The Effects of Petroleum Exposure on the Feeding Behaviour of Rainbow Trout (*Oncorhynchus mykiss*) Using the Water-soluble Fraction of Norman Wells Crude Oil

A thesis submitted to the Faculty of Graduate studies in partial fulfillment of the requirements for the degree Master of Science

by

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Abstract

The effects of exposure to the water-soluble fraction (WSF) of Norman Wells crude oil (NWC) on the feeding behaviour of rainbow trout were studied. Trout were exposed to WSF of NWC mixed at 10, 25, 50, 100, 200 and 400 mg/L nominal concentrations. Exposures lasted 14 days with complete, daily renewal of the WSF in a static, non-aerated system. Nominal hydrocarbon (HC) concentrations were compared with measured concentrations by extracting water with hexane and analyzing the samples with mass spectroscopy gas chromatography.

At 400 mg/L nominal (8.27 mg/L measured) HC concentrations, all the fish died within 96 hours. Between 50 mg/L and 200 mg/L nominal concentrations (0.55-1.92 mg/L measured concentrations), feeding became noticeably slower within 2 days and stopped completely by 3 days. The fish displayed signs of narcosis and swimming impairment with >92% decrease in food consumption. The 'lowest observable effect' concentration (LOEC) was 25 mg/L nominal (0.31 mg/L measured). Feeding slowed after 5 days resulting in a decrease in food consumption >54% after 14 days when compared to controls. No effects on feeding behaviour or body water content were evident at the 'no observable effect' concentration (NOEC) of 10 mg/L nominal (0.14 mg/L measured) HC concentrations. The maximum allowable toxicant concentration (MATC) for the alteration of feeding behaviour in young rainbow trout ranged between NOEC and LOEC at 0.14 to 0.31 mg measured HC per litre of water.

No significant trend was seen in the wet weight and lengths of the fish during the experiment probably because of the short exposure duration (14 days). There was a small increase in body water content in the exposed fish relative to unexposed controls.

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1.0 INTRODUCTION

The discharge of petroleum hydrocarbons (HC) into the environment often causes considerable ecological damage for extended periods of time. In 1989, the *Exxon Valdez* ran aground in Prince William Sound, Alaska, releasing 40 million litres of crude oil into a coastal shoreline area extending over a distance of 700 kilometers (Sumich, 1992). The spill was blamed for the deaths of over 33,000 sea birds, several thousand commercial and non-commercial fish, sea otters, seals and possibly some cetaceans (Sumich, 1992; Loughlin, 1994). Studies on the biological impact around Prince William Sound continue to this day. During 1997, a total of 136 major oil spills introduced an estimated 222 million litres of petroleum into the aquatic habitats worldwide (Cutter Information Co., 1998).

In actuality, oil spills make up only a fraction of the total amount of hydrocarbons entering the environment annually. Human activities are believed to make up about 76% of the total hydrocarbons entering the sea (Table 1). Municipal runoff and industrial pollution also contribute significant amounts of petroleum hydrocarbons to aquatic environments. San Francisco Bay, in 1979, was receiving municipal inputs of grease and oil sludge amounting to 72,400 pounds per day (Whipple et al., 1981).

Natural oil seeps, such as those found in the Gulf of Mexico, are also common sources of petroleum inputs into aquatic habitats. A conservative estimate of 40 million litres of seeps oil flows into the Gulf every decade which is almost the equivalent amount of oil released by the *Exxon Valdez* spill in 1989 (MacDonald, 1998). Although the total quantities are the same, the rates of

Table 1. Estimated inputs of petroleum from all sources.
(Modified from Etkin et al., 1998)

	Amount of Input by year (tonnes)			Amount of Input by year (as percentage of total)		
Source	**1973**	**1981**	**1990**	**1973**	**1981**	**1990**
Municipal/Industrial	2,700,000	1,230,000	1,175,000	44.2	38.4	50
Transportation (including spills)	2,130,000	1,420,000	564,000	34.9	44.4	24
Atmosphere (deposition)	600,000	300,000	305,500	9.8	9.4	13
Natural sources	600,000	200,000	258,500	9.8	6.4	11
Offshore production/ Exploration	80,000	500,000	47,000	1.3	1.6	2
Total	**6,110,000**	**3,200,000**	**2,350,000**	**100**	**100**	**100**

input are not. The open sea may be able to tolerate such massive inputs over the course of time whereas the same amount released into a smaller water body at a single point in time can be devastating. Natural seepages have also been documented along the Mackenzie River near the Norman Wells area of the Northwest Territories, Canada (Bone and Mahnic, 1984). A rough estimate of 600 barrels was calculated as the annual input of seepage oil into the river (Sikstrom, 1998). Such constant, chronic discharges of petroleum into the environment may influence aquatic life not adapted to HC exposure.

Concerns about natural seepage effects on fish in the Mackenzie River around Norman Wells were investigated in the 1980s by the Canadian Department of Fisheries and Oceans. Expansion of the Norman Wells drilling site, by Imperial Oil Resources, coincided with reports of deteriorating fish quality. Burbot (*Lota lota*) livers were reported to be small, dark and unfit for consumption while complaints were also made about the watery texture of whitefish (*Coregonus clupeaformis*) by local residents downstream from the oil field (Lockhart et al., 1987a). Studies done by Lockhart et al. (1987a) concluded that burbot were being influenced by a loss in liver fat. The effects of this loss made the livers small and dark compared to edible livers which are larger and pale colored. The watery texture of whitefish was not thoroughly investigated although the body moistures reported were among the highest recorded for North American whitefish (Lockhart et al., 1989a). Hydrocarbons were detected in burbot and whitefish tissues which may be explained by a general aerial dispersal and deposition of HC laden particles (Juttner, 1986). However, larger amounts of low boiling point aromatic compounds were detected in fish tissues during the winter season suggesting that a source of hydrocarbons existed under the ice covered water (Lockhart et al., 1989a). It was

hypothesized that the unusual occurrences in fish could have been due to contamination by Norman Wells crude oil seeping into the Mackenzie River.

The routes of hydrocarbon uptake into fish are primarily across the gills during respiration, through the gut after ingestion of tainted food (as oil is adsorbed onto particles) and through the skin (Vandermeulen, 1987). Partitioning of hydrocarbons between the environment and fish is regulated by simple concentration equilibria (Malins and Hodgins, 1981). There is no known mechanism for actively transporting HC across the gill or dermal epithelium. Bioavailability and uptake of the HC is linked to the molecular structure of the individual compounds (Vandermeulen, 1987; Whipple et al., 1981).

Several other factors modify uptake of HC by fish, including type of exposure (WSF 'inhalation', adsorbed to food and ingested, as droplet-particulates), type of soluble components, concentration, duration of exposure, temperature, salinity, alkalinity and the presence of other pollutants which may be synergistic/antagonistic. Research suggests that respiration provides the primary intake route while ingestion and especially the dermal routes are very limited in their assimilation of HC into the body (Whipple et al., 1981).

The physiological state of the fish is also important in controlling the uptake of petroleum hydrocarbons. Larval fish are far more susceptible to HC contamination than older juveniles because they accumulate hydrocarbons in their yolk faster than adults accumulate in their tissues (Thomas et al., 1989). The gender of the fish also dictates uptake rates with females generally accumulating HC faster than males, while size and reproductive state also play a role (Whipple et al., 1981).

Hydrocarbon uptake is rapid and can be detected within fish tissues after several minutes of exposure (Lee et al., 1972) but few studies have measured rates of uptake. Maximum uptake rate is often reached within 2-24 hours of continuous exposure while the maximum concentration in organs is reached in 48-72 hours (Whipple et al., 1981). Equilibrium occurs when the organism reaches a balance between rates of uptake and depuration. Changes in fish behaviour and physiology can become evident in similar periods of time; after minutes to hours of exposure.

There are several reports which document lethal and sub-lethal changes in fish exposed to the water-soluble fraction of crude oils. Exposed fish exhibit a greater demand for oxygen, increase breathing rates and begin excess "coughing". Increasing the motion of gills in water increases the exposure to the fish by accelerating HC uptake into the body (Rice et al., 1975). Higher hydrocarbon concentrations may cause spasms and death (Lockhart et al., 1987b; Patten, 1977), while sub-lethal effects include narcosis, cellular morphology changes such as neoplasia (Malins et al., 1984) and possible avoidance behaviour based on chemosensory damage (Birtwell et al., 1996; Patten, 1977). Two crucial neurological effects include disorientation inhibiting swimming (Carls, 1987; Patten, 1977; Steadman et al., 1991) and a loss of appetite (Collodi et al., 1984; Carls, 1987).

The loss of appetite in fish exposed to a WSF has been documented frequently but rarely investigated. Folmar et al. (1981) determined that the WSF of Cook Inlet crude oil significantly impaired the capturing of prey by Coho salmon (*Oncorhynchus kisutch*) noting that there was a loss of interest in feeding. Moles et al. (1981) also reported "less aggressive" feeding behaviour in Coho salmon fry exposed to toluene and naphthalene in

aqueous solutions. Carls (1987) investigated the effects of both dietary and water-borne oil exposure on larval Pacific herring (*Clupea harengus pallasi*). Data were reported only on the percentage of fish that reduced feeding within three concentrations of 0.3, 0.5 and 0.9 ppm WSF. These data were further correlated with decreased larval growth and mortality (Carls, 1987). No studies have been reported that measured the levels of hydrocarbons that induce this sub-lethal effect on appetite.

A decrease in the food consumption caused by HC contamination could have an impact on fish and the fishing industry. Fish that starve for excessive periods of time tend to exhibit an increase in body water content which may change the tissue texture (Reinitz, 1983). The fish body reacts to a decrease in its food consumption by utilizing its carbohydrates, fat and then protein for energy causing dry weight to decrease while wet weights remain relatively constant (Moles and Rice, 1983; Groves, 1970). This phenomenon has been observed in rainbow trout (Reinitz, 1983; Denton and Yousef, 1976), pink salmon (*Oncorhynchus gorbuscha*; Moles and Rice, 1983) and burbot (Lockhart et al., 1989a). Larval trout exposed chronically to NWC oil also developed edema but it was not clear whether this was a response to the oil or a response due to reduced feeding (Lockhart et al., 1996).

Fish tissue contaminated by hydrocarbons may also retain an oily taste and odour (Murray, 1984; Lockhart et al., 1989b) marking the fish as hazardous and unfit for human consumption. Fish exposed to a WSF have also been reported to have significant decreases in long-term and short-term growth (Lockhart et al., 1996; Moles and Rice, 1983). Contamination of fish by petroleum hydrocarbons influences feeding behaviour

and ultimately growth, quality and even mortality of the animals which could generate a significant loss to recreational and commercial fishing (Schneider, 1993).

Hydrocarbons in solution, or dispersed as fine particulates, in high enough concentrations may elicit adverse physiological responses. The 'maximum allowable toxicant concentration' (MATC) for such physiological responses to occur allows direct comparisons between water analysis and fish toxicity. The MATC is defined as the hypothetical toxic threshold concentration range falling between the lowest concentration showing an observable effect (LOEC) and the lowest concentration showing no observable effects (NOEC) (Landis and Yu, 1995). Woodward et al. (1981) studied the effects of WSF toxicity on cutthroat trout (*Salmo clarki*) and found a LOEC of 39 *u*g/L and a NOEC of 24 *u*g/L, suggesting a MATC between 24-39 *u*g soluble HC per litre of water to avoid any biological effects on fish.

The disappearance of HC from tissues and organs over time suggests some metabolism and/or excretion of HC by fish (Rice et al., 1975). The metabolism of naphthalene has been demonstrated in 3 species of marine fish (Lee et al., 1972) and metabolism of benzopyrene occurs in rainbow trout (Pederson and Hershberger, 1974). The metabolism of HC by fish primarily occurs in the liver through the mixed function oxygenase (MFO) system. MFOs work on a large number of highly lipophilic compounds and are present in most tissues but are most abundant in the liver and kidney. They are part of a complex, broad response enzyme system which functions to detoxify foreign compounds making lipid soluble chemicals more water-soluble and hence more readily excreted (Vandermeulen, 1987; Payne and Penrose, 1975).

MFOs can be induced by a variety of compounds including aliphatic and aromatic HC or similarly structured molecules. This enhancement has been demonstrated in most vertebrates from eels to humans (Vandermeulen, 1987). Induction is measurably observed within 24 hours of exposure to petroleum products (Payne and Penrose, 1975).

When crude oil and water are mixed, two primary phases are formed in the process. The first phase consists of crude oil mixed with water to form a thick brown surface mixture termed "chocolate mousse". The mousse is a thick emulsion that coats everything it contacts. As the crude oil becomes 'weathered', volatile components are lost to the surrounding environment. Heavy waxes and asphaltenes eventually group together forming "tar balls". These balls are usually neutrally buoyant and may remain in the water column for long periods and may eventually wash ashore coating shoreline sediment (Mackay, 1987). Both the mousse and the tar balls can be physically extracted from a water medium resulting in a significant recovery of spilled material. Mousse and tar ball formations comprise the most visual aspects of an oil spill.

The second phase formed by an oil spill is less obvious. Some hydrocarbons with low molecular weights form a true aqueous solution while others become suspended as fine particulates or droplets (Vandermeulen, 1987). This mixture has been called the 'water-soluble fraction' (WSF). The dissolved and suspended hydrocarbons in a WSF are not readily removed by physical means. The WSF disperses throughout the water column coming into more intimate contact with aquatic organisms. It consists primarily of volatile (low boiling point), aromatic hydrocarbons such as benzene, toluene, ethylbenzene and xylene (BTEX). The amount of aromatic compounds in a WSF depends

on the type of oil used as well as the oil loading rate (Blenkinsopp et al., 1996). Norman Wells crude oil (NWC), for example, consists of approximately 12% aromatic hydrocarbons while Louisiana crude contains approximately 25% (Jokuty et al., 1996). Exposure to WSF in the environment is probably responsible for many of the toxic effects on fish.

A WSF would not normally persist in an aquatic environment for an extended period of time due to the volatility of its components. Under ideal conditions, aromatic hydrocarbons would be removed naturally from the aquatic medium in a variety of ways including volatilization, photolysis, microbial degradation, emulsification and dispersion (Figure 1; Mackay, 1987; Blumer et al., 1973) or diluted below limits of detection. The sum of these processes is termed 'weathering'. Weathering is a major factor in determining the persistence of petroleum hydrocarbons in an environment over time. The environmental fates of two light paraffinic crude oils stranded on beaches in Massachusetts and Bermuda were observed over 16 months (Blumer et al., 1973). These two sites maintained a difference in climates with similar beach characteristics expected to facilitate the weathering process. Lower boiling point components were evaporated within several months. The oils were also partially degraded by microbial action. However, after the 16 month period the oil residues were not highly weathered, inert asphalts. An unanticipated perseverance of the oil and its higher boiling components, including aromatic compounds was evident. It was concluded that a considerable and biologically important fraction of the crude oils was retained over the observation period giving evidence to the persistence of oil over time even under favorable weathering conditions (Blumer et al., 1973).

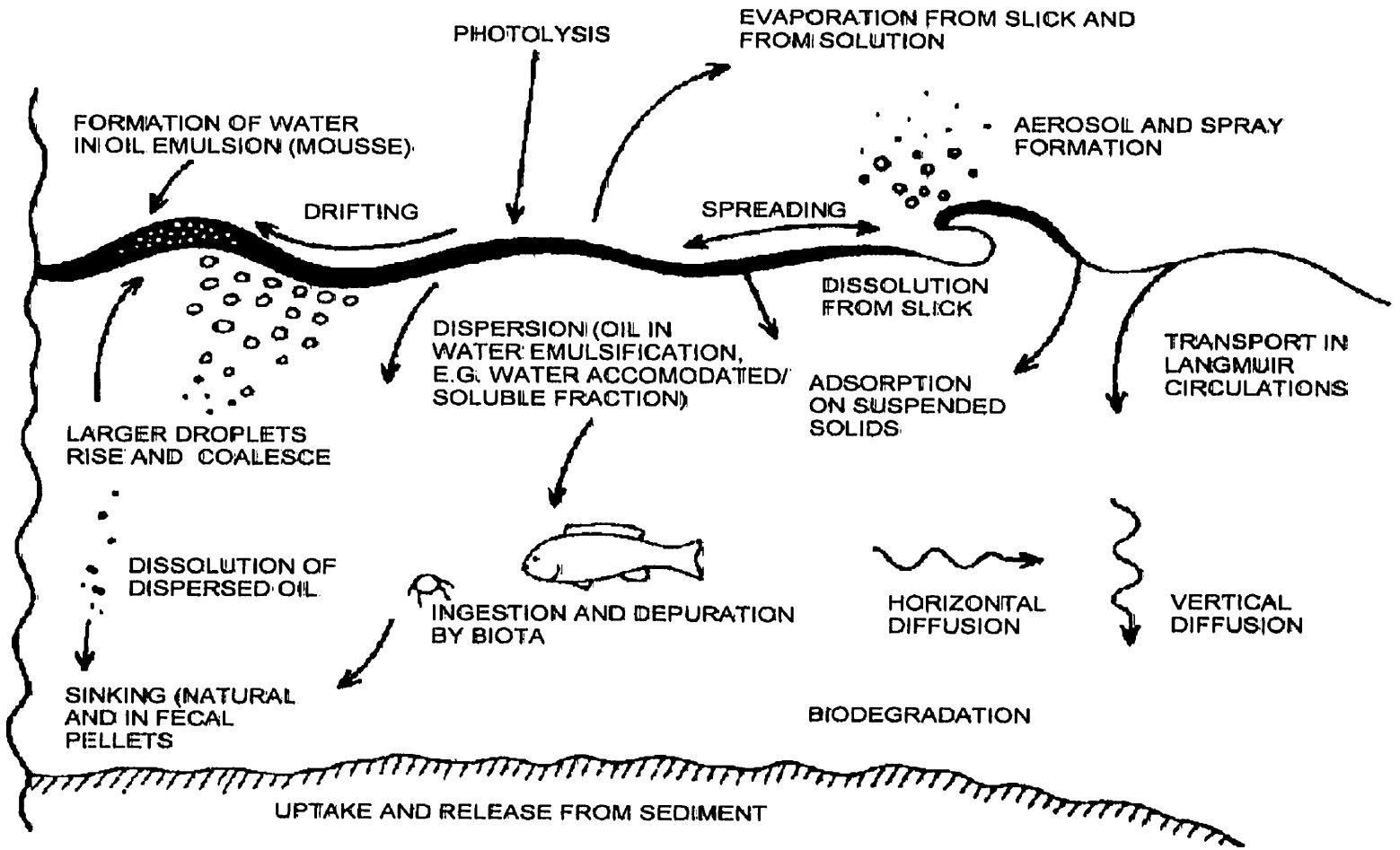


Figure 1. Various biotic and abiotic factors affecting weathering (photolysis, biodegradation, evaporation, dissolution), sources of exposure to biota and oil in water formations (mousse, dispersions such as water-soluble fraction, aerosol). (Modified from Mackay, 1987)

A constant concentration of petroleum hydrocarbons may persist in certain areas for several reasons. Locations downstream of natural oil seeps or municipal sewage plants may receive a steady input of hydrocarbons (Hoffman and Quinn, 1987) and hence retain elevated WSF levels. Soluble petroleum hydrocarbons may also persist after an oil spill in locations such as wetlands or lakes since their small volume, as compared to a marine environment, decreases the amount of dilution (Vandermeulen, 1987). An environment which inhibits volatilization also promotes perseverance of the WSF. For example, petroleum caught under winter ice (Figure 2; Mackay, 1987) would be hindered from weathering since this relatively static environment allows for virtually no evaporation (Mackay, 1987; Dickens et al., 1981). The Mackenzie River (NWT, Canada) has a constant source of hydrocarbons from natural seeps and is ice-capped in winter. These two conditions create an environment with potential for persistence of toxic, soluble petroleum hydrocarbons.

Accurate analytical methods are required to detect low concentrations of hydrocarbons in water. The concentrations of water-soluble hydrocarbons can be analyzed using several gas chromatography (GC) methods. A common method, known as microextraction, uses a very small volume of organic solvent to extract hydrocarbons from the water. The Murray microextraction method was derived in 1979 to find a faster, cost-effective analytical procedure for determining the concentrations of sparingly soluble hydrocarbons in solution (Murray, 1979). The microextraction had the advantage of being simple, economic and quick relative to other analysis routines (Murray, 1979). It improved recovery percentages by eliminating the solvent concentration step used by

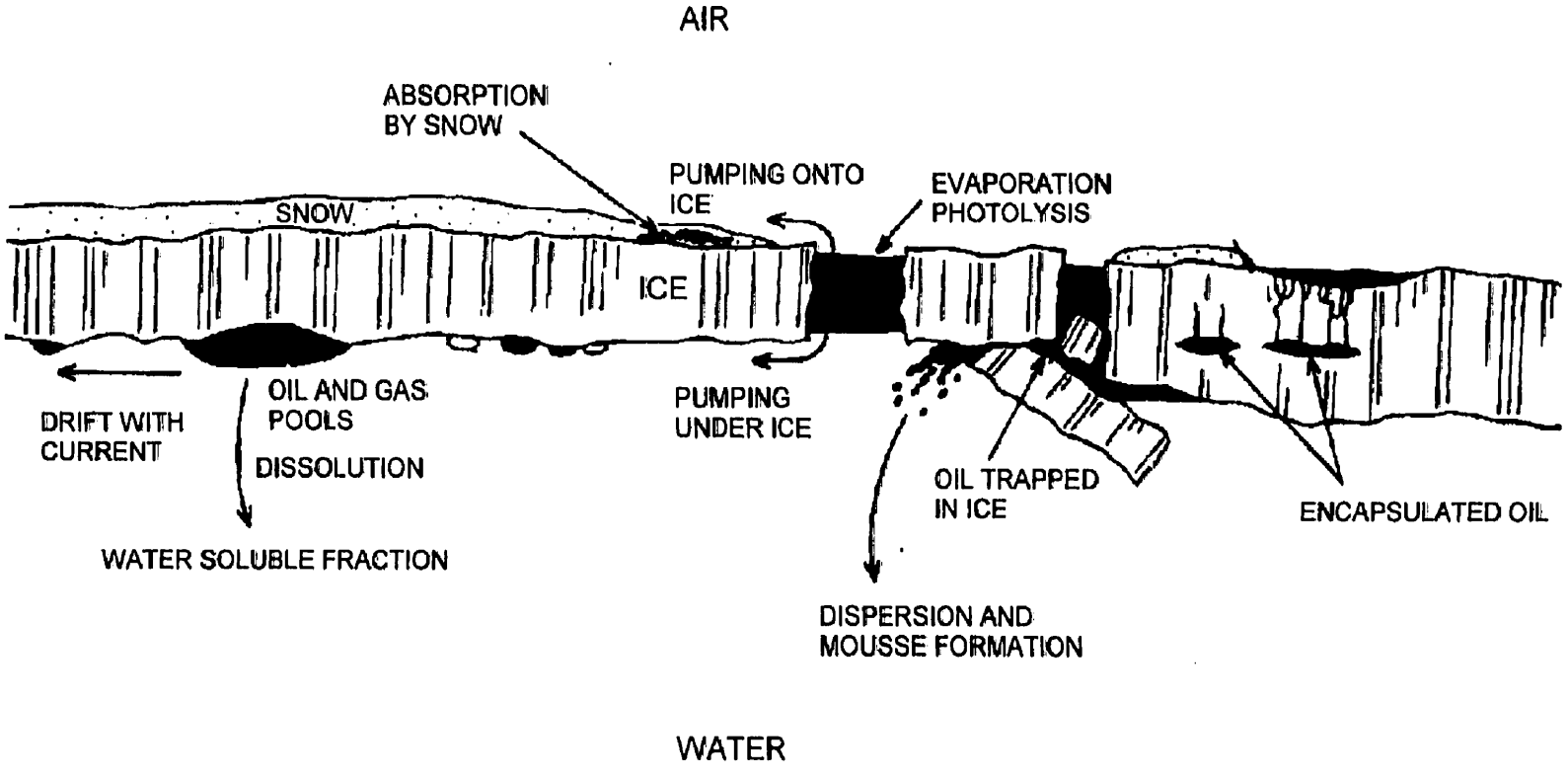


Figure 2. The role of ice in retaining volatile properties of petroleum. (Modified from Mackay, 1987)

earlier protocols. Analyses that used rotary evaporation, Snyder column or blowing nitrogen over a sample showed analyte losses between 27-66% (Murray and Lockhart, 1981). The Murray extraction, with the optimum solvent to sample ratio, increased analyte recoveries to values as high as 74% (Murray, 1984). The Murray method could be used for extracting low molecular weight (C6-C14) aromatic compounds such as those found in a water-soluble fraction. It is hypothesized that aromatic hydrocarbons are responsible for most of the tainting and physiological changes found in contaminated fish.

Fish exposed to the WSF of crude oil become contaminated with hydrocarbons which modify feeding behaviour and causes several other adverse physiological responses. A loss of appetite leading to starvation may decrease the quality of fish ultimately affecting their survival. Chronic exposure to seepages of Norman Wells crude oil in the Mackenzie River in high enough concentrations may be initiating this effect on local fish. The exposure concentrations and durations affecting appetite loss in fish tainted by WSF contamination have never been measured quantitatively.

The primary purpose of this thesis was to determine the 'lowest' and the 'no observable effect' concentrations (LOEC/NOEC), of soluble petroleum hydrocarbons from Norman Wells crude oil, that altered the feeding behaviour of rainbow trout under static conditions. The secondary purpose was to express quantitatively the degree of this altered feeding behaviour. The responses reported include LOEC, NOEC and the maximum allowable toxicant concentration range (MATC). Other responses recorded include the quantities of food consumed at LOEC and NOEC, lengths, weights and fish body water content.

2.0 MATERIALS AND METHODS

2.1 Water-soluble Fraction (WSF) Preparation

The mixing protocol to prepare WSF conformed to that of Blenkinsopp et al. (1996) with modifications in order to fit experimental design requirements. The amount of oil loaded into the test water, referred to as the 'oil loading rate' or 'nominal concentration', provided a range of concentrations of WSF. The relationship between nominal oil concentration and actual hydrocarbon (HC) concentrations 'measured' by gas chromatography (GC) were described with a linear regression (Pearson correlation and Bartlett's chi square statistic) using Systat®.

The oil loading rates used were 400, 200, 100, 50, 25 and 10 mg Norman Wells crude oil (NWC; Imperial Resources, Calgary, Alberta) per litre of test water. Oil was weighed by displacement by measuring the weight of a syringe filled with oil before and after the addition of oil to the test water. Weight measurements were made using a Sartorius top loading balance for large loading rates or a Mettler microbalance for small loading rates. The weighed oil sample was transferred to a 20 L glass carboy filled with test water and sealed with a size 12 neoprene stopper. There was less than 1.5 L of headspace in the sealed carboy. Solutions were stirred for 18 h in the dark, at approximately 10 °C, followed by a 3 h settling time. The settling time was necessary because the magnetic stirrers produced a spinning vortex of approximately 20-25% of the carboy height which is greater than the 10% vortex suggested by Bleinkinsopp et al. (1996). Prior to each use, carboys were first washed three times with Sparkleen® detergent followed by rinses with distilled water and finally with test water. Analyses of

samples taken of distilled water from the carboys between preparations but after washes revealed no evidence of residual hydrocarbons.

The experimental WSF preparations used dechlorinated (with sodium thiosulphate) and micro-filtered City of Winnipeg water.

The WSF was removed from under the oil layer in the carboys and placed directly into the non-control/experimental fish tanks. A new WSF was mixed every 24 h. Water samples of the control and WSF solutions were taken daily, immediately following the settling period, and analyzed.

2.2 WSF Extraction and Analysis

Water samples were stored in glass bottles with Teflon®-lined lids with no headspace at 4 °C until analyzed. All samples were analyzed within 7 days of extraction. A modified 100 mL volumetric flask (Figure 3) was used to extract a 90 mL water sample with 400 *u*L of HPLC-grade hexane (Murray et al., 1984). This mixture was shaken vigorously for one minute then left to stand for ten minutes to allow the collection of hexane droplets on the surface of the sample. The flask was tilted and distilled water was added to bring the hexane up the capillary tube. The hexane was removed to a GC autosampling vial and fortified with 10 *u*L of 5 deuterated compounds; toluene, ethylbenzene, naphthalene, 1,4 dichlorobenzene (1,4-di*Cl*-benzene) and chlorobenzene as internal standards. A 1 *u*L injection of the hexane fraction was analyzed for 12 compounds using a Hewlett Packard 5890 GC with an HP 5970 mass selective detector. The compounds included toluene, ethylbenzene, *m*- and *p*-xylene (combined data),

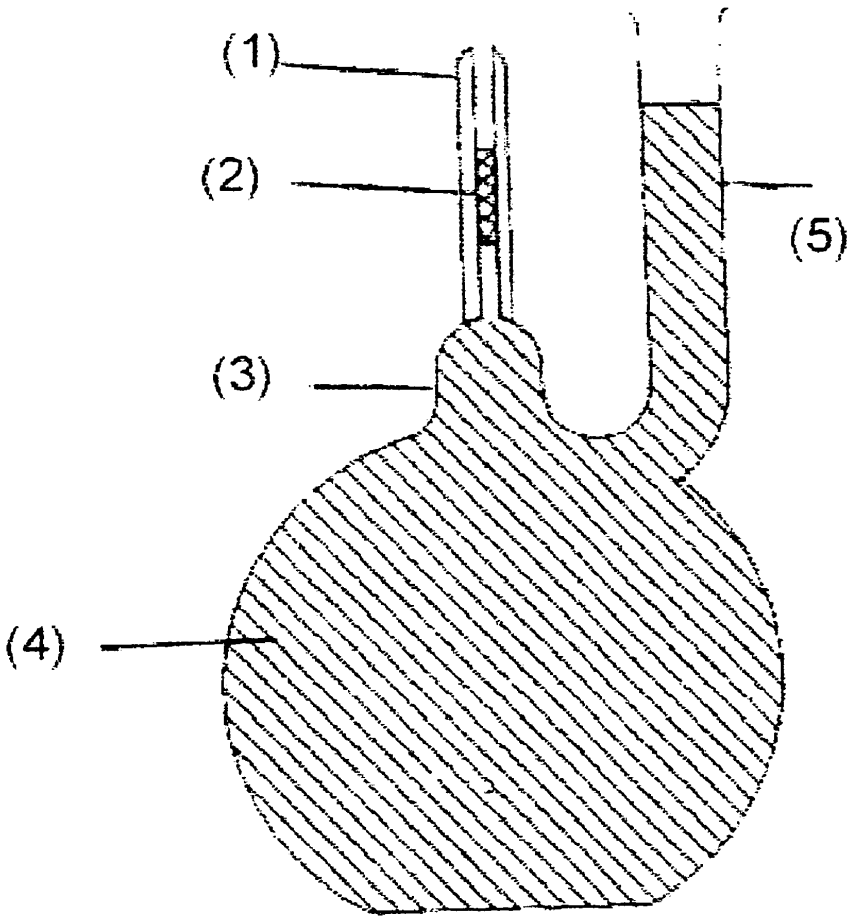


Figure 3 Murray's modified flask for micro-extraction. WSF sample and hexane are mixed in the flask. The solvent hexane coalesces in the collection bulb and is pushed up the capillary tube by the addition of distilled water down the flask arm. (1) Capillary tube, (2) hexane solvent, (3) collection bulb, (4) modified 100 mL volumetric flask with water sample, (5) flask arm.

o-xylene, isopropylbenzene, *n*-propylbenzene, 1,3-trimethylbenzene, 1,4-di*Cl*-benzene, naphthalene, 2-methylnaphthalene and 1-methylnaphthalene.

The specifications of the analysis program were:

Injection: 1 *u*L using HP 7673 autosampler,

Column: J&W DB-5MS, 0.25 mm I.D., 0.25 *u*m film thickness, 30 m capillary column,

Temperature program: start 35 °C increasing at 4 °C per min to 100 °C, then increasing at 10 °C per minute to 200 °C,

Inj temp: 250 °C, Detector temp: 290 °C, Carrier: UHP-grade helium, Flow rate: 34 cm/s, Column flow rate: 0.9 mL/min, Split flow: 40mL/min, Split ratio: 45:1.

Blank samples were analyzed to calculate the limits of detection by integrating the area under the curve at the baseline for the 12 analytes. The limits of detection were reported as the integrated concentration plus 3 times the standard deviation (Keith, 1990).

BTEX (benzene, toluene, ethylbenzene, xylene) certified standards (Ultrachek®- Fisher Scientific) were also analyzed to determine the percent of hydrocarbons recovered while using this extraction process. The standards were used to fortify blank water samples followed by extraction with the Murray method. GC measured concentrations were compared to expected concentrations to calculate percent recovery of analytes.

Losses of HC during sample storage before GC analysis were determined. A WSF was prepared using 1000 mg/L NWC in distilled water and analyzed on a Hewlett Packard GC with a flame ionization detector (FID). The specifications of the analysis program were:

Injection: 1*u*L manual injection,

Column: Quadrex, high temp., Al-clad, bonded methyl-silicone 25 m, 0.25 mm I.D., capillary column

Temperature program: start 40 °C increasing at 5 °C per min to 100 °C, then increasing at 10 °C per minute to 200 °C,

Injector temp.: 290 °C, Detector temp: 400 °C, Carrier: UHP-grade helium,

Note: Two different GCs were used in the analyses due to the location of the experiments. Fish experiments were performed at the Freshwater Institute in Winnipeg, Manitoba which had a GC-MS while the WSF-only experiments were performed at the Imperial Oil Research Centre in Calgary, Alberta which had a GC-FID.

Samples were taken immediately following the WSF settling period and stored over one week in Teflon®-lined sample jars at 5 °C, designated as "closed containers". Samples were analyzed daily, and compared to day zero concentrations. Concentrations of HC in an open container (20 L carboys) without fish, were monitored over 7 days to predict WSF loss in experimental tanks before solution renewal. Several samples were taken from tanks containing fish, 24 h after the addition of WSF and prior to WSF solution renewal during the feeding experiments. The percent recoveries were applied as correction factors in determining the actual concentration of HC in each sample.

The calculation for the measured HC concentrations was as follows:

(GC output (ng) / 0.09 L (90 mL) of WSF sample) / (1,000,000 ng : 1 mg)

= concentration of HC unadjusted (mg/L)

HC unadjusted (mg/L) * recovery factor

= actual HC concentration (this value may be further adjusted by estimated values for potential loss during sample storage and hence does not reflect 'actual' HC concentrations, the value is further referred to as 'measured' HC)

Note: The percent recovery was based on the average of five known compounds (ethylbenzene, toluene, *m*-, *p*- and *o*-xylene and assumed to be similar for the other HC.

2.3 Rainbow trout Satiation Feeding Experiments

Several 6 L, non-aerated, glass tanks were set up so that each contained five rainbow trout (Kamloops strain, one year old fingerlings, 2-4 g, from Glacier Springs Hatchery, Manitoba) for feeding experiments in a controlled environment room at 10 °C. Fish were originally kept in holding tanks at 10 °C and fed starter food (Martin Mills, Elmira, Ontario) daily at approximately 2% of their total body weight. Special food scoops were constructed from acrylic to measure out approximately 15 mg and 50 mg of food per scoop. The first satiation experiment began with a 48 h starvation period but since a large peak in food consumption was noticed in the first day of the experiment, following experiments used only 24 h starvation prior to testing. After the starvation period, fish were then fed Martin Mills Trout starter food twice per day to satiation for 14 days. The amount of food consumed by the fish in a tank was measured as a percentage of the total weight of fish in that tank. This was divided by the number of fish in the tank to produce a percentage of body weight of food consumed daily per fish. The endpoint for feeding was reached when greater than 40% of the last scoop of food remained on the

surface for 5 minutes with no evidence of active feeding by more than one fish. Analysis of variance combined with Tukey's Honestly Significant Difference (HSD) post-hoc comparison was used to test for significant differences between satiation feeding experiments before and after exposure to WSF and between replicates of individual tanks.

Fish wet weights and lengths were recorded before and after the experiments. Dissolved oxygen, pH and temperatures were measured daily. Dissolved oxygen never fell below the recommended 5 mg/L for cold freshwater fish (APHA/AWWA/WPCF, 1985) and pH values ranged between 7.4 and 7.6. Wastes were siphoned and tank water was changed daily. All the experiments included a replicate tank.

Note: Martin Mills starter food has a guaranteed analysis of 52 % (min.) crude protein, 15 % (min.) crude fat, 1.5 % (max.) crude fibre, 0.7 % sodium, 1.5 % calcium, 1.0 % phosphorous, 10,000 IU/kg (min.) Vit A, 3000 IU/kg (min.) Vit D3, 110 IU/kg (min.) Vit E, and 300 IU/kg (min.) Vit C.

2.4 WSF Exposure and Feeding Experiments

The 6 L fish tanks were set up with the same method as the satiation feeding experiments (Section 2.3). Control tanks were filled with test water. The WSF was removed from under the oil layer in the carboys and placed directly into the non-control tanks. A new WSF was mixed every 24 h. Both control and WSF tank solutions were replaced daily and wastes were siphoned. Water samples of the control and WSF solutions were taken daily and analyzed (Section 2.2).

The oil loading rates selected (10, 25, 50, 100, 200 and 400 mg/L) were based on research done by Lockhart et al. (1996), who reported a lethal level of NWC for rainbow

trout could be obtained by mixing a WSF of 249 mg/L NWC. Based on this information, the WSF feeding experiments utilized a lethal loading rate (400 mg/L) as well as a graded series of smaller loading rates of NWC to generate sub-lethal levels of the WSF.

Fish were subjected to the same definition of the endpoint of feeding as in the satiation experiments except an upper limit of 1.2% of the body weight per fish had been arbitrarily set based on the data obtained in the satiation feeding experiments (Section 2.3 and 3.2.2). Trout were originally fed twice per day but excessive algal growth occurred in tanks that contained fish with suppressed feeding. To decrease the algal growth the minimum ration was cut to only one food measure per day on the condition that an observable amount of food remained in the tanks since the first feeding. Fish were fed a minimum of one scoop of 15 mg of food per day per tank regardless of observations of behaviour. At the end of fifteen days, fish were killed by a blow to the head and measured for wet weight and length. Dry weight measurements were taken by placing the fish in an oven at 85 °C for 48 h then re-weighing them. Dissolved oxygen, pH and temperatures were measured daily.

Differences in body water content, wet weights and lengths were analyzed statistically in Systat® to determine effects of WSF on body composition and growth. Average body water content was compared between exposed and control tanks and against their paired replicates, within experiments, using a one-way analysis of variance (ANOVA) with Tukey's HSD post hoc test. Exposed tanks were also compared to control groups outside of their experiments. The one-way ANOVA was also used to detect differences between average lengths and wet weights of fish before and after the 14 day experiments (Appendix). The ANOVA analysis assumes errors are normally distributed,

have constant variance and are independent. These assumptions were checked graphically and statistically using Durbin Watson and First Order autocorrelation values (Appendix).

3.0 RESULTS AND DISCUSSION

3.1 Water-soluble Fraction (WSF) analysis

The composition of the WSF of NWC was dominated by toluene, *m*-, *p*-, and *o*-xylene, ethylbenzene, substituted benzenes and naphthalenes (Table 2 and Figure 4). Measured water-soluble HC concentrations ranged from 0.16 mg/L to 2.15 mg/L in the experiments depending on oil loading rates or "nominal" concentrations (Table 2). Doubling the oil loading rate effectively doubled the concentration of WSF compounds with the exception of the 100 mg/L data. A plot of nominal versus measured concentrations revealed a high linear correlation (R^2= 0.92, P=0.008; Figure 5).

Samples at the high concentration 400 mg/L loading rate appeared to 'overload' the GC column producing a large variation in results. This may have been caused by small oil droplets coalescing within the samples. Water-soluble fractions may be more properly termed 'water-accommodated' fractions due to microscopic oil droplets (estimated less than 1.2 *u*m in diameter) that are stably dispersed but undissolved in the water after mixing (Maher, 1986; Girling, 1989).

The concentrations of hydrocarbons are underestimated partly because benzene was not included in the analysis (Table 2). Benzene is a known constituent of Norman Wells crude oil (Jokuty et al., 1996) and is expected to be present in a WSF of NWC (Murray et al., 1984) in about the same proportion as ethylbenzene. The BTEX portion of NWC consists of 44.7% xylenes, 35.8% toluene and 9.6% of both benzene and

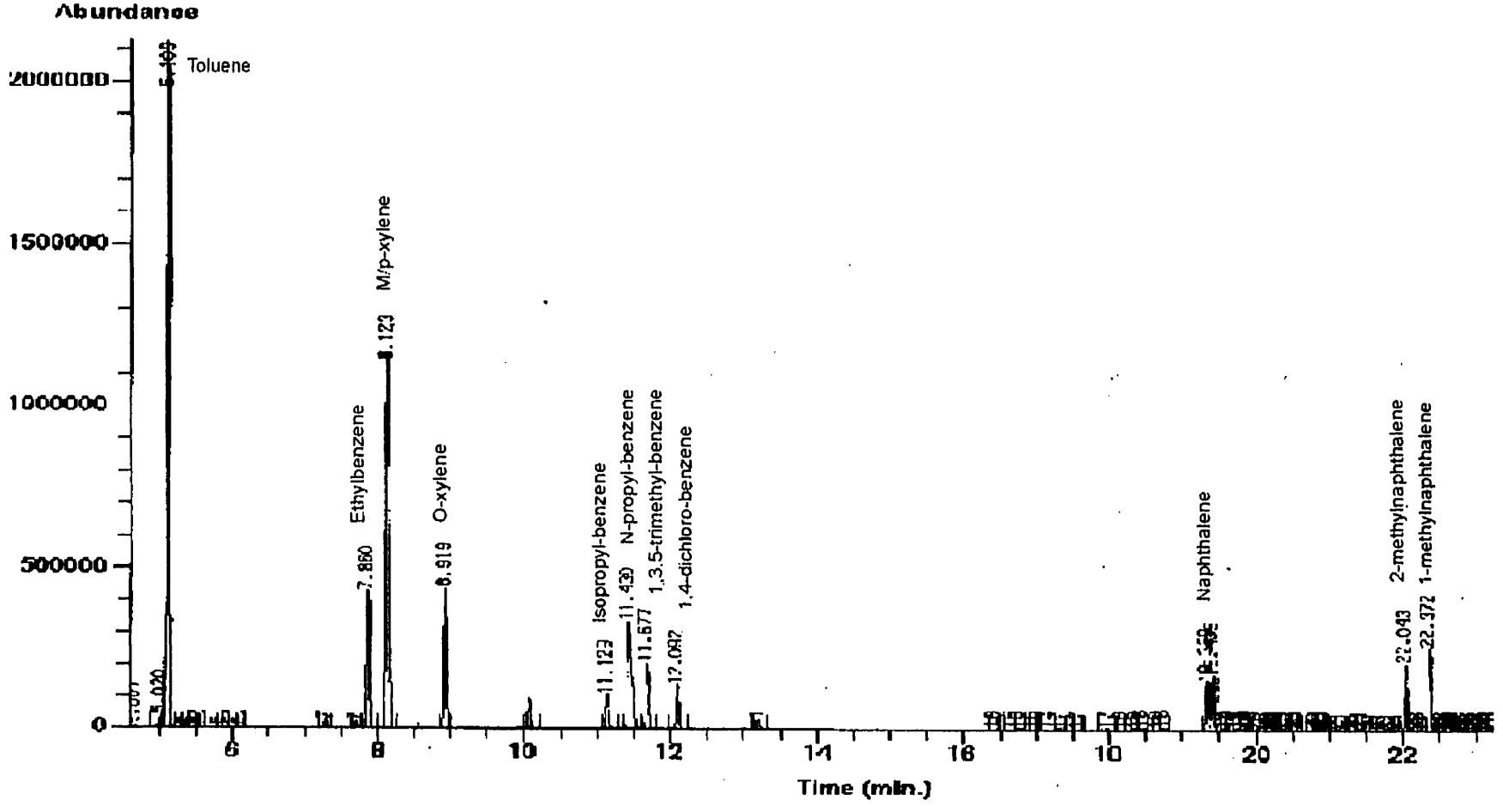


Figure 4 Chromatogram from GC-MS showing analyte peaks and retention times.

Table 2 Average measured concentrations of individual hydrocarbons in WSF by oil loading rates (oil:water ratio).

Loading rates	**10 mg/L**	**25 mg/L**	**50 mg/L**	**100 mg/L**	**200 mg/L**	**400 mg/L**
Compound	**Measured concentrations in solution (mg/L)***					
Toluene	0.040	0.086	0.225	0.240	0.621	1.411
Ethylbenzene	0.010	0.021	0.037	0.040	0.091	0.133
m- & *p*-xylene	0.048	0.089	0.167	0.180	0.331	0.443
o-xylene	0.012	0.026	0.051	0.065	0.111	0.093
Isopropylbenzene	0.004	0.007	0.011	0.009	0.014	1.450
n-propylbenzene	0.006	0.010	0.013	0.008	0.017	1.914
1,3,5-trimethylbenzene	0.009	0.015	0.023	0.021	0.027	2.783
1,4-di*Cl* benzene	0.000	0.000	0.000	0.000	0.000	0.007
Naphthalene	0.003	0.029	0.007	0.002	0.014	0.016
2-methylnaphthalene	0.009	0.018	0.010	0.001	0.688	0.015
1-methylnaphthalene	0.007	0.013	0.010	0.002	0.005	0.007
Total	**0.141**	**0.316**	**0.554**	**0.569**	**1.919**	**8.271**
Std Deviation	**0.003**	**0.008**	**0.022**	**0.035**	**0.182**	**10.572**
N	**8**	**7**	**10**	**8**	**8**	**3**

* adjusted for extraction recoveries (Table 3)

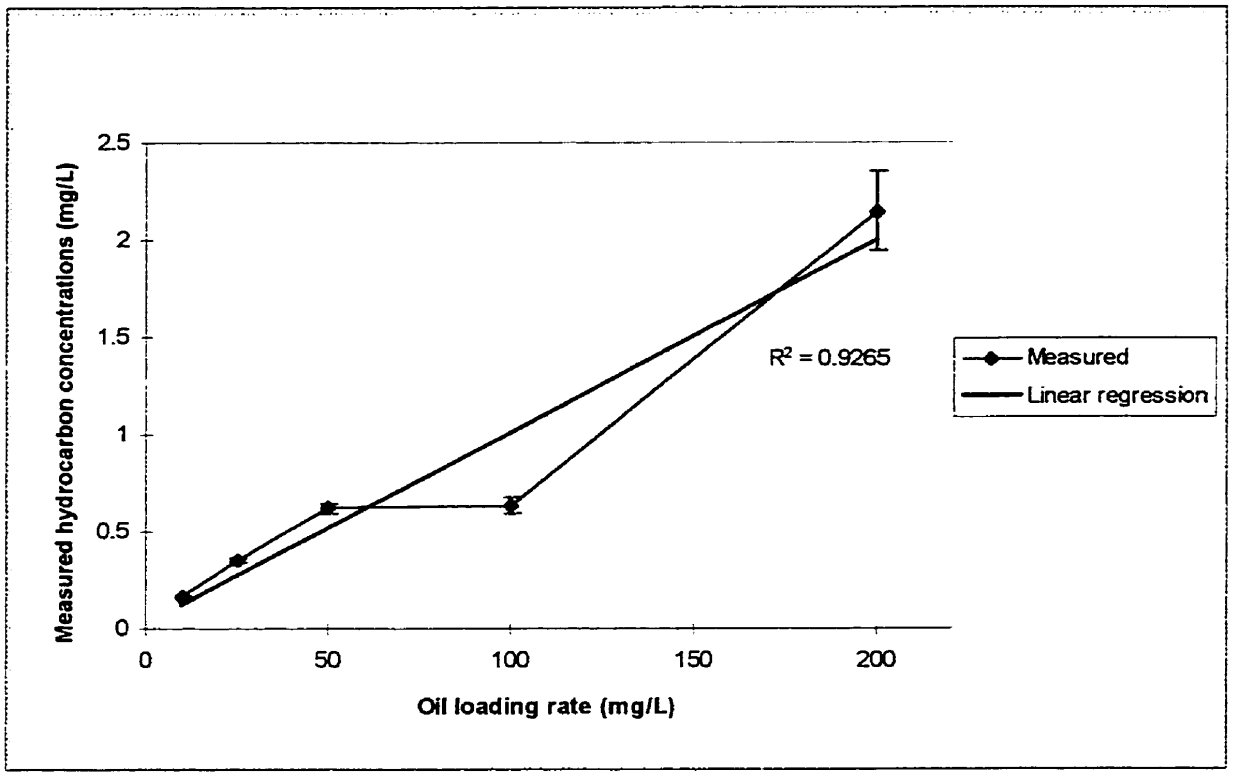


Figure 5 Oil loading rates vs. measured hydrocarbon concentrations (error bars represent standard deviation).

ethylbenzene (Jokuty et al., 1996). The extraction solvent, hexane, eluted from the GC column with the same retention time as benzene so any traces of benzene were masked by the large hexane peak. Benzene is a significant contributor to a WSF solution and would have increased the measured HC values. Limits of detection ranged from 0.014 *u*g/L for *n*-propylbenzene to 9.69 *u*g/L for toluene with a summed limit of 9.92 *u*g/L or approximately 0.01 mg/L (Table 3).

Percent recoveries were used as correction factors in calculating measured HC concentrations. Using the Murray extraction method (Murray et al., 1984), recoveries using samples spiked with known concentrations of the analytes are listed in Table 3. These recoveries are improved compared to a previously reported recovery average of 40% (Murray and Lockhart, 1981).

A correction factor for the potential loss of WSF during storage is also reported and can be used to adjust measured HC concentrations. Storage of a WSF is not recommended due to the volatility of WSF components (Blenkinsopp et al., 1996; Heras et al., 1995; Aurand and Coelho, 1996) although some success has occurred in retaining WSF in Teflon ® lined bags (Aurand and Coelho, 1996). WSF samples were retained for several days before analysis and the US-EPA maximum recommended time until analysis for volatile hydrocarbons, such as BTEX, is 7 days (Aurand and Coelho, 1996). Storage bottles were tested under holding conditions and an average loss over 7 days was determined. The experiments provided an average HC loss in storage containers of 12 % over 7 days (Figure 6) although final values of 20 to 25% were recorded on day 7. Of the individual HC measured (toluene, ethylbenzene, xylene), no differences are evident in volatilization in either closed or open containers.

The volatility of low boiling hydrocarbons suggests that persistence of a WSF is unlikely over a long period even under laboratory conditions. Estimations of the half-life, (time for 50% of the substance to volatilize from water), of some aromatic hydrocarbons have been calculated by using equations of Fick's law of diffusion and Henry's law constant along with solubilities, mass transfer coefficients and vapor pressures of individual compounds assuming certain depth, temperature and turbidity within a solution. The half-life for toluene, ethylbenzene and xylene (assuming 25 °C and 1 m depth) are 2.9, 3.1 and 3.2 hours respectively (Thomas, 1982). Lower temperatures would be expected to extend a half-life proportionately (Figure 6).

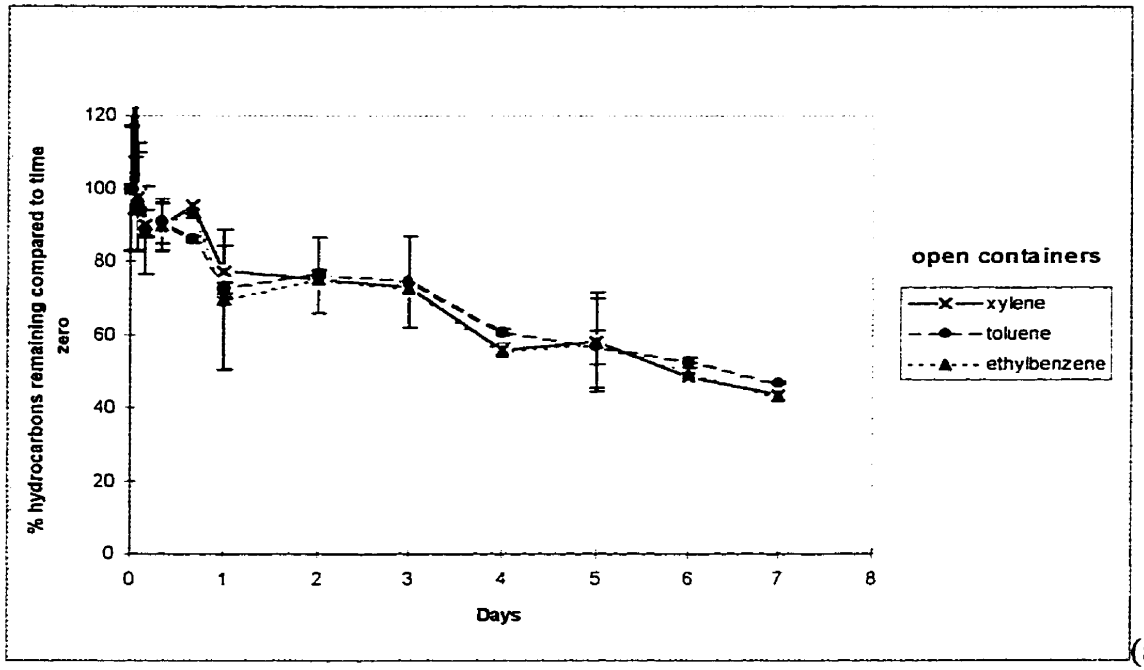
When compared to actual observations, theoretical estimations of the volatilization rates of hydrocarbons often have substantial error. Open containers without fish were measured for WSF loss at 5 °C over 24 hours up to 7 days to observe the volatilization under static conditions (without aeration). The loss of hydrocarbons in an open jar without fish was significant (55%) but 45% of the aromatic compounds including toluene, ethylbenzene and xylene, still remained after 7 days (Figure 6). This gives an estimated half life of approximately 6 days at 5 °C providing evidence for the persistence of a water-soluble fraction under some circumstances. Similar experiments measured a 40% loss of the WSF of Cook Inlet crude at 8 °C after four and a half days (Rice et al., 1977). Depending on the type of oil and environmental conditions, weathering of a WSF can be slow. In low energy shore areas near spill sites such as Chedabucto Bay (Nova Scotia) and the *Arrow* spill (1970), or the *Metula* spill (1974) (Strait of Magellan, South America), oil had remained "buried in intertidal beaches for years, often retaining its high boiling aromatic hydrocarbons" (Clark and Finley, 1977).

Table 3 GC-MS detection limits (+/- S.D.), calculated limits of detection and extraction recoveries of the HC analytes in the WSF.

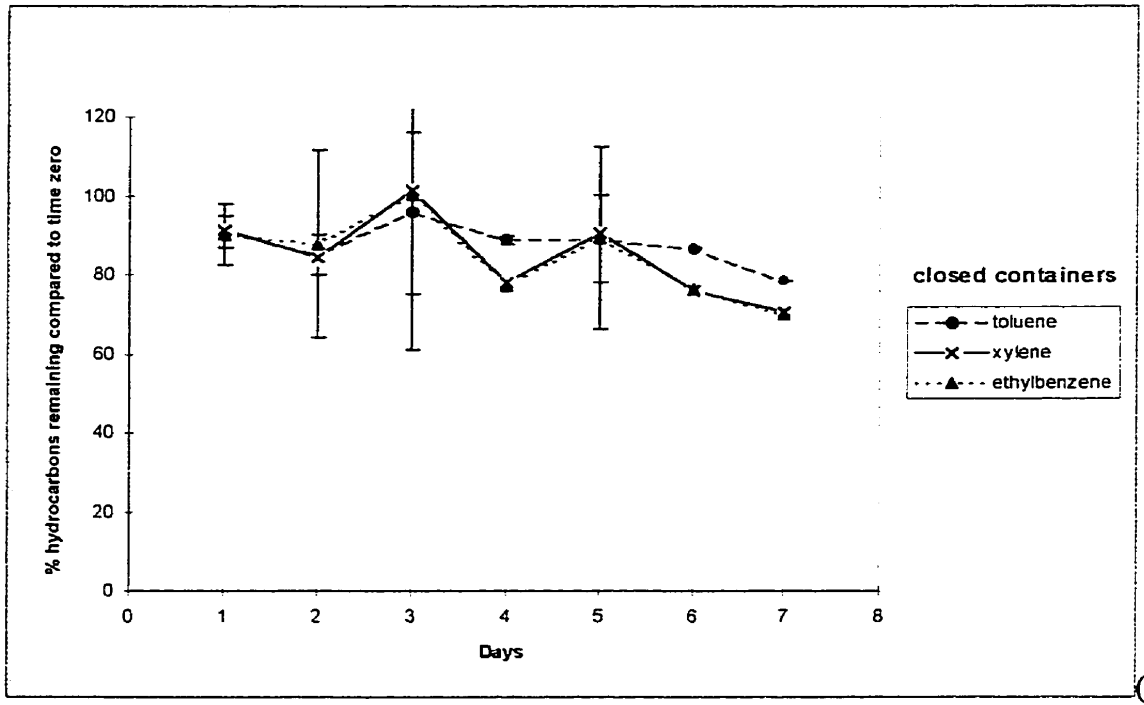
Compound	**Detection limit (*u*g/L)**	**Std Deviation**	**Limit of Detection* (*u*g/L)**	**Percent recoveries**
Toluene	7.59	0.70	9.69	14.8
ethyl benzene	0.01	<0.01	0.02	45.0
m- & *p*-xylene	0.02	<0.01	0.03	48.7
o-xylene	0.01	<0.01	0.01	59.3
isopropyl benzene	<0.01	<0.01	0.02	45.0**
n-propyl benzene	0.01	<0.01	0.01	45.0**
1,3,5-trimethyl-benzene	0.01	<0.01	0.02	45.0**
1,4-di*Cl* benzene	0.01	0.01	0.03	45.0**
Naphthalene	0.01	<0.01	0.02	45.0**
2-methyl naphthalene	0.01	0.01	0.04	45.0**
1-methyl naphthalene	0.01	0.01	0.04	45.0**
Total	**7.67**		**9.92**	

* calculated as detection limit plus 3 times the std. deviation (Keith, 1990)

**based on the average recovery for toluene, ethylbenzene, *m*- & *p*-xylene and *o*-xylene



(a)



(b)

Figure 6 Hydrocarbon loss at 5 °C from a WSF of NWC (1000 mg/L loading rate) in open tanks (a) and closed sample storage bottles (b). (Closed sample analysis began after 24 hours; error bars represent standard deviation)

The calculated HC values are overestimated in regards to the amount of WSF that fish were exposed to over 24 hours before solution renewal. The partial replacement design required the renewal of the WSF solution every 24 hours. The reported HC values are averages of samples taken after renewal (i.e. samples of the fresh WSF solution; Table 2). Although concentrations of soluble HC were still measurable in tanks after 24 h before replacement (Table 4), there was substantial loss within the 24 h. Experiments with open tanks and no fish showed greater amounts of WSF remained after 24 h (approximately 75% of time zero concentrations, Figure 6). Experiments with open tanks containing fish retained an average of 11.1 % (range of 2.5 to 18.6 %) of starting concentrations (Table 4). This value is considerably smaller than the WSF remaining in the open tank without fish suggesting that the low retention in the water was probably due to the uptake of hydrocarbons by the fish. High variability between replicate tanks may be explained by the individuality of the fish. Some fish take up less WSF than others. Considerable variation in HC tolerance among individuals was also reported for exposed pink salmon fry (Birtwell et al., 1996).

Table 4 WSF concentrations remaining after 24 hours before solution replacement in tanks containing fish.

Loading rate (mg/L)	**Total WSF (mg/L)** Time zero (start) (single WSF mix)	24 hour (end) (replicate tanks)	**% of start concentration** (after 24 hours)
25	0.336		
25		0.056	16.74
25		0.043	12.78
25	0.251		
25		0.007	2.59
25		0.045	18.08
25	0.243		
25		0.006	2.51
25		0.056	23.20
10	0.168		
10		0.013	7.74
10		0.027	16.07
10	0.155		
10		0.029	18.63
10		0.006	4.03
10	0.150		
10		0.014	9.03
10		0.002	1.58
		Average	**11.08**

3.2 Rainbow Trout Satiation Feeding Experiments

Rainbow trout displayed several behavioural patterns during the satiation feeding experiments, which were conducted to estimate how much food the fish would consume under these experimental conditions. The object of estimating a satiation value was to create a baseline from which the decrease in food consumption by fish during the WSF experiments could be measured. The amount of food required to reach satiation was first determined.

The feeding frequency was set at twice per day to satiation. This frequency was based on research by Grayton and Beamish (1977) who showed the maximum daily food intake for rainbow trout occurred with only two feedings to satiation per day. Fish that were fed to satiation three or more times per day did not have a significantly different body composition from fish fed twice per day (Grayton and Beamish, 1977). A similar feeding frequency was shown by green sunfish (*Apomotis cyanellus*) which accepted food once or twice a day and rarely more often (Moore, 1941). Experimental rainbow trout fed twice a day to satiation would be expected to have similar body composition, over time, assuming all other aspects (i.e. levels of stress, individual appetite) were equal.

Grayton and Beamish (1977) discovered that feeding frequency in rainbow trout did not directly affect body composition but it did influence food intake (percentage of body weight consumed) increasing to some daily maximum. Moore (1941) recognized a tendency for both perch (*Perca flavescens*) and green sunfish to "maintain a quantitatively constant weekly level of food ingestion, regardless of whether the feedings are once or several times daily".

Grayton and Beamish (1977) defined fish as being fed to satiation when "[fish] allowed food pellets, dropped singly onto the water surface, to sink to the bottom of the tank and the fish failed to consume them within one minute". This interpretation of satiation was re-defined in this experiment as "greater than 40% of the last scoop of food remaining on the surface and not consumed within 5 minutes with no evidence of active feeding by greater than one fish". This helped to eliminate feeding variability among fish and to account for the finely ground starter food used.

3.2.1 Rainbow Trout Feeding Behaviour

Some fish appeared to be dominant and territorial, guarding food at the water surface while they fed. There appeared to be hierarchical behaviour in which dominant individuals fed first, yet size did not necessarily predict dominance. Rainbow trout have previously been shown to be territorial and hierarchical in behaviour (Landless, 1976). Dominant fish feed first and most aggressively although dominance is not necessarily determined by size (Landless, 1976). Certain fish, regardless of size, exhibited signs of distinct individual appetite. It is not uncommon to see a high variability among fish in feeding preferences (Grayton and Beamish, 1977). Regardless of dominance, all fish were observed to feed either from the surface or from sinking particles and sometimes from the bottom. No observations of cannibalism occurred in experimental tanks although some incidents did occur in the holding tanks. There is no evidence to suggest that non-dominant fish in a group are deprived of food (Landless, 1976).

3.2.2 Food Consumption Rates

The average daily food consumption rates for trout in control Tanks 1, 2, and 3, were 1.82%, 1.36% and 1.30% of body weight per day, respectively (Figure 7). Similar average daily consumptions for rainbow trout were noted by Boujard and Leatherland (1992) ranging between 1.5-5% of body weight while Grayton and Beamish (1977) reported a daily food demand between 2.8-3.5%. Tank 3 was a satiation experiment performed four months after the WSF experiments were completed to determine whether the satiation level of the fish had changed over the experimental period. The average daily consumption in Tank 3 (and replicate) was not significantly different from the averages in Tanks 1 and 2 ($P=0.13$; Appendix). The second satiation feeding experiment (Tank 3) was ended on day 10 before the 14 d test period because signs of an infection became apparent in both a replicate tank and holding tank fish. Tank 3 (replicate 2) did not appear to be affected but the experiment was concluded regardless. Nonetheless, the average food consumption rates among all three sets of tanks were similar. The food consumption averages before and after 4 months were not significantly different indicating there was no change in the feeding behaviour of rainbow trout during the test period.

A distinct pattern of periodic fasting followed by a period of hyperphagia (ingestion of abnormally large amounts of food) occurred in all tanks which is consistent with other research on fish (Moore, 1941) including rainbow trout (Grayton and Beamish, 1977). The fish consumed larger quantities of food per body weight per day over several days and then decreased their food consumption for several days (Figure 7). Rainbow trout also feed in diel cycles with the majority of food consumed in early morning hours

with a small peak again at dusk (Boujard and Leatherland, 1992) which may account for variations of food consumption within each day. All the tanks showed a prominent increase in feeding a few days after the start of an experiment. Within the first five days, Tank 3 (Figure 7) showed a less prominent consumption peak than tanks 1 and 2. Fish in tank 3 (2.4% peak body weight consumed) had only 24 h for starvation before the experiments as opposed to tanks 1 and 2 (3.75% and 2.7% peak body weight consumed) when a 48 h starvation protocol was used (Section 2.3). The daily food consumption rates were averaged to be 1.6% of body weights during satiation feeding with the highest measured consumption at 3.75% and the lowest recorded at 0.5% (Figure 7).

The Dallal plot (Figure 8) shows the relative distribution of daily food consumption from all three satiation feeding tanks. The Dallal plot showed that during the satiation feeding experiments, fish consumed no less than 75% of the satiation value approximately 77.5% of the time (Figure 8). Based on this distribution, it was decided arbitrarily that 75% of the average daily satiation value (75% of 1.6%), or 1.2% of body weight, would be used as a reference value in the WSF feeding behaviour experiments to remove variability generated from hyperphagia. Consistent deviation below this value would suggest that the fish were not consuming normal amounts of food on a daily basis.

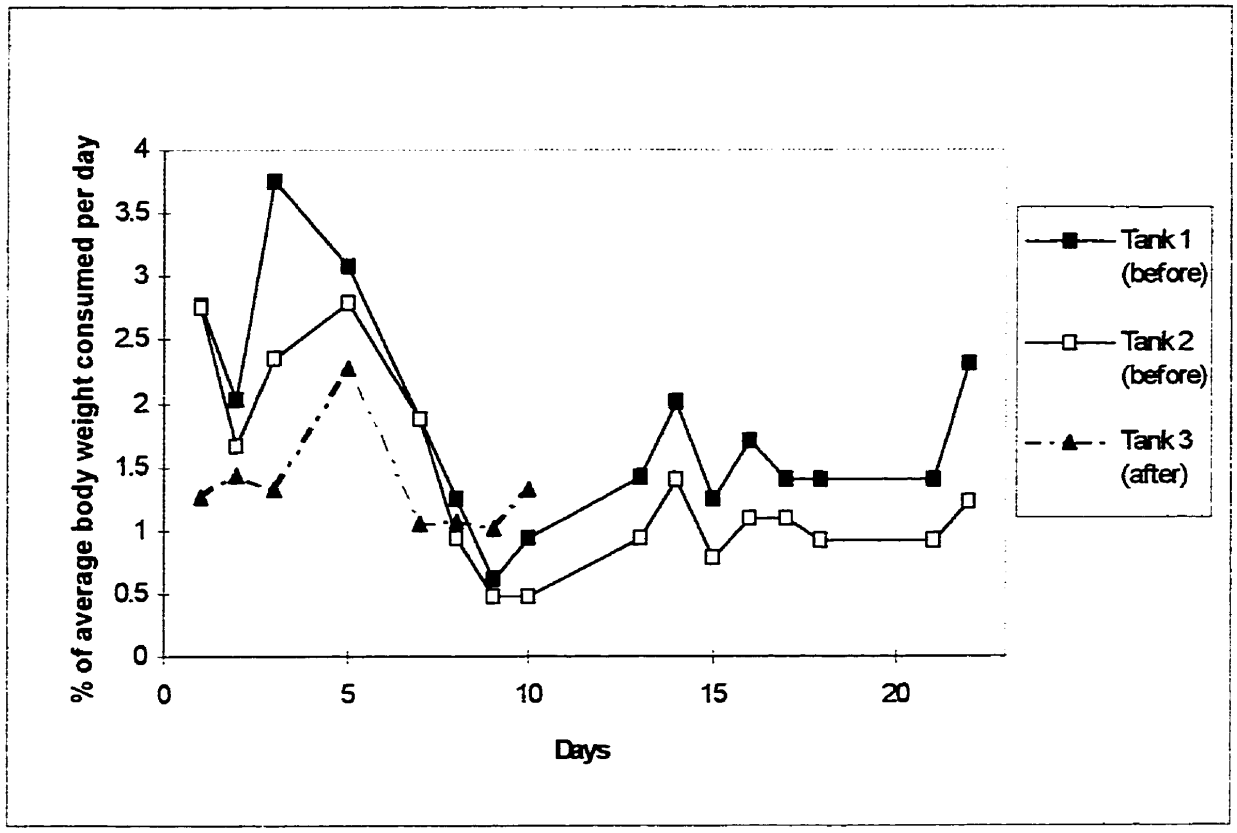


Figure 7 Satiation feeding of rainbow trout expressed as the percentage of fish body weight consumed daily.

Note: Tanks one and two represent experiments performed prior to WSF experiments and tank three represents a replicate satiation feeding verification experiment immediately after the conclusion of WSF experiments (approx. 3 months).

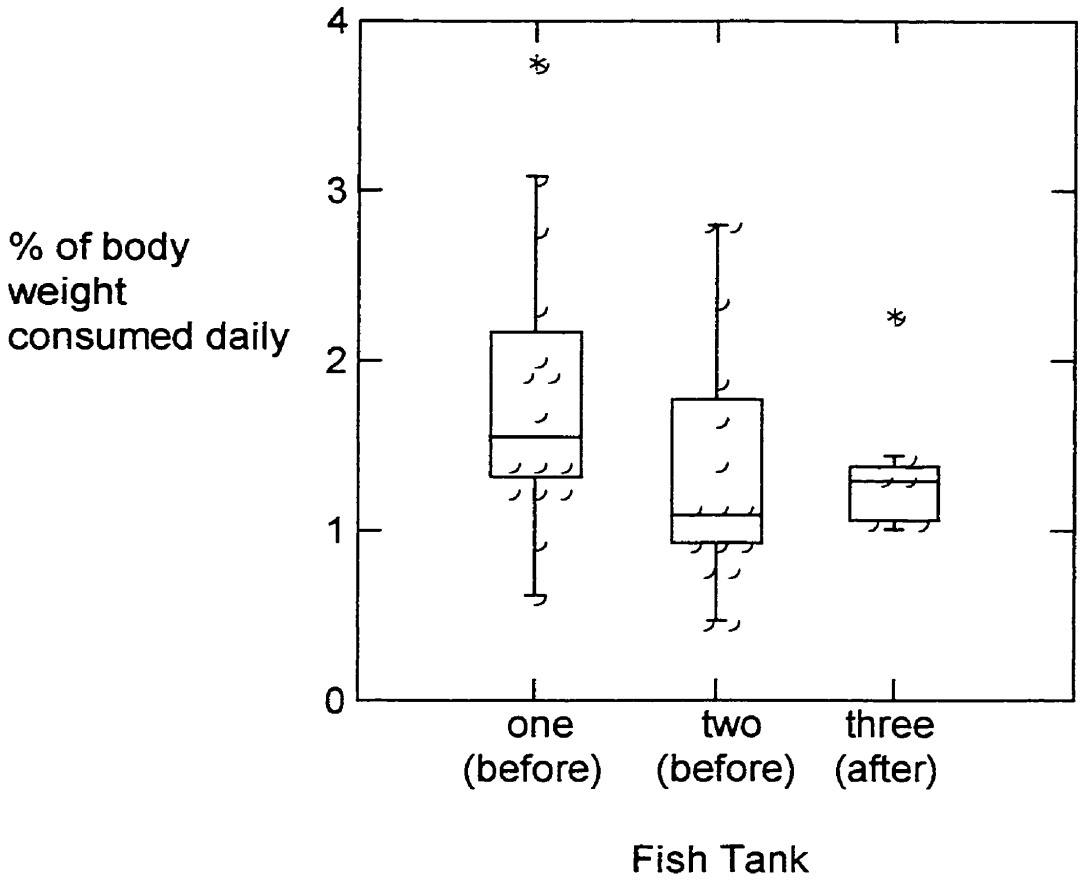


Figure 8 Dallal plot (dot-box) of the range of food consumed daily expressed as a percent of the average individual fish body weights. The middle line indicates the median while the box indicates the range of 50% of the data with the whiskers representing 75% of the data range. Quarter circles designate actual data points and asterisks are outliers.

3.3 WSF Exposure and Rainbow Trout Feeding Experiments

During the WSF experiments, a restricted ration was used instead of satiation feeding for several reasons. Feeding the fish a restricted ration would not only serve as a baseline for comparison throughout the experiments, but it would also limit growth. A restricted ration diet did not have an appreciable effect on mean weights or body moisture in feeding experiments with rainbow trout, but growth efficiencies were slightly lower than fish fed to satiation (Grayton and Beamish, 1977). It was beneficial to keep the fish from growing too large for the experimental tanks being used. Consequently, fish were fed 75% of a pre-determined feeding satiation value (Section 3.2.2).

3.3.1 WSF Exposure and Feeding Behaviour

Rainbow trout exposed to the WSF of NWC displayed pronounced physiological and behavioural effects depending on HC concentrations. Trout in the 400 mg/L tanks began convulsing immediately after immersion in the WSF and died within 4 days. The measured concentration of 8.27 mg/L from the 400 mg/L tank is comparable to the 48 h LC50 of 11.6 mg/L for rainbow trout larvae exposed to the WSF of NWC (Lockhart et al., 1987b).

Fish exposed to 100 and 200 mg/L (0.57 and 1.92 mg/L measured concentrations) loading rates appeared narcotized and began swimming near the water surface within 24 h (Table 5). After several days, the fish did not swim and remained on the bottom. Fish made no attempts to feed over the experimental period. Similar behaviour patterns occurred after 3 days in 50 mg/L tanks (0.55 mg/L measured concentration; Table 5). Steadman et al. (1991) exposed rainbow trout to loading rates between 25 to 50 mg/L

Table 5 Observed behavioural effects on rainbow trout after exposure to varying concentrations of the WSF of Norman Wells crude oil.

Loading rate (mg/L)	Measured HC concentration (mg/L)	Behavioural effects observed				
		Feeding	Narcosis[a]	Equilibrium loss[b]	Swimming impairment[c]	Death
400	8.27	n	Y	y	y	y
200	1.29	n	Y	y	y	n
100	0.65	n	Y	y	y	n
50	0.55	n*	y*	y*	y	n
25	0.32	y	y**	n	n	n
10	0.14	y	N	n	n	n

*occurred after 3 days
**occurred inconsistently after 5 days
[a] characterized by lack of reaction to stimulus (i.e. tapping on glass tank)
[b] characterized by struggling to remain upright and vertical swimming
[c] characterized by convulsions and spinosis

of #2 fuel oil in water and observed fish consistently swimming at the surface followed by loss of equilibrium beginning after nine days of exposure. It was also reported that fish exposed to 100 mg/L WSF of #2 fuel oil were similarly affected after only 2 days. This included a gradual progression from loss of equilibrium to death in concentrations above 100 mg/L nominal (Steadman et al., 1991). Moles and Rice (1983) reported comparable behaviour when pink salmon juveniles were exposed to 0.38 mg/L (measured concentration) Cook Inlet crude WSF concentrations over forty days.

The rainbow trout exposed to 25 and 10 mg/L (0.32 and 0.14 mg/L measured concentration) WSF of NWC exhibited no signs of equilibrium loss or swimming impairment over the 14 day experiment (Table 5). Food consumption was mildly affected at the 25 mg/L loading rate (Table 5). The 25 and 10 mg/L loading rates were designated as the lowest effect concentration (LOEC) and the no effect concentration (NOEC) respectively, based on these results. The maximum acceptable toxicant concentration (MATC) falls between the NOEC and LOEC (0.14 to 0.32 mg/L measured concentrations).

The neurotoxicity of hydrocarbons is evident by behavioural changes in the trout beginning with loss of appetite and narcosis, followed by loss of equilibrium and eventual impairment of swimming (Moles and Rice 1983; Folmar et al., 1981; Malins and Hodgins, 1981). Swimming impairment may be caused by neurotoxic disorientation or possibly gas bladder problems although this has never been investigated.

Fish exposed to the WSF of a crude oil assimilate hydrocarbons into the fish body where neurotoxic effects occur creating adverse behavioural change. Carls (1987)

described the observed behaviour changes as being due to accumulated hydrocarbons rather than exposure concentrations. Fish exposed to high concentrations for short period exhibit similar effects to fish exposed to lower levels for longer periods.

Hydrocarbons enter the fish body primarily through the gills and are promptly detected in organs and tissues. The fact that they distribute so rapidly infers that the blood transports them. Once across the gill membrane, the parent hydrocarbons readily solubilize in cell membranes including those of the red blood cells. Some monoaromatic hydrocarbons are also carried by lipoproteins and leukocytes in the blood. Hydrocarbons will partition to lipid-rich substances like adipose tissues, and lipid containing organs such as the liver and brain as shown in fish that retained C14 labeled naphthalene after exposure (Thomas and Rice, 1981). The first organs to be contaminated are gills, gut (adipose tissue) and liver shortly followed by flesh (heart muscle), nervous tissue and the gall bladder (Lee et al., 1972). High levels of hydrocarbons in the liver and gall bladder are not surprising since they are organs that metabolize and excrete parent compounds and metabolites. Since the transport of HC occurs in the blood, high levels are also expected in the heart and the brain which receives blood, oxygenated and containing HC, straight from the gills (Anderson, 1977). During transport or after storage, HC in the fish have several fates. They can be put back into circulation, carried to the liver and metabolized or excreted via bile and urine (Lee et al., 1972).

Several variables affect the uptake of HC in fish based on the chemical nature of the WSF and the physiology of the fish. Uptake and persistence of HC in tissues is partly regulated by the structures of the hydrocarbons. Uptake increases with the decreasing number of aromatic rings. For example, Coho salmon and starry flounder (*Platichthys*

stellatus) accumulated alkyl substituted monoaromatics over non-substituted diaromatics (Roubal et al., 1978). Uptake rates follow a pattern according to increasing complexity: alkylated benzenes > indans > tetralins > fluorene > alkylated naphthalene where alkylated benzene is a monocyclic aromatic (one ring) and alkylated naphthalene is a diaromatic (two rings) hydrocarbon (Vandermeulen, 1987; Figure 9).

Metabolism of these complex hydrocarbons occurs by means of a group of enzymes collectively called mixed function oxygenases (MFO). They catalyze the degradation of both endogenous and exogenous compounds through oxidative pathways by incorporating one atom of molecular O_2 into the organic substrate. The metabolism of HC creates intermediate products which are more water-soluble and hence are more readily excreted than the parent compounds. Many metabolites of petroleum compounds are not detected by the same analytical methods as the parent substance. Inferring depuration of oil from tissues, while using the same analysis method as used for the parent compound, is inappropriate. The analysis of HC in the body is compromised when a detection method does not account for its metabolites. For example, a known product of 3,4-benzopyrene metabolism is 7,8-dihydro-7,8-dihydroxybenzopyrene which has 4 intermediates. A major product of naphthalene is 1,2-dihydro-1,2-dihydroxynapthalene, with 4 intermediates and several glycoside and sulfate conjugates (Lee et al., 1972). These two examples show that a large number of intermediates can be produced in the metabolic process. Research has not been able to identify more than a few of the compounds produced during HC metabolism.

These metabolites are very different from the parent compounds and therefore produce a variety of new problems within the fish. Some metabolites can interact with

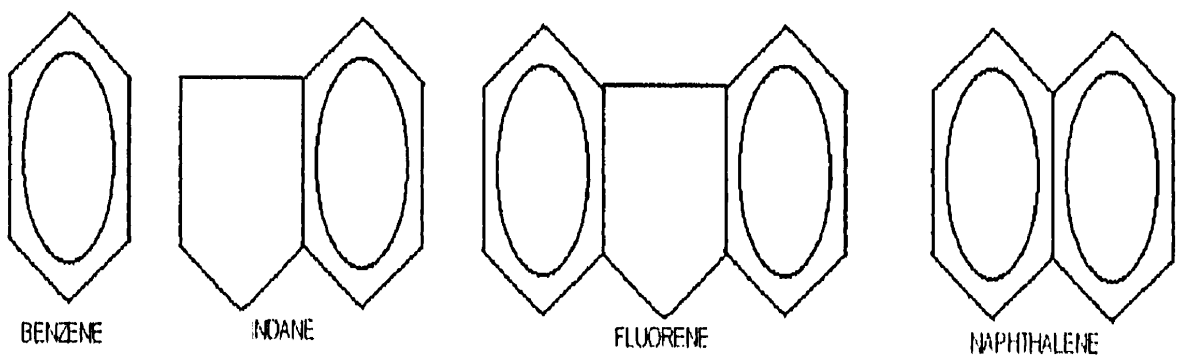


Figure 9 Chemical structure of four petroleum hydrocarbons.

nucleic macromolecules (i.e. DNA, RNA) resulting in genotoxicity. The MFO-mediated metabolism of HC causes a concern because it has been implicated in the activation of potential carcinogens through the formation of mutagenic metabolites (Vandermeulen, 1987; Collodi et al., 1984). It may be some of these same metabolites that cause the tainting of flesh leading to an unpalatable meal and closures of fisheries (Murray, 1984). Hydrocarbons are lipophilic compounds which makes the phospholipid cell membrane susceptible to physical disruption. Physiological processes depend on the integrity of the cell membrane and lipophilic substances can distort the physical structure causing alterations in permeability (Boyles, 1980). Disruption of the cell wall may cause leakage of electrolytes, critical process suppression (i.e. protein formation, ion regulation) and even inhibition of cellular mitosis. At an organismal level, a range of effects will become evident such as increased metabolism and oxygen consumption, anesthesia and possibly carcinogenicity. For example, the narcotic effect of certain hydrocarbons is explained by "the formation of micro-crystalline hydrates which disrupt nerve responses" (Boyles, 1980). Many of the toxic aspects of hydrocarbons and their derivatives can be related to one or more disruptions in the cell membrane systems. The specific fates of MFO/HC metabolites in fish is still largely unknown.

Some controversy exists over which hydrocarbon compounds influence behavioural change. Some researchers report higher amounts of parent compounds in tissues of exposed fish that are unaffected by change (Folmar et al., 1981). This suggests that the metabolic products of crude oil may have been responsible for the cessation of feeding in fish exposed to the WSF. This is in contrast with findings that acute neurotoxic

effects and behavioural changes were related to accumulation of parent compounds rather than non-conjugated metabolites (Folmar et al., 1981).

The exact mechanism of HC effects on fish feeding behaviour has not been determined. Effects of WSF on invertebrate feeding behaviour is, however, described as being similar (Rogerson et al., 1982). A decrease in feeding by the freshwater rotifer, *Asplanchna sieboldi*, exposed to NWC oil, and the manifestation of neuroparalytic effects on petroleum exposed shrimps (*Leander adspersus*), suggests a common neurotoxic or physiological effect between species (Rogerson et al., 1982; Mazmanidi and Kovaleva, 1975).

Evidence suggests that suppression of feeding is not necessarily caused by swimming impairment. Larval herring exposed to Cook Inlet crude had reductions in feeding prior to inhibited swimming (Carls, 1987). Swimming impairment also did not occur before feeding suppression at LOEC in this experiment but instead occurred at the next highest concentration (Table 5). Narcosis was a symptom that did occur consistently with decreased food consumption. This suggests that swimming impairment is not the primary explanation for decreased food consumption by fish exposed to hydrocarbons in the WSF.

Other potential causes for the suppression of feeding in fish exposed to soluble petroleum hydrocarbons include impaired visual acuity and chemosensory damage. Apart from hunger, the feeding response of rainbow trout is highly affected by visual stimuli (Adron et al., 1973). Concentrations between 0.15 to 0.45 mg/L WSF of Wyoming crude induced lesions in the eyes of cutthroat trout (Woodward et al., 1981). These lesions

caused retinal edema and changes in the eye lens fibers. The morphological alteration of the lens may cause distortion or loss of visual acuity that would adversely affect the feeding behaviour in fish. The larval herring used by Carls (1987) exposed to 0.9 ppm Cook Inlet crude continued to exhibit strike behaviour for food at the water surface although unsuccessfully. This suggested the fish experienced a loss of vision without changes in appetite resulting in a reduction in food consumption.

Consequently, it has been suggested that low levels of hydrocarbons may not be detected by fish senses (Khan and Kiceniuk, 1983). Pink salmon fry pre-exposed to a petroleum WSF had reduced ability to detect and avoid dissolved hydrocarbons (Birtwell et al., 1996). Periodic travel through contaminated areas may damage the olfactory senses with possible consequences to homing, feeding behaviour and predator avoidance. Fish may remain in contaminated areas containing low levels of dissolved hydrocarbons for extended periods uptaking hydrocarbons to the point of neurotoxicity causing adverse changes in chemosensory ability and behaviour.

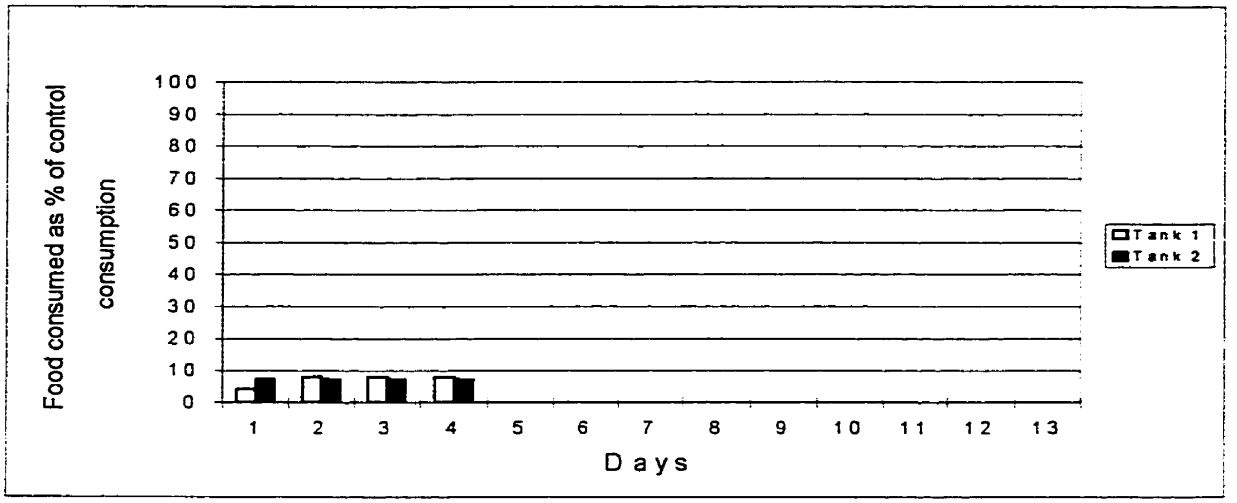
3.3.2 Food Consumption Rates

Rainbow trout exposed to the water-soluble fraction of Norman Wells crude oil exhibited a suppression in feeding behaviour as observed by reductions in daily food consumption. All fish in the 100 and 200 mg/L tanks survived the 14 days but did not consume any food. However, values of 0.1 to 0.12% body weight, or 7-15% of control tanks food consumption (Figure 10), were recorded due to the minimum daily feeding allocation. Fish in the 50 mg/L loading tank ate inconsistently over three days

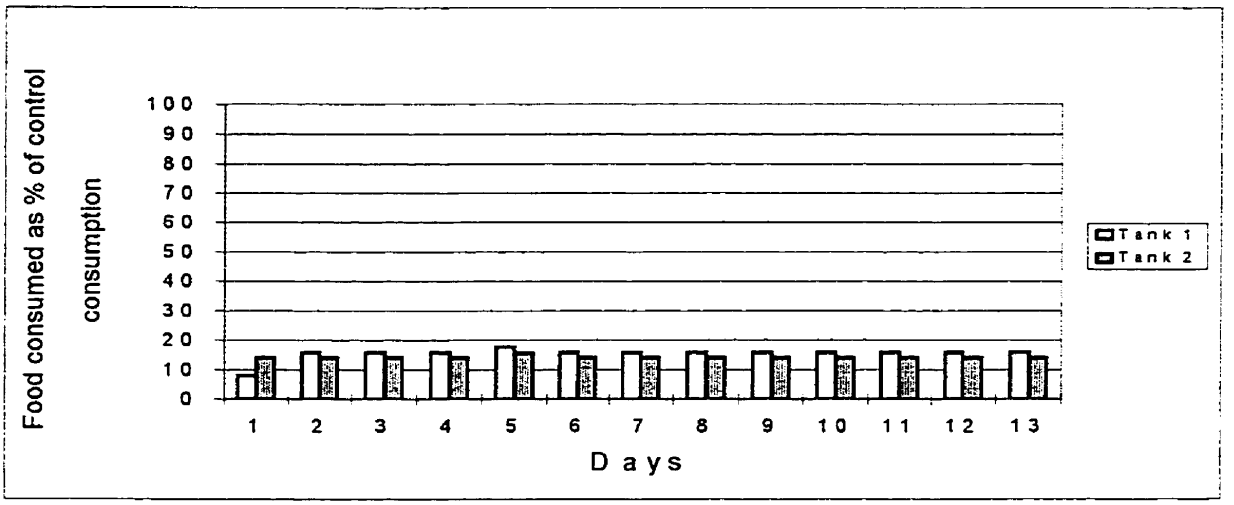
and then did not consume any more food for the duration of the experiment (Figure 11).

The 50 to 200 mg/L data showed food consumption of approximately 7-15% of control fish consumption, but this was an overestimate. The fish displayed a consistent suppression of appetite and did not actually consume a full 7 or 15% of the control values per day. This overestimation reflected the minimum daily food allocation used to check for appetite suppression. One food measure approximated 7% of the fish body weight in a tank compared to controls (two food measures totaled approximately 15%). The difference between 7% and 15% reflected a small change in the experimental design between experiments. Fish were originally fed twice per day but excessive algal growth occurred in tanks that contained fish with suppressed feeding. The feeding protocol was modified so that fish that exhibited a suppression of appetite were fed only one food measure per day on the condition that an observable amount of food remained in the tanks since the first feeding (Section 2.4). Data from the fish exposed to 50 mg/L displayed the same overestimation after day 3.

Trout in the 25 mg/L tanks ate the same amount of food as the control fish for the first 4 days. After day 5, these fish did not consume as much as control fish (only 85% of control levels on day 5) and a slow decrease in the amount of food consumed was evident over the 14 day period (Figure 12). The fish ate as little as 39-46% and no higher than 85-88% of control values after day 5 with an average of only 65 and 70% for each replicate tank over the 14 days. This loading rate provided the lowest observable effect concentration (LOEC) of hydrocarbons that elicited a change in feeding



(a)



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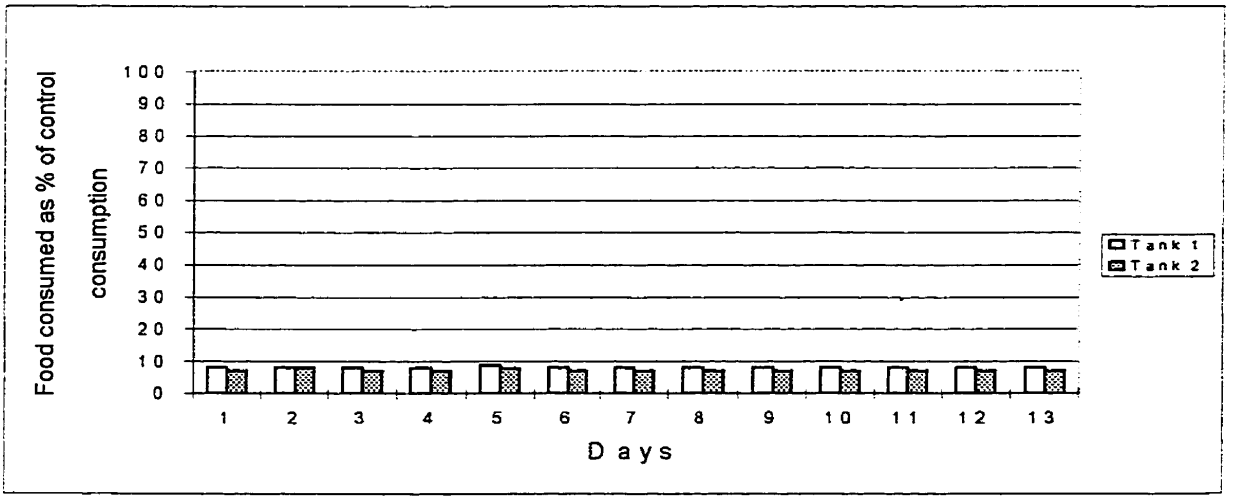

(c)

Figure 10 Food consumption per day (as a percentage of control tanks) by rainbow trout using (a) 400, (b) 200 and (c) 100 mg/L loading rates of Norman Wells crude (duplicate tanks).

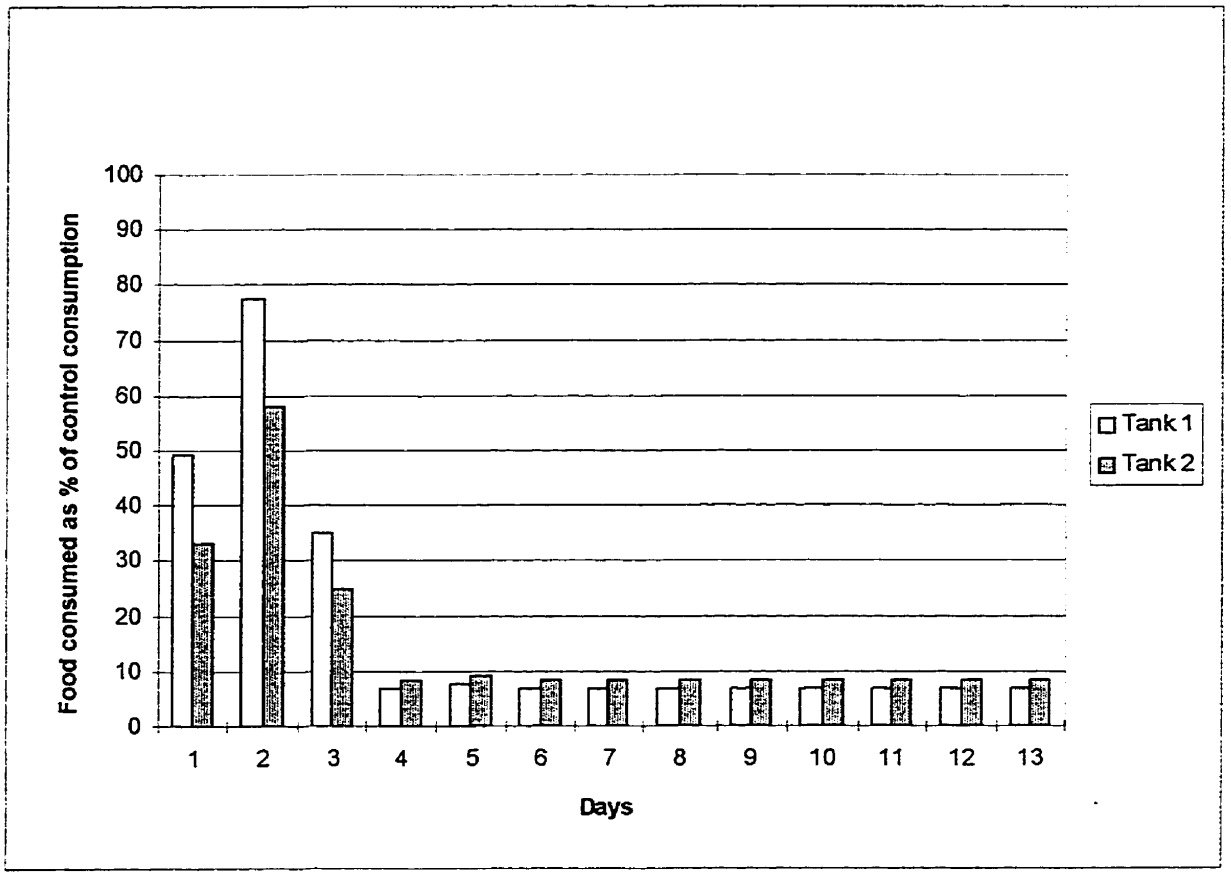

Figure 11 Food consumption per day (as a percentage of control tanks) by rainbow trout using 50 mg/L oil loading rate of Norman Wells crude (duplicate tanks).

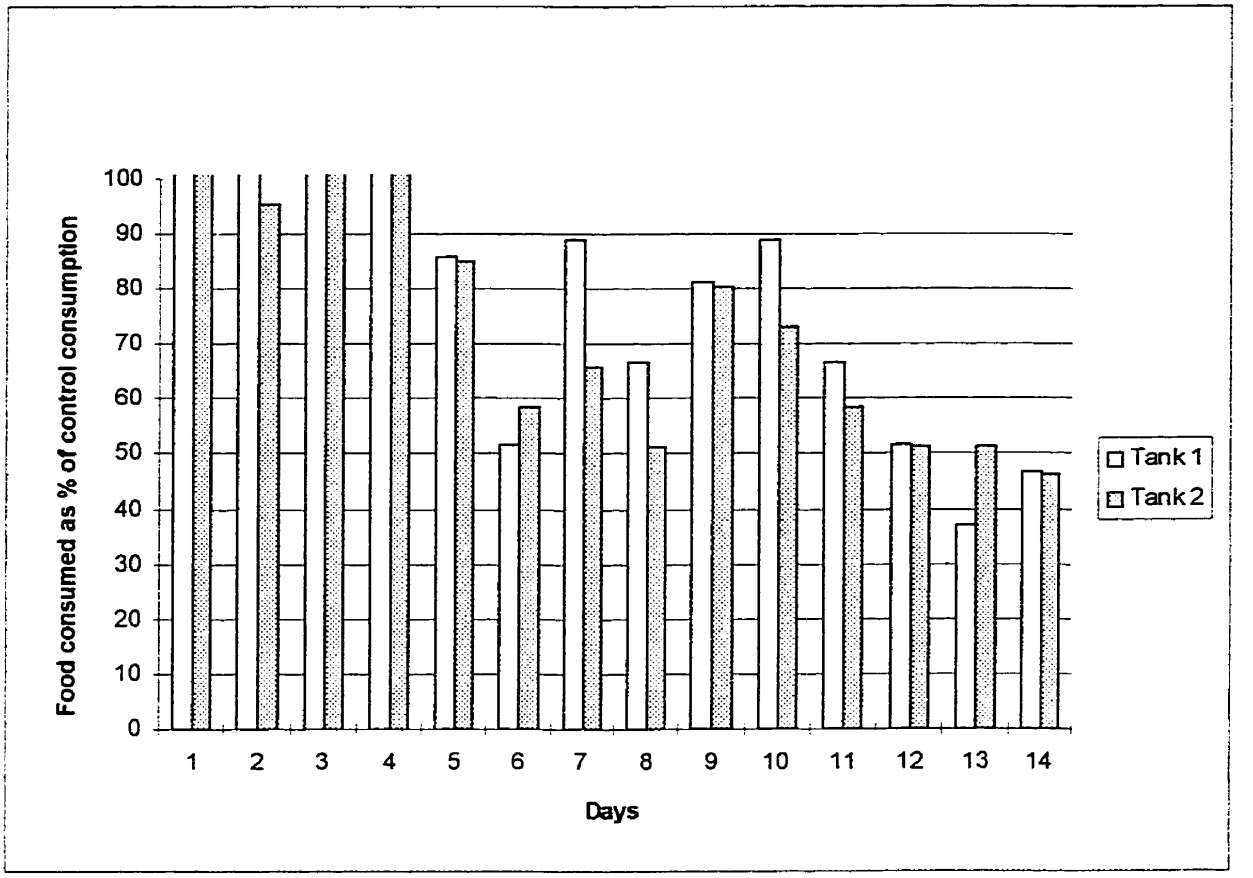

Figure 12 Food consumption per day (as a percentage of control tanks) by rainbow trout using 25 mg/L oil loading rate of Norman Wells crude (duplicate tanks).

behaviour (0.32 mg/L measured HC concentration; Table 5) among the concentrations tested. After 14 days at LOEC, the daily consumption by the fish was approximately 46% of the amounts consumed by controls resulting in a decrease in food consumption >54%.

There was no change in feeding behaviour at the no observable effect concentration (NOEC) of 10 mg/L (0.14 mg/L measured HC concentration; Table 5). Trout subjected to 10 mg/L nominal concentrations ate the same amounts as controls with only a small decrease in one tank on day 8 (Figure 13). The fish exhibited no signs of stress or change in swimming patterns (Table 5). The maximum allowable toxicant concentration (MATC) range falls between the NOEC and LOEC (0.14 and 0.32 mg/L) as measured by the extractable hydrocarbons.

Similar observations on the suppression of feeding behaviour have been reported for other species of fish. A decrease in feeding for Atlantic cod (*Gadus morhua*) exposed to 0.15 to 0.3 ppm of Hibernia crude oil over 91 days was observed by Khan and Kiceniuk (1983). It was noted that exposed cod ate 103 g/kg of food per week while control fish ate 195 g/kg of food per week calculating to a decrease of 47% in food consumption. The time of onset of this behavioural change was not included in the report. Pink salmon juveniles exposed to Cook Inlet crude at 0.21 mg/L fed normally throughout a 40 d exposure although at higher concentrations (>0.38 mg/L), feeding behaviour was affected and fish consumed only 80% of dispensed food (Moles and Rice, 1983). Folmar et al. (1981) reported similar values for adult Coho salmon stating an effective MATC range of 0.23 to 0.53 mg/L and concluded that this exposure can significantly impair the capturing of prey by Coho salmon. Carls' (1987) research on larval Pacific herring observed feeding inhibition by 50% using a WSF concentration of 0.9 ppm of Cook Inlet

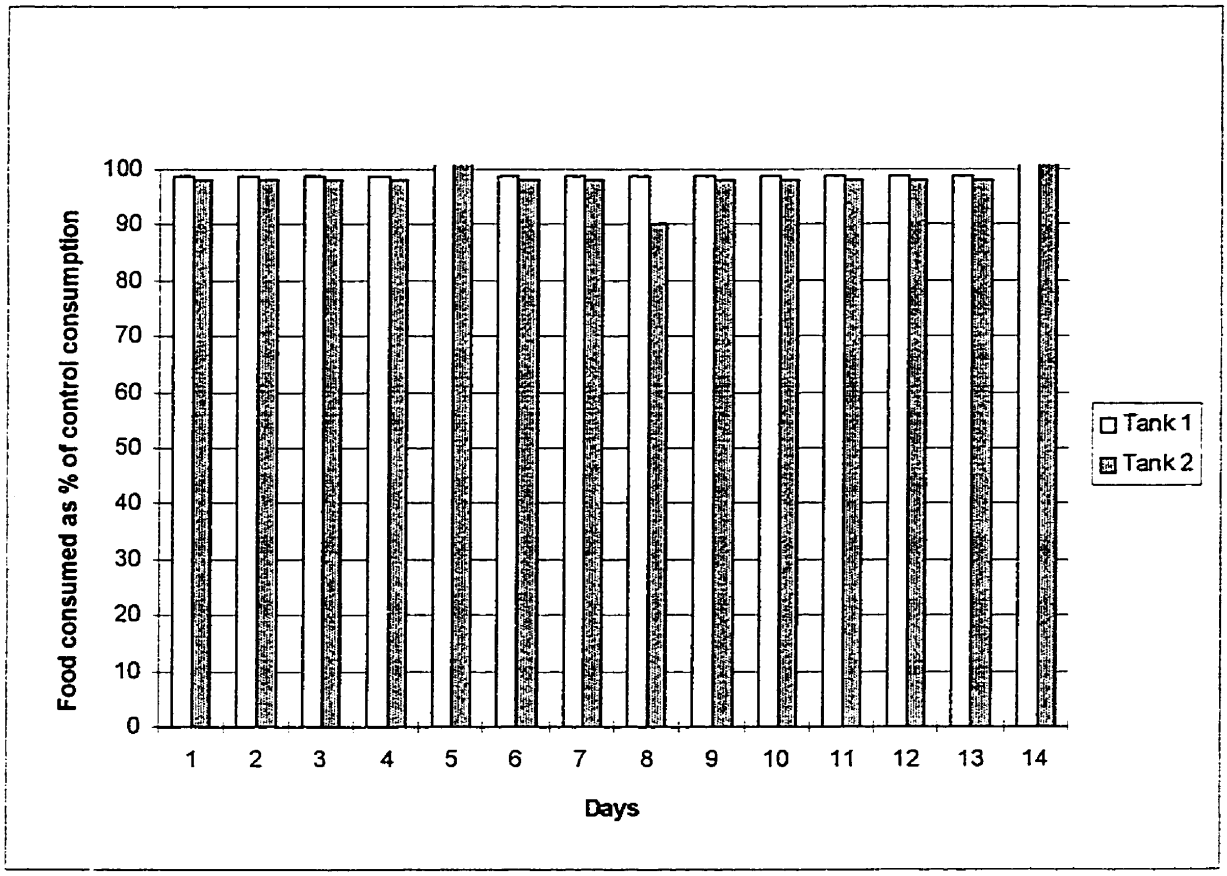

Figure 13 Food consumption per day (as a percentage of control tanks) by rainbow trout using 10 mg/L oil loading rate of Norman Wells crude (duplicate tanks). The loss of appetite occurred within one day of exposure and ceased completely within 10 days.

The decrease in food consumption by trout in the WSF experiments is underestimated because of the restricted daily food ration. The food ration limited control tanks to eating a maximum of approximately 1.2% body weight per day. If fed to satiation, these tanks may have consumed more food on any particular day due to periodic hyperphagia (Section 3.2.2). When compared to controls, the WSF tanks that contained fish with suppressed feeding would have an estimated loss of consumption greater than what is reported. The food ration limit, used as a reference, allows the quantification of the loss in food consumption while at the same time putting a limitation on how accurately the loss can be reported.

A summary of the food consumption rates is as follows; the rainbow trout were observed to have a >92% decrease in food consumption when exposed to the WSF of NWC at nominal concentrations of 50, 100 and 400 mg/L (0.55, 0.57, and 8.27 mg/L measured concentrations). The 200 mg/L (1.92 mg/L measured concentration) loading rate tanks had a decrease of more than 85%. The 25 mg/L (0.32 mg/L measured) loading rate tanks had a decrease in food consumption of approximately 54% while fish in 10 mg/L (0.14 mg/L measured) did not suffer any acute effects on feeding over the 14 day test period.

3.3.3 Body Moisture Analysis

Analysis of fish wet and dry weights revealed a small increase in average water content at the 50 mg/L to 200 mg/L loading rates of approximately 1-1.5% after 14 days (Figure 14; Table 6). The ANOVA analysis showed that the averages were not significantly different (lowest P=0.07) from the controls or lower concentration tanks with one exception between the pooled data of the 50 mg/L tank and pooled data from Control C (P=0.02; Appendix). Control C was a control tank outside of the 50 mg/L experiment. The 100 mg/L data set displayed an abnormally narrow data spread due to an error in the weigh scale calibration before the experiment which was corrected prior to the end of the test period.

Body composition is often used as an indicator of fish health and quality (Reinitz, 1983). Wet weights, lengths and dry weights were measured in order to assess any short term changes in body water content over two weeks while stressed by the WSF. Increases in fish water content were evident by edema (swelling) that occurred in larval rainbow trout exposed to NWC at 30 ppm nominal WSF (0.15 ppm measured HC) for 4 days with a 10 day clearance period (Duncan, 1984). It was suggested the large increase in body moisture in fish exposed to WSF was caused by impaired osmoregulation (Duncan, 1985; Lockhart et al., 1996) possibly caused by cellular damage to kidneys and gills (Khan and Kiceniuk, 1983; Birtwell et al, 1996). However, experiments using fish in tritium-labeled water failed to show any significant differences in water uptake-equilibration rates between exposed and control fish (Duncan, 1985). A correlation can be made between the noted lack of feeding and an increase in water body contents in fish deprived of food (Denton and Yousef, 1976). Reinitz (1983) studied the starvation effects on rainbow trout

and reported a trend of increasing body water contents of approximately 3% after 28 days starvation. After 140 days starvation, fish body water content had increased 16%. The increasing body moisture was inversely related with decreasing body fat. It was concluded that a starved fish utilizes its energy stores to survive while replacing that material with fluids (Grayton and Beamish, 1977; Reinitz, 1983; Groves, 1970; Denton and Yousef, 1976).

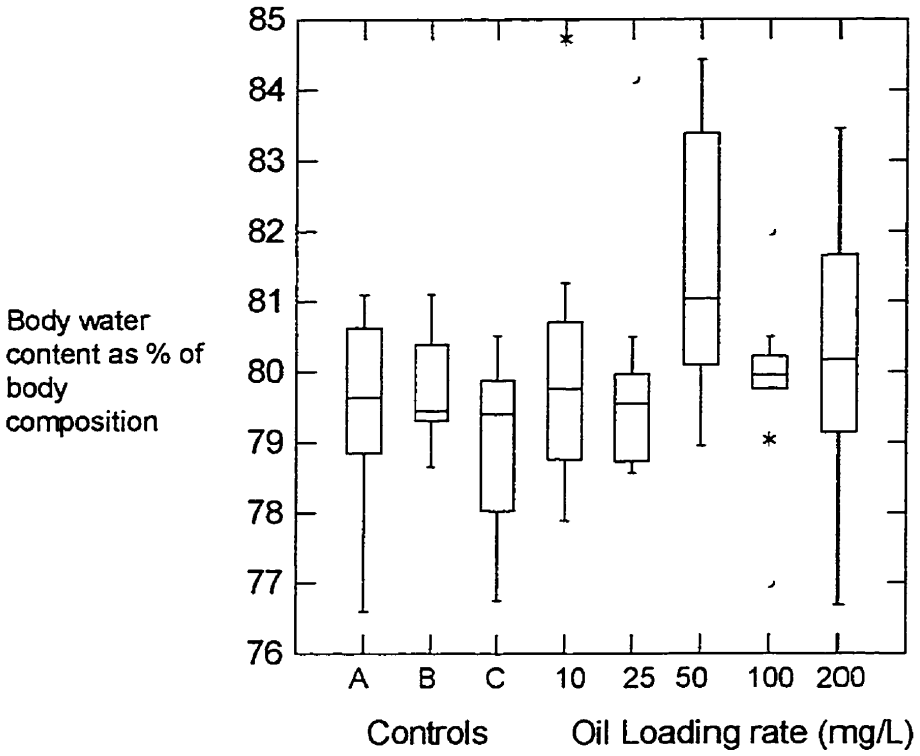

Figure 14 Box plot of experimental and control tanks (pooled data) displaying moisture content averages after exposure to WSF concentrations for 14 days. The middle line indicates the median while the box indicates the range of 50% of the data with the whiskers representing 75% of the data range. Quarter circles designate extreme outliers and asterisks are mild outliers.

Table 6 The average fish body moistures per loading rate (replicate tanks pooled data).

Experimental tank	Control A	Control B	Control C	10 mg/L	25 mg/L	50 mg/L	100 mg/L	200 mg/L
Avg. % wet weight	79.4	79.8	79.0	79.5	79.8	81.4	79.8	80.2
Standard Dev (+/-)	1.43	0.84	1.16	1.95	1.64	1.82	1.33	1.93

3.3.4 Growth Analysis

Measurements of fish lengths and weights were taken both before and after the experiments to estimate growth. There were no significant differences detected between before and after lengths of fish (P=0.21 ANOVA). However, the controls (A, B, C), 10 and 25 mg/L tanks showed a slightly greater median in lengths from start to end while the 50, 100 and 200 mg/L tanks showed a slightly smaller median after the test period (Figure 15).

There was a mild but significant difference between start and end averages of fish wet weights (P=0.03). All tanks except Control C displayed a decrease in weights from start to end although the 25 to 200 mg/L levels show a more significant loss from start averages as compared to controls (Figure 16). Measuring error and instrument calibration can account for some individual significance in the results that may have affected figures for the 100 mg/L data set causing an abnormal spread of data points previously detected in body water content (Section 3.3.3). The overall indication was that little or no growth occurred over two weeks by either the control or exposed fish.

Exposing fish to a WSF can inhibit growth. Moles et al. (1981) noted that using concentrations of 0.8 mg/L of naphthalene over 40 days changed feeding behaviour of Coho salmon fry which was the most probable reason for decrease in growth as measured by lengths and dry weights. They concluded that chronic exposures to concentrations as low as 0.4 mg/L soluble hydrocarbons is likely to inhibit growth of salmonids (Moles and Rice, 1983). Some species of fish are even more sensitive to toxic hydrocarbons as shown by the reported MATC of 24-39 *u*g/L for cutthroat trout exposed to a refined oil (Woodward et al., 1983).

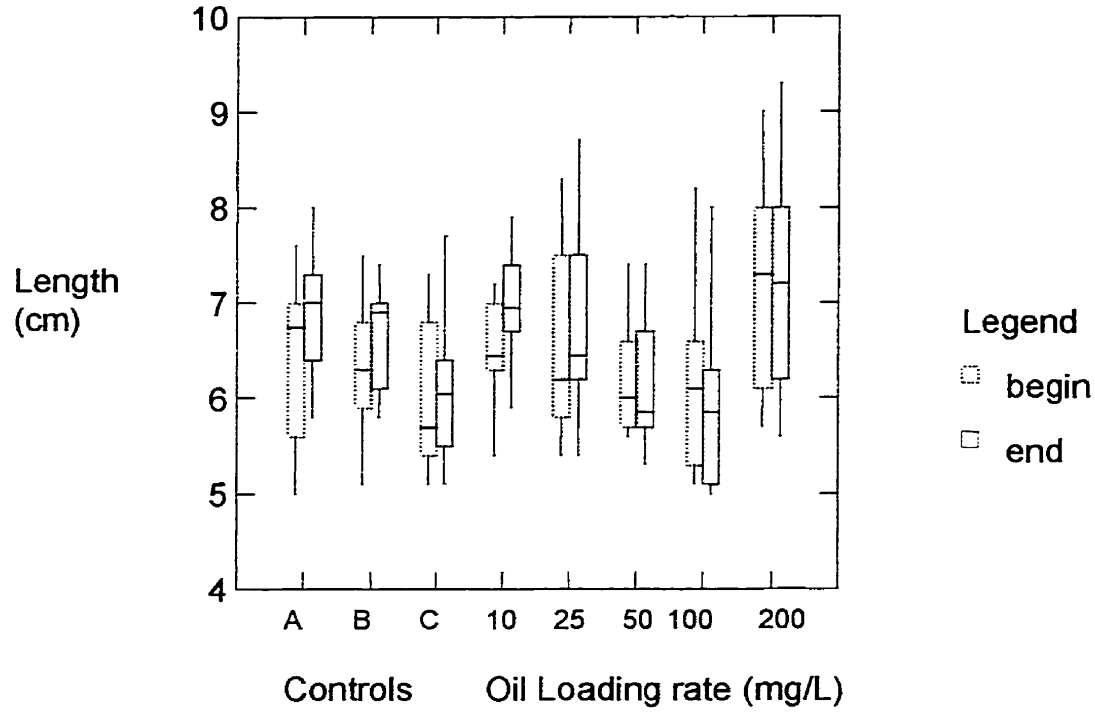

Figure 15 Boxplot of fish lengths by WSF loading rates before and after 14 days exposure. The middle line indicates the median while the box indicates the range of 50% of the data with the whiskers representing 75% of the data range.

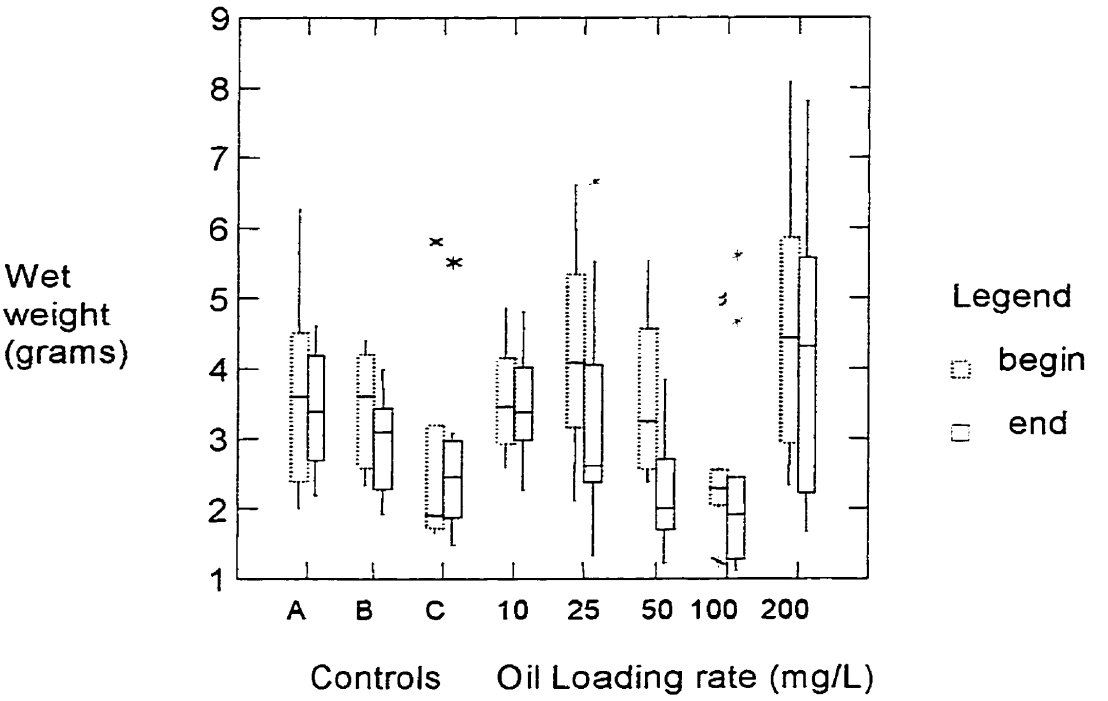

Figure 16 Boxplot of fish weights by WSF nominal concentration before and after 14 days exposure. The middle line indicates the median while the box indicates the range of 50% of the data with the whiskers representing 75% of the data range. Asterisks are mild outliers.

Carls (1987) also showed Pacific larval herring growth was significantly reduced by exposure to a WSF which was correlated with reduced feeding. Feeding incidence was a sensitive and easily measured indicator of WSF effects that accurately predicted mortality and growth.

Fish that retain normal feeding habits could still exhibit significant losses in growth when exposed to a WSF. This may be due to the induction of energy intensive processes such as the MFO system for the depuration of hydrocarbons or by inhibition of glycogen metabolism due to hydrocarbon hepatotoxicity although the former hypothesis is more probable than the latter.

Starvation will lead to the suppression of mixed function oxygenases such as aryl hydrocarbon hydroxylase (AHH) but the presence of toxic compounds in the fish system would induce it (Collodi et al., 1984). The result would be a reduction in energy stores normally used for fish growth (Moles and Rice, 1983). Collodi et al. (1984) noted that Coho salmon did not feed or grow and starvation led to suppression of AHH when exposed to 0.58 ppm of Cook Inlet crude. Fish eventually resumed production of AHH as exposure to the WSF continued. Fish increased their metabolic rate to metabolize and excrete hydrocarbons allocating less energy for growth and more for homeostatic maintenance.

A decrease in growth may also be attributed to the disruption of glycogen metabolism in tissues. The hepatotoxic effects of hydrocarbons were revealed in young adult porgy (*Spicara smaris*), Black Sea sole (*Solea lascaris*) and shrimps (*Leander adspersus*) chronically exposed to petroleum products (Mazmanidi and Kovaleva, 1975). A loss in glycogen stores after exposure created large carbohydrate losses in the liver

which were connected to the detoxification mechanisms that include glycolysis as a basic component. It was also noted that some concentrations, 0.05 mg/L for the sole, induced a phenomenon similar to glycogenesis causing glycogen to accumulate in all tissues especially in the liver (Mazmanidi and Kovaleva, 1975). This suggests that sub-acute poisoning disrupts glycogen metabolism in metabolically active fish tissues.

The metabolism of hydrocarbons creates a strain on fish. Reports of increased oxygen consumption in exposed fish supports evidence for increased physiological activities pertaining to the metabolism and excretion of HCs (Rice et al., 1975). Energy is required to synthesize large quantities of MFO enzymes although the exact energetics of this process have not been reported in terms of caloric intake and usage. However, pink salmon exposed to naphthalene and toluene activated an MFO system resulting in higher O_2 use and food consumption (Rice et al., 1975). An increase in energy demand causes a subsequent increase in food intake, which again may be a source of contamination. The exact amount of energy used by fish in producing MFO enzymes during exposure periods has not been measured quantitatively. Body fat tends to be used at higher rates in exposed fish but this may be due to an altered behavioural feeding effect as opposed to a physiological enzyme response (Lockhart et al., 1996).

4.0 CONCLUSIONS

Immersion of rainbow trout in the water-soluble fraction of Norman Wells crude oil had an adverse effect on feeding behaviour. Rainbow trout exposed to the MATC range (0.14 to 0.32 mg/L) of WSF over two weeks had decreased food consumption >54% that coincided with increasing concentrations of hydrocarbons in the water. At higher concentrations, the fish displayed signs of narcosis and swimming impairment with a reduction in food consumption >85%. The short experimental time allowed little or no trends to be seen in the increasing body water content of starved fish without any appreciable decrease or increase in growth. The decrease in feeding in WSF-exposed rainbow trout may have been caused by narcosis, loss of visual acuity, olfactory disruption (cell morphology changes) or loss of equilibrium leading to movement impairment. Body moisture increases were hypothesized to be attributed to a suspected decrease in body fat caused by increased energy requirements and decreased food intake in exposed trout. An increased energy requirement is an artifact of MFO production which is used for petroleum HC oxidation and subsequent excretion. Loss in body fat is also a consequential effect of suppressed feeding behaviour.

Petroleum hydrocarbon concentrations have been recorded near oil spill sites that ranged upwards of 0.1 mg/L, three months after the *Arrow* spill near Chedabucto Bay, Nova Scotia (1970), to 0.5 mg/L in estuaries near the *Amoco Cadiz* spill (1978) off the coast of France (NRC/OSBC/PSMR, 1985). These values fall within the MATC range for feeding behaviour alteration in rainbow trout and are analogous concentrations that induce adverse effects in other fish. Such concentrations may exist for periods long

enough to have adverse effects on the feeding behaviour of fish that fail to avoid such low environmental levels of hydrocarbons. The extent to which these concentrations may continue to exist highly depends on environmental factors. The factors that retard volatilization, such as low energy shores and over ice conditions in winter, extend fish exposures to a WSF. Consistent inputs from either natural or industrial sources can also lead to chronic exposures at low levels. Chronic exposures may be immediately lethal to aquatic organisms or create changes in the cellular morphology of fish over time (Khan and Kiceniuk, 1983). In turn, this can induce sub-lethal behavioural modifications, such as loss of appetite, which will eventually affect the overall health, growth and reproductivity of individual fish and the possibly the population. Successive consequences could be expected in commercial and recreational fisheries within several years (Paine et al., 1988; Carls, 1987).

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APPENDIX:

Systat output for analysis of variance tests

Pearson correlation matrix for measured vs. nominal hydrocarbon concentrations

	ACTUALHC	LOADRATE
ACTUALHC	1.0000	
LOADRATE	0.9625	1.0000

Bartlett Chi-square statistic: 6.525 df=1 Prob= 0.011
Number of observations: 5

Regression on actual vs. nominal
Dep Var: ACTUALHC N: 5 Multiple R: 0.9625 Squared multiple R: 0.9265

Adjusted squared multiple R: 0.9020 Standard error of estimate: 0.2465

Effect	Coefficient	Std Error	Std Coef	Tolerance	t	P(2 Tail)
CONSTANT	0.0258	0.1656	0.0	.	0.1557	0.8861
LOADRATE	0.0099	0.0016	0.9625	1.0000	6.1479	0.0087

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	2.2958	1	2.2958	37.7962	0.0087
Residual	0.1822	3	0.0607		

*** WARNING ***
Case 4 is an outlier (Studentized Residual = -19.8373)
Case 5 has large leverage (Leverage = 0.8416)
Case 5 is an outlier (Studentized Residual = 2.6609)
Case 5 has large influence (Cook distance = 6.2145)

Durbin-Watson D Statistic 2.776
First Order Autocorrelation -0.455

ANOVA using Tukey's HSD post-hoc test on Satiation feeding (testing for differences between tanks)

Categorical values encountered during processing are:
TANK$ (3 levels)
one, three, two

Dep Var: FOODAVG N: 40 Multiple R: 0.3226 Squared multiple R: 0.1041

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
TANK$	2.2856	2	1.1428	2.1488	0.1310
Error	19.6775	37	0.5318		

Least squares means.

		LS Mean	SE	N
TANK$	=one	1.8237	0.1823	16
TANK$	=three	1.2960	0.2578	8
TANK$	=two	1.3591	0.1823	16

*** WARNING ***
Case 3 is an outlier (Studentized Residual = 3.0136)

Durbin-Watson D Statistic 0.856
First Order Autocorrelation 0.546
COL/
ROW TANK$
1 one
2 three
3 two
Using least squares means.
Post Hoc test of FOODAVG

Using model MSE of 0.532 with 37 df.
Matrix of pairwise mean differences:

	1	2	3
1	0.0		
2	-0.5278	0.0	
3	-0.4646	0.0631	0.0

Tukey HSD Multiple Comparisons.
Matrix of pairwise comparison probabilities:

	1	2	3
1	1.0000		
2	0.2296	1.0000	
3	0.1830	0.9782	1.0000

ANOVA analysis on fish MOISTURE CONTENT (testing for differences between tanks, experimental and controls and replicates)

Categorical values encountered during processing are:

TANK$ (8 levels)
R1, R2, R3, _ten, _twentyfive, fifty, onehun, twohun

Dep Var: WETWGT N: 80 Multiple R: 0.4083 Squared multiple R: 0.1667

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
TANK$	34.5656	7	4.9379	2.0576	0.0593
Error	172.7923	72	2.3999		

Least squares means.

		LS Mean	SE	N
TANK$	=R1	79.4047	0.4899	10
TANK$	=R2	79.7976	0.4899	10
TANK$	=R3	79.0580	0.4899	10
TANK$	=_ten	80.0351	0.4899	10
TANK$	=_twentyfive	79.8866	0.4899	10
TANK$	=fifty	81.4415	0.4899	10
TANK$	=onehun	79.8544	0.4899	10
TANK$	=twohun	80.2629	0.4899	10

*** WARNING ***
Case 10 is an outlier (Studentized Residual = 3.4203)

Durbin-Watson D Statistic 1.702
First Order Autocorrelation 0.141
COL/
ROW TANK$
1 R1
2 R2
3 R3

4 _ten
5 _twentyfive
6 fifty
7 onehun
8 twohun
Using least squares means.
Post Hoc test of WETWGT

Using model MSE of 2.400 with 72 df.
Matrix of pairwise mean differences:

	1	2	3	4	5
1	0.0				
2	0.3928	0.0			
3	-0.3467	-0.7396	0.0		
4	0.6303	0.2375	0.9771	0.0	
5	0.4818	0.0890	0.8286	-0.1485	0.0
6	2.0367	1.6439	2.3835	1.4064	1.5549
7	0.4496	0.0568	0.7964	-0.1807	-0.0322
8	0.8581	0.4653	1.2049	0.2278	0.3763

	6	7	8
6	0.0		
7	-1.5871	0.0	
8	-1.1786	0.4085	0.0

Tukey HSD Multiple Comparisons.
Matrix of pairwise comparison probabilities:

	1	2	3	4	5
1	1.0000				
2	0.9992	1.0000			
3	0.9996	0.9615	1.0000		
4	0.9842	1.0000	0.8496	1.0000	
5	0.9969	1.0000	0.9305	1.0000	1.0000
6	0.0795	0.2705	0.0207	0.4695	0.3386
7	0.9980	1.0000	0.9432	1.0000	1.0000
8	0.9174	0.9975	0.6621	1.0000	0.9994

	6	7	8
6	1.0000		
7	0.3129	1.0000	
8	0.6864	0.9989	1.0000

ANOVA analysis of fish LENGTH averages (testing for differences between tanks, experimental and controls and replicates)

(before and after experiments)

CONCEN$ (8 levels)
  fifty, onehun, r1, r2, r3, ten, twentyfive, twohun
BEFAFT$ (2 levels)
  end, start
2 case(s) deleted due to missing data.

Dep Var: LNGTH N: 158 Multiple R: 0.4327 Squared multiple R: 0.1872

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
CONCEN$	21.4933	7	3.0705	4.1089	**0.0004**
(is a non consequential value since BEFAFT was non-significant)					
BEFAFT$	1.1645	1	1.1645	1.5584	**0.2140**
CONCEN$*BEFAFT$	1.7625	7	0.2518	0.3369	0.9359
Error	106.1121	142	0.7473		

Least squares means.

		LS Mean	SE	N
CONCEN$	=fifty	6.1750	0.1933	20
CONCEN$	=onehun	6.1400	0.1933	20
CONCEN$	=r1	6.6600	0.1933	20
CONCEN$	=r2	6.4900	0.1933	20
CONCEN$	=r3	6.0150	0.1933	20
CONCEN$	=ten	6.7350	0.1933	20
CONCEN$	=twentyfive	6.7000	0.1933	20
CONCEN$	=twohun	7.2333	0.2038	18

BEFAFT$	=end	6.6044	0.0973	79
BEFAFT$	=start	6.4326	0.0973	79

CONCEN$	=fifty			
BEFAFT$	=end	6.1300	0.2734	10

CONCEN$	=fifty			
BEFAFT$	=start	6.2200	0.2734	10
CONCEN$	=onehun			
BEFAFT$	=end	6.0800	0.2734	10
CONCEN$	=onehun			
BEFAFT$	=start	6.2000	0.2734	10
CONCEN$	=r1			
BEFAFT$	=end	6.8900	0.2734	10
CONCEN$	=r1			
BEFAFT$	=start	6.4300	0.2734	10
CONCEN$	=r2			
BEFAFT$	=end	6.6700	0.2734	10
CONCEN$	=r2			
BEFAFT$	=start	6.3100	0.2734	10
CONCEN$	=r3			
BEFAFT$	=end	6.0600	0.2734	10
CONCEN$	=r3			
BEFAFT$	=start	5.9700	0.2734	10
CONCEN$	=ten			
BEFAFT$	=end	6.9400	0.2734	10
CONCEN$	=ten			
BEFAFT$	=start	6.5300	0.2734	10
CONCEN$	=twentyfive			
BEFAFT$	=end	6.8100	0.2734	10
CONCEN$	=twentyfive			
BEFAFT$	=start	6.5900	0.2734	10
CONCEN$	=twohun			
BEFAFT$	=end	7.2556	0.2881	9
CONCEN$	=twohun			
BEFAFT$	=start	7.2111	0.2881	9

Durbin-Watson D Statistic 2.147
First Order Autocorrelation -0.081

ANOVA on trout WET WEIGHT averages (testing for differences between tanks, experimental and controls and replicates)

(before and after experiments)

Dep Var: WGTS N: 159 Multiple R: 0.4770 Squared multiple R: 0.2276

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
CONCEN$	52.6842	7	7.5263	4.6567	**0.0001**
(is a mildly significant value since BEFAFT was significant)					
BEFAFT$	7.6871	1	7.6871	4.7561	**0.0308**
CONCEN$*BEFAFT$	7.3061	7	1.0437	0.6458	0.7174
Error	231.1237	143	1.6162		

Least squares means.

		LS Mean	SE	N
CONCEN$	=fifty	2.9039	0.2843	20
CONCEN$	=onehun	2.5580	0.2843	20
CONCEN$	=r1	3.5356	0.2843	20
CONCEN$	=r2	3.2116	0.2843	20
CONCEN$	=r3	2.6087	0.2843	20
CONCEN$	=ten	3.4650	0.2843	20
CONCEN$	=twentyfive	3.8046	0.2843	20
CONCEN$	=twohun	4.3794	0.2921	19

BEFAFT$	=end	3.0884	0.1431	79
BEFAFT$	=start	3.5283	0.1421	80

CONCEN$	=fifty			
BEFAFT$	=end	2.2537	0.4020	10
CONCEN$	=fifty			
BEFAFT$	=start	3.5541	0.4020	10
CONCEN$	=onehun			
BEFAFT$	=end	2.3989	0.4020	10
CONCEN$	=onehun			
BEFAFT$	=start	2.7172	0.4020	10

CONCEN$	=r1			
BEFAFT$	=end	3.4290	0.4020	10
CONCEN$	=r1			
BEFAFT$	=start	3.6421	0.4020	10
CONCEN$	=r2			
BEFAFT$	=end	2.9504	0.4020	10
CONCEN$	=r2			
BEFAFT$	=start	3.4729	0.4020	10
CONCEN$	=r3			
BEFAFT$	=end	2.6544	0.4020	10
CONCEN$	=r3			
BEFAFT$	=start	2.5631	0.4020	10
CONCEN$	=ten			
BEFAFT$	=end	3.4319	0.4020	10
CONCEN$	=ten			
BEFAFT$	=start	3.4981	0.4020	10
CONCEN$	=twentyfive			
BEFAFT$	=end	3.3576	0.4020	10
CONCEN$	=twentyfive			
BEFAFT$	=start	4.2517	0.4020	10
CONCEN$	=twohun			
BEFAFT$	=end	4.2314	0.4238	9
CONCEN$	=twohun			
BEFAFT$	=start	4.5274	0.4020	10

Durbin-Watson D Statistic 2.071
First Order Autocorrelation -0.067