possible. The fish were killed with a blow on the head, gutted, and thoroughly washed with clean tap water before dissection. Whole muscle tissue samples of about 6 cm in width were taken from the middle area of the half fillet, and included belly flaps, dorsal white muscle and skin. Equal amounts of whole muscle tissue from each of the three fish were immediately frozen at -35°C until analysis. The lipid content of skin-on Atlantic salmon whole muscle was determined in triplicate according the procedure of Bligh & Dyer (1959).

**Quantitation of Total WSF-HC in Seawater**

The WSF-HC in seawater were extracted immediately according to the Murray microextraction procedure (Murray 1979, Murray and Lockhart 1981, Murray et al. 1984). The extraction of WSF-HC from seawater, GLC-FID conditions and quantitative analyses of the extracted WSF-HC have been described elsewhere (Section 4.3.2).

**Recoveries of WSF-HC from Atlantic Salmon Whole Muscle Tissue by Steam Distillation**

The WSF-HC in the whole muscle tissue of Atlantic salmon were recovered by a steam distillation procedure modified from that of Ackman and Noble (1973). The detailed procedures on hydrocarbon recovery by steam distillation have been described in Section 3.3.3 (condition IV).

The efficiency of recovering hydrocarbons from homogenized Atlantic salmon muscle tissue by steam distillation was evaluated by spiking 16 standard hydrocarbons into the control muscle samples, encompassing most of the hydrocarbons present in the WSF of K-D diluent. The spiked hydrocarbons were recovered following the above procedures (in quadruplicate).

The GLC-FID analyses of the recovered hydrocarbons have been described in Section 3.3.4. The amounts of tainting WSF-HC were calculated according to the internal standard added, the recovery efficiency and the GLC response factors of individual hydrocarbons (Table A2) (Section 4.3.2). The amounts of hydrocarbons in the control fish were used as background levels and were subtracted from those in the tainted samples. Extraction of WSF-HC from the exposed fish muscle and from the control whole muscle tissue was carried out in triplicate.

**Identification of Hydrocarbons by Gas-Liquid Chromatography-Mass Spectrometry (GLC-MS)**

The GLC-MS system consisted of a Perkin-Elmer 990 gas chromatograph and a Finnigan MAT Model 700 Ion Trap Detector (ITD) (Finnigan MAT, San Jose, CA). The GLC was equipped with a fused-silica capillary column similar to that in the GLC-FID system, passed through a transfer line directly into the ITD inlet. The GLC was operated using helium carrier gas (ultra high purity grade) at a pressure of 138 kPa. The

injector temperature was held at 320°C and the GLC-MS transfer line was maintained at 280°C. The column and oven temperature programs were the same as the GLC-FID system. The mass spectrometer scanned from m/z 50 to 400. The identification of hydrocarbons was carried out with the assistance of the NBS library (National Bureau of Standards) and authentic hydrocarbon standards. Numerous minor components including phenolic aromatics, sulphur- and/or nitrogen-substituted PAHs, etc., were also identified but were not included in this report.

**Statistical Analysis**

Statistical analysis was performed by one way analysis of variance, with the aid of Minitab software (Minitab Release 7.2, Minitab, Inc.).

### 4.7.3 Results

#### 4.7.3.1 Characterization of K-D Diluent and Its Water Soluble Fraction

The principal recognizable components of K-D diluent are alkanes rather than aromatic hydrocarbons (Fig. 4.20). The alkanes are dominated by a straight chain *n*-alkane series and ranged from *n*-heptane ($C_7$) to *n*-pentacosane ($C_{25}$). However, a few branched alkanes, particularly pristane and phytane (Fig. 4.20), are present in relatively high amounts. The diluent WSF-HC are primarily those present in the complex envelope emerging between n-dodecane and *n*-heneicosane in the GLC chromatograms.

A typical GLC chromatogram of the WSF-HC of K-D diluent prepared at 3 °C in a sealed brown bottle is shown in Fig. 4.20. Aromatic hydrocarbons were the major components present in the WSF-HC although a small proportion of low molecular weight alkanes (molecular weight less than *n*-nonane, including cyclics and branched chain hydrocarbons) were detected (Table 4.9). The most noticeable difference in the

Table 4.9. Concentrations of selected hydrocarbons of K-D diluent WSF prepared in a ratio of 100 : 1 (seawater : K-D diluent) at 23°C in a sealed brown bottle and at 16°C in a 450 L stainless steel mixing tank for the exposure studies. Values are the average ± standard deviations of 6 analyses (by brown bottles) and duplicate analyses (by stainless steel mixing vessel) respectively.

| WSF-HC | Concentration in Seawater, ppm (with % in brackets) | |
|---|---|---|
| | Brown Bottles | Stainless Steel Mixing Vessel |
| Benzene | 0.209 ± 0.024 (6.59) | 0.073 ± 0.005 (2.64) |
| Cyclohexane | 0.136 ± 0.025 (4.29) | 0.040 ± 0.001 (1.44) |
| 2-Methylhexane | 0.015 ± 0.001 (0.47) | bdl |
| Isooctane | 0.067 ± 0.007 (2.11) | 0.022 ± 0.003 (0.79) |
| *n*-Heptane | bdl[a] | bdl |
| Methylcyclohexane | 0.059 ± 0.005 (1.86) | 0.042 ± 0.004 (1.52) |
| Toluene | 0.382 ± 0.029 (12.05) | 0.258 ± 0.023 (9.32) |
| Dimethylcyclohexanes | 0.028 ± 0.007 (0.88) | 0.026 ± 0.002 (0.94) |
| *n*-Octane | bdl | 0.014 ± 0.002 (0.51) |
| Ethylcyclohexane | 0.021 ± 0.002 (0.66) | 0.021 ± 0.001 (0.76) |
| Ethylbenzene | 0.053 ± 0.005 (1.67) | 0.069 ± 0.005 (2.49) |
| *m*+*p*-Xylenes | 0.082 ± 0.008 (2.59) | 0.083 ± 0.004 (3.00) |
| *o*-Xylene | 0.058 ± 0.005 (1.83) | 0.061 ± 0.007 (2.20) |
| *n*-Nonane | bdl | bdl |
| Isopropylbenzene | 0.006 ± 0.001 (0.19) | 0.007 ± 0.002 (0.25) |
| Propylbenzene | 0.009 ± 0.001 (0.28) | 0.014 ± 0.002 (0.51) |
| 1-Ethyl-3-methylbenzene | 0.025 ± 0.002 (0.79) | 0.025 ± 0.004 (0.90) |
| 1-Ethyl-4-methylbenzene | 0.010 ± 0.001 (0.32) | 0.012 ± 0.004 (0.43) |

Table 4.9 (continued)

| WSF-HC | Concentration in Seawater, ppm (with % in brackets) | |
|---|---|---|
| | Brown Bottles | Stainless Steel Mixing Vessel |
| 1,3,5-Trimethylbenzene | 0.014 ± 0.002 (0.44) | 0.016 ± 0.004 (0.58) |
| 1-Ethyl-2-methylbenzene | 0.021 ± 0.002 (0.66) | 0.025 ± 0.003 (0.90) |
| 1,2,4-Trimethylbenzene | 0.021 ± 0.002 (0.66) | 0.023 ± 0.003 (0.83) |
| *n*-Decane | bdl | bdl |
| 1,2,3-Trimethylbenzene | 0.031 ± 0.003 (0.98) | 0.043 ± 0.001 (1.55) |
| *n*-Hendecane | bdl | bdl |
| Tetramethylbenzenes | 0.014 ± 0.001 (0.44) | 0.017 ± 0.002 (0.61) |
| Naphthalene | 0.052 ± 0.005 (1.64) | 0.056 ± 0.004 (2.02) |
| 2-Methylnaphthalene | 0.082 ± 0.009 (2.59) | 0.085 ± 0.007 (3.07) |
| 1-Methylnaphthalene | 0.058 ± 0.005 (1.83) | 0.062 ± 0.005 (2.24) |
| 2,6-Dimethylnaphthalene | 0.052 ± 0.005 (1.64) | 0.056 ± 0.007 (2.02) |
| Total PAHs (naphthalene to $n$-$C_{21}$) | 1.158 ± 0.301 (36.52) | 1.315 ± 0.095 (47.49) |
| Other aromatics | 0.752 ± 0.058 (23.72) | 0.563 ± 0.044 (20.33) |
| **Total** | **3.171 ± 0.261** | **2.769 ± 0.186** |

[a] bdl = below detection limits

hydrocarbon profiles between K-D diluent and its WSF-HC was the presence of high concentrations of alkylated benzenes in the seawater. These were only minor components in the original K-D diluent (Fig. 4.20). It is quite obvious that toluene ($>11.2\%$) was the major WSF-HC component, and benzene ($>6.3\%$), cyclohexane ($>4.3\%$), and xylenes ($>3.9\%$) were also present in fairly high amounts.

The symmetrical baseline envelope of unresolved hydrocarbons in the chromatogram of K-D diluent was also represented in the seawater WSF-HC (Fig. 4.20). However, the WSF-HC envelope became less prominent than that of the original diluent and the centre of the WSF-HC envelope was slightly shifted towards a lower retention time. The WSF-HC displayed a characteristic fingerprint spectrum of PAHs, which became the most interesting components for the exposure studies (Table 4.9). Identification of individual PAHs by GLC-MS revealed phenolics, normal PAHs, and also sulphur- and nitrogen-substituted PAHs. The amounts of PAHs in the WSF-HC were very high, accounting for 37.8%, 38.6% and 36.5% of the total WSF-HC prepared at 3°C, 8.5°C and 23°C respectively. These WSF-HC represented the major tainting hydrocarbons available to exposed marine organisms.

Solubilities of hydrocarbons depend on both the temperature of seawater and the mixing ratio of seawater and crude petroleum. WSF-HC prepared at 3°C, 8.5°C and 23°C were significantly different from each other with respect to the total hydrocarbons dissolved in seawater ($p<0.05$). At the mixing ratio of 100:1, the total WSF-HC concentrations increased from 2.25 ppm to 2.74 ppm and 3.17 ppm as the preparation temperatures changed from 3.0°C to 8.5°C and 23.0°C respectively (Fig. 4. 21a). The actual WSF-HC concentrations in seawater were not proportional to the increases of temperature. The incremental rate for the total amount of hydrocarbons solubilized in seawater decreased as the temperature increased (Fig. 4.21a). The effect of temperature on the transfer of individual volatile WSF-HC such as toluene into the water column (Fig. 4.21b) was similar to that of total WSF-HC in the seawater.

The mixing ratio of seawater and K-D diluent had a strong effect on both the total

amount of hydrocarbons dissolved in seawater and their relative proportions (Table 4.10). At the temperature of 8.5°C, the WSF-HC concentrations for the mixing ratios of 100:1, 1000:1 and 4000:1 were significantly different from each other ($p < 0.05$). The amount of total hydrocarbons dissolved in seawater decreased from 2.74 ppm to 1.83 ppm and then to 1.37 ppm as the ratio of seawater to diluent increased from 100:1 to 1000:1 and 4000:1 (Fig. 4.21c). However, the influence of mixing ratio on individual hydrocarbons in WSF depended on both their abundance in the diluent and their molecular weights. Toluene is present in only trace amounts in K-D diluent (Fig. 4.20) and is one of the components most soluble in seawater. Its concentration in seawater decreased from 0.334 ppm to 0.059 ppm (Fig. 4.21d), 5.7 times lower, when the seawater proportions increased from 100:1 to 4000:1, while the total WSF-HC concentrations decreased only 2 times for the same level of changes in mixing ratio. The faster decrease rates of the concentrations of lower molecular weight WSF-HC than of those of higher molecular weight resulted in the increases in the relative proportions of higher molecular weight WSF-HC (e.g., PAHs) in the total WSF-HC (Table 4.10). The proportion of PAHs in the total WSF-HC went up from 38.6% to 54.0% when the ratio of seawater and diluent increased from 100:1 to 4000:1 (Table 4.10).

The overall hydrocarbon profile of WSF-HC stock solution used for the exposure of Atlantic salmon was similar to that of the WSF-HC prepared on the pilot scale. However there were slightly higher proportions of PAHs and lower proportions of alkylated benzenes in the WSF-HC stock solution prepared from the pilot scale for the exposure studies (Table 4.9), i.e., there was 47.5% PAHs in the stock WSF-HC solution prepared on a pilot scale at 23°C compared with 36.5% PAHs in the total WSF-HC from a sealed bottle. The concentration of total stock WSF-HC was 2.77 ppm sampled at 0 h of exposure.

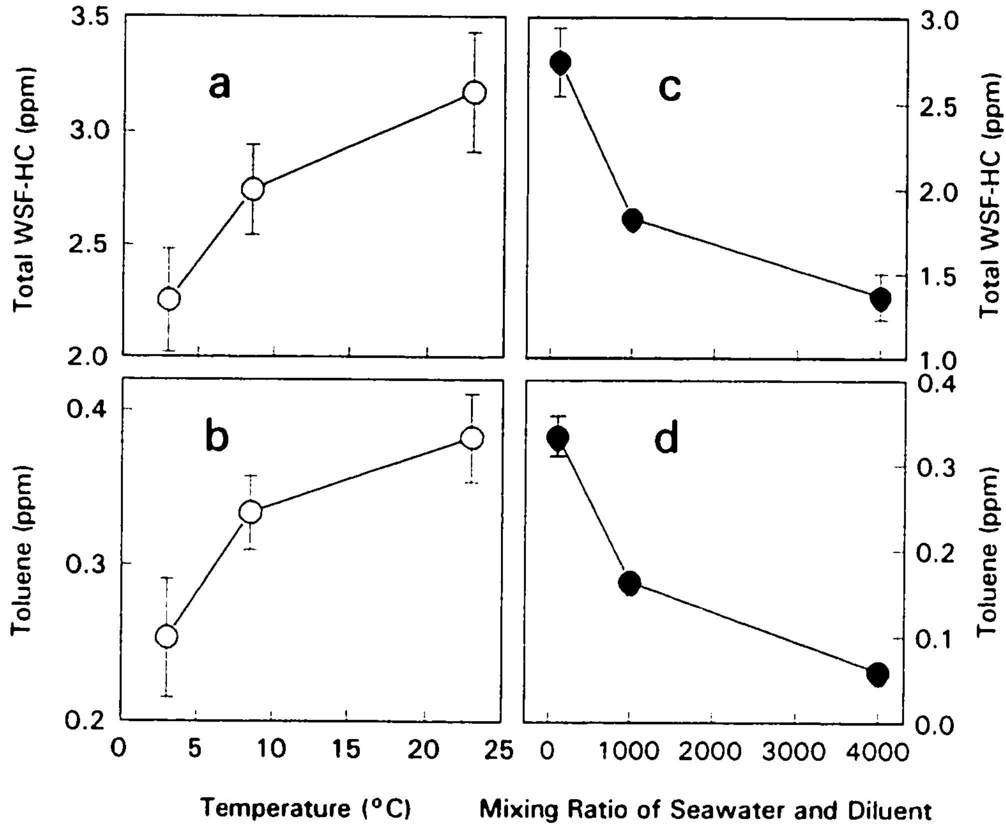


Fig. 4.21. Effects of preparation temperature and mixing ratio of seawater and K-D diluent on the concentrations of total WSF-HC and of toluene dissolved in seawater.

Table 4.10. WSF composition of K-D diluent prepared in three different mixing ratios of seawater and K-D diluent at 8.5°C in sealed brown bottles. Values of hydrocarbon concentrations (% of total WSF-HC) in seawater are the average of triplicate analyses ± standard deviation.

| Hydrocarbons | Mixing ratios (seawater : K-D diluent) | | |
|---|---|---|---|
| | 100:1 | 1000:1 | 4000:1 |
| Benzene | 6.34 ± 0.58 | 3.61 ± 0.11 | 1.53 ± 0.15 |
| Cyclohexane | 4.34 ± 0.29 | 1.09 ± 0.22 | 0.37 ± 0.07 |
| 2-Methylhexane | 0.51 ± 0.04 | 0.49 ± 0.05 | 0.37 ± 0.07 |
| Isooctane | 2.41 ± 0.22 | 3.28 ± 0.16 | 2.48 ± 0.22 |
| *n*-Heptane | 1.31 ± 0.11 | 0.82 ± 0.05 | 0.66 ± 0.15 |
| Methylcyclohexane | 2.51 ± 0.26 | 2.90 ± 0.11 | 2.92 ± 0.29 |
| Toluene | 12.28 ± 0.87 | 8.97 ± 0.05 | 4.31 ± 0.73 |
| Dimethylcyclohexanes | 1.17 ± 0.18 | 1.04 ± 0.00 | 1.24 ± 0.15 |
| *n*-Octane | 0.69 ± 0.07 | 0.49 ± 0.16 | 0.37 ± 0.15 |
| Ethylcyclohexane | 0.73 ± 0.07 | 0.49 ± 0.00 | 0.44 ± 0.07 |
| Ethylbenzene | 2.22 ± 0.15 | 2.41 ± 0.05 | 1.83 ± 0.29 |
| *m*+*p*-Xylenes | 2.51 ± 0.15 | 2.84 ± 0.05 | 2.26 ± 0.37 |
| *o*-Xylene | 1.71 ± 0.11 | 1.97 ± 0.00 | 1.46 ± 0.29 |
| *n*-Nonane | bdl | bdl | bdl |
| Isopropylbenzene | 0.18 ± 0.04 | 0.38 ± 0.16 | 0.44 ± 0.15 |
| Propylbenzene | 0.40 ± 0.04 | 0.49 ± 0.05 | 0.58 ± 0.07 |
| 1-Ethyl-3-methylbenzene | 0.73 ± 0.07 | 1.26 ± 0.00 | 1.31 ± 0.22 |
| 1-Ethyl-4-methylbenzene | 0.33 ± 0.04 | 0.44 ± 0.05 | 0.51 ± 0.07 |
| 1,3,5-Trimethylbenzene | 0.47 ± 0.04 | 0.66 ± 0.05 | 0.66 ± 0.07 |
| 1-Ethyl-2-methylbenzene | 0.62 ± 0.04 | 0.71 ± 0.05 | 0.88 ± 0.22 |
| 1,2,4-Trimethylbenzene | 0.62 ± 0.04 | 0.88 ± 0.05 | 0.95 ± 0.15 |

Table 4.10 (continued)

| Hydrocarbons | Mixing ratios (seawater : K-D diluent) | | |
|---|---|---|---|
| | 100:1 | 1000:1 | 4000:1 |
| *n*-Decane | bdl | bdl | bdl |
| 1,2,3-Trimethylbenzene | 0.87 ± 0.07 | 1.26 ± 0.05 | 1.46 ± 0.22 |
| *n*-Hendecane | bdl | bdl | bdl |
| Tetramethylbenzenes | 0.40 ± 0.04 | 0.55 ± 0.05 | 0.66 ± 0.07 |
| Naphthalene | 1.31 ± 0.11 | 1.81 ± 0.11 | 1.90 ± 0.22 |
| 2-Methylnaphthalene | 2.22 ± 0.18 | 2.68 ± 0.05 | 3.29 ± 0.44 |
| 1-Methylnaphthalene | 1.49 ± 0.11 | 2.02 ± 0.05 | 3.07 ± 0.37 |
| 2,6-Dimethylnaphthalene | 1.38 ± 0.11 | 1.70 ± 0.05 | 2.12 ± 0.22 |
| Total PAHs (naphthalene to n-$C_{21}$) | 38.63 ± 3.28 | 44.53 ± 1.64 | 54.05 ± 4.82 |
| Other aromatics | 18.00 ± 1.49 | 18.44 ± 1.20 | 18.26 ± 2.19 |
| **Total (ppm)** | **2.744 ± 0.198** | **1.828 ± 0.059** | **1.369 ± 0.135** |

bdl = below detectable limits

#### 4.7.3.2 Recovery Efficiency of Spiked Hydrocarbons from Whole Muscle Tissue

Generally the efficiency of recovering hydrocarbons from tissue samples should be evaluated whenever different species of aquatic organisms or organism of different sizes are examined for hydrocarbon concentrations by steam distillation. This is simply because different species of aquatic organisms or organism of different sizes might be different in their lipid contents, which is an important factor affecting the efficiency of recovering hydrocarbons from tissue samples.

Recoveries of the spiked hydrocarbons from whole muscle tissue of Atlantic salmon used for this study by steam distillation are reported in Table 4.11. The results are similar to those reported in Section 3.4.2 with the minimum recovery of over 60% for the aromatic standard spiked. The lower molecular weight hydrocarbons, such as toluene and xylenes, tended to have higher recoveries than higher molecular weight hydrocarbons.

#### 4.7.3.3 Background Hydrocarbons in the Control Atlantic Salmon

The average lipid content of the pooled Atlantic salmon whole muscle tissue was 8.26 ± 0.98%. Since Atlantic salmon used for the exposure trials had been held in the Dalhousie Aquatron aquaria for about two years, tainting by hydrocarbons of petroleum origin in these fish could be excluded. The chromatograms of hydrocarbons recovered from the fish muscle (Fig. 4.22c) showed that they were quite "clean" with regard to both n-alkanes and to the complex envelope of commonly occurring petroleum hydrocarbons detected in fish tainted from exposure to the WSF-HC of crude petroleum (Heras et al. 1992). Several volatile aromatics, such as toluene, xylenes, ethyl-methylbenzenes, were present in very small quantities and were subtracted from the total hydrocarbons in the calculation of tainting amounts of WSF-HC in the exposed Atlantic salmon. Pristane at 20.51 ppm was the obvious and major natural hydrocarbon found

Table 4.11. Recoveries of spiked hydrocarbon standards from the muscle tissue of Atlantic salmon by steam distillation. Values are the average of quadruplicate analyses ± standard deviations.

| Hydrocarbons | Spiked ($\mu$g) | Recoveries (%) |
|---|---|---|
| Toluene | 6.25 | 84.3 ± 8.3 |
| *n*-Octane | 1.93 | 68.7 ± 8.7 |
| Ethylbenzene | 3.80 | 77.2 ± 7.1 |
| *m*+*p*-Xylene | 3.54 | 87.1 ± 7.3 |
| *o*-Xylene | 2.92 | 81.5 ± 7.3 |
| *n*-Nonane | 2.02 | 58.1 ± 9.7 |
| Isopropylbenzene | 3.55 | 73.0 ± 5.9 |
| Propylbenzene | 3.78 | 69.3 ± 6.5 |
| 1,3,5-Trimethylbenzene | 3.70 | 70.3 ± 6.0 |
| 1,2,4-Trimethylbenzene | 3.79 | 68.5 ± 5.4 |
| *n*-Decane | 1.70 | 54.1 ± 6.8 |
| *n*-Hendecane | 1.54 | 47.6 ± 5.8 |
| 1,2,4,5-Tetramethylbenzene | 3.07 | 70.3 ± 5.6 |
| Naphthalene | 2.49 | 77.3 ± 5.5 |
| 2-Methylnaphthalene | 4.52 | 69.4 ± 4.0 |
| 1-Methylnaphthalene | 4.11 | 66.9 ± 2.9 |

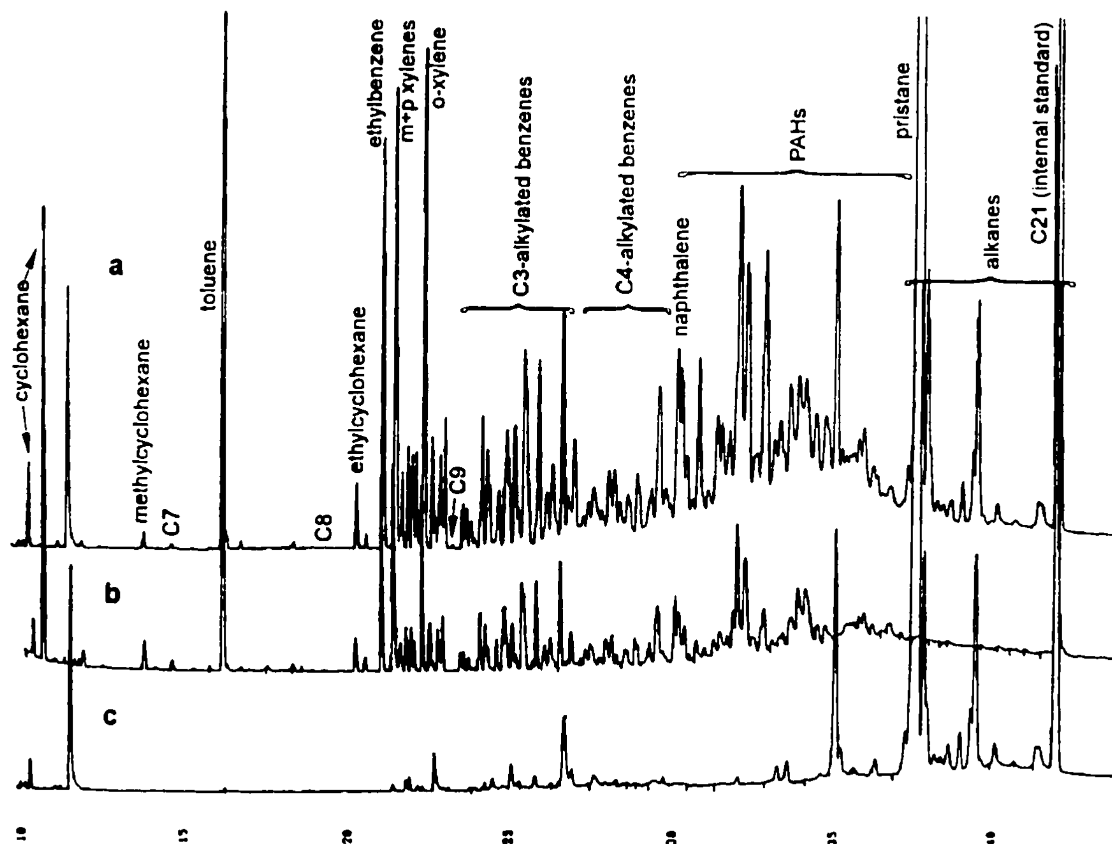


Fig. 4.22. Gas-liquid chromatograms of tainting WSF in the whole muscle tissue of the 8 h exposed Atlantic salmon (a), of the corresponding stock solution of WSF sampled at the beginning of the exposure period (b), and of the hydrocarbons recovered from the control salmon (c).

in the control salmon whole muscle tissue. Herring oil and fish meal lipids in the diets were the obvious sources of the pristane (Ackman 1971). There were several other components present in relatively lesser concentrations, particularly those between pristane and n-heneicosane (Fig. 4.22c), which were identified as unspecified aliphatic hydrocarbons by GLC-MS. These were all excluded in the calculation of total tainting WSF-HC and PAHs.

#### 4.7.3.4 Short Term Exposure - Accumulation and Depuration of WSF-HC of K-D Diluent in the Whole Muscle Tissue of Atlantic Salmon

The average WSF-HC concentration in the 8 h exposure tank was 0.503 ppm including 0.239 ppm total PAHs. At the end of exposure, the fish were heavily tainted by the WSF-HC of K-D diluent, and 12.50 ppm of the total WSF-HC and 7.96 ppm of PAHs were found to have accumulated in the whole muscle tissue. These are respectively 25 and 33 times as much as the concentrations in the WSF-HC of the exposure water (Table 4.12). The GLC pattern of tainting WSF-HC accumulated in the whole muscle tissue was similar to the original WSF-HC (Figs. 4.22a and 4.22b). PAHs in the fish accounted for 63.5% of the total tainting WSF-HC (Table 4.12), compared with only 47.5% in the exposure water. There were virtually no alkanes longer than n-nonane accumulated in the whole muscle tissue (Table 4.12). Toluene and xylenes were the major components among the volatile WSF-HC in the whole muscle tissue. The unresolved baseline envelope of the GLC chromatograms emerging prior to pristane appeared to be the same as that in the WSF-HC of K-D diluent (Figs. 4.22a and 4.22b).

Fig. 4.23a shows the rapid depuration of total tainting WSF-HC and PAHs from the whole muscle tissue. After 7 days in clean water, the tainting WSF-HC in the whole muscle tissue of the Atlantic salmon exposed for 8 h sharply decreased from 12.50 ppm to only 0.88 ppm. The amounts of volatile alkylated benzenes were reduced to the same levels as in the fish exposed for 96 h with lower level of WSF-HC and depurated for 21

Table 4.12. Concentrations (ppb, wet tissue basis) of selected tainting WSF-HC of K-D diluent accumulated and retained in the whole muscle tissue of Atlantic salmon after 8 h and 96 h of exposures and during the depuration in clean seawater. Values are the average of triplicate analyses ± standard deviations. Samples from three fish were pooled for analyses.

| Hydrocarbons | Depuration Time (8 h exposure) | | Depuration Time (96 h exposure) | | | |
|---|---|---|---|---|---|---|
| | 0 Day | 7 Days | 0 Day | 7 Days | 14 Days | 21 Days |
| Toluene | 490 ± 27 | tr | 9 ± 2 | 3 ± 1 | 7 ± 1 | tr |
| Dimethylcyclohexanes | 17 ± 2 | 12 ± 9 | 20 ± 7 | 21 ± 3 | 11 ± 7 | 10 ± 4 |
| *n*-Octane | 9 ± 3 | tr | tr | tr | tr | tr |
| Ethylcyclohexane | 55 ± 2 | 9 ± 7 | 24 ± 7 | 11 ± 8 | 14 ± 11 | 8 ± 5 |
| Ethylbenzene | 253 ± 13 | tr | 17 ± 3 | tr | tr | tr |
| *m*+*p*-Xylenes | 303 ± 15 | 5 ± 0 | 82 ± 15 | 5 ± 1 | tr | tr |
| *o*-Xylene | 249 ± 13 | tr | 63 ± 10 | tr | tr | tr |
| *n*-Nonane | tr[b] | tr | tr | tr | tr | tr |
| Isopropylbenzene | 27 ± 2 | tr | 7 ± 1 | tr | tr | tr |
| Propylbenzene | 49 ± 4 | tr | tr | tr | tr | tr |
| 1-Ethyl-3-methylbenzene | 138 ± 7 | tr | 36 ± 5 | tr | tr | tr |
| 1-Ethyl-4-methylbenzene | 43 ± 5 | tr | 10 ± 2 | tr | tr | tr |
| 1,3,5-Trimethylbenzene | 97 ± 10 | tr | 41 ± 5 | 12 ± 3 | 16 ± 14 | tr |
| 1-Ethyl-2-methylbenzene | 259 ± 80 | tr | 94 ± 14 | 9 ± 2 | tr | tr |
| 1,2,4-Trimethylbenzene | 167 ± 14 | tr | 39 ± 5 | tr | tr | tr |

Table 4.12 (continued)

| Hydrocarbons | Depuration Time (8 h exposure) | | Depuration Time (96 h exposure) | | | |
|---|---|---|---|---|---|---|
| | 0 Day | 7 Days | 0 Day | 7 Days | 14 Days | 21 Days |
| *n*-Decane | 15 ± 2 | tr | tr | tr | tr | tr |
| 1,2,3-Trimethylbenzene | 221 ± 13 | 20 ± 4 | 89 ± 13 | tr | tr | tr |
| *n*-Hendecane | tr | tr | tr | tr | tr | tr |
| Tetramethylbenzenes | 110 ± 9 | 13 ± 3 | 44 ± 4 | 27 ± 1 | 17 ± 6 | 12 ± 3 |
| Naphthalene | 216 ± 53 | 15 ± 2 | 79 ± 9 | 27 ± 2 | 16 ± 2 | 8 ± 2 |
| 2-Methylnaphthalene | 804 ± 42 | 98 ± 13 | 236 ± 30 | 121 ± 10 | 63 ± 5 | 56 ± 7 |
| 1-Methylnaphthalene | 592 ± 31 | 108 ± 11 | 188 ± 26 | 137 ± 12 | 71 ± 5 | 41 ± 4 |
| 2,6-Dimethylnaphthalene | 451 ± 32 | 53 ± 7 | 145 ± 15 | 69 ± 8 | 34 ± 2 | 33 ± 4 |
| Pristane[a] | 21,700 ± 829 | 19,300 ± 2,040 | 12,800 ± 2,020 | 21,600 ± 840 | 19,900 ± 1,700 | 19,900 ± 2,130 |
| PAHs in the range of naphthalene to pristane | 7,960 ± 490 | 713 ± 104 | 2,800 ± 283 | 1,040 ± 180 | 520 ± 79 | 503 ± 78 |
| Other tainting aromatics | 2,038 ± 195 | 90 ± 9 | 785 ± 85 | 159 ± 19 | 156 ± 11 | 91 ± 12 |
| Total tainting WSF-HC | 12,500 ± 740 | 880 ± 116 | 4,160 ± 440 | 1,300 ± 193 | 741 ± 131 | 624 ± 130 |

[a] originally present in the muscle tissue, not from K-D diluent

[b] trace (<5 ppb)

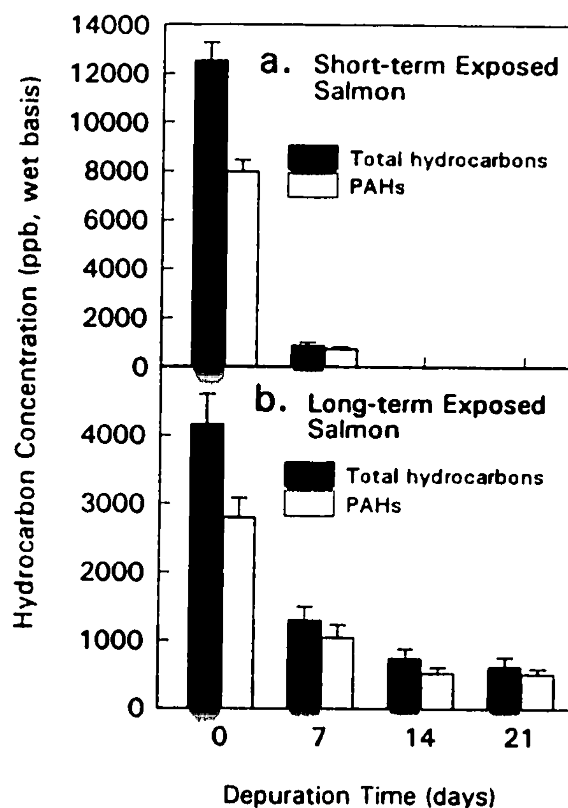


Fig. 4.23. Depuration of total tainting WSF and PAHs in the whole muscle tissue of Atlantic salmon exposed to the WSF of K-D diluent for 8 h (a) and 96 h (b).

days (Table 4.12). The residual tainting WSF-HC were mainly PAHs (81%).

#### 4.7.3.5 Long Term Exposure - Accumulation and Depuration of WSF-HC in the Whole Muscle Tissue of Atlantic Salmon

The average concentrations of total WSF-HC and of the total PAHs in the exposure water column were 0.077 ppm and 0.040 ppm respectively during the 96 h exposure period. At the end of exposure, 4.16 ppm of total tainting WSF-HC and 2.80 ppm of total PAHs were accumulated in the whole muscle tissue; these levels are respectively 54 and 70 times higher than those in the water column and are twice as high as those in the salmon muscle exposed for 8 h. The WSF-HC profile accumulated in the whole muscle tissue was also quite similar to the WSF-HC present in the exposure water. However, compared with fish exposed for 8 h, the most volatile alkylated benzenes in the 96 h exposed Atlantic salmon were present in much lower proportions (Table 4.12). For example, toluene was present at the level of only 9.3 ppb in the whole muscle tissue of the 96 h exposed fish, accounting for only 0.2% of the total accumulated WSF-HC whereas in the 8 h exposed salmon it was 3.9%.

The depuration pattern of the accumulated WSF-HC in the salmon exposed for 96 h was substantially different from that of the 8 h exposed fish. During the first 7 days of depuration, Atlantic salmon were freed of the tainting WSF-HC at a slower rate than from the fish exposed for 8 h. The content fell from 4.16 ppm to 1.30 ppm, accounting for 69% of the total accumulated WSF-HC, while for the 8 h exposed salmon, as much as 93% of the total WSF-HC was depurated during the same period of time. Fig. 4.23b shows the changes in concentrations of total tainting WSF-HC and PAHs in the whole muscle tissue during the 21 days of depuration. The lower molecular weight aromatic hydrocarbons, mainly alkylated benzenes, were depurated from the whole muscle tissue much more rapidly than other WSF-HC and after only 7 days of depuration approached the levels finally achieved after 21 days (Table 4.12). The

remaining tainting WSF-HC at the end of 7 days of depuration were mainly composed of PAHs (80%).

The depuration rate for the remaining higher molecular weight tainting WSF-HC was much reduced after day 7 and entered a slow depuration stage. Only 42.8% of the remaining tainting WSF-HC and 50.0% of the total PAHs were depurated between day 7 and day 14. During the depuration period from day 14 to day 21, the depuration rates further decreased and there were only 15.7% and 3.2% reductions from day 14 levels of the total remaining tainting WSF-HC and PAHs respectively (Fig. 4.23b). Fig. 4.24 compares the partial chromatograms of hydrocarbons isolated from a series of depurated muscle tissues of the long term exposed fish, emphasizing the regions of four carbon alkylated benzenes and PAHs. It is quite clear that despite the rapid discharge of accumulated PAHs during the first 7 days of depuration the complete clearance of PAHs could certainly take months.

The changes in individual WSF-HC during the depuration period display trends similar to those of the total or groups of WSF-HC. Figs. 4.25a and 4.25b show the depuration of two typical volatile WSF-HC (toluene and o-xylene) and two PAHs representatives (1-methylnaphthalene and 2,6-dimethylnaphthalene) from both the short term and the long term exposed fish. Toluene and o-xylene in both the 8 h and the 96 h trials were completely depurated in the first 7 days of depuration (Fig. 4.25a). The difference in the depuration of tainting WSF-HC between the short term and the long term exposed fish was well demonstrated by the depuration of 1-methylnaphthalene and 2,6-dimethylnaphthalene (Table 4.12, Fig. 4.25b). Although the amounts of these two PAHs in the short term exposed fish were initially over three times higher than those in the long term exposed fish, 7 days of depuration eventually reversed the order, i.e., there was then less 1-methylnaphthalene and 2,6-dimethylnaphthalene in the muscle of the short term exposed fish than remained in the muscle of the long term exposed fish. The overall depuration rates of these two PAHs in the 96 h exposed salmon were rather slow and reflects their persistent retention, potentially a characteristic of marine organisms

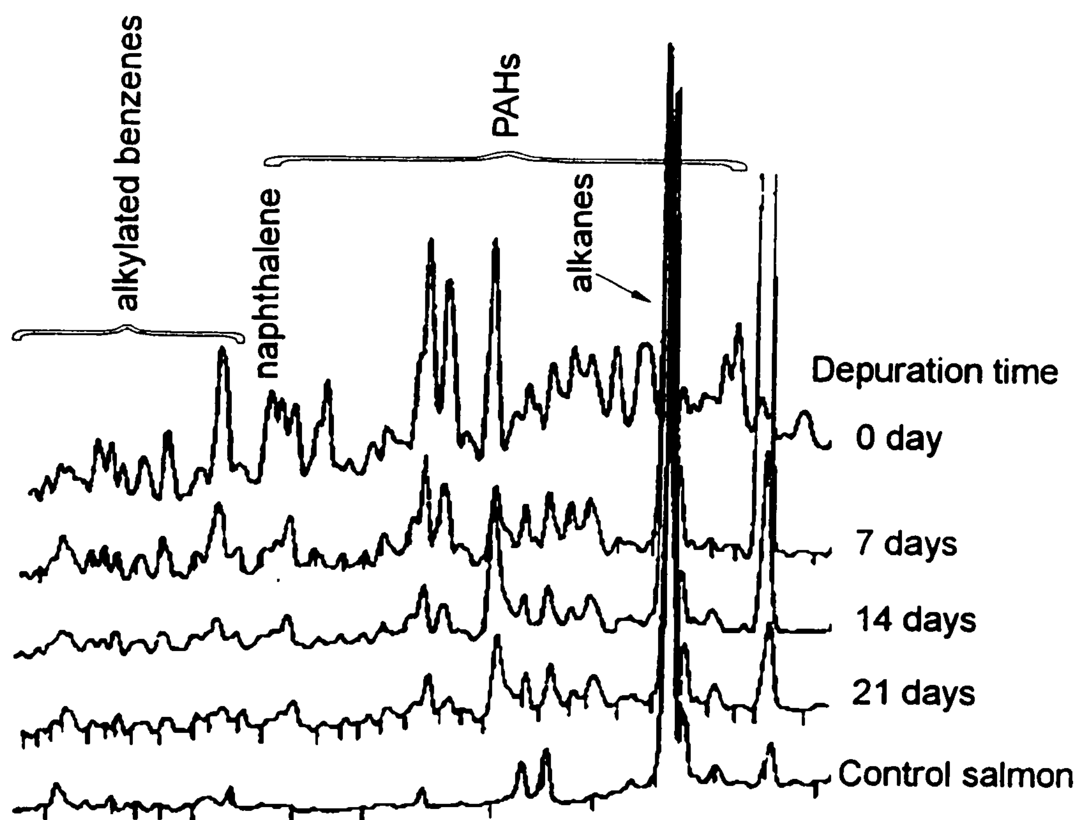


**Fig. 4.24. Partial GLC chromatograms of hydrocarbons recovered from the whole muscle tissue of the control and the tainted Atlantic salmon sampled at different depuration stages. The fish were exposed to 0.077 ppm WSF of K-D diluent for 96 h.**

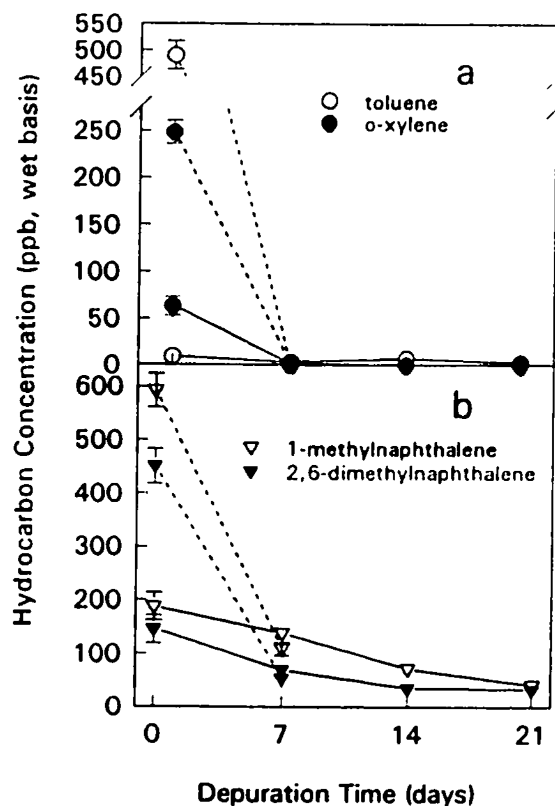


Fig. 4.25. Changes in the concentrations of individual volatile aromatics (toluene, o-xylene) and individual PAH (1-methylnaphthalene, 2,6-dimethylnaphthalene) recovered from the whole muscle tissue of both the short term (8 h, dotted line) and the long term (96 h, solid line) exposed Atlantic salmon during the 21 days of depuration period.

chronically exposed to such materials.

### 4.7.4 Discussion

The composition of the WSF-HC of K-D diluent depends on a combination of several factors: mixing ratio of the diluent and seawater, temperature, air exchange inside and outside the mixing tank, degree and duration of agitation, settling time, storage time before being admitted to the exposure tank etc. (Heras et al. 1995). The higher the temperature, the proportion of diluent to seawater, or the degree of agitation, the more WSF-HC are apt to partition into the seawater from the bulk hydrocarbon phase. There is also a possible effect of light, hence our use of brown glass bottles in the initial studies.

A wide range of aliphatic and aromatic hydrocarbons are present in the K-D diluent. The actual hydrocarbon profile of those parts of K-D diluent in the water phase is largely determined by the solubilities of hydrocarbons in water rather than by the abundance of individual hydrocarbons in the diluent. The virtual absence of *n*-alkanes higher than n-nonane in the water phases is primarily due to their very low solubilities in water compared with those of the aromatic hydrocarbons (McAuliffe 1966, Sutton and Calder 1974, Burris and MacIntyre 1984). Among the aromatics, it is the number of aromatic rings and the number of alkyl carbons substituted on the rings which determine their solubilities in the water phase. The fewer the aromatic rings and the alkyl carbon substituents on the rings, the higher are their solubilities in water. On the other hand, lower molecular weight aromatics, i.e., alkylated benzenes, have relatively higher vapour pressures and therefore are lost more easily in an open environment than the higher molecular weight PAHs. Considering the faster depuration rates of the lower molecular weight aromatics accumulated in marine organisms, the predominant detection of PAHs in naturally tainted marine organisms (Vassilaros et al. 1982, Lawrence and Weber 1984, Granby and Spliid 1995) is understandable. Long term storage of the WSF-HC stock

solutions in the open before admission to the exposure tank would also result in relatively more volatile WSF-HC evaporating from the stock WSF-HC solution. Our introduction of the "bag" storage reduced this effect (Heras et al. 1995).

Water solubilities of hydrocarbons increase with an increase of temperature. Although the influence of temperature is expected to be different on individual hydrocarbons there were no large proportional differences among individual hydrocarbons between the WSF-HC prepared at 3°C, 8.5°C and 23°C. Thus, the effect of changes in temperature would mainly contribute to the changes in total WSF-HC concentrations rather than to the proportions of individual WSF-HC for the WSF-HC prepared in an isolated environment.

The profile of crude petroleum WSF-HC created by natural processes in an open sea environment would be different from that of the laboratory-prepared WSF-HC. The major difference would be the rapid evaporation of volatile hydrocarbons from seawater and K-D diluent because of wind and wave movement, a large water surface area exposed to air, the very low ratio of crude petroleum relative to the volume of seawater and any effect of UV light. In some circumstances a continuous supply of hydrocarbons such as those of K-D diluent to the WSF-HC from underwater oil seeps could be a complicating factor. In assessing natural contamination from a persistent source, fish from more remote locations could reflect exposure to WSF-HC mainly composed of high molecular weight hydrocarbons, especially PAHs with little or no volatile aromatics. Oil spills from marine disasters near shorelines can be reinforced by shore deposits when tides are high but presumably these would also be low in volatiles and high in PAHs.

Some of the background hydrocarbons in the non-exposed salmon could have come from the continuous supply of sand-filtered seawater which included very low "normal" seawater levels of dissolved hydrocarbons (Parrish et al. 1992). A more likely source of the accumulated background of hydrocarbons would be the commercial maintenance diet. Both the fish meal and fish oil (probably herring oil) ingredients would be suspect as sources of natural and/or petrogenic hydrocarbons, particularly

pristane, a common component of fish oils (Ackman 1971). Contamination by diesel fuels or machinery lubricants could take place during transportation or fish reduction for meal and oil, and diet pellet fabrication.

Both the short term and the long term exposure demonstrated that the WSF of K-D diluent hydrocarbons were readily accumulated in the muscle tissue of Atlantic salmon. The hydrocarbons extracted from the salmon muscle tissue also displayed a profile identical to those transferred from K-D diluent to seawater. The substantially higher concentrations of WSF-HC in the whole muscle tissue than of those in the water column are primarily due to the strong lipophilic nature of various organic components in the muscle tissue compared with those of the water molecules. This effect is particularly evident for the muscle tissue of farmed Atlantic salmon in which high proportions (10-16%) of lipid are normally found. It is primarily the difference in the lipid contents of marine organisms that causes the species-specific characteristics of hydrocarbon accumulation and retention (Ernst et al. 1987, Ernst et al. 1989, Heras et al. 1992).

Although fish can rapidly take up hydrocarbons from water, the attainment of equilibrium of hydrocarbon partitioning between seawater and tissue fluids, and even between different compartments (mainly adipocytes, muscle cells) in the tissue, would be a long term process during hydrocarbon exposure. The lower bioconcentration factor of WSF-HC accumulation in the whole muscle tissue during the short term exposure suggests that 8 h exposure was far from reaching an equilibrium between the WSF-HC in the exposure water and the WSF-HC in the muscle tissue.

The uptake and depuration of WSF-HC by marine organisms proceeds through dynamic processes and the net effect of inward and outward transfer of WSF-HC through fish tissues at the early stage of exposure leads to the continuous accumulation of WSF-HC in the muscle tissue. It should however be recalled that the water content of muscle includes extracelluar fluids which are in effect seawater. The fluids inside cells are similar but behind a lipophilic barrier. The inward transfer begins immediately when the fish are placed in the exposure tanks. The volatile hydrocarbons in water usually

decrease somewhat in concentration as the exposure proceeds; therefore, due to the constant solubilities of PAHs in seawater, an outward transfer of WSF-HC during the exposure period will eventually result in a higher proportion of volatile WSF-HC than of the less volatile ones being released back to the seawater. This leads to the increase of PAH proportions in the total WSF-HC concentrated in the tissue as the exposure proceeds. The longer the exposure period, the higher the proportion of PAHs accumulated in the tissues (Fig. 4.23).

The two basic cell compartments present in the muscle tissue are muscle cells and adipocytes. The muscle cells constitute the major part of the muscle tissue. Previous studies with salmon (Section 4.4) have shown that both adipocytes and normal muscle cells can be heavily tainted by WSF-HC at the end of a 96 h exposure but these WSF-HC were found to be much more concentrated in the adipocytes than in the muscle cells. On the other hand, the release of WSF-HC tended to be much faster from the muscle cells than from the adipocytes. Consequently, the rapid release of WSF-HC from the whole muscle tissue at the initial stage of depuration mainly reflects the discharge of WSF-HC from inter- and intracellular fluids of the muscle cells.

The WSF-HC clearing processes take place in two ways: (1) Physical transference of WSF-HC from the tissue to the water column by partition and diffusion, and (2) transfer of WSF-HC and metabolites to the liver and finally, by excretion of metabolic conversion products in the bile. The latter process is normally much slower than the physical discharge of WSF-HC in the heavily tainted fish. It is clear from our exposure trials that WSF-HC in the long term exposed fish tended to be retained in the muscle tissue for a longer period time than those in the short term exposed fish; in other words, the clearing processes for the long term accumulated WSF-HC were much slower than those for those accumulated in the short term exposure. This is primarily due to the non-equilibrium partitioning of WSF-HC between the muscle cells and adipocytes in the muscle tissue at the end of short term exposure since transportation of hydrocarbons from blood to the muscle cells seems to be easier than into the adipocytes. As a result, a

higher proportion of the total WSF-HC in the muscle tissue would be present in the muscle cells or intercellular fluids after the 8 h exposure than that of the 96 h exposure. Due to the hydrophillic characteristic of the muscle cells, WSF-HC in these compartments would be released back to the water column much faster than those that had dissolved into the adipocytes.

The large difference in the depuration rate of WSF-HC between the 8 h and 96 h exposed fish relates to the commonly expected demand for speed in cleaning up the obvious accumulated hydrocarbons of any petrogenic oil spill. In the case of a large area oil spill, traces of WSF-HC would be present in the spill or nearby areas for a long period of time. Fish might avoid the most contaminated area, but they are less likely to escape from a wider area where trace WSF-HC levels would be present in the water. Such WSF-HC materials would be expected to be present in the water for a fairly long time period after the spill although the amount might be below chemical detection limits. As a result, the fish would in fact experience a much longer exposure period than the 96 h exposure and hydrocarbons in the fish would subsequently be released in a much slower way. Chronic exposure is the most adverse circumstance as demonstrated by our comparison of short term and long term exposure of Atlantic salmon.

# 5. PREPARATION AND CHARACTERIZATION OF A WATER-SOLUBLE FRACTION OF CRUDE OIL BY A KARR RECIPROCATING-PLATE COUNTERCURRENT EXTRACTION COLUMN

## 5.1 Introduction

The pollution of the aquatic environment caused by oil spills has led to serious problems for aquatic life. Investigation of the toxicity of crude oil contamination has revealed that the toxicity of oils is apparently due to the water-soluble fraction rather than to dispersed droplets (Rice et al. 1977). The most immediate effect of oil pollution once a slick is dispersed is the intimate contact of the WSF-HC with fish or other marine organisms.

Many experiments have been conducted on the exposure of different marine and freshwater organisms to various dilutions of the WSF-HC (Davis et al. 1992, Heras et al. 1992, Lockhart and Danell 1992, Vignier et al. 1992); the preparation of the WSF-HC is one of the critical steps necessary to obtain reproducible and precise results. It is difficult to prepare a reproducible WSF-HC stock solution and to continuously supply a fresh stock solution in order to maintain a stable chemical environment in exposure tanks. Different laboratories have used a variety of methods to prepare a WSF-HC, with a wide range of mixing forces, water to oil ratios, and times of settlement (Boylan and Tripp 1971, Katz 1973, Anderson et al. 1974, Smith and Cameron 1979, Heras et al. 1992). Maher (1982) tended to justify his choice of a mixing method based on gentle oscillation rather than turbulent stirring as this appeared to simulate the most probable environmental situation. However, many different mixing mechanisms occur in the marine environment and it is impossible to duplicate these natural processes in a laboratory.

Most of the current methods for making a WSF-HC are batch operations, and the stock solution is usually metered into the exposure tanks either continuously (Heras et

al. 1992) or at certain time intervals (Keck et al. 1978). The exposure may also be conducted with no addition of WSF-HC during the entire experimental period (Lee and Nicol 1977). The concentration and composition of the stock solution can change due to any or all of evaporation, biodegradation, photo-oxidation, and adsorption of the WSF-HC on surfaces during the experimental period (Rice et al. 1977; Moles et al. 1979). This will result in serious variations in the desired WSF-HC concentration during an experiment, especially when a longer-term exposure is required. Continuous-flow test systems have been suggested by Nunes et al. (1978) and Johannessen (1983) to solve these problems, but owing to complications associated with the physical and chemical properties of oils, they have not been widely adopted.

We have, therefore, investigated the continuous production of a WSF-HC. Operation of continuous countercurrent extraction is conducted when one of the phases is dispersed and allowed to pass continuously and countercurrently through the other phase, which is not dispersed. This operational system theoretically gives maximum "efficiency" under equilibrium states (Treybal 1973). Equipment in this category that has been studied and developed includes mechanically assisted gravity devices and centrifugal extractors (Goldberg 1973). Performance data for a 3-inch diameter open type of reciprocating extraction column were first reported by Karr (1959). Since then, various studies on the performance and applications of this type of column have been conducted, including those with a laboratory scale 1-inch diameter column (Lo and Karr 1972). These investigations revealed that the reciprocating extraction column had features of simple construction and easy operation, high versatility and extraction efficiency, and reasonable capacity. The purpose of our work was to investigate the suitability of using a reciprocating plate extraction column for the preparation of WSF-HC. This would provide fresh, reproducible, petroleum water-soluble fractions on a demand basis as stock solutions for long-term exposures of marine organisms to WSF-HC.

## 5.2 Materials and Methods

### 5.2.1 Preparation of Water-Soluble Fraction

Flotta North Sea crude oil, obtained from the Dartmouth, Nova Scotia refinery of Esso Petroleum Canada, was used to prepare the water-soluble fraction. Sand-filtered seawater was supplied by the Aquatron Laboratory of Dalhousie University.

Fig. 5.1 is an outline of the method for the preparation of the WSF of hydrocarbons from crude oil using a Karr reciprocating plate extraction column. This column was a product of PEGASUS Industrial Specialties Ltd., Agincourt, Ontario (Model KC-1-6, diameter 2.54 cm, plate spacing 5.3 cm, plate stack height 1.83 m). It was fitted with a reciprocating speed adjustment device. The stroke amplitude of the reciprocating plates was set at 2.54 cm. A stainless steel Duplex pump (FR 221-A-117 Frame A, Milton Roy Industries LTD), and a Masterflex pump (Cole-Parmer Instrument Co., Niles, IL) with a standard pump head (Model 6212-14) and Viton tubing, were used to supply seawater and crude oil respectively. The seawater was metered into the column via the nozzle above the reciprocating plates. Similarly, the crude oil entered the column at a controlled rate via the nozzle below the reciprocating plates. Before admitting crude oil, the entire column was filled with seawater. The interface was established at the midpoint of the upper separation section by adjusting the hand-controlled valve in the bottom discharge line through which the stock WSF-HC solution exited from the column. The reciprocation speeds were varied with the speed adjusting handwheel. Three flow rates of seawater (220, 130, and 66 mL/min), including the maximum and minimum speeds achievable with this pump, were selected and a flow rate of 2.9 mL/min was chosen for crude oil. Reciprocation speeds of 55, 130, 170, 220, 260 strokes per min corresponding to the scale readings of 2, 4, 5, 6, 7 were adopted for the experiments. The extractions were conducted at room temperature (25 °C). After equilibrium the stock WSF-HC solution was collected into a separatory funnel (8 L) and

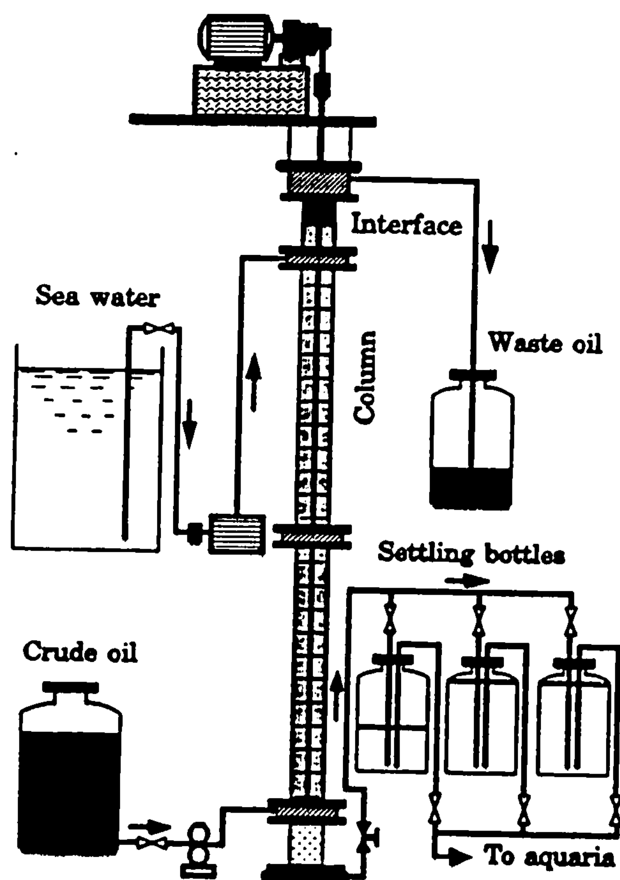


Fig. 5.1. An outline of equipment used for the preparation of water-soluble fraction of hydrocarbons from crude oil using Karr reciprocating plate extraction column.

settled in a cold room (5°C) for 5 h before analyses. A water-soluble fraction was also prepared by a normal batch extraction method (400 L per batch) as reported by Heras et al. (1992).

### 5.2.2 Extraction of WSF-HC for Gas-Liquid Chromatography

The WSF-HC in the stock solution were recovered according to the Murray microextraction procedure (Murray 1979; Murray et al. 1984); an extraction flask of 1 L capacity was constructed with a side arm and a capillary tube at the top-centre of the flask as described by Murray (1979). The detailed procedures for the extraction of WSF-HC from water has been described in Section 4.3.2.

### 5.2.3 Gas-Liquid Chromatography

The WSF-HC analyses were analyzed by GLC-FID. The analysis conditions were the same as described in Section 4.3.2.

## 5.3 Results and Discussion

### 5.3.1 Extraction of the Water-Soluble Fraction

Many experimental parameters affect the extraction of WSF-HC from crude oil, i.e. type of oil, degree and duration of agitation, ratio of crude oil to seawater, and settling time required to achieve a stable distribution of hydrocarbons between the aqueous and oil phases. The results showed that the concentrations of continuously extracted WSF-HC were principally related to the reciprocation speeds of the column agitator, whereas the throughputs of the stock solution had little effect under the experimental conditions (Fig. 5.2). Two critical reciprocation speeds were observed.

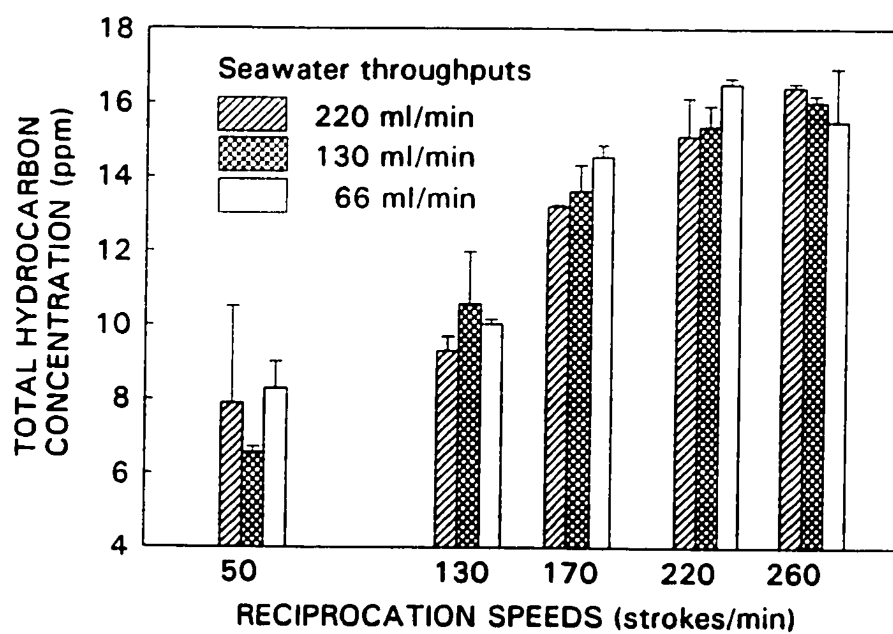


Fig. 5.2. Effect of reciprocation speeds and seawater throughput on the extraction of WSF from crude oil.

Below 130 strokes/min the crude oil droplets were not well dispersed and much less WSF-HC was extracted compared to higher speeds. At or above 170 strokes/min dispersion of the crude oil was excellent and the concentration of the lower molecular weight hydrocarbons increased sharply (Fig. 5.3). At the reciprocation speed of 170 strokes/min the effect of seawater flow rate on the extraction of hydrocarbons into WSF was very low, with coefficients of variation of only 4% (flow rate was varied from 66 mL/min to 220 mL/min).

The reproducibility for the extraction of WSF-HC using this column was tested. The results of Table 5.1 revealed that the reproducibility of this column method was much better than that produced by normal batch extraction method (400 L each time). The total WSF-HC concentration was 13.5 $\pm$ 0.3 mg/kg (n=6) extracted at reciprocation speeds of 170 strokes/min and a seawater flow rate of 220 mL/min; with the normal batch extraction it was only 11.03 $\pm$ 2.33 (n=12).

### 5.3.2 Separation of Dispersed Crude Oil from the Aqueous Phase

A period of time is needed for the subsequent separation of dispersed oil droplets from the water phase. Two well-resolved GC peaks are convenient for this assessment. The ratio of $C_{13}$ *n*-alkane + 2-methylnaphthalene to 1-methylnaphthalene was used as an indicator for the involvement of undissolved oil droplets in seawater as it is well recognized that the *n*-alkanes from $C_{11}$ upward generally have a very low solubility in water compared with aromatic hydrocarbons (McAuliffe 1966, Sutton and Calder 1974, Clark et al. 1977, Burris and MacIntyre 1984). After one hour of settlement, many oil droplets were still present as indicated by the high ratio of alkanes to aromatic hydrocarbons, but the stock solution was almost free of oil droplets after two hours of settlement. Further settlement continuously decreased crude oil inclusion but at a very slow rate. The stock solution was considered to be essentially free of crude oil droplets after five hours of settlement.

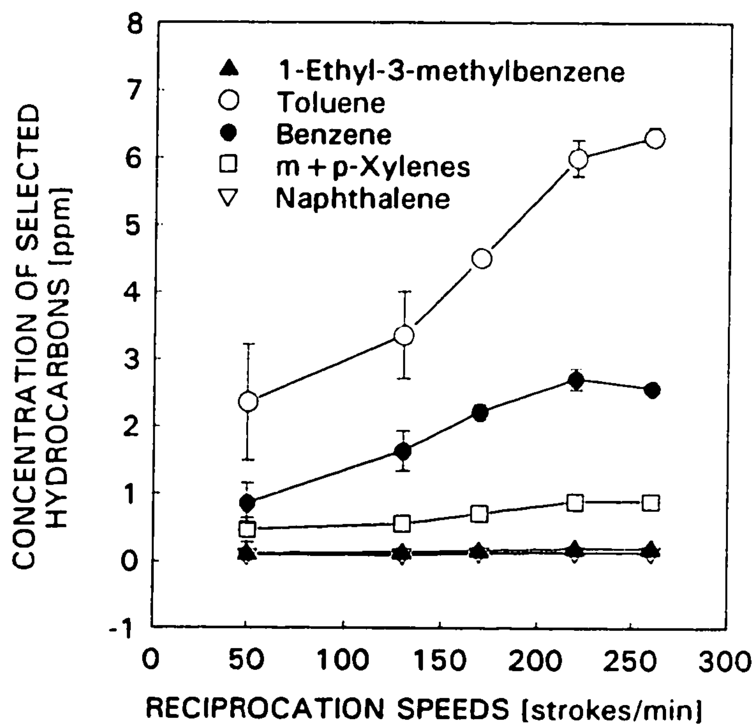


Fig. 5.3. Effect of reciprocation speeds on the extraction of individual hydrocarbons from Flotta North Sea crude oil.

Table 5.1. Relative concentration of hydrocarbon components in stock water-soluble fraction extracted from Flotta North Sea crude oil by a Karr reciprocating plate countercurrent extraction column and by a usual batch extraction method. Reciprocation speed, 170 strokes/min; throughput, 220 mL/min. Values are mean ± SD of results from three separate extractions.

| Component | Concentration | |
|---|---|---|
| | Column method | Batch method |
| Total hydrocarbons (mg/kg) | 13.51 ± 0.30 (n=6) | 11.03 ± 2.33 (n=12) |
| Aromatics (wt%) | | |
| Benzene | 15.82 ± 0.67 | 22.24 ± 5.14 |
| Toluene | 36.21 ± 1.64 | 31.94 ± 2.55 |
| Ethylbenzene | 3.64 ± 0.16 | 4.99 ± 0.53 |
| *m+p* Xylene | 5.38 ± 0.24 | 7.69 ± 0.92 |
| *o*-Xylene | 3.31 ± 0.16 | 4.80 ± 0.61 |
| Isopropylbenzene | 0.37 ± 0.01 | 0.65 ± 0.18 |
| Propylbenzene | 0.73 ± 0.03 | 1.16 ± 0.25 |
| 1-Ethyl-3-methylbenzene | 1.18 ± 0.06 | 2.22 ± 0.70 |
| 1-Ethyl-4-methylbenzene | 0.44 ± 0.03 | 0.40 ± 0.29 |
| 1,3,5-Trimethylbenzene | 0.17 ± 0.01 | 0.27 ± 0.05 |
| 1-Ethyl-2-methylbenzene | 0.60 ± 0.03 | 0.94 ± 0.15 |
| 1,2,4-Trimethylbenzene | 0.72 ± 0.04 | 1.13 ± 0.18 |
| 1,2,3-Trimethylbenzene | 0.60 ± 0.04 | 1.00 ± 0.21 |
| Tetramethylbenzenes | 0.09 ± 0.00 | 0.15 ± 0.02 |
| $C_4$-benzenes | 0.07 ± 0.03 | 2.80 ± 1.41 |
| Naphthalene | 0.88 ± 0.07 | 0.94 ± 0.67 |

Table 5.1 (continued)

| Component | Concentration | |
|---|---|---|
| | Column method | Batch method |
| 2-Methylnaphthalene | 0.55 ± 0.03 | 0.64 ± 0.22 |
| 1-Methylnaphthalene | 0.45 ± 0.01 | 0.56 ± 0.29 |
| 1,1'-Biphenyl | 0.02 ± 0.00 | 0.03 ± 0.02 |
| Ethylnaphthalene | 0.12 ± 0.01 | 0.05 ± 0.04 |
| Dimethylnaphthalenes | 0.32 ± 0.05 | 0.54 ± 0.26 |
| Propylnaphthalene | 0.13 ± 0.07 | 0.00 ± 0.00 |
| $C_3$-Naphthalenes | 0.12 ± 0.07 | 0.28 ± 0.07 |
| Total aromatics | 71.92 ± 3.46 | 85.42 ± 14.76 |
| Alkanes(wt%) | | |
| Cyclohexane | 11.05 ± 0.65 | 4.88 ± 3.71 |
| 2-Methylhexane | 0.63 ± 0.04 | 0.48 ± 0.47 |
| Isooctane | 1.35 ± 0.13 | 1.25 ± 0.50 |
| *n*-Heptane | 2.01 ± 0.08 | 0.38 ± 0.05 |
| Methylcyclohexane | 4.41 ± 0.09 | 2.33 ± 0.34 |
| Dimethylcylcohexanes | 1.00 ± 0.07 | 0.91 ± 0.19 |
| *n*-Octane | 0.62 ± 0.10 | 0.27 ± 0.07 |
| Ethylcyclohexane | 0.27 ± 0.02 | 0.23 ± 0.06 |
| *n*-Nonane | 0.06 ± 0.01 | 0.02 ± 0.01 |
| *n*-Decane | 0.20 ± 0.02 | 0.17 ± 0.05 |
| *n*-Hendecane | 0.11 ± 0.03 | 0.14 ± 0.03 |
| *n*-Dodecane | 0.02 ± 0.00 | 0.04 ± 0.03 |
| Total Alkanes[a] | 21.73 ± 1.24 | 11.10 ± 5.51 |
| Unknown (wt%) | 5.12 | 3.48 |

[a] except alkanes of $C_{13}$ upward.

### 5.3.3 Characteristics of the Water-Soluble Fraction

The two methods presented the same profile of water-soluble hydrocarbons dissolved in water (Table 5.1) but the column method had slightly lower aromatic hydrocarbon percentages and higher alkane percentages (71.92% and 21.73%) compared with those from the normal batch extraction method (85.42% and 11.1%). The slight differences of WSF-HC composition were probably caused by the evaporation of low boiling alkane components during the long term mixing (24 h) and settling periods (48 h) of the normal batch extraction method. In both methods, the aromatic hydrocarbons were highly enriched (see column WSF-HC, Fig. 5.4b) compared with the crude oil hydrocarbon profile (Fig. 5.4a). The alkanes from $C_{11}$ upward were negligible in the WSF-HC solution, which means that it was essentially free of crude oil suspension. The monoaromatic hydrocarbons such as benzene, toluene, xylene and ethylbenzene, were the main components among the aromatic hydrocarbons. These are the major sources of the toxicity of crude oil and were largely responsible for the fluctuations of WSF-HC concentration in different batch extractions. Our experimental results on composition of the WSF-HC extracted from Flotta North Sea crude oil were quite similar to those previously reported by Heras et al. (1992) and by Widdows et al. (1982) who worked with the same oil. Fig. 5.5 is a typical GLC chromatogram of the water-soluble hydrocarbons extracted by the reciprocating extraction column.

Fig. 5.3 shows the partitioning equilibrium between oil and water attained at different reciprocation rates for individual WSF-HC. Benzene is the most soluble component and the solubility of the five compounds shown in Fig. 5.3 decreases in the following order: benzene, toluene, *m+p*-xylene, 1-ethyl-3-methylbenzene and naphthalene (Clark et al. 1977). Due to the low solubility of naphthalene and 1-ethyl-3-methylbenzene in seawater, partitioning equilibrium is rapidly attained even at low reciprocation speeds (Fig. 5.3). Toluene showed the largest change in concentration in seawater with increases in reciprocation speeds, although its solubility was three times

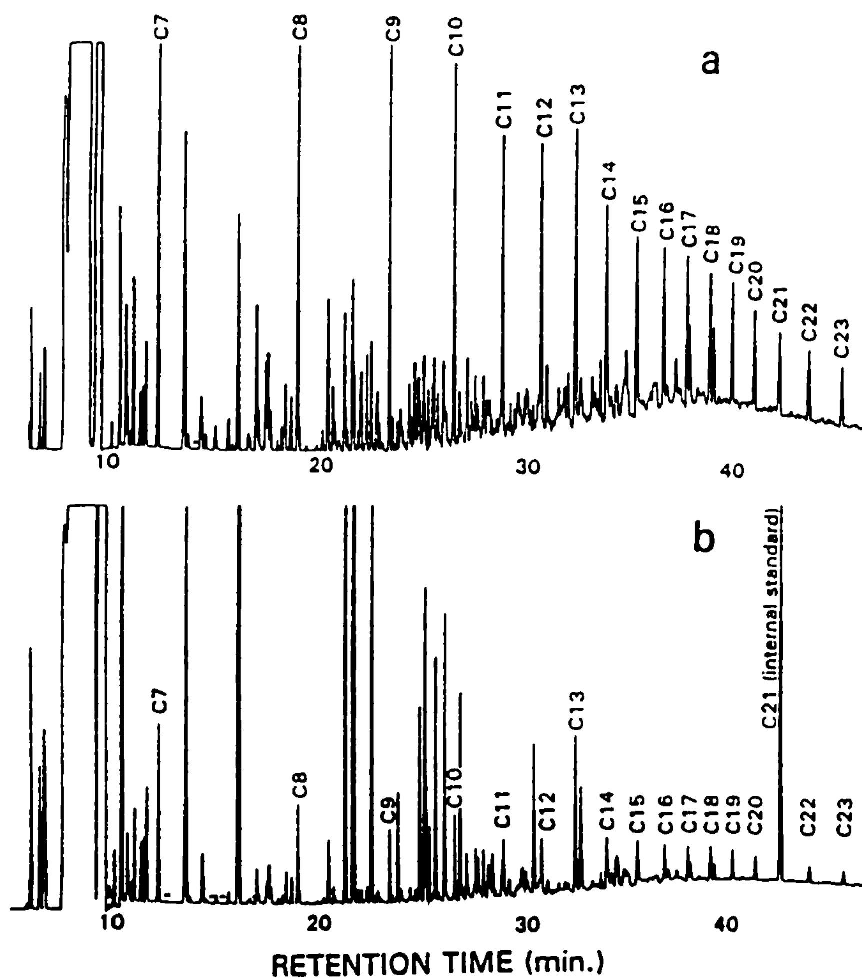


Fig. 5.4. (a) GLC chromatogram of Flotta North Sea crude oil; (b) GLC chromatogram of water-soluble fraction extracted from the same crude oil by reciprocating plate extraction column under conditions of an excessive reciprocation speed (260 strokes/min) at a flow rate of seawater of 220 mL/min and a flow rate of crude oil of 2.9 mL/min; settling time, 5 h at 5 °C.

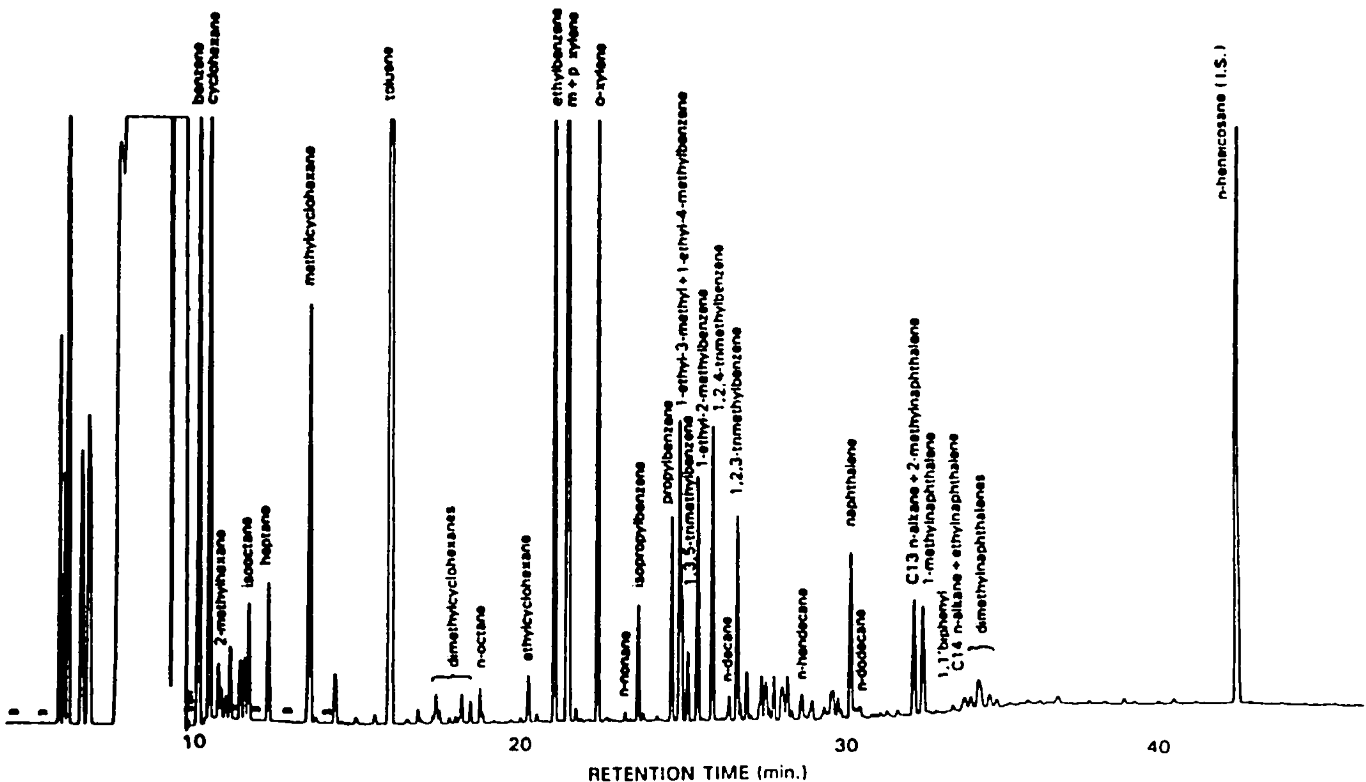


Fig. 5.5. GLC chromatogram of water-soluble fraction extracted by reciprocating plate countercurrent column under ideal conditions of reciprocation speed (170 strokes/min) at a flow rate of seawater of 220 mL/min and a flow rate of crude oil of 2.9 mL/min; settling time, 5 h at 5°C.

less than that of benzene. There is much more toluene than benzene present in crude oil and the combination of solubility in water and amount in crude oil results in a higher concentration of toluene present in the WSF-HC. The increase in reciprocation speeds has a direct effect on the contacting area between the two phases. The higher the contacting area, the faster the mass transfer. Fig. 5.3 also shows that reciprocation speeds between 130-220 strokes/min were more effective for the extraction of low boiling aromatics than other hydrocarbons. At reciprocation speeds above 220 strokes/min, this benefit diminished. The fine oil droplets produced under higher reciprocation speeds caused serious problems in the separation of these droplets from seawater and larger amounts of crude oil were still present in the water phase even after five h of settlement. Fig. 5.4b is a GLC chromatogram of the water soluble fraction prepared at a reciprocation speed of 260 strokes/min analyzed after five h of settlement at 5°C. It still somewhat resembles the parent crude oil (Fig. 5.4a) in respect to n-alkanes (compare Fig. 5.5 of a superior WSF-HC also prepared with the Karr column).

### 5.3.4 Practical Applications

It is difficult to compare the experimental results reported for bioassay or exposure studies because of different oils and methods used in the preparation of a WSF-HC, and consequent variations in the concentration and composition of a WSF-HC from batch to batch. It was clear that the major variation is related to the more volatile components, *i.e.*, benzene and toluene. There are two types of biological effects of the WSF-HC on marine organisms, namely acute and chronic; the former is caused primarily by the low boiling aromatics, whereas the latter might be caused by all fractions, but the most important substances are probably still those with relatively low volatility. It is quite obvious that a preparation of a WSF-HC with a reproducible high amount of lower boiling aromatics is very important for the exposure or bioassay experiments.

Another recurring difficulty has been the effect of vapor losses from the WSF-HC

solution during exposure or bioassay experiments, resulting in exposure to decreasing concentrations of toxicants. Anderson et al. (1974) found that gentle aeration of the oil-in-water dispersions resulted in a loss of 80 to 90% of the aqueous hydrocarbons in 24 h. Craddock (1977) recommended that additional aeration should not be used with volatile toxicants, but suggested that if the dissolved oxygen levels are below saturation, aeration may be used. To solve these problems, a fresh, reproducible stock WSF-HC solution should be continuously added to the exposure tanks. When diluted with fresh seawater it can bring enough oxygen to support the experimental organisms and avoid stress. In contrast to the large (70 L) WSF-HC storage reservoirs from our batch operations (Heras et al. 1992) a number of 4 L settling bottles were used for the collection from the column, settling (2 h), and continuous supplying of WSF-HC to our fish exposure tanks. Only 30 min to 2 h were needed to empty one bottle of stock WSF-HC solution. During this short time the composition was stable. Extraction of crude oil using a reciprocating countercurrent column could provide an ideal way to meet all requirements of continuous preparation of WSF-HC if a continuous centrifuge step was also provided for fast removal of the fine droplets from the WSF-HC.

The efficiency of the extraction of WSF-HC by the reciprocating plate column depends on the flow rate of seawater, ratio of seawater to crude oil, and stroke amplitude, but particularly on the reciprocation speed as also reported by Lo and Karr (1972). Both high and low reciprocation speeds are not suitable due to the incomplete extraction of crude oil, high variations in the WSF-HC concentration, and the formation of emulsions. Irrespective of different column models, the most suitable speed would be the point at which the crude oil is just completely dispersed into droplets, and no ascent of the crude oil along the column wall or reciprocating rod is observed. If the ratio of water to crude oil is to be further increased, i.e. by decreasing the flow rate of crude oil or by increasing the flow rate of seawater, it would also be expected that the WSF-HC solution would present the same composition and no large difference in the concentration would be observed. However, an increase in flow rate of seawater

increased the linear speed of water inside of the column which resulted in more fine droplets of crude oil leaving the column with the seawater. Physical inclusion of crude oil in water could also be observed at high reciprocation speeds.

Oil can become associated with an aqueous phase in a variety of different ways, such as an emulsion, a dispersion (Peake and Hodgson 1966), or dissolved in water. Since some of the hydrocarbons in the water phase were not present in a truly dissolved form, the hydrocarbon composition in these stock solutions closely resembled that of the parent oil. This phenomenon was also observed in the WSF-HC solution prepared by batch extraction methods with violent mixing instead of gentle stirring (Boylan and Tripp 1971, Gordon et al. 1973, Anderson et al. 1974). Shaw (1977) pointed out that unless the settling time was quite long (on the order of weeks or months), the stirring of crude oil and seawater would result in a non-equilibrium, non-steady system containing hydrocarbons associated with water in various ways. However, under the optimized countercurrent extraction conditions (170 strokes/min), most of the crude oil was disengaged from the water phase after only two h of settlement.

Siron et al. (1987) analyzed both total hydrocarbons and non-hydrocarbon components dissolved in seawater, and found that very polar oil compounds were present in the resulting WSF-HC of crude oil. They observed that the solubles included a strong unresolved envelope of a complex mixture of less-volatile hydrocarbons that appeared in the GLC chromatogram after the more volatile hydrocarbons. In the WSF-HC prepared by the column extraction method only a very small envelope of late-eluting materials was found on the GLC chromatograms of the extracts from the cleared stock solution; it became larger as more crude oil remained in the water phase. The envelope of such materials could be removed through treatment of the aqueous extract on active silica gel (Boylan and Tripp 1971) and was due mostly to polar aromatic materials. These materials are presumably a function of the hydrocarbon mixture used and are not associated with the particular method of preparation of the WSF-HC.

## 5.4 Conclusions

The reciprocating plate extraction column for the production of stock WSF-HC solutions provided very stable and reproducible WSF-HC characteristics. The equilibrium rates of extraction of different hydrocarbons were inversely related to their solubilities in the seawater. Higher proportions of low-boiling aromatics were transferred to the water-soluble fraction, an important consideration for tainting or toxicity tests of the WSF-HC of crude petroleum or petroleum products. Compared to batch extraction methods more *n*-alkanes were found in the WSF-HC from the column.

The reciprocating plate countercurrent column is in one sense a duplication of surface mixing in oil spill situations. It is capable of semicontinuously supplying fresh WSF-HC stock solutions which are especially suitable for flow-through experiments of long term exposure or bioassay where the requirement is to continuously provide a metered input of WSF-HC with low fluctuations in composition and concentration during the experiments.

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# 7 APPENDIX

Table A1. Concentrations of spiked hydrocarbon standards in tissues for recovery studies by steam distillation.

| Spiked Hydrocarbons | Concentrations in Tissue Samples (ppm, wet tissue basis) |
|---|---|
| Benzene | 1.31 |
| Cyclohexane | 0.95 |
| *n*-Heptane | 0.73 |
| Methylcyclohexane | 0.65 |
| Toluene | 1.88 |
| *n*-Octane | 0.58 |
| Ethylbenzene | 1.14 |
| *m*+*p* Xylene | 1.06 |
| *o*-Xylene | 0.88 |
| *n*-Nonane | 0.61 |
| Isopropylbenzene | 1.67 |
| Propylbenzene | 1.14 |
| 1,3,5-Trimethylbenzene | 1.11 |
| 1,2,4-Trimethylbenzene | 1.14 |
| *n*-Decane | 0.51 |
| *n*-Undecane | 0.46 |
| 1,2,4,5-Tetramethylbenzene | 0.92 |
| Naphthalene | 0.75 |
| *n*-Dodecane | 0.57 |
| 2-Methylnaphthalene | 1.36 |
| 1-Methylnaphthalene | 1.23 |

Table A2. GLC-FID responses and response factors of hydrocarbon standards. Values are the average of triplicate analyses ± standard deviations.

| Hydrocarbon Standards | GLC-FID Responses[a] | GLC-FID Response Factors[b] |
|---|---|---|
| Benzene | 1.17 | 0.85 |
| Cyclohexane | 1.06 | 0.94 |
| *n*-Heptane | 1.07 | 0.93 |
| Methylcyclohexane | 1.09 | 0.92 |
| Toluene | 1.15 | 0.87 |
| *n*-Octane | 1.07 | 0.93 |
| Ethylbenzene | 1.13 | 0.88 |
| *m+p* Xylene | 1.10 | 0.91 |
| *o*-Xylene | 1.11 | 0.90 |
| *n*-Nonane | 1.11 | 0.90 |
| Isopropylbenzene | 1.14 | 0.88 |
| Propylbenzene | 1.10 | 0.91 |
| 1,3,5-Trimethylbenzene | 1.11 | 0.90 |
| 1,2,4-Trimethylbenzene | 1.10 | 0.91 |
| *n*-Decane | 1.13 | 0.88 |
| *n*-Undecane | 1.04 | 0.96 |
| 1,2,4,5-Tetramethylbenzene | 1.11 | 0.90 |
| Naphthalene | 1.11 | 0.90 |
| *n*-Dodecane | 1.05 | 0.95 |
| 2-Methylnaphthalene | 1.07 | 0.93 |
| 1-Methylnaphthalene | 1.10 | 0.91 |

[a] GLC-FID response = $A \times m_{IS}/A_{IS} \times m$; where: A and m represent the peak area and injection amount of hydrocarbon standard, $A_{IS}$ and $m_{IS}$ are the peak area and injection amount of internal standard n-heneicosane.

[b] GLC-FID response factor = 1/GLC-FID response

Table A3. Recoveries of spiked hydrocarbon standards from white muscle, dark muscle, belly flap and mesentery adipose tissue of Atlantic salmon by steam distillation under experimental design IV (refer to Table 3.1). Values are the average of triplicate analyses ± standard deviations. Different transverse lettering superscripts associated with the percent recovery of each hydrocarbon indicate significant differences between the means of recovery efficiency at $p<0.05$ (only those with superscript letters were tested for statistical significance).

| Spiked Hydrocarbons | White Muscle | Dark Muscle | Belly Flap | Mesentery |
|---|---|---|---|---|
| Benzene | $92.07\pm3.56^{a}$ | $81.80\pm4.94^{ab}$ | $80.05\pm2.50^{b}$ | $74.66\pm3.47^{b}$ |
| Cyclohexane | 86.70±4.26 | 87.68±2.30 | 69.52±3.69 | 63.22±3.28 |
| *n*-Heptane | 81.42±3.90 | 74.22±4.97 | 83.20±1.78 | 66.74±2.54 |
| Methylcyclohexane | 80.51±4.97 | 82.97±3.18 | 77.60±3.27 | 63.03±1.65 |
| Toluene | $83.33\pm4.69^{ab}$ | $88.28\pm2.85^{b}$ | $77.48\pm1.73^{a}$ | $74.83\pm4.21^{a}$ |
| *n*-Octane | 83.49±4.29 | 76.99±5.14 | 66.82±1.65 | 65.34±3.01 |
| Ethylbenzene | 81.03±2.01 | 80.82±3.11 | 70.54±4.72 | 84.32±4.00 |
| *m+p* Xylene | 82.90±5.53 | 72.21±5.88 | 76.62±3.12 | 70.44±2.79 |
| *o*-Xylene | 85.35±5.61 | 74.83±2.21 | 67.09±2.91 | 74.23±3.29 |
| *n*-Nonane | 78.23±3.39 | 73.37±4.13 | 62.22±2.94 | 64.99±2.73 |
| Isopropylbenzene | 76.40±0.41 | 84.70±3.85 | 68.26±1.24 | 63.09±2.10 |
| Propylbenzene | $72.20\pm3.59^{a}$ | $72.46\pm1.77^{a}$ | $73.95\pm2.58^{a}$ | $66.90\pm3.37^{a}$ |
| 1,3,5-Trimethylbenzene | 74.36±4.12 | 84.88±1.32 | 70.55±3.75 | 60.22±1.14 |
| 1,2,4-Trimethylbenzene | 75.90±4.22 | 77.64±2.25 | 68.51±4.34 | 67.03±3.58 |
| *n*-Decane | 67.22±2.23 | 70.66±1.03 | 60.43±3.22 | 62.40±3.39 |
| *n*-Undecane | $60.05\pm1.35^{a}$ | $76.19\pm3.46^{b}$ | $62.79\pm1.96^{a}$ | $53.00\pm2.10^{c}$ |
| 1,2,4,5-Tetramethylbenzene | 65.79±0.67 | 70.10±3.02 | 58.33±2.59 | 52.09±3.59 |
| Naphthalene | 72.73±4.52 | 63.08±2.76 | 55.66±1.97 | 50.32±1.44 |
| *n*-Dodecane* | - | - | - | - |
| 2-Methylnaphthalene | 63.46±3.31 | 69.40±0.97 | 55.33±1.84 | 43.48±3.05 |
| 1-Methylnaphthalene | $69.99\pm2.15^{a}$ | $62.32\pm1.25^{b}$ | $51.53\pm4.31^{c}$ | $40.10\pm2.40^{d}$ |

* n-dodecane overlapped with an unknown component from tissues.

Table A4. Recoveries of spiked hydrocarbon standards from cod muscle, scallop muscle, and lobster meat by steam distillation under experimental design IV (refer to Table 3.1). Values are the average of triplicate analyses ± standard deviations. Different transverse lettering superscripts associated with the percent recovery of each hydrocarbon indicate significant differences between the means of recovery efficiency at $p<0.05$ (only those with superscript letters were tested for statistical significance).

| Spiked Hydrocarbons | Cod Muscle | Scallop Muscle | Lobster Meat |
|---|---|---|---|
| Benzene | $89.41 \pm 3.85^{a}$ | $84.49 \pm 3.71^{ab}$ | $79.17 \pm 2.68^{b}$ |
| Cyclohexane | 88.34±2.47 | 78.18±3.79 | 56.76±2.97 |
| *n*-Heptane | 85.86±2.49 | 77.80±5.44 | 67.68±2.75 |
| Methylcyclohexane | 88.05±4.18 | 77.63±5.05 | 67.98±1.46 |
| Toluene | $90.93 \pm 2.89^{a}$ | $85.38 \pm 1.62^{a}$ | $88.93 \pm 1.07^{a}$ |
| *n*-Octane | 77.66±2.48 | 80.53±4.18 | 77.18±4.22 |
| Ethylbenzene | 89.42±3.01 | 82.11±4.01 | 87.82±1.69 |
| *m+p* Xylene | 90.02±2.69 | 83.00±3.38 | 96.61±2.01 |
| *o*-Xylene | 86.98±1.35 | 85.07±3.24 | 92.29±1.14 |
| *n*-Nonane | 84.29±3.97 | 76.49±3.20 | 83.64±1.30 |
| Isopropylbenzene | 88.26±1.30 | 78.85±2.44 | 87.17±5.91 |
| Propylbenzene | $84.01 \pm 3.01^{a}$ | $76.79 \pm 2.26^{b}$ | $87.46 \pm 2.58^{a}$ |
| 1,3,5-Trimethylbenzene | 82.18±0.91 | 78.66±2.38 | 86.41±1.77 |
| 1,2,4-Trimethylbenzene | 81.98±3.33 | 76.92±2.67 | 84.13±4.89 |
| *n*-Decane | 81.54±11.08 | 76.55±3.57 | 74.02±3.45 |
| *n*-Undecane | $66.58 \pm 4.22^{a}$ | $63.65 \pm 1.43^{a}$ | $69.56 \pm 3.15^{a}$ |
| 1,2,4,5-Tetramethylbenzene | 76.76±2.08 | 69.14±2.65 | 83.31±1.91 |
| Naphthalene | 72.26±0.40 | 65.74±2.32 | 74.69±4.92 |
| *n*-Dodecane | 58.89±2.51 | 55.74±0.69 | 74.88±2.81 |
| 2-Methylnaphthalene | 63.18±1.98 | 55.75±2.40 | 67.87±2.18 |
| 1-Methylnaphthalene | $62.38 \pm 2.57^{a}$ | $54.11 \pm 2.77^{b}$ | $62.73 \pm 1.61^{a}$ |

Table A5-1. Concentrations of tainting WSF-HC accumulated in the belly flap of Atlantic salmon muscle tissue after 8 h of exposure and those retained in the belly flap after 1 day and 4 days of depuration in clean seawater. Values are the average ± standard deviations of triplicate analyses (ppm, $\mu g/g$, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 0 | 1 | 4 |
| Total Tainting WSF-HC | 62.4±12.1 | 32.9±5.2 | 15.8±5.2 |
| **Aromatics** | | | |
| benzene | 9.810±1.008 | 0.990±0.418 | 0.020±0.008 |
| toluene | 23.657±3.198 | 10.383±2.220 | 1.673±0.980 |
| ethylbenzene | 3.660±0.562 | 2.830±0.516 | 1.537±0.560 |
| *m+p*-xylenes | 5.813±1.254 | 4.347±0.823 | 2.653±0.825 |
| *o*-xylene | 3.550±0.688 | 2.723±0.464 | 1.600±0.498 |
| isopropylbenzene | 0.347±0.068 | 0.337±0.121 | 0.203±0.054 |
| propylbenzene | 0.487±0.116 | 0.407±0.061 | 0.317±0.095 |
| 1-ethyl-3(4)-methylbenzenes | 0.920±0.163 | 0.783±0.151 | 0.511±0.220 |
| 1,3,5-trimethylbenzene | 0.210±0.054 | 0.137±0.029 | 0.117±0.029 |
| 1-ethyl-2-methylbenzene | 0.693±0.186 | 0.530±0.110 | 0.427±0.107 |
| 1,2,4-trimethylbenzene | 0.820±0.237 | 0.587±0.100 | 0.487±0.103 |
| 1,2,3-trimethylbenzene | 0.690±0.232 | 0.597±0.127 | 0.493±0.084 |
| tetramethylbenzenes | 0.297±0.141 | 0.177±0.021 | 0.130±0.016 |
| $C_4$-benzenes[a] | 0.427±0.125 | 0.207±0.084 | 0.137±0.059 |
| naphthalene | 0.843±0.180 | 0.670±0.112 | 0.633±0.071 |
| 2-methylnaphthalene | 0.757±0.237 | 0.473±0.127 | 0.470±0.046 |
| 1-methylnaphthalene | 0.520±0.115 | 0.503±0.189 | 0.473±0.059 |
| other PAHs[b] | 0.954±0.133 | 0.573±0.221 | 0.343±0.130 |
| Total Tainting Aromatics | 54.455 | 27.254 | 12.254 |

Table A5-1 (continued)

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 0 | 1 | 4 |
| **Aliphatics** | | | |
| cyclohexane | 1.073±0.087 | 1.033±0.202 | 0.333±0.143 |
| 2-methylhexane | 0.090±0.037 | 0.080±0.071 | 0.020±0.028 |
| isooctane | 0.183±0.017 | 0.157±0.058 | 0.133±0.099 |
| *n*-heptane | 0.153±0.012 | 0.187±0.074 | 0.147±0.089 |
| methylcyclohexane | 0.667±0.084 | 0.683±0.247 | 0.797±0.438 |
| dimethylcyclohexanes | 0.337±0.176 | 0.340±0.050 | 0.310±0.179 |
| *n*-octane | 0.103±0.058 | 0.090±0.028 | 0.050±0.022 |
| ethylcyclohexane | 0.220±0.045 | 0.207±0.111 | 0.127±0.087 |
| *n*-nonane | 0.077±0.045 | 0.023±0.017 | 0.013±0.012 |
| *n*-decane | 0.040±0.024 | 0.021±0.014 | tr[c] |
| Total Tainting Aliphatics | 2.943 | 2.821 | 1.930 |
| **Unknown** | **4.999±0.758** | **2.808±0.211** | **1.573±0.478** |

[a] excludes tetramethylnaphthalenes

[b] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

[c] trace (< 0.01 ppm)

Table A5-2. Concentrations of tainting WSF-HC retained in the belly flap of Atlantic salmon after 10, 17 and 31 days of depuration in clean seawater. Values are the average ± standard deviations of triplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 10 | 17 | 31 |
| Total Tainting WSF-HC | 7.1±1.0 | 2.4±0.7 | 1.8±1.2 |
| **Aromatics** | | | |
| benzene | 0.035±0.015 | 0.013±0.018 | tr |
| toluene | 0.017±0.009 | tr | tr |
| ethylbenzene | 0.186±0.093 | 0.023±0.010 | 0.014±0.004 |
| *m*+*p*-xylenes | 0.455±0.205 | 0.068±0.015 | 0.043±0.019 |
| *o*-xylene | 0.226±0.114 | 0.025±0.014 | 0.019±0.007 |
| isopropylbenzene | 0.082±0.024 | 0.023±0.021 | tr |
| propylbenzene | 0.171±0.037 | 0.056±0.032 | 0.041±0.028 |
| 1-ethyl-3(4)-methylbenzenes | 0.320±0.068 | 0.099±0.020 | 0.050±0.008 |
| 1,3,5-trimethylbenzene | 0.070±0.011 | 0.024±0.020 | tr |
| 1-ethyl-2-methylbenzene | 0.204±0.043 | 0.063±0.022 | 0.049±0.043 |
| 1,2,4-trimethylbenzene | 0.263±0.060 | 0.074±0.008 | 0.031±0.009 |
| 1,2,3-trimethylbenzene | 0.277±0.037 | 0.090±0.015 | 0.015±0.014 |
| tetramethylbenzenes | 0.061±0.002 | 0.027±0.011 | 0.031±0.009 |
| $C_4$-benzenes[a] | 0.090±0.008 | 0.039±0.004 | 0.037±0.018 |
| naphthalene | 0.279±0.050 | 0.064±0.018 | 0.037±0.032 |
| 2-methylnaphthalene | 0.414±0.120 | 0.208±0.036 | 0.146±0.116 |
| 1-methylnaphthalene | 0.443±0.121 | 0.145±0.022 | 0.075±0.012 |
| other PAHs[c] | 0.322±0.145 | 0.090±0.051 | 0.035±0.027 |
| Total Tainting Aromatics | 3.915 | 1.131 | 0.639 |

Table A5-2 (continued)

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 10 | 17 | 31 |
| **Aliphatics** | | | |
| cyclohexane | 0.057±0.021 | tr | tr |
| 2-methylhexane | tr[b] | tr | tr |
| isooctane | 0.082±0.014 | 0.038±0.002 | tr |
| *n*-heptane | 0.155±0.062 | 0.140±0.042 | 0.204±0.148 |
| methylcyclohexane | 0.538±0.141 | 0.194±0.030 | 0.158±0.045 |
| dimethylcyclohexanes | 0.304±0.054 | 0.175±0.040 | 0.178±0.113 |
| *n*-octane | 0.051±0.007 | 0.032±0.007 | 0.040±0.018 |
| ethylcyclohexane | 0.137±0.028 | 0.075±0.014 | 0.014±0.004 |
| *n*-nonane | tr | tr | tr |
| *n*-decane | tr | tr | tr |
| Total Tainting Aliphatics | 1.324 | 0.654 | 0.594 |
| **Unknown** | **1.835±0.247** | **0.575±0.085** | **0.615±0.185** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A6-1. Concentrations of tainting WSF-HC accumulated in the dark muscle of Atlantic salmon after 8 h of exposure and those retained in the dark muscle after 1 day and 4 days of depuration in clean seawater. Values are the average ± standard deviations of triplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 0 | 1 | 4 |
| Total Tainting WSF-HC | 60.6±3.4 | 25.6±3.0 | 7.7±2.1 |
| **Aromatics** | | | |
| benzene | 6.410±0.824 | 0.290±0.134 | 0.013±0.005 |
| toluene | 23.223±1.792 | 4.403±1.731 | 0.273±0.063 |
| ethylbenzene | 5.060±0.260 | 2.130±0.428 | 0.520±0.022 |
| *m*+*p*-xylenes | 7.237±0.894 | 3.700±0.671 | 0.977±0.012 |
| *o*-xylene | 4.513±0.500 | 2.100±0.399 | 0.540±0.014 |
| isopropylbenzene | 0.410±0.036 | 0.290±0.014 | 0.160±0.034 |
| propylbenzene | 0.557±0.034 | 0.453±0.062 | 0.213±0.074 |
| 1-ethyl-3(4)-methylbenzenes | 0.983±0.090 | 0.980±0.254 | 0.654±0.064 |
| 1,3,5-trimethylbenzene | 0.163±0.021 | 0.203±0.057 | 0.057±0.017 |
| 1-ethyl-2-methylbenzene | 0.677±0.082 | 0.710±0.022 | 0.323±0.024 |
| 1,2,4-trimethylbenzene | 0.800±0.115 | 0.837±0.126 | 0.427±0.040 |
| 1,2,3-trimethylbenzene | 0.810±0.153 | 0.657±0.052 | 0.290±0.036 |
| tetramethylbenzenes | 0.120±0.052 | 0.277±0.085 | 0.093±0.012 |
| $C_4$-benzenes[a] | 0.317±0.042 | 0.247±0.111 | 0.101±0.032 |
| naphthalene | 0.810±0.219 | 0.773±0.148 | 0.323±0.026 |
| 2-methylnaphthalene | 0.507±0.090 | 0.440±0.092 | 0.350±0.094 |
| 1-methylnaphthalene | 0.497±0.091 | 0.473±0.101 | 0.273±0.061 |
| other PAHs[b] | 0.694±0.199 | 0.407±0.103 | 0.204±0.066 |
| Total Tainting Aromatics | 53.788 | 19.370 | 5.791 |

Table A6-1 (continued)

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 0 | 1 | 4 |
| **Aliphatics** | | | |
| cyclohexane | 1.630±0.335 | 0.660±0.131 | 0.193±0.021 |
| 2-methylhexane | 0.127±0.075 | 0.117±0.098 | tr[c] |
| isooctane | 0.517±0.094 | 0.297±0.107 | 0.063±0.021 |
| *n*-heptane | 0.297±0.071 | 0.373±0.119 | 0.123±0.029 |
| methylcyclohexane | 1.397±0.878 | 0.813±0.085 | 0.347±0.033 |
| dimethylcyclohexanes | 0.520±0.022 | 0.210±0.163 | 0.080±0.017 |
| *n*-octane | 0.183±0.025 | 0.160±0.086 | 0.023±0.021 |
| ethylcyclohexane | 0.227±0.048 | 0.140±0.054 | 0.090±0.027 |
| *n*-nonane | 0.047±0.034 | 0.030±0.042 | tr |
| *n*-decane | 0.030±0.014 | 0.012±0.004 | tr |
| Total Tainting Aliphatics | 4.975 | 2.812 | 0.919 |
| **Unknown** | **1.814±0.244** | **3.375±0.412** | **0.950±0.258** |

[a] excludes tetramethylnaphthalenes

[b] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

[c] trace (< 0.01 ppm)

Table A6-2. Concentrations of tainting WSF-HC retained in the dark muscle of Atlantic salmon after 10, 17 and 31 days of depuration in clean seawater. Values are the average ± standard deviations of triplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 10 | 17 | 31 |
| Total Tainting WSF-HC | 3.7±1.2 | 1.4±1.1 | 0.6±0.8 |
| **Aromatics** | | | |
| benzene | tr[b] | tr | tr |
| toluene | 0.023±0.004 | tr | tr |
| ethylbenzene | 0.056±0.009 | tr | tr |
| *m*+*p*-xylenes | 0.115±0.030 | 0.026±0.024 | 0.018±0.019 |
| *o*-xylene | 0.050±0.015 | 0.013±0.007 | tr |
| isopropylbenzene | 0.052±0.004 | tr | tr |
| propylbenzene | 0.099±0.048 | 0.026±0.024 | 0.010±0.012 |
| 1-ethyl-3(4)-methylbenzenes | 0.302±0.027 | 0.048±0.010 | 0.017±0.004 |
| 1,3,5-trimethylbenzene | 0.031±0.033 | 0.010±0.008 | tr |
| 1-ethyl-2-methylbenzene | 0.145±0.112 | 0.011±0.008 | tr |
| 1,2,4-trimethylbenzene | 0.194±0.083 | 0.037±0.018 | 0.021±0.009 |
| 1,2,3-trimethylbenzene | 0.145±0.041 | 0.026±0.016 | tr |
| tetramethylbenzenes | 0.039±0.007 | 0.026±0.008 | 0.012±0.007 |
| $C_4$-benzenes[a] | 0.024±0.006 | 0.020±0.014 | 0.012±0.011 |
| naphthalene | 0.123±0.015 | 0.040±0.013 | 0.014±0.004 |
| 2-methylnaphthalene | 0.282±0.030 | 0.134±0.040 | 0.064±0.014 |
| 1-methylnaphthalene | 0.203±0.111 | 0.104±0.032 | 0.036±0.048 |
| other PAHs[c] | 0.161±0.056 | 0.011±0.008 | tr |
| Total Tainting Aromatics | 2.044 | 0.532 | 0.204 |

Table A6-2 (continued)

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 10 | 17 | 31 |
| **Aliphatics** | | | |
| cyclohexane | 0.030±0.007 | tr | tr |
| 2-methylhexane | tr | tr | tr |
| isooctane | 0.039±0.028 | 0.011±0.002 | tr |
| *n*-heptane | 0.134±0.015 | 0.076±0.008 | 0.030±0.005 |
| methylcyclohexane | 0.247±0.135 | 0.068±0.044 | 0.022±0.004 |
| dimethylcyclohexanes | 0.148±0.038 | 0.073±0.032 | 0.034±0.006 |
| *n*-octane | 0.072±0.017 | 0.024±0.010 | tr |
| ethylcyclohexane | 0.056±0.010 | 0.026±0.013 | 0.010±0.004 |
| *n*-nonane | tr | tr | tr |
| *n*-decane | tr | tr | tr |
| Total Tainting Aliphatics | 0.726 | 0.278 | 0.096 |
| **Unknown** | **0.917±0.122** | **0.545±0.124** | **0.336±0.096** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A7-1. Concentrations of tainting WSF-HC accumulated in the lower flank muscle of Atlantic salmon after 8 h of exposure and those retained in the lower flank muscle after 1 day and 4 days of depuration in clean seawater. Values are the average ± standard deviations of triplicate analyses (ppm, $\mu g/g$, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 0 | 1 | 4 |
| Total Tainting WSF-HC | 56.2±4.4 | 18.8±4.3 | 10.3±1.0 |
| **Aromatics** | | | |
| benzene | 4.533±0.214 | 0.147±0.082 | tr[b] |
| toluene | 18.293±0.457 | 2.973±0.980 | 0.440±0.026 |
| ethylbenzene | 4.700±0.494 | 1.697±0.257 | 0.738±0.014 |
| *m*+*p*-xylenes | 7.540±0.740 | 3.027±0.324 | 1.511±0.014 |
| *o*-xylene | 4.570±0.518 | 1.810±0.233 | 1.081±0.227 |
| isopropylbenzene | 0.523±0.112 | 0.313±0.090 | 0.136±0.035 |
| propylbenzene | 0.703±0.109 | 0.383±0.031 | 0.229±0.024 |
| 1-ethyl-3(4)-methylbenzenes | 1.234±0.097 | 0.760±0.118 | 0.488±0.073 |
| 1,3,5-trimethylbenzene | 0.207±0.039 | 0.147±0.019 | 0.112±0.032 |
| 1-ethyl-2-methylbenzene | 0.743±0.042 | 0.477±0.026 | 0.371±0.095 |
| 1,2,4-trimethylbenzene | 1.440±0.165 | 0.573±0.125 | 0.566±0.218 |
| 1,2,3-trimethylbenzene | 1.120±0.041 | 0.517±0.072 | 0.389±0.082 |
| tetramethylbenzenes | 0.210±0.036 | 0.103±0.034 | 0.068±0.013 |
| $C_4$-benzenes[a] | 0.463±0.050 | 0.183±0.057 | 0.143±0.073 |
| naphthalene | 0.907±0.066 | 0.457±0.052 | 0.318±0.075 |
| 2-methylnaphthalene | 0.887±0.179 | 0.537±0.102 | 0.510±0.137 |
| 1-methylnaphthalene | 0.710±0.141 | 0.427±0.082 | 0.319±0.067 |
| other PAHs[c] | 0.747±0.254 | 0.543±0.403 | 0.220±0.088 |
| Total Tainting Aromatics | 49.530 | 15.074 | 7.639 |

Table A7-1 (continued)

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 0 | 1 | 4 |
| **Aliphatics** | | | |
| cyclohexane | 1.127±0.103 | 0.343±0.096 | 0.142±0.060 |
| 2-methylhexane | 0.033±0.034 | tr | tr |
| isooctane | 0.190±0.029 | 0.087±0.040 | 0.049±0.015 |
| *n*-heptane | 0.167±0.042 | 0.217±0.122 | 0.175±0.017 |
| methylcyclohexane | 0.890±0.205 | 0.407±0.087 | 0.239±0.103 |
| dimethylcyclohexanes | 0.463±0.119 | 0.173±0.065 | 0.095±0.037 |
| *n*-octane | 0.057±0.012 | 0.047±0.012 | 0.050±0.014 |
| ethylcyclohexane | 0.173±0.049 | 0.093±0.031 | 0.056±0.009 |
| *n*-nonane | 0.010±0.000 | tr | tr |
| *n*-decane | tr | tr | tr |
| Total Tainting Aliphatics | 3.110 | 1.367 | 0.806 |
| **Unknown** | **3.547±0.471** | **2.363±0.217** | **1.888±0.417** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A7-2. Concentrations of tainting WSF-HC retained in the lower flank muscle of Atlantic salmon after 10, 17 and 31 days of depuration in clean seawater. Values are the average ± standard deviations of triplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 10 | 17 | 31 |
| Total Tainting WSF-HC | 3.2±1.6 | 1.5±1.1 | 1.3±1.8 |
| **Aromatics** | | | |
| benzene | tr[b] | tr | tr |
| toluene | tr | tr | tr |
| ethylbenzene | 0.050±0.012 | tr | tr |
| *m*+*p*-xylenes | 0.128±0.032 | 0.023±0.023 | 0.017±0.018 |
| *o*-xylene | 0.063±0.022 | 0.010±0.008 | tr |
| isopropylbenzene | 0.037±0.018 | tr | tr |
| propylbenzene | 0.092±0.017 | 0.032±0.024 | 0.022±0.009 |
| 1-ethyl-3(4)-methylbenzenes | 0.169±0.086 | 0.044±0.008 | 0.021±0.010 |
| 1,3,5-trimethylbenzene | 0.039±0.009 | tr | tr |
| 1-ethyl-2-methylbenzene | 0.091±0.018 | 0.023±0.015 | 0.023±0.026 |
| 1,2,4-trimethylbenzene | 0.125±0.061 | 0.057±0.018 | 0.049±0.053 |
| 1,2,3-trimethylbenzene | 0.102±0.027 | 0.016±0.010 | tr |
| tetramethylbenzenes | 0.045±0.019 | 0.019±0.010 | 0.022±0.007 |
| $C_4$-benzenes[a] | 0.082±0.016 | 0.063±0.027 | 0.055±0.013 |
| naphthalene | 0.087±0.016 | 0.035±0.025 | 0.018±0.004 |
| 2-methylnaphthalene | 0.352±0.082 | 0.125±0.113 | 0.111±0.024 |
| 1-methylnaphthalene | 0.242±0.063 | 0.119±0.037 | 0.075±0.017 |
| other PAHs[c] | 0.178±0.107 | 0.071±0.021 | tr |
| Total Tainting Aromatics | 1.882 | 0.637 | 0.413 |

Table A7-2 (continued)

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 10 | 17 | 31 |
| **Aliphatics** | | | |
| cyclohexane | 0.027±0.006 | tr | tr |
| 2-methylhexane | tr | tr | tr |
| isooctane | 0.031±0.001 | 0.016±0.005 | tr |
| *n*-heptane | 0.115±0.019 | 0.098±0.062 | 0.184±0.040 |
| methylcyclohexane | 0.168±0.039 | 0.100±0.031 | 0.134±0.122 |
| dimethylcyclohexanes | 0.100±0.027 | 0.052±0.055 | 0.063±0.035 |
| *n*-octane | 0.044±0.005 | 0.031±0.016 | 0.039±0.015 |
| ethylcyclohexane | 0.049±0.018 | 0.025±0.034 | 0.032±0.017 |
| *n*-nonane | tr | tr | tr |
| *n*-decane | tr | tr | tr |
| Total Tainting Aliphatics | 0.534 | 0.322 | 0.452 |
| **Unknown** | **0.744±0.062** | **0.534±0.057** | **0.393±0.121** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A8-1. Concentrations of tainting WSF-HC accumulated in the dorsal white muscle of Atlantic salmon after 8 h of exposure and those retained in the dorsal white muscle after 1 day and 4 days of depuration in clean seawater. Values are the average ± standard deviations of triplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 0 | 1 | 4 |
| Total Tainting WSF-HC | 29.7±3.7 | 4.1±0.3 | 1.5±1.0 |
| **Aromatics** | | | |
| benzene | 2.680±0.440 | 0.035±0.005 | tr[b] |
| toluene | 9.565±1.525 | 0.410±0.207 | 0.039±0.010 |
| ethylbenzene | 2.230±0.330 | 0.245±0.121 | 0.078±0.014 |
| *m*+*p*-xylenes | 3.835±0.335 | 0.430±0.211 | 0.142±0.032 |
| *o*-xylene | 2.285±0.345 | 0.285±0.139 | 0.103±0.010 |
| isopropylbenzene | 0.220±0.030 | 0.035±0.017 | 0.025±0.007 |
| propylbenzene | 1.570±1.200 | 0.065±0.031 | 0.042±0.006 |
| 1-ethyl-3(4)-methylbenzenes | 0.675±0.115 | 0.100±0.047 | 0.053±0.026 |
| 1,3,5-trimethylbenzene | 0.125±0.025 | 0.030±0.014 | 0.011±0.012 |
| 1-ethyl-2-methylbenzene | 0.450±0.070 | 0.105±0.050 | 0.056±0.013 |
| 1,2,4-trimethylbenzene | 0.685±0.105 | 0.160±0.075 | 0.088±0.047 |
| 1,2,3-trimethylbenzene | 0.540±0.040 | 0.115±0.054 | 0.061±0.010 |
| tetramethylbenzenes | 0.060±0.025 | 0.045±0.022 | 0.012±0.012 |
| $C_4$-benzenes[a] | 0.155±0.055 | 0.100±0.057 | 0.031±0.044 |
| naphthalene | 0.550±0.070 | 0.110±0.052 | 0.054±0.013 |
| 2-methylnaphthalene | 0.385±0.025 | 0.130±0.062 | 0.058±0.014 |
| 1-methylnaphthalene | 0.305±0.071 | 0.095±0.045 | 0.044±0.013 |
| other PAHs[c] | 0.270±0.030 | 0.160±0.092 | 0.029±0.030 |
| Total Tainting Aromatics | 26.585 | 2.655 | 0.926 |

Table A8-1 (continued)

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 0 | 1 | 4 |
| **Aliphatics** | | | |
| cyclohexane | 0.440±0.060 | 0.065±0.031 | 0.025±0.008 |
| 2-methylhexane | tr | tr | tr |
| isooctane | 0.130±0.030 | 0.070±0.046 | tr |
| *n*-heptane | 0.160±0.020 | 0.070±0.041 | 0.053±0.047 |
| methylcyclohexane | 0.495±0.155 | 0.130±0.063 | 0.068±0.016 |
| dimethylcyclohexanes | 0.170±0.030 | 0.095±0.069 | 0.039±0.029 |
| *n*-octane | 0.015±0.005 | tr | tr |
| ethylcyclohexane | 0.070±0.010 | 0.045±0.022 | 0.020±0.016 |
| *n*-nonane | tr | tr | tr |
| *n*-decane | tr | tr | tr |
| Total Tainting Aliphatics | 1.48 | 0.475 | 0.205 |
| **Unknown** | **1.625±0.286** | **0.980±0.117** | **0.334±0.087** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A8-2. Concentrations of tainting WSF-HC retained in the dorsal white muscle of Atlantic salmon after 10, 17 and 31 days of depuration in clean seawater. Values are the average ± standard deviations of triplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 10 | 17 | 31 |
| Total Tainting WSF-HC | 0.6±0.9 | 0.3±0.7 | 0.2±0.5 |
| **Aromatics** | | | |
| benzene | tr[b] | tr | tr |
| toluene | tr | tr | tr |
| ethylbenzene | tr | tr | tr |
| *m*+*p*-xylenes | 0.010±0.011 | tr | tr |
| *o*-xylene | tr | tr | tr |
| isopropylbenzene | tr | tr | tr |
| propylbenzene | 0.028±0.037 | 0.010±0.023 | tr |
| 1-ethyl-3(4)-methylbenzenes | 0.021±0.011 | 0.015±0.015 | 0.010±0.010 |
| 1,3,5-trimethylbenzene | tr | tr | tr |
| 1-ethyl-2-methylbenzene | 0.015±0.004 | 0.010±0.017 | tr |
| 1,2,4-trimethylbenzene | 0.033±0.011 | 0.021±0.016 | 0.012±0.003 |
| 1,2,3-trimethylbenzene | 0.011±0.006 | tr | tr |
| tetramethylbenzenes | tr | tr | tr |
| $C_4$-benzenes[a] | 0.016±0.010 | 0.010±0.005 | tr |
| naphthalene | 0.017±0.014 | 0.011±0.009 | 0.010±0.014 |
| 2-methylnaphthalene | 0.057±0.012 | 0.027±0.018 | 0.019±0.014 |
| 1-methylnaphthalene | 0.050±0.023 | 0.025±0.024 | tr |
| other PAHs[c] | tr | tr | tr |
| Total Tainting Aromatics | 0.258 | 0.129 | 0.066 |

Table A8-2 (continued)

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 10 | 17 | 31 |
| **Aliphatics** | | | |
| cyclohexane | tr | tr | tr |
| 2-methylhexane | tr | tr | tr |
| isooctane | tr | tr | tr |
| *n*-heptane | 0.048±0.007 | 0.021±0.012 | 0.011±0.002 |
| methylcyclohexane | 0.057±0.014 | 0.026±0.020 | 0.027±0.004 |
| dimethylcyclohexanes | 0.010±0.011 | tr | 0.019±0.005 |
| *n*-octane | tr | tr | tr |
| ethylcyclohexane | tr | 0.010±0.007 | tr |
| *n*-nonane | tr | tr | tr |
| *n*-decane | tr | tr | tr |
| Total Tainting Aliphatics | 0.115 | 0.057 | 0.057 |
| **Unknown** | **0.201±0.042** | **0.108±0.040** | **0.132±0.109** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A9-1. Concentrations of tainting WSF-HC accumulated in the mesenteric adipose tissue of Atlantic salmon after 8 h of exposure and those retained in the mesenteric adipose tissue after 1 day and 4 days of depuration in clean seawater. Values are the average ± standard deviations of triplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 0 | 1 | 4 |
| Total Tainting WSF-HC | 163.0±12.6 | 89.5±5.4 | 63.7±3.5 |
| **Aromatics** | | | |
| benzene | 23.340±1.590 | 2.915±0.545 | 0.020±0.008 |
| toluene | 63.935±6.895 | 27.690±1.560 | 4.975±0.735 |
| ethylbenzene | 10.725±1.445 | 8.000±0.980 | 6.245±0.105 |
| *m*+*p*-xylenes | 15.125±1.975 | 11.340±0.530 | 10.440±0.290 |
| *o*-xylene | 9.605±1.305 | 6.890±0.350 | 5.995±0.365 |
| isopropylbenzene | 0.910±0.060 | 0.945±0.025 | 1.070±0.060 |
| propylbenzene | 1.125±0.127 | 1.440±0.130 | 1.745±0.125 |
| 1-ethyl-3(4)-methylbenzenes | 2.045±0.245 | 1.685±0.165 | 2.085±0.615 |
| 1,3,5-trimethylbenzene | 0.395±0.065 | 0.240±0.020 | 0.315±0.035 |
| 1-ethyl-2-methylbenzene | 1.785±0.166 | 1.865±0.125 | 2.190±0.110 |
| 1,2,4-trimethylbenzene | 2.015±0.145 | 2.220±0.240 | 2.805±0.135 |
| 1,2,3-trimethylbenzene | 1.530±0.420 | 1.810±0.190 | 2.155±0.174 |
| tetramethylbenzenes | 0.395±0.155 | 0.215±0.045 | 0.280±0.036 |
| $C_4$-benzenes[a] | 0.450±0.130 | 0.480±0.050 | 0.505±0.045 |
| naphthalene | 1.400±0.304 | 1.960±0.220 | 2.245±0.135 |
| 2-methylnaphthalene | 1.195±0.245 | 0.725±0.315 | 0.880±0.160 |
| 1-methylnaphthalene | 1.170±0.100 | 1.120±0.370 | 1.265±0.195 |
| other PAHs[b] | 1.660±0.899 | 1.301±0.566 | 0.891±0.223 |
| Total Tainting Aromatics | 139.705 | 72.841 | 46.106 |

Table A9-1 (continued)

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 0 | 1 | 4 |
| **Aliphatics** | | | |
| cyclohexane | 4.570±0.510 | 3.565±0.955 | 1.620±0.100 |
| 2-methylhexane | 0.315±0.025 | 0.260±0.110 | 0.105±0.035 |
| isooctane | 0.555±0.045 | 0.460±0.050 | 0.505±0.165 |
| *n*-heptane | 0.545±0.042 | 0.730±0.130 | 0.970±0.230 |
| methylcyclohexane | 3.155±0.125 | 3.440±0.460 | 3.905±0.205 |
| dimethylcyclohexanes | 2.765±1.445 | 1.075±0.115 | 1.340±0.180 |
| *n*-octane | 0.300±0.050 | 0.215±0.035 | 0.225±0.025 |
| ethylcyclohexane | 0.545±0.055 | 0.505±0.085 | 0.520±0.060 |
| *n*-nonane | 0.130±0.035 | 0.125±0.025 | 0.100±0.010 |
| *n*-decane | 0.080±0.044 | 0.090±0.015 | 0.095±0.009 |
| Total Tainting Aliphatics | 12.960 | 10.465 | 9.385 |
| **Unknown** | **10.310±2.144** | **6.144±0.744** | **8.224±1.257** |

[a] excludes tetramethylnaphthalenes

[b] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A9-2. Concentrations of tainting WSF-HC retained in the mesenteric adipose tissue of Atlantic salmon after 10, 17 and 31 days of depuration in clean seawater. Values are the average ± standard deviations of triplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 10 | 17 | 31 |
| Total Tainting WSF-HC | 37.9±8.3 | 12.6±0.8 | 3.5±1.5 |
| **Aromatics** | | | |
| benzene | 0.294±0.043 | tr[b] | tr |
| toluene | 0.033±0.010 | 0.021±0.010 | tr |
| ethylbenzene | 0.828±0.180 | 0.072±0.020 | 0.011±0.012 |
| *m*+*p*-xylenes | 2.050±0.544 | 0.217±0.043 | 0.032±0.029 |
| *o*-xylene | 0.854±0.322 | 0.108±0.027 | 0.017±0.004 |
| isopropylbenzene | 0.638±0.160 | 0.106±0.018 | 0.016±0.009 |
| propylbenzene | 1.375±0.369 | 0.297±0.023 | 0.063±0.018 |
| 1-ethyl-3(4)-methylbenzenes | 1.624±0.280 | 0.329±0.054 | 0.098±0.038 |
| 1,3,5-trimethylbenzene | 0.315±0.030 | 0.078±0.026 | 0.026±0.024 |
| 1-ethyl-2-methylbenzene | 1.368±0.385 | 0.331±0.022 | 0.085±0.018 |
| 1,2,4-trimethylbenzene | 2.383±0.337 | 0.592±0.040 | 0.040±0.030 |
| 1,2,3-trimethylbenzene | 1.607±0.325 | 0.406±0.013 | 0.021±0.005 |
| tetramethylbenzenes | 0.366±0.072 | 0.185±0.016 | 0.075±0.016 |
| $C_4$-benzenes[a] | 0.555±0.210 | 0.305±0.070 | 0.143±0.032 |
| naphthalene | 1.133±0.322 | 0.406±0.024 | 0.048±0.011 |
| 2-methylnaphthalene | 1.274±0.242 | 0.674±0.090 | 0.165±0.030 |
| 1-methylnaphthalene | 1.366±0.346 | 0.760±0.070 | 0.123±0.031 |
| other PAHs[c] | 0.724±0.213 | 0.261±0.058 | 0.033±0.030 |
| Total Tainting Aromatics | 18.787 | 5.148 | 0.996 |

Table A9-2 (continued)

| Hydrocarbon Components | Depuration Time (Days) | | |
|---|---|---|---|
| | 10 | 17 | 31 |
| **Aliphatics** | | | |
| cyclohexane | 0.239±0.015 | 0.007±0.004 | tr |
| 2-methylhexane | tr | tr | tr |
| isooctane | 0.433±0.063 | 0.071±0.014 | tr |
| *n*-heptane | 1.462±0.371 | 0.358±0.042 | 0.430±0.130 |
| methylcyclohexane | 3.298±0.563 | 0.537±0.012 | 0.247±0.067 |
| dimethylcyclohexanes | 1.478±0.434 | 0.608±0.058 | 0.255±0.067 |
| *n*-octane | 0.292±0.137 | 0.044±0.005 | 0.057±0.036 |
| ethylcyclohexane | 0.590±0.177 | 0.296±0.057 | 0.159±0.032 |
| *n*-nonane | 0.173±0.043 | 0.054±0.074 | tr |
| *n*-decane | 0.052±0.008 | tr | tr |
| Total Tainting Aliphatics | 8.017 | 1.975 | 1.148 |
| **Unknown** | **11.070±2.077** | **5.508±3.524** | **1.335±0.755** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A10-1. Accumulation of tainting WSF-HC in the dorsal white muscle of Atlantic salmon after 1 h, 3 h and 6 h of exposure to the WSF-HC of Flotta North Sea crude oil. Values are the average ± standard deviations of triplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Exposure Time (Hours) | | |
|---|---|---|---|
| | 1 h | 3 h | 6 h |
| Total Tainting WSF-HC | 1.884±0.062 | 2.266±0.250 | 3.921±0.032 |
| **Aromatics** | | | |
| benzene | 0.205±0.017 | 0.278±0.027 | 0.547±0.014 |
| toluene | 0.612±0.056 | 0.834±0.124 | 1.564±0.060 |
| ethylbenzene | 0.135±0.005 | 0.172±0.021 | 0.274±0.010 |
| *m*+*p*-xylenes | 0.189±0.001 | 0.229±0.029 | 0.361±0.013 |
| *o*-xylene | 0.196±0.018 | 0.195±0.024 | 0.317±0.011 |
| isopropylbenzene | 0.023±0.011 | 0.018±0.001 | 0.033±0.001 |
| propylbenzene | 0.021±0.001 | 0.027±0.006 | 0.029±0.002 |
| 1-ethyl-3(4)-methylbenzenes | 0.033±0.001 | 0.040±0.006 | 0.048±0.004 |
| 1,3,5-trimethylbenzene | 0.010±0.000 | 0.010±0.000 | tr |
| 1-ethyl-2-methylbenzene | 0.034±0.004 | 0.041±0.005 | 0.056±0.006 |
| 1,2,4-trimethylbenzene | 0.013±0.007 | 0.013±0.011 | 0.036±0.008 |
| 1,2,3-trimethylbenzene | 0.084±0.009 | 0.063±0.016 | 0.069±0.018 |
| tetramethylbenzenes | tr[b] | tr | tr |
| $C_4$-benzenes[a] | tr | tr | tr |
| naphthalene | tr | tr | 0.010±0.001 |
| 2-methylnaphthalene | 0.012±0.001 | tr | 0.013±0.001 |
| 1-methylnaphthalene | 0.013±0.005 | tr | 0.011±0.004 |
| other PAHs[c] | tr | tr | tr |
| Total Tainting Aromatics | 1.570 | 1.912 | 3.364 |

Table A10-1 (continued)

| Hydrocarbon Components | Exposure Time (Hours) | | |
|---|---|---|---|
| | 1 h | 3 h | 6 h |
| **Aliphatics** | | | |
| cyclohexane | 0.115±0.004 | 0.117±0.009 | 0.136±0.018 |
| 2-methylhexane | tr | tr | tr |
| isooctane | tr | tr | tr |
| *n*-heptane | tr | tr | tr |
| methylcyclohexane | 0.050±0.002 | 0.060±0.007 | 0.074±0.002 |
| dimethylcyclohexanes | tr | tr | tr |
| *n*-octane | tr | tr | tr |
| ethylcyclohexane | tr | tr | tr |
| *n*-nonane | tr | tr | tr |
| *n*-decane | tr | tr | tr |
| Total Tainting Aliphatics | 0.165 | 0.177 | 0.210 |
| **Unknown** | **0.149±0.021** | **0.177±0.004** | **0.347±0.129** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A10-2. Accumulation of tainting WSF-HC in the dorsal white muscle of Atlantic salmon after 12 h and 24 h of exposure to the WSF-HC of Flotta North Sea crude oil. Values are the average ± standard deviations of triplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Exposure Time (Hours) | |
|---|---|---|
| | 12 h | 24 h |
| Total Tainting WSF-HC | 4.390±0.040 | 3.105±0.285 |
| **Aromatics** | | |
| benzene | 0.590±0.060 | 0.295±0.015 |
| toluene | 1.630±0.070 | 0.945±0.085 |
| ethylbenzene | 0.315±0.015 | 0.230±0.030 |
| *m*+*p*-xylenes | 0.410±0.020 | 0.310±0.040 |
| *o*-xylene | 0.380±0.020 | 0.300±0.040 |
| isopropylbenzene | 0.040±0.010 | 0.045±0.005 |
| propylbenzene | 0.045±0.005 | 0.035±0.005 |
| 1-ethyl-3(4)-methylbenzenes | 0.050±0.000 | 0.045±0.005 |
| 1,3,5-trimethylbenzene | 0.010±0.000 | 0.010±0.000 |
| 1-ethyl-2-methylbenzene | 0.070±0.005 | 0.065±0.006 |
| 1,2,4-trimethylbenzene | 0.065±0.005 | 0.040±0.010 |
| 1,2,3-trimethylbenzene | 0.085±0.005 | 0.080±0.020 |
| tetramethylbenzenes | 0.010±0.000 | 0.010±0.010 |
| $C_4$-benzenes[a] | 0.020±0.007 | 0.015±0.005 |
| naphthalene | 0.010±0.003 | 0.010±0.007 |
| 2-methylnaphthalene | 0.045±0.005 | 0.060±0.020 |
| 1-methylnaphthalene | 0.035±0.005 | 0.040±0.010 |
| other PAHs[c] | tr[b] | tr |
| Total Tainting Aromatics | 3.810 | 2.530 |

Table A10-2 (continued)

| Hydrocarbon Components | Exposure Time (Hours) | |
|---|---|---|
| | 12 h | 24 h |
| **Aliphatics** | | |
| cyclohexane | 0.115±0.055 | 0.155±0.015 |
| 2-methylhexane | tr | tr |
| isooctane | tr | tr |
| *n*-heptane | tr | tr |
| methylcyclohexane | 0.090±0.010 | 0.075±0.005 |
| dimethylcyclohexanes | tr | tr |
| *n*-octane | tr | tr |
| ethylcyclohexane | tr | tr |
| *n*-nonane | tr | tr |
| *n*-decane | tr | tr |
| Total Tainting Aliphatics | 0.205 | 0.230 |
| **Unknown** | **0.375±0.185** | **0.345±0.045** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A11-1. Accumulation of tainting WSF-HC in the dark muscle of Atlantic salmon after 1 h, 3 h and 6 h of exposure to the WSF-HC of Flotta North Sea crude oil. Values are the average ± standard deviations of triplicate analyses (ppm, $\mu g/g$, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Exposure Time (Hours) | | |
|---|---|---|---|
| | 1 h | 3 h | 6 h |
| Total Tainting WSF-HC | 7.540±0.615 | 13.065±0.362 | 13.382±0.696 |
| **Aromatics** | | | |
| benzene | 1.093±0.026 | 1.923±0.041 | 1.963±0.109 |
| toluene | 3.272±0.196 | 5.728±0.109 | 5.962±0.178 |
| ethylbenzene | 0.540±0.037 | 0.907±0.028 | 0.953±0.088 |
| *m*+*p*-xylenes | 0.680±0.066 | 1.131±0.034 | 1.145±0.093 |
| *o*-xylene | 0.578±0.061 | 0.928±0.026 | 0.924±0.073 |
| isopropylbenzene | 0.075±0.003 | 0.081±0.002 | 0.061±0.007 |
| propylbenzene | 0.060±0.008 | 0.102±0.006 | 0.090±0.006 |
| 1-ethyl-3(4)-methylbenzenes | 0.107±0.019 | 0.163±0.005 | 0.154±0.009 |
| 1,3,5-trimethylbenzene | 0.023±0.009 | 0.042±0.002 | 0.036±0.010 |
| 1-ethyl-2-methylbenzene | 0.119±0.021 | 0.171±0.002 | 0.162±0.008 |
| 1,2,4-trimethylbenzene | 0.062±0.014 | 0.129±0.009 | 0.068±0.013 |
| 1,2,3-trimethylbenzene | 0.069±0.034 | 0.180±0.001 | 0.166±0.009 |
| tetramethylbenzenes | 0.016±0.001 | 0.022±0.001 | 0.039±0.016 |
| $C_4$-benzenes[a] | 0.115±0.020 | 0.089±0.024 | 0.090±0.014 |
| naphthalene | 0.034±0.006 | 0.043±0.007 | 0.053±0.018 |
| 2-methylnaphthalene | 0.047±0.012 | 0.041±0.004 | 0.046±0.006 |
| 1-methylnaphthalene | 0.045±0.020 | 0.055±0.002 | 0.080±0.005 |
| other PAHs[c] | tr[b] | 0.046±0.012 | 0.057±0.006 |
| Total Tainting Aromatics | 6.930 | 11.776 | 12.040 |

Table A11-1 (continued)

| Hydrocarbon Components | Exposure Time (Hours) | | |
|---|---|---|---|
| | 1 h | 3 h | 6 h |
| **Aliphatics** | | | |
| cyclohexane | 0.159±0.021 | 0.199±0.014 | 0.260±0.052 |
| 2-methylhexane | tr | tr | tr |
| isooctane | tr | 0.098±0.014 | 0.112±0.012 |
| *n*-heptane | tr | tr | tr |
| methylcyclohexane | 0.108±0.011 | 0.210±0.009 | 0.208±0.024 |
| dimethylcyclohexanes | tr | 0.092±0.009 | 0.066±0.012 |
| *n*-octane | tr | 0.014±0.014 | tr |
| ethylcyclohexane | tr | 0.026±0.009 | 0.031±0.005 |
| *n*-nonane | tr | tr | tr |
| *n*-decane | tr | tr | tr |
| Total Tainting Aliphatics | 0.267 | 0.639 | 0.677 |
| **Unknown** | **0.343±0.105** | **0.650±0.095** | **0.665±0.215** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A11-2. Accumulation of tainting WSF-HC in the dark muscle of Atlantic salmon after 12 h and 24 h of exposure to the WSF-HC of Flotta North Sea crude oil. Values are the average ± standard deviations of triplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Exposure Time (Hours) | |
|---|---|---|
| | 12 h | 24 h |
| Total Tainting WSF-HC | 20.143±2.470 | 15.956±1.893 |
| **Aromatics** | | |
| benzene | 2.384±0.149 | 1.649±0.015 |
| toluene | 8.077±0.762 | 6.118±0.703 |
| ethylbenzene | 1.474±0.195 | 1.136±0.133 |
| *m*+*p*-xylenes | 1.871±0.248 | 1.383±0.191 |
| *o*-xylene | 1.564±0.207 | 1.241±0.141 |
| isopropylbenzene | 0.151±0.020 | 0.134±0.012 |
| propylbenzene | 0.174±0.023 | 0.141±0.012 |
| 1-ethyl-3(4)-methylbenzenes | 0.297±0.043 | 0.238±0.023 |
| 1,3,5-trimethylbenzene | 0.074±0.013 | 0.055±0.009 |
| 1-ethyl-2-methylbenzene | 0.316±0.041 | 0.285±0.007 |
| 1,2,4-trimethylbenzene | 0.245±0.058 | 0.150±0.030 |
| 1,2,3-trimethylbenzene | 0.322±0.045 | 0.295±0.022 |
| tetramethylbenzenes | 0.057±0.014 | 0.058±0.027 |
| $C_4$-benzenes[a] | 0.261±0.049 | 0.235±0.079 |
| naphthalene | 0.030±0.001 | 0.039±0.010 |
| 2-methylnaphthalene | 0.088±0.004 | 0.063±0.010 |
| 1-methylnaphthalene | 0.090±0.004 | 0.086±0.015 |
| other PAHs[c] | 0.094±0.001 | 0.088±0.003 |
| Total Tainting Aromatics | 17.527 | 13.387 |

Table A11-2 (continued)

| Hydrocarbon Components | Exposure Time (Hours) | |
|---|---|---|
| | 12 h | 24 h |
| **Aliphatics** | | |
| cyclohexane | 0.291±0.018 | 0.294±0.002 |
| 2-methylhexane | tr[b] | tr |
| isooctane | 0.175±0.002 | 0.238±0.048 |
| *n*-heptane | tr | tr |
| methylcyclohexane | 0.345±0.046 | 0.338±0.001 |
| dimethylcyclohexanes | 0.161±0.025 | 0.188±0.016 |
| *n*-octane | 0.025±0.025 | tr |
| ethylcyclohexane | 0.064±0.008 | 0.082±0.009 |
| *n*-nonane | tr | tr |
| *n*-decane | tr | tr |
| Total Tainting Aliphatics | 1.061 | 1.140 |
| **Unknown** | **1.555±0.482** | **1.429±0.280** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A12-1. Accumulation of tainting WSF-HC in the belly flap of Atlantic salmon after 1 h, 3 h and 6 h of exposure to the WSF-HC of Flotta North Sea crude oil. Values are the average ± standard deviations of triplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Exposure Time (Hours) | | |
|---|---|---|---|
| | 1 h | 3 h | 6 h |
| Total Tainting WSF-HC | 3.944±0.794 | 9.141±0.176 | 15.161±0.667 |
| **Aromatics** | | | |
| benzene | 0.954±0.170 | 2.001±0.229 | 3.488±0.061 |
| toluene | 1.619±0.357 | 3.930±0.394 | 6.878±0.357 |
| ethylbenzene | 0.193±0.043 | 0.461±0.029 | 0.778±0.064 |
| *m*+*p*-xylenes | 0.297±0.067 | 0.604±0.027 | 0.928±0.094 |
| *o*-xylene | 0.191±0.036 | 0.469±0.003 | 0.701±0.082 |
| isopropylbenzene | 0.016±0.003 | 0.071±0.012 | 0.055±0.012 |
| propylbenzene | 0.023±0.008 | 0.058±0.002 | 0.079±0.007 |
| 1-ethyl-3(4)-methylbenzenes | 0.023±0.010 | 0.080±0.004 | 0.113±0.012 |
| 1,3,5-trimethylbenzene | 0.016±0.005 | 0.032±0.003 | 0.037±0.001 |
| 1-ethyl-2-methylbenzene | 0.048±0.012 | 0.114±0.007 | 0.119±0.005 |
| 1,2,4-trimethylbenzene | 0.062±0.013 | 0.108±0.002 | 0.111±0.021 |
| 1,2,3-trimethylbenzene | 0.036±0.004 | 0.080±0.012 | 0.098±0.020 |
| tetramethylbenzenes | 0.011±0.002 | 0.041±0.028 | 0.027±0.004 |
| $C_4$-benzenes[a] | 0.053±0.001 | 0.097±0.040 | 0.057±0.014 |
| naphthalene | 0.034±0.006 | 0.019±0.004 | 0.029±0.011 |
| 2-methylnaphthalene | 0.060±0.012 | 0.073±0.023 | 0.118±0.012 |
| 1-methylnaphthalene | 0.037±0.009 | 0.046±0.005 | 0.038±0.005 |
| other PAHs[c] | tr[b] | tr | 0.039±0.008 |
| Total Tainting Aromatics | 3.672 | 8.275 | 13.651 |

Table A12-1 (continued)

| Hydrocarbon Components | Exposure Time (Hours) | | |
|---|---|---|---|
| | 1 h | 3 h | 6 h |
| **Aliphatics** | | | |
| cyclohexane | 0.124±0.031 | 0.207±0.002 | 0.431±0.077 |
| 2-methylhexane | tr | tr | tr |
| isooctane | tr | 0.023±0.013 | 0.100±0.023 |
| *n*-heptane | tr | 0.010±0.008 | 0.014±0.004 |
| methylcyclohexane | 0.056±0.018 | 0.132±0.008 | 0.252±0.022 |
| dimethylcyclohexanes | tr | tr | 0.095±0.073 |
| *n*-octane | tr | tr | tr |
| ethylcyclohexane | tr | 0.031±0.021 | 0.071±0.034 |
| *n*-nonane | tr | tr | 0.013±0.005 |
| *n*-decane | tr | tr | tr |
| Total Tainting Aliphatics | 0.180 | 0.403 | 0.976 |
| **Unknown** | **0.092±0.008** | **0.463±0.309** | **0.534±0.128** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A12-2. Accumulation of tainting WSF-HC in the belly flap of Atlantic salmon after 12 h and 24 h of exposure to the WSF-HC of Flotta North Sea crude oil. Values are the average ± standard deviations of triplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Exposure Time (Hours) | |
|---|---|---|
| | 12 h | 24 h |
| Total Tainting WSF-HC | 20.223±1.438 | 25.049±2.041 |
| **Aromatics** | | |
| benzene | 4.343±0.417 | 4.924±0.209 |
| toluene | 9.453±0.554 | 12.098±1.040 |
| ethylbenzene | 1.061±0.056 | 1.381±0.147 |
| *m+p*-xylenes | 1.238±0.064 | 1.522±0.164 |
| *o*-xylene | 0.977±0.056 | 1.373±0.094 |
| isopropylbenzene | 0.090±0.004 | 0.118±0.002 |
| propylbenzene | 0.100±0.004 | 0.122±0.007 |
| 1-ethyl-3(4)-methylbenzenes | 0.147±0.010 | 0.183±0.012 |
| 1,3,5-trimethylbenzene | 0.042±0.004 | 0.055±0.002 |
| 1-ethyl-2-methylbenzene | 0.172±0.010 | 0.204±0.006 |
| 1,2,4-trimethylbenzene | 0.148±0.018 | 0.153±0.003 |
| 1,2,3-trimethylbenzene | 0.139±0.013 | 0.199±0.006 |
| tetramethylbenzenes | 0.037±0.004 | 0.055±0.002 |
| $C_4$-benzenes[a] | 0.084±0.026 | 0.176±0.018 |
| naphthalene | 0.023±0.012 | 0.082±0.014 |
| 2-methylnaphthalene | 0.122±0.003 | 0.188±0.011 |
| 1-methylnaphthalene | 0.093±0.023 | 0.183±0.019 |
| other PAHs[c] | 0.016±0.016 | 0.039±0.008 |
| Total Tainting Aromatics | 18.279 | 23.047 |

Table A12-2 (continued)

| Hydrocarbon Components | Exposure Time (Hours) | |
|---|---|---|
| | 12 h | 24 h |
| **Aliphatics** | | |
| cyclohexane | 0.410±0.013 | 0.505±0.086 |
| 2-methylhexane | tr[b] | tr |
| isooctane | 0.133±0.034 | 0.108±0.044 |
| *n*-heptane | 0.040±0.011 | tr |
| methylcyclohexane | 0.283±0.024 | 0.335±0.072 |
| dimethylcyclohexanes | 0.113±0.011 | 0.136±0.035 |
| *n*-octane | 0.033±0.011 | tr |
| ethylcyclohexane | 0.088±0.008 | 0.140±0.036 |
| *n*-nonane | 0.020±0.002 | tr |
| *n*-decane | tr | tr |
| Total Tainting Aliphatics | 1.120 | 1.224 |
| **Unknown** | **0.824±0.160** | **0.778±0.148** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A13-1. Accumulation of tainting WSF-HC in the subdermal fat tissue of Atlantic salmon after 1 h, 3 h and 6 h of exposure to the WSF-HC of Flotta North Sea crude oil. Values are the average ± standard deviations of duplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Exposure Time (Hours) | | |
|---|---|---|---|
| | 1 h | 3 h | 6 h |
| Total Tainting WSF-HC | 17.376±0.946 | 28.975±2.495 | 49.380±1.620 |
| **Aromatics** | | | |
| benzene | 2.945±0.195 | 4.705±0.365 | 7.650±0.450 |
| toluene | 6.810±0.400 | 13.155±1.295 | 21.600±1.290 |
| ethylbenzene | 1.049±0.099 | 2.085±0.125 | 3.700±0.180 |
| *m*+*p*-xylenes | 1.300±0.070 | 2.500±0.220 | 4.470±0.210 |
| *o*-xylene | 1.098±0.187 | 1.890±0.140 | 3.375±0.165 |
| isopropylbenzene | 0.125±0.025 | 0.295±0.045 | 0.305±0.065 |
| propylbenzene | 0.330±0.050 | 0.375±0.005 | 0.580±0.050 |
| 1-ethyl-3(4)-methylbenzenes | 0.130±0.050 | 0.245±0.005 | 0.515±0.035 |
| 1,3,5-trimethylbenzene | 0.030±0.010 | 0.050±0.010 | 0.125±0.015 |
| 1-ethyl-2-methylbenzene | 0.375±0.005 | 0.290±0.020 | 0.615±0.075 |
| 1,2,4-trimethylbenzene | 0.215±0.025 | 0.300±0.040 | 0.680±0.080 |
| 1,2,3-trimethylbenzene | 0.306±0.016 | 0.465±0.055 | 0.810±0.035 |
| tetramethylbenzenes | 0.020±0.010 | 0.040±0.020 | 0.055±0.015 |
| $C_4$-benzenes[a] | 0.470±0.060 | 0.460±0.040 | 0.580±0.050 |
| naphthalene | 0.150±0.040 | 0.175±0.045 | 0.100±0.010 |
| 2-methylnaphthalene | 0.290±0.080 | 0.270±0.050 | 0.140±0.010 |
| 1-methylnaphthalene | 0.045±0.025 | 0.110±0.020 | 0.175±0.005 |
| other PAHs[c] | tr[b] | tr | tr |
| Total Tainting Aromatics | 15.686 | 27.410 | 45.480 |

Table A13-1 (continued)

| Hydrocarbon Components | Exposure Time (Hours) | | |
|---|---|---|---|
| | 1 h | 3 h | 6 h |
| **Aliphatics** | | | |
| cyclohexane | 0.325±0.035 | 0.390±0.040 | 0.600±0.150 |
| 2-methylhexane | tr | tr | tr |
| isooctane | 0.250±0.020 | 0.065±0.025 | 0.105±0.025 |
| *n*-heptane | tr | tr | 0.015±0.005 |
| methylcyclohexane | 0.420±0.050 | 0.385±0.035 | 0.700±0.070 |
| dimethylcyclohexanes | 0.020±0.011 | 0.015±0.005 | 0.165±0.005 |
| *n*-octane | 0.025±0.015 | 0.025±0.005 | 0.075±0.005 |
| ethylcyclohexane | tr | 0.130±0.040 | 0.080±0.020 |
| *n*-nonane | tr | 0.095±0.015 | 0.085±0.005 |
| *n*-decane | tr | tr | tr |
| Total Tainting Aliphatics | 1.040 | 1.105 | 1.825 |
| **Unknown** | **0.650±0.060** | **0.460±0.060** | **2.075±0.145** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A13-2. Accumulation of tainting WSF-HC in the subdermal fat tissue of Atlantic salmon after 12 h and 24 h of exposure to the WSF-HC of Flotta North Sea crude oil. Values are the average ± standard deviations of duplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Exposure Time (Hours) | |
|---|---|---|
| | 12 h | 24 h |
| Total Tainting WSF-HC | 56.509±1.155 | 84.773±3.185 |
| **Aromatics** | | |
| benzene | 7.530±0.480 | 7.735±0.155 |
| toluene | 24.790±1.220 | 36.360±2.090 |
| ethylbenzene | 4.750±0.110 | 7.410±0.360 |
| *m*+*p*-xylenes | 5.630±0.160 | 8.800±0.320 |
| *o*-xylene | 4.835±0.025 | 7.300±0.350 |
| isopropylbenzene | 0.445±0.105 | 0.760±0.110 |
| propylbenzene | 0.715±0.035 | 1.025±0.015 |
| 1-ethyl-3(4)-methylbenzenes | 0.625±0.055 | 0.915±0.005 |
| 1,3,5-trimethylbenzene | 0.105±0.015 | 0.140±0.020 |
| 1-ethyl-2-methylbenzene | 0.795±0.085 | 1.430±0.090 |
| 1,2,4-trimethylbenzene | 0.495±0.095 | 1.335±0.135 |
| 1,2,3-trimethylbenzene | 0.935±0.045 | 1.655±0.095 |
| tetramethylbenzenes | 0.090±0.010 | 0.065±0.025 |
| $C_4$-benzenes[a] | 0.315±0.105 | 0.735±0.095 |
| naphthalene | 0.095±0.035 | 0.360±0.040 |
| 2-methylnaphthalene | 0.415±0.005 | 0.315±0.095 |
| 1-methylnaphthalene | 0.455±0.085 | 0.670±0.050 |
| other PAHs[c] | 0.154±0.036 | 0.188±0.041 |
| Total Tainting Aromatics | 53.174 | 77.198 |

Table A13-2 (continued)

| Hydrocarbon Components | Exposure Time (Hours) | |
|---|---|---|
| | 12 h | 24 h |
| **Aliphatics** | | |
| cyclohexane | 0.990±0.100 | 0.160±0.040 |
| 2-methylhexane | 0.040±0.020 | 0.190±0.110 |
| isooctane | 0.125±0.025 | 0.245±0.065 |
| *n*-heptane | tr[b] | tr |
| methylcyclohexane | 0.840±0.030 | 1.470±0.190 |
| dimethylcyclohexanes | 0.245±0.045 | 0.345±0.015 |
| *n*-octane | tr | tr |
| ethylcyclohexane | 0.170±0.060 | 0.375±0.035 |
| *n*-nonane | 0.045±0.045 | tr |
| *n*-decane | tr | tr |
| Total Tainting Aliphatics | 2.455 | 2.785 |
| **Unknown** | **0.880±0.250** | **4.790±0.410** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A14-1. Accumulation of tainting WSF-HC in the mesenteric adipose tissue of Atlantic salmon after 1 h, 3 h and 6 h of exposure to the WSF-HC of Flotta North Sea crude oil. Values are the average ± standard deviations of duplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Exposure Time (Hours) | | |
|---|---|---|---|
| | 1 h | 3 h | 6 h |
| Total Tainting WSF-HC | 5.603±0.297 | 23.500±1.010 | 20.425±1.155 |
| **Aromatics** | | | |
| benzene | 0.955±0.065 | 3.480±0.150 | 4.835±0.165 |
| toluene | 1.475±0.155 | 6.785±0.295 | 7.945±0.375 |
| ethylbenzene | 0.175±0.035 | 0.900±0.030 | 0.850±0.050 |
| *m*+*p*-xylenes | 0.350±0.040 | 1.350±0.050 | 1.055±0.055 |
| *o*-xylene | 0.155±0.015 | 0.900±0.030 | 0.715±0.045 |
| isopropylbenzene | 0.025±0.005 | 0.085±0.005 | 0.075±0.005 |
| propylbenzene | 0.015±0.005 | 0.145±0.005 | 0.075±0.005 |
| 1-ethyl-3(4)-methylbenzenes | 0.020±0.010 | 0.155±0.005 | 0.070±0.010 |
| 1,3,5-trimethylbenzene | 0.023±0.003 | 0.120±0.000 | 0.050±0.010 |
| 1-ethyl-2-methylbenzene | 0.350±0.050 | 1.890±0.090 | 1.135±0.175 |
| 1,2,4-trimethylbenzene | 0.275±0.035 | 1.375±0.055 | 0.595±0.085 |
| 1,2,3-trimethylbenzene | 0.040±0.010 | 0.405±0.015 | 0.150±0.010 |
| tetramethylbenzenes | tr[b] | 0.200±0.010 | 0.080±0.020 |
| $C_4$-benzenes[a] | 0.015±0.005 | 0.810±0.140 | 0.150±0.010 |
| naphthalene | 0.075±0.015 | 0.260±0.030 | 0.020±0.010 |
| 2-methylnaphthalene | 0.075±0.015 | 0.170±0.020 | 0.180±0.060 |
| 1-methylnaphthalene | 0.020±0.020 | 0.070±0.030 | 0.075±0.015 |
| other PAHs[c] | tr | 0.135±0.015 | 0.080±0.010 |
| Total Tainting Aromatics | 4.043 | 19.235 | 18.135 |

Table A14-1 (continued)

| Hydrocarbon Components | Exposure Time (Hours) | | |
|---|---|---|---|
| | 1 h | 3 h | 6 h |
| **Aliphatics** | | | |
| cyclohexane | 0.195±0.045 | 0.275±0.045 | 0.395±0.005 |
| 2-methylhexane | 0.070±0.020 | 0.170±0.025 | 0.090±0.020 |
| isooctane | 0.075±0.045 | 0.085±0.005 | 0.095±0.045 |
| *n*-heptane | 0.480±0.010 | 0.055±0.015 | 0.025±0.015 |
| methylcyclohexane | 0.065±0.015 | 0.245±0.035 | 0.280±0.020 |
| dimethylcyclohexanes | 0.015±0.005 | 0.390±0.020 | 0.185±0.045 |
| *n*-octane | 0.025±0.005 | 0.055±0.025 | 0.055±0.005 |
| ethylcyclohexane | tr | 0.065±0.005 | 0.075±0.005 |
| *n*-nonane | tr | 0.070±0.020 | 0.060±0.010 |
| *n*-decane | tr | tr | tr |
| Total Tainting Aliphatics | 0.925 | 1.410 | 1.260 |
| **Unknown** | **0.635±0.095** | **2.855±0.195** | **1.030±0.160** |

[a] excludes tetramethylnaphthalenes

[b] trace (< 0.01 ppm)

[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

Table A14-2. Accumulation of tainting WSF-HC in the mesenteric adipose tissue of Atlantic salmon after 12 h and 24 h of exposure to the WSF-HC of Flotta North Sea crude oil. Values are the average ± standard deviations of duplicate analyses (ppm, μg/g, wet tissue basis). Samples from three fish were pooled for analyses.

| Hydrocarbon Components | Exposure Time (Hours) | |
|---|---|---|
| | 12 h | 24 h |
| Total Tainting WSF-HC | 43.625±1.295 | 61.710±2.980 |
| **Aromatics** | | |
| benzene | 8.095±0.305 | 9.825±0.425 |
| toluene | 17.865±0.815 | 27.285±2.005 |
| ethylbenzene | 2.165±0.095 | 4.040±0.190 |
| *m+p*-xylenes | 2.560±0.150 | 4.250±0.190 |
| *o*-xylene | 1.970±0.120 | 3.860±0.210 |
| isopropylbenzene | 0.200±0.000 | 0.565±0.045 |
| propylbenzene | 0.245±0.005 | 0.400±0.040 |
| 1-ethyl-3(4)-methylbenzenes | 0.240±0.010 | 0.380±0.010 |
| 1,3,5-trimethylbenzene | 0.105±0.005 | 0.105±0.005 |
| 1-ethyl-2-methylbenzene | 1.905±0.025 | 0.695±0.075 |
| 1,2,4-trimethylbenzene | 1.010±0.020 | 1.015±0.125 |
| 1,2,3-trimethylbenzene | 0.395±0.015 | 0.795±0.025 |
| tetramethylbenzenes | 0.115±0.025 | 0.060±0.010 |
| $C_4$-benzenes[a] | 0.585±0.095 | 0.590±0.030 |
| naphthalene | 0.055±0.005 | 0.170±0.010 |
| 2-methylnaphthalene | 0.340±0.040 | 0.710±0.110 |
| 1-methylnaphthalene | 0.190±0.040 | 0.350±0.030 |
| other PAHs[c] | 0.155±0.015 | 0.110±0.020 |
| Total Tainting Aromatics | 38.195 | 55.205 |

Table A14-2 (continued)

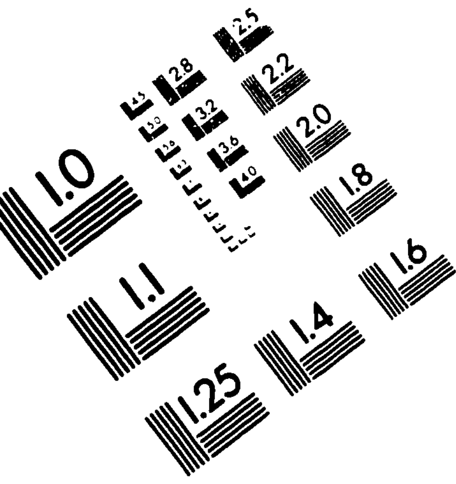
| Hydrocarbon Components | Exposure Time (Hours) | |
|---|---|---|
| | 12 h | 24 h |
| **Aliphatics** | | |
| cyclohexane | 0.865±0.025 | 1.735±0.185 |
| 2-methylhexane | 0.155±0.025 | 0.125±0.025 |
| isooctane | 0.105±0.005 | 0.220±0.030 |
| *n*-heptane | 0.035±0.005 | tr |
| methylcyclohexane | 0.710±0.020 | 1.185±0.075 |
| dimethylcyclohexanes | 0.460±0.040 | 0.615±0.055 |
| *n*-octane | 0.080±0.010 | tr |
| ethylcyclohexane | 0.120±0.040 | 0.300±0.030 |
| *n*-nonane | 0.070±0.010 | 0.050±0.010 |
| *n*-decane | tr[b] | tr |
| Total Tainting Aliphatics | 2.600 | 4.230 |
| **Unknown** | **2.830±0.110** | **2.275±0.075** |

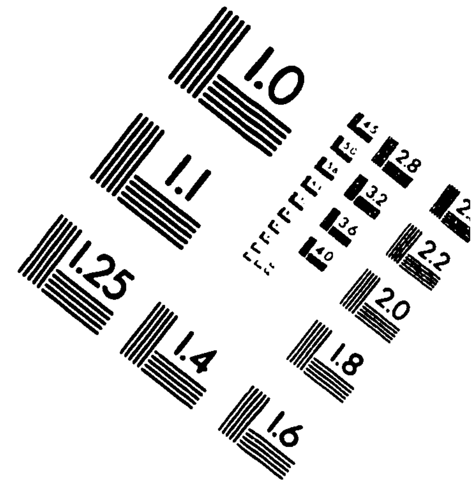
[a] excludes tetramethylnaphthalenes

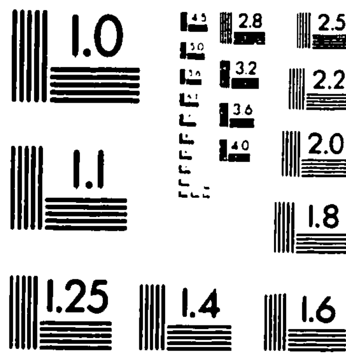
[b] trace (< 0.01 ppm)

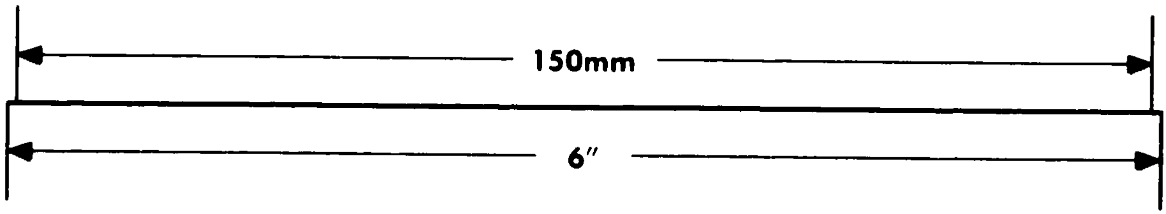
[c] other polyaromatic hydrocarbons including ethylnaphthalene, dimethylnaphthalene and $C_3$-naphthalenes

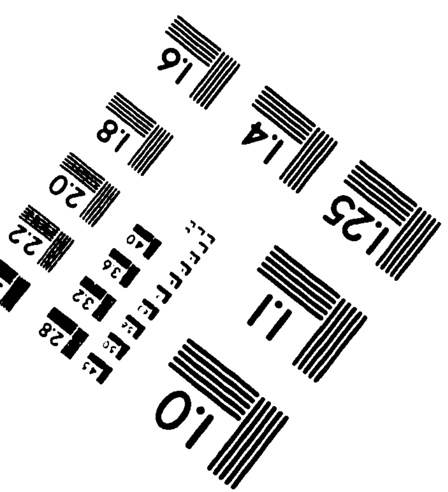
IMAGE EVALUATION
TEST TARGET (QA-3)

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