THE EFFECT OF PETROLEUM HYDROCARBONS ON A FRESHWATER ENVIRONMENT, THE SPRING GULCH WETLANDS, CANADIAN FORCES BASE GOOSE BAY, LABRADOR USING PEARL DACE (MARGARISCUS (SEMOTILUS) MARGARITA) AS AN ENVIRONMENTAL INDICATOR

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by

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Abstract

A study was conducted to determine the impact of petroleum hydrocarbons on pearl dace, *Margariscus (=Semotilus) margarita,* inhabiting stillwaters (SW) 1-4 of the Spring Gulch Wetlands, Canadian Forces Base Goose Bay, Labrador. The results of ten (10) biomarkers were compared to results for pearl dace captured from three reference sites (R1-3) at varying distances from CFB Goose Bay.

Evidence supporting the premise that the extinction of pearl dace from the Spring Gulch Wetlands as a consequence of the chronic effects of petroleum hydrocarbon contamination was collected. The petroleum hydrocarbon contaminant levels in SW1, 3 and 4 impaired reproduction in pearl dace populations. There was a predominance of female fish in SW1, 3 and 4 and they were producing mature eggs in 1995 but none had spawned. There were none, or very few, juvenile fish in SW1, 3 and 4, suggesting reproductive failure and/or low reproductive survival rates. Fish collected from SW1, 3 and 4 were also longer, heavier and had higher condition factors than fish sampled from three reference sites (R1-3). There were more, and greater degrees, of histopathological lesions in fish sampled from SW1, 3 and 4 compared to the reference sites. These lesions included interlamellar hyperplasia, hepatic lipid vacuolation, pigmented and non-pigmented splenic melanomacrophage aggregates and significant splenic concentrations of hemosiderin. Mixed function oxygenase activity (7-EROD) was elevated only in samples of liver taken from pearl dace from SW4 in 1996 but not in 1995. It is possible that estrogenic hormonal levels disrupted 7-EROD induction in 1995.

The absence of juvenile fish, high prevalence of histopathological lesions, delay in seasonal maturity, and the complete disappearance of pearl dace from SW1, 2 and 3 over the two-year study period suggests that the pearl dace population at SW4 also faces eventual extinction.

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List of Abbreviations and Acronyms

BSA	Bovine serum albumin
CFB	Canadian Forces Base
DND	Department of National Defence
EROD	Ethoxyresorufin-O-deethylase
MFO	Mixed function oxygenase
MMOU	Multinational Memorandum of Understanding
Mn	Manganese
NADPH	B-Nicotinamide adenine dinucleotide phosphate, reduced form
NATO	North Atlantic Treaty Organization
NBF	Neutral buffered formalin
PAH	Polycyclic aromatic hydrocarbon
SW	Stillwater
TPH	Total petroleum hydrocarbon
Xenobiotic	Substance foreign to biological systems

1.0 Introduction

1.1 Purpose of Study

The province of Newfoundland and Labrador, with its vast marine coastline and abundant freshwater habitat, is a large area that needs to be protected from contamination. Aquatic-based industries are continually expanding into numerous areas including aquaculture, hydroelectric generation and countless tourism industries, such as whale and iceberg watching from boat charters, kayaking, adventure tourism and ecological reserve tourism. Facilitating industrial prosperity, while simultaneously protecting Newfoundland and Labrador's environment and natural resources, is the responsibility of government and individuals. An important example of this is the development and expansion of the petroleum industry in Newfoundland and Labrador.

With oil production ongoing at Hibernia, Terra Nova and White Rose oil and gas fields in various stages of development, and the Port aux Port peninsula being probed for oil and gas reserves, the petroleum industry within Newfoundland and Labrador, along with its related infrastructure, will be a driving economic force for decades. The responsibility, however, for ensuring that all aspects of the petroleum industry are managed in the most efficient and environmentally sound manner possible lies with government, industry and residents of the province. One only has to read any of the numerous journals or newspaper articles available to discover what acute effects spilled petroleum hydrocarbons have had on the aquatic environment. This extensive media coverage of recent oil disasters has lead to increased awareness by not just the public, but

also environmental groups and governments of the acute effects of spilled petroleum hydrocarbons.

Concern at the levels and effects of chronic - low level - petroleum contamination in the aquatic environment does not receive the same level of attention regardless of the possible significant effects of such pollution on both human health and the well-being of animal and plant populations. Since the seas and lakes, via rivers and other water courses, are the final resting point for pollutants, it is in such environments that we might expect to find the first warnings of environmental catastrophe (Kime, 1995). Establishing, however, the cause and effect relationship between chronic, low-level, hydrocarbon pollution and its effect on the aquatic environment is not as well established.

The inevitable consequence of polluting an aquatic environment is that the water quality is diminished and inhabiting organisms become stressed (Adams, 1990). Canadian Forces Base Goose Bay, Labrador (Figure 1.1) is an excellent site for studying the effects of chronic petroleum hydrocarbon contamination on freshwater aquatic environments and how stress induction in fish by hydrocarbon pollution affects the immune system in animals and alters a fish's defence mechanism (Zeeman and Brindley, 1981; Wojdani and Alfred, 1984; Anderson, 1990).

The Spring Gulch Wetlands (Figure 1.2), part of 5 Wing Goose Bay, became contaminated through the disposal and spillage of petroleum hydrocarbons into both surface water and sediment during the course of operations from the beginning of World War II to the end of the Cold War (1939-1989). In an effort to quantify the chronic effects

Figure 1.1: Aerial photograph of Canadian Forces Base (CFB) Goose Bay. Note the yellow outline of the Spring Gulch Wetlands, and the labeled major local water bodies, Terrington Basin, Lake Melville (the western end of Hamilton Inlet) and the Churchill River. Photo courtesy of CFB Goose Bay.

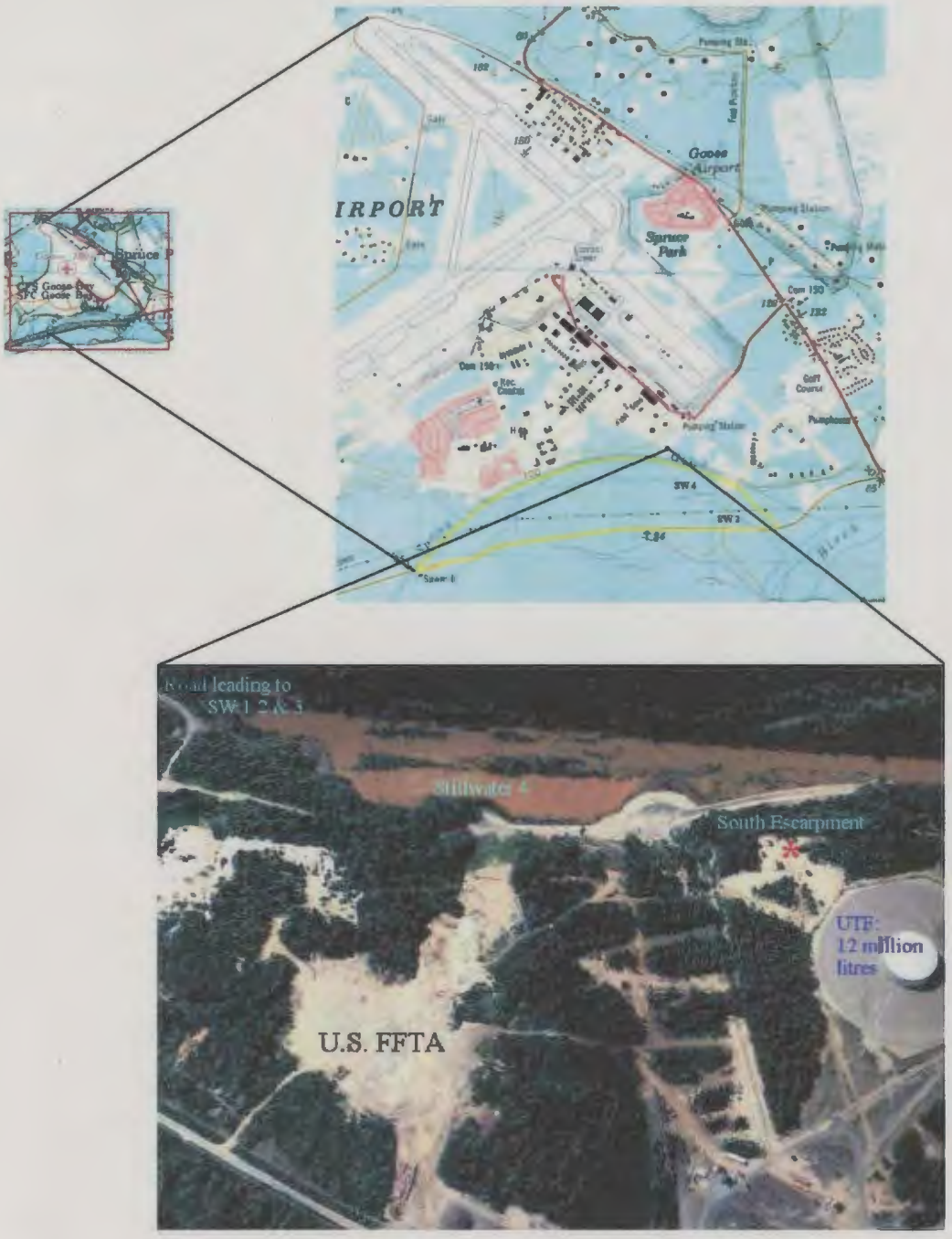

Figure 1.2: Location of Spring Gulch Wetlands, CFB Goose Bay. In aerial photograph note water colour of SW4, 12 million litre Upper Tank Farm (UTF) on top of South Escarpment, U.S. Fire Fighter Training Area (FFTA), access road leading to SW1, 2 and 3 and red asterisk, indicative of field lab site. Photo courtesy of CFB Goose Bay.

of petroleum hydrocarbon contamination on a freshwater aquatic environment, a biological indicator species was required. A small teleost, pearl dace (*Margariscus (Semotilus) margarita*), was used as the bioindicator for this study. Numerous consultants contracted by the CFB Goose Bay's Environment Office have conducted detailed sampling of soil, vegetation, water, fish and invertebrates throughout the Spring Gulch Wetlands. Their findings indicated that although metal contamination in certain areas appeared to be above acceptable levels, total petroleum hydrocarbons (TPH) were the major contaminants (Beatty Franz and Assoc. Ltd., 1995).

The purpose of this study was twofold. First, using pearl dace as a biological indicator and a multifaceted approach, to ascertain the impact of chronic petroleum hydrocarbon contamination on four stillwaters (SW) at CFB Goose Bay by comparison to three reference sites. Second, investigate the potential of using pearl dace as an environmental indicator of petroleum hydrocarbon contamination in an aquatic environment.

1.2 Biomarkers and Determinants of Petroleum Hydrocarbon Exposure

Biomarkers - the use of biochemical and biological techniques to measure chronic toxicity - can be powerful tools in detecting exposure to sublethal concentrations of xenobiotics in fish and the destruction of immunobiochemical signals that eventually permit the demise of an individual fish and subsequently the population (Ewald, 1995). Biomarkers are excellent scientific methods to predict potential adverse effects on survival, growth and reproduction of individuals based on the

premise that non-acute, or chronic, affects at the cellular or organ level can result in an effect on the integrated organism functions (Ewald, 1995). Coupled with histopathology, organ somatic indices, determination of condition factor and new fields in molecular biology (i.e. genotoxicology), these processes can provide very strong, corroborating evidence to aid researchers in determining the effects of a hydrocarbon pollutant on a selected fish species. In short, stress-related changes in fish species, such as pearl dace, represent attempts by the organism to maintain homeostasis.

Sub-lethal quantities of petroleum hydrocarbons, in general, within an aquatic ecosystem, are deleterious in that they produce a stress response. The most toxic family of petroleum hydrocarbons, the polycyclic aromatic hydrocarbons (PAHs), such as benzo(a)pyrene, are mutagenic and carcinogenic, and pose a significant hazard to aquatic organisms (Connell and Miller, 1980; Tuvikene, 1995; Khan, 1999a).

Theoretically, any measurable biochemical function of fish can be used as a biomarker. However, use of these functions as a measure of stress must be accompanied by a thorough knowledge of the importance of the biochemical function at a higher organizational level (Ewald, 1995). To date, one of the most extensively used biomarkers is the induction of the mixed function oxygenase (MFO) system, but other biomarkers are quickly gaining notoriety including acetylcholinesterase (ACHE), antioxidant enzymes and DNA adducts.

1.2.1 Mixed Function Oxygenase (MFO)

When fish are exposed to contaminants, the toxins are taken up into the bloodstream where the rate of pollutant distribution to specific tissues is determined by the regional blood

flow through each tissue. Organs with high blood flow, such as the liver, tend to accumulate xenobiotics most readily (Tuvikene, 1995). Accordingly, the liver is the primary organ of pollutant schismatics. Lipophilic PAHs move via passive diffusion through the hepatic cellular membranes which are also mostly composed of lipids, and are bound to the cytosolic aromatic hydrocarbon (Ah)-receptor (Tuvikene, 1995). The key components in the MFO system are the monooxygenase reactions mediated by enzymes referred to as cytochrome P450, specifically, P4501A1 (CYP1A1) and P4501A2 (CYP1A2) which belong to the sub-family CYP1A (Tuvikene, 1995). The level of PAH-MFO induction is measured as the catalytic activity of specific forms of P4501A1, namely, 7-ethoxyresorufin-O-deethylase (EROD), aryl-hydrocarbon-hydroxylase (AHH) and ethoxycoumarin-O-deethylase (ECOD). The result of these enzymatic reactions is oxidation of the substrate into polar products which are more easily conjugated or excreted than the original PAH (Khan and Thulin, 1991; Addison, 1992).

Using EROD values as biomarkers to identify areas of PAH pollution in aquatic environments has lately become widespread mainly because of its rapid induction - within four days (Payne *et al.*, 1987; Lindström-Seppä and Oikari, 1988; 1990; Ewald, 1995; Burgeot *et al.*, 1996). EROD activity is associated with genotoxicity in fish (Masfaraud *et al.*, 1992); however, its high specificity can be regarded as both an advantage and a disadvantage. For example, the effects of non-aromatic hydrocarbon contamination on fish species are not detected by this method and thus go unnoticed.

Another very important point to consider when using induction of the MFO system to quantify the effect of petroleum contamination on a fish is that hormones such as estradiol and

cortisol have a significant impact upon the levels of MFO induced. Tuvikene (1995) reported that many endogenous and exogenous factors may modulate the activity of the MFO system in fish tissues. Steroid hormones, such as cortisol, have been suggested to be one of the modulating factors usually promoting induction of the MFO system (Devaux *et al.*, 1992). Estradiol has also been shown to be a regulator of monooxygenase activity in that it suppresses P4501A (Stegeman and Hahn, 1994). Consequently, when studying MFO induction in fish that may have high levels of these hormones, researches have to be cognizant of the biochemical interrelationships.

1.2.2 Histopathology

Histological changes are not as readily and objectively assessed as MFO induction and the interpretation of results requires significant experience before one becomes competent in identification of histopathological anomalies. This notwithstanding, since organ damage is a clear indication of exposure to toxic substances and is also considered as indisputable evidence of an adverse effect on an organism and its environment, the results concluding histological trauma due to sublethal exposure to pollutants are very difficult to dispute and consequently are powerful indicators of prior exposure to environmental stressors (Hinton and Laurén, 1990; Ewald, 1995).

1.2.2.1 Liver

The liver is one of the principal organs affected by exposure to toxic pollutants since it functions as the primary site of detoxification / metabolism for most chemicals. As a result, the liver is also the first internal organ to be exposed to any toxic petroleum hydrocarbon

metabolites. Furthermore, the liver converts food materials, stores glycogen and produces bile and plasma proteins (Goede and Barton, 1990). Given the importance of its multifunctional role, damage to the liver will adversely affect all other hepatic functions (Köhler and Pluta, 1995). Consequently, the liver is the most extensively studied organ in fish toxicology (Ewald, 1995). A variety of histological changes occur in the liver of fish as a result of exposure to petroleum hydrocarbon contamination: vacuolation of hepatocytes, presence of melanomacrophage centres, white blood cell foci, bile ductule hyperplasia, and cellular necrosis (Khan and Kiceniuk, 1983; Tuvikene, 1995).

Recent studies have demonstrated a relationship between the occurrence of neoplastic lesions and petroleum pollution, mainly due to PAHs. Malins *et al.* (1985) cited an example of this in English sole (*Parophrys vetulus*) and Myers *et al.* (1998) another when they reported that the prevalence of hepatic neoplasms in marine bottomfish species was statistically associated with exposure to PAHs.

Liver anomalies, such as accumulation of lipid, lead to injury in cellular components such as enzyme systems and impairment of catabolism of organic pollutants (Krahn *et al.*, 1986). With the relatively large amount of blood flow through the liver and the continual detoxification of petroleum hydrocarbons into biproducts, the unfortunate consequence is that, with most pollutants, the resulting metabolites are just as, or more, toxic than the original compound (Krahn *et al.*, 1986).

1.2.2.2 Kidney and Spleen

The kidney and spleen are supplementary organs for evaluating the effects of pollutants

on fish. The findings from histological examination of these organs usually corroborate the results found in the liver and consequently they serve to strengthen the argument that hydrocarbon pollution has a deleterious effect upon fish.

The fish spleen is primarily an organ of blood storage and blood cell production and it also degrades red blood cells and releases hemoglobin (Goede and Barton, 1990). The kidney on the other hand, has a major function in osmoregulation. In freshwater, it excretes water and in marine fish, it functions to expel magnesium and sulfate ions. Furthermore, the kidney also acts as a hemopoietic organ in fish (Goede and Barton, 1990).

There are a number of anomalies that can be noted within the kidney and spleen, after exposure to petroleum hydrocarbons, especially PAHs. These include the proliferation of melanomacrophage aggregates (MMAs) from the aggregates of iron residues due to the destruction of red blood cells and also the presence of clear cell foci, hemorrhages, cellular necrosis and various other lesions (Khan and Kiceniuk, 1983).

1.2.2.3 Gills

The morphology of the gills is a useful indicator of the general well being of a fish. At the level of the branchial lamellae, only a semipermeable membrane separates blood from water. Movements of molecules across the membrane may be affected by a variety of biochemical and physiological processes and alteration of any of these processes may result in structural change, which may be expressed in a number of ways (Goede and Barton, 1990).

Exposure of fish to petroleum hydrocarbons culminates in several gill epithelial lesions. Subsequent histological examination of branchial tissue sections clearly reveal histopathological

changes. Khan and Kiceniuk (1984) found increased mucus production, increased mucus-producing epithelial cells, capillary dilation, lamellar hyperplasia and fusion of adjacent filaments in the gills in Atlantic cod (*Gadus morhua*) exposed to Hibernia crude oil.

1.2.3 Organ Somatic Indices

Somatic indices, particularly those that are representative of the whole fish, can be used to relate the consequences of biochemical and physiological alterations in order to observe changes in the individual and sometimes the population (Goede and Barton, 1990). Organosomatic indices produce ratios of organ weight to body weight (eviscerated) that have been used in various stress-related studies. The most frequently used of these is the hepatosomatic index (HSI) or otherwise called the liver somatic index (LSI). Other organ weight ratios used in stress investigations are the gonadosomatic index (GSI) and kidney (KSI) or splenic somatic index (SSI).

The ratio of the liver weight to eviscerated body weight is used by toxicologists as one method of determining whether a pollutant has had any effect upon a fish when it is compared to a fish captured from a reference location. A reduction in the HSI has been demonstrated in cod (*Gadus morhua*) populations under stress from oil pollutants but conversely, there can also be an increase in liver weight due to hyperplasia or hypertrophy as an adaptive response to increase the capacity of the liver to detoxify foreign compounds via the MFO system (Kiceniuk and Khan, 1987; Goede and Barton, 1990). Increased HSI was reported by Everaarts *et al.* (1993) after sunfish (*Alectis ciliaris*) and hardhead catfish (*Arius felis*) had been exposed to PAH-contaminated sites. Theodorakis *et al.* (1992) also reported a significant increase in HSI

in bluegill sunfish (*Lepomis macrochirus*) exposed to contaminated sediments. Baumann *et al.* (1991) documented elevated HSIs in brown bullhead (*Ameiurus nebulosus*) from polluted sites in the tributaries of the Laurentian Great Lakes. Fletcher *et al.* (1982) reported that following exposure of winter flounder to oiled sediments, there was a significant increase in the weight of the liver. Given the contrasting results between Kiceniuk and Khan (1987) and the other reports, there are other factors at play. There may be species differentiation with respect to the effects of petroleum hydrocarbons on fish HSIs, marine and freshwater fish species may exhibit differing responses or other factors may be involved (i.e. a reduction in feed intake is associated with hepatic shrinkage) (Khan, pers comms).

Organosomatic indices display seasonal variability depending upon the distribution of energy available in the fish and also with the sex and life history of the fish (Goede and Barton, 1990). The gonadosomatic index is a particularly good example of this. Depending upon the season, a species of fish may or may not have spawned or molted and, as a result this weight change, introduces seasonal variation. If this variation can not be removed, then conclusions regarding pollution effects based on fish GSIs calculated from different seasons can be inaccurate because of the difference in weights of the gonads in prespawners and spawners.

Knowledge of the impact of seasonal variation on GSI, however, does not introduce scepticism into any studies that have used this index. Lopez *et al.* (1981) reported a low GSI in plaice (*Pleuronectes platessa*) after exposure to oil spilled from the *Amoco Cadiz* wreck along the coast of France. Similarly, Payne *et al.* (1978) reported that after a six-month exposure of cunners (*Tautogolabrus adspersus*) to Venezuelan crude oil there were significant differences

in the male GSI when compared to the control fish. Striving to understand all factors relating GSI to fishes' health can only aid in comprehending the entire dynamics of an organisms health.

1.2.4 Condition (K) Factor

According to Khan (1999a), given the weight (W) and length (L) of a fish species, such as winter flounder (*Pleuronectes americanus*), the condition (K) factor can be determined ($K=W/L^3$). Comparison of the condition factor is a reliable method for investigating the relative health of a fish population when compared to a reference population (Khan, 1998; 1999b; George-Nascimento *et al.*, 2000; Barker *et al.*, 1994). Condition factor was consequently used to determine the relative health of the pearl dace populations sampled.

1.2.5 Digital Image Analysis (DIA)

According to Agius and Agbede (1984), hemosiderin is derived from the breakdown of hemoglobin from effete erythrocytes. Khan and Nag (1993) first used DIA to quantify hemosiderin deposits within the splenic cells of seabirds and fish exposed to petroleum. In 1994, using DIA, Khan *et al.*, further provided evidence of a quantitative nature that the histopathology of winter flounder living adjacent to a pulp and paper mill was significantly altered from that of a reference population. Consequently, in an effort to demonstrate the effectiveness of this method and expand its use, DIA was employed during this study.

1.3 History and Ecology of CFB Goose Bay, Labrador

In 1938, the British government decided that by 1940 it required 26,000 aircraft from Canada and the U.S.A. to keep itself on par with the ever-expanding German Luftwaffe (MacLeod, 1986). Britain decided that the best method of transporting these planes across the Atlantic Ocean was to build a series of fortified landing stations in Labrador, Greenland and Iceland and have pilots "leap-frog" the planes to Great Britain (MacLeod, 1986). Stations were already built in Greenland and Iceland so the construction of a Base in Goose Bay, Labrador was delegated to the Canadian government. By August 1941, construction of the airstrip began and the end of November was chosen as the deadline for having the site operational. The deadline was met and a U.S. Army aircraft landed at Goose Bay on 6 November, 1941 (Jennings, 1991).

The decision to build a Base at Goose Bay initiated a series of events that, in four short months, would convert a Labrador cranberry patch into the largest airport in the world, at the time (Cardoulis, 1990). After the Japanese bombed Pearl Harbor, the United States was officially brought in to World War II and the decision to construct a U.S. Base on the southeast side of the Goose Bay airfield was reached (Cardoulis, 1990). At the end of the war, Goose Bay was a fully developed air force station. The airport itself was and still is massive, covering 1600 acres including three concrete runways, each over 2950 metres long, and 340 permanent buildings.

1.3.1 Cold War Years

The years between 1950 and 1973 were the peak period of the Cold War between the NATO Alliance and the Warsaw Pact counties (Rowell, 1990). The year 1951 brought

about revised American military requirements and the Canadian and American governments signed the Pine Tree Agreement for defending North America (Rowell, 1990). This agreement permitted the establishment of American-manned early warning radar sites in Labrador. A year later, a 20-year lease for the continued American use of the sixteen square kilometers comprising the American side of Goose Bay airfield was signed (Rowell, 1990).

Between 1952 and 1958, the Americans invested heavily in Goose Bay and the site became a fortress of U.S. air power (Cardoulis, 1990). Its primary role was to enable in-flight refueling for the fleet of American long-range strategic nuclear bombers. Fulfilling this mandate, enormous volumes of jet fuel were stored on the Base. Fuel storage was accomplished by using both above and underground tank farms. Supply of fuel to these tank farms was via a pipeline from the docking terminal located in Terrington Basin (Figure 1.1) Over its operational period from 1952 to 1973, large fuel spills were, unfortunately commonplace. Such was the case in 1962 when over five million gallons (21 million litres) of jet fuel were spilled due to a structural failure in one of the aboveground storage tanks (Lammey, pers. comms). The primary source of petroleum contamination into the Spring Gulch Wetlands was thus from the aboveground tank farm (Upper Tank Farm – UTF) located atop the southern escarpment (Figures 1.2 and 1.3). Secondarily, as a means of fire fighter training, it was common for Bases to have an established Fire Fighter Training Area (FFTA). The U.S. FFTA was a series of large mockups for military fire fighters to set on fire, using large amounts of petroleum, for

practice extinguishing the fires (see Figures 1.2 and 1.4). This method of training has been stopped recently, however, the environmental legacy of damage is something DND is still trying to resolve. Lastly, with thought and due diligence for the environment commonly lacking during this period of history, it is not surprising that the accumulation from many small petroleum spills over the twenty-one year operation of the Base is currently posing a significant and expensive problem for DND to rectify (Lammey, pers. comms).

Between 1952 and 1973 the ecosystems surrounding Goose Bay sustained the greatest amount of environmental damage (Lammey, pers. comms). Environmental consultants, Newfoundland Geosciences Ltd. (1994), reported that under the UTF three plumes of free hydrocarbon products, covering 41 hectares and comprising 2.4 to 3.9 million litres were observed. These plumes continue to discharge into the Spring Gulch Wetlands and specifically SW4 directly.

1.3.2 New Roles for Goose Bay

In the 1980's, NATO recognized the opportunity afforded by the terrain and climate of the Quebec-Labrador peninsula for low-level flight training. The West German Air Force was the first to receive permission from the Canadian government to begin flight training in Labrador. Soon after, the United States Air Force, Royal Air Force, Canadian Air Force and Royal Netherlands Air Force started flying from CFB Goose Bay (Rowell, 1990). In 1990, after the end of the Cold War, the United States Air Force announced the withdrawal of its personnel and aircraft stationed at the Base. Essentially, this brought an end to almost 50 years of American association with Goose Bay.

Figure 1.3: Photograph of Upper Tank Farm (UTF) storage facility (12 million litres, Jet Fuel - JP4) located on top of south escarpment.

Figure 1.4: Photograph of south escarpment with FFTA on right side, stillwater (SW) 4 at the base of the south escarpment and Officer's Quarters in background.

1.3.3 Ecology of CFB Goose Bay

Canadian Forces Base Goose Bay is located within the community of Happy Valley-Goose Bay in the province of Newfoundland and Labrador. Within central Labrador, CFB Goose Bay is situated upon a plateau high above Terrington Basin (53°25'N 060°30'W) on the south shore of Goose Bay near the junction of the Churchill River and the western end of Hamilton Inlet / Lake Melville. The bulk of the airfield runways, technical support facilities (including fuel storage) and airbase ancillary areas are located on top of the "northern escarpment" which runs in a west-northwest direction.

Goose Bay is within the Northeastern Forest region of the eleven divisions of the Canadian Climate Region. The plateau, however, upon which CFB Goose Bay is constructed, is a "sub-arctic environmental zone" influenced by continental polar air from the west and maritime polar air from the North Atlantic. The forest and vegetation consist of a coniferous forest woodland with black spruce (*Picea mariana*) as the dominant species and balsam fir (*Abies balsamea*), white spruce (*Picea glauca*), tamarack (*Larix laricina*) and white birch (*Betula papyrifera* var. *cordifolia*) as the other abundant species present (Newfoundland Geosciences Limited, 1994).

Climate of the CFB Goose Bay region is typically sub-arctic. Mean annual precipitation is 959.5mm with the maximum amount of precipitation falling in July as rain. Snow has been recorded to fall in every month except July and August with an annual average of 463.8cm. The temperature range in Goose Bay is extreme. In January, the average daily temperature is -17.3^{0}C and in July it is 9.8^{0}C. The record minimum low

temperature for Goose Bay is -39.4^{0}C and the record maximum high is 37.8^{0}C, for a range of 77.2^{0}C (Environment Canada, 1998).

The Goose Bay region is a vast block of Canadian Shield composed of Archean gneisses and granites. The plateau upon which CFB Goose Bay was built is uplifted with the highest elevation in the southeast. Glaciated in the Pleistocene Epoch (1.65 million years ago until 10,000 years ago), Goose Bay has large deposits of glacial till and sand accumulations which occurred as a result of rivers reworking this till over thousands of years (Newfoundland Geosciences Ltd., 1994).

The main water bodies (including lakes and rivers) in the Goose Bay region had, and still have, great effects upon all aspects of the regional geography. Goose Bay is located at the western end of Lake Melville and is 24km (15 miles) long and 12.8km (8 miles) wide (Newfoundland Geosciences Ltd., 1994). Lake Melville terminates in a small shallow named Terrington Basin. Grand Lake is the largest freshwater body in the area and is 64km (40 miles) long, 4km (2.5 miles) wide and up to 122m (400 feet) deep (Newfoundland Geosciences Ltd., 1994). Prior to glaciating, the area was a large valley; however, during the glacial period, it was further deepened and widened. North West River is one of three major rivers in the region. North West River enters Lake Melville via Little Lake and contains all the river water that flows into Grand Lake. Goose River is the second major river in the region and it flows into Terrington Basin. The Churchill River is the most important and largest river in the area and on the entire Labrador coast. The Churchill River receives all the waters from the impacted stillwater sites. The mouth of the

river is a series of terraces, rising 45m (150ft) above sea level (Newfoundland Geosciences Ltd., 1994). The community of Happy Valley-Goose Bay is built upon an ancient delta of the Churchill River, which was deposited with the rest of the sand when the river was enlarged due to the glacial meltdown. The Churchill River still deposits a large amount of sand at its mouth and the area has to be continually dredged to maintain the merchant shipping lanes navigable.

1.4 Biology of Pearl Dace

Margariscus (=Semotilus) margarita (Cope, 1869) is a member of the Kingdom Animalia, Phylum Chordata, Class Actinopterygii, Order Cypriniformes and Family Cyprinidae (Smith, 1985). Cyprinids share several common taxonomic characteristics, which distinguish them from the fish in other families. External features include: scaleless head, toothless jaws, lack of adipose fin, lack of appendages at the base of the pelvic fins, and a single, soft dorsal fin of less than 10 rays. Internal anatomical features include 10 teeth in any row on the pharyngeal arch, an enlarged intestine instead of a true stomach and a series of bones called the weberian ossicles that form a rudimentary ear (Scott and Crossman, 1973).

In spite of its long-term importance as a bait-fish for sport fishing, the biology of the pearl dace is not very well known (Scott and Crossman, 1973). In Canada specifically, the life history of this species is poorly known. In general, most data gathered are from south-central United States populations where climatic conditions and fish community

structures are quite different from those of central and northeastern Canada (Tallman *et al.*, 1984).

According to Tallman (1980), pearl dace are not abundant in the southern portion of its range, but are very abundant in many northern streams and lakes. Ironically, they have a wide Canadian distribution. They occur from the maritime provinces west to the Peace River system of British Columbia and from the Arctic tundra south to Montana in the west and to Pennsylvania and Virginia in the east (Tallman, 1980). They are most commonly found in small, cool, sandhill streams at sites high in the drainage system near the stream's cold headwaters. The streams are usually narrow and shallow with deeper pools – up to several yards wide and several centimeters to a metre deep. There are four subspecies of *S. margarita* including: *S. m. margarita* (Cope, 1869), *S. m. nachtriebi* (Cox, 1869), *S. m. koelzi* (Hubbs and Lagler, 1949) and *S. m. athabascae* (Bajkov, 1927).

Physically, the pearl dace is a stout-bodied minnow with an average length of 7.6-10.2cm. Appearing almost cylindrical in cross section, the terminal mouth is nearly horizontal with the upper jaw separated from the snout by a groove. It is dusky mottled, almost dark olive in colour on the upper side and silvery-white on the lower sides and abdomen. The lateral line is distinct on the young, fades in adults but is usually complete. In spawning season, males have a pinkish-orange stripe along their lower sides (Figure 1.5).

Pearl dace have an unusually broad feeding niche for a temperate climate species (Tallman and Gee, 1982). They consume anything from terrestrial insects, winged insects,

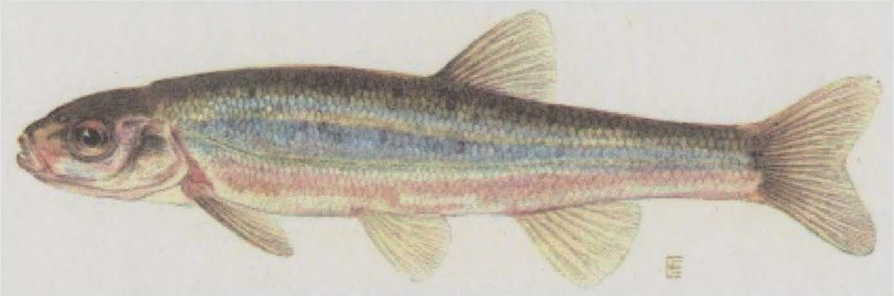

Figure 1.5: Male pearl dace (*Margariscus margarita*) in spawning season (actual size). Note the prominent pinkish-orange lateral stripe (Smith, 1985).

small benthic invertebrates, benthic macro-invertebrates, detritus and aquatic plants to terrestrial plant seeds and berries (Tallman, 1980).

Pearl dace may live to age four (4) but few individuals survive beyond age three (3) in northern waters (Tallman, 1980). Females usually have a longer lifespan, grow faster and reach greater maximum lengths than males. Young of the year (age 0 fish) have the greatest growth rate and over 75% of the yearly growth occur from May to November (Tallman, 1980). Sex ratios of adults (age 1 and older) vary from 2:1 (male:female) to 1.1 depending upon the subspecies. According to Tallman (1980), pearl dace spawn in early spring, about the time of the spring melt and ice-off occurs in many northern lakes. Spawning occurs in tributary streams or in the vegetation on the periphery of lakes. Sexual maturity is reached during the second summer of life (age 1). The reproductive behavior of pearl dace was summarized by Langlois (1929).

1.5 Pearl Dace as an Environmental Indicator

According to the National Research Council (1987), biological indicators were originally defined as measures of body fluids, cells, tissues, or other biotic variables that indicate the presence and magnitude of stress responses. Boudou and Ribeyre (1989) reported that the relative susceptibility, or tolerance, of individual species to toxic pollutants (i.e. stressors) forms the fundamental basis for the indicator species concept. They went on to state that "positive bioindicators" are the tolerant species or populations that are found in contaminated ecosystems. In contrast, "negative bioindicators" are

represented by sensitive species found to occur in low abundance or even to be absent from polluted areas, or the relative abundance of which in the community sharply decreases in a gradient of increasing pollution. Adams (1990), however, reported the now widely accepted view that the biological indicator approach employs selected indicators of stress at each of several levels of biological organization to evaluate and predict the effects of stressed-induced changes before irreversible damage occurs at the population or community level. In fact, by employing a multidimensional approach to evaluate the effect of petroleum hydrocarbon pollution on populations of pearl dace, it was the aim of this study to provide evidence to support Adam's (1990) suggestion that sublethal stress is generally expressed first at the molecular and biochemical levels by factors such as alterations in the functions of enzymes, cell membranes, or genetic material. These changes, then, induce a series of structural or functional responses at the next higher level of biological organization inducing responses that impair integrated processes such as hormonal regulation, metabolism, osmoregulation and immunological regulation. These effects, in turn, may affect the organism's ability to survive, grow, or reproduce.

As a general rule, indicator organisms should be abundant, if not, they should be the dominant species where they occur and widely distributed in order to avoid the risk that the population will be affected by sampling (Adams, 1990). Secondly, the species should be relatively sedentary and easy to identify. Thirdly, the species should belong to an important economic, scientific or aesthetic group since they are the most important to protect and for which there is the most scientific information. The bioindicator species

should also be a convenient size. Payne (1984) further identified that any environmental indicator responses should be economical, sensitive, and relatively selective, as well as relatively insensitive to any generalized stress associated with sample collection.

Margariscus margarita is a good potential bioindicator since it meets these criteria. This species, relative to other teleosts does not migrate and because of its small size, is easy to capture and transport. Additionally, also due to its small size, individuals can be pooled to provide adequate material for analysis. Tallman (1980) stated that bait fish such as pearl dace are one of the first species impacted by pollution.

2.0 Materials and Methods

2.1 Description of Stillwater and Reference Sites

All fish sampled in this study were collected from the region surrounding Happy Valley-Goose Bay, Labrador. Sampling was conducted from August 29 to September 01, 1995, and July 10 to August 05, 1996. Sampling periods were deliberately staggered so as to collect samples from the same sites over the largest portion of the summer months possible and thus perhaps better ascertain the reproductive dynamics of the pearl dace populations at CFB Goose Bay during the summer season. Seven (7) sites were sampled between August 1995 and August 1996: four stillwater (SW1-4) and three reference (R1-3) sites. Sites SW1-4 were located below the southern escarpment of CFB Goose Bay in the Spring Gulch Wetlands. The Spring Gulch Wetlands consist of a series of wetlands and the four stillwaters that are located in the Churchill River floodplain. Stillwaters are not ponds, but are small water pools that differ from ponds by having negligible water inflow or outflow. The stillwaters were formed by the cutoff meanders of the Churchill River, located 1.8km to the south. The entire wetland area slopes gently from the toe of the south escarpment to the river. In August 1995, four stillwater sites (SW1-4) were sampled, along with one reference site (R3). Pearl dace were captured at all sites except SW2. In 1996, in addition to sampling the four stillwater sites (SW1-4) again, two additional reference sites (R1 and R2) were chosen and sampled (Figures 2.1 and 2.2).

From the Base perspective, the northwest corner and entire upper section of SW1 is isolated with very limited access. This area of SW1 is located east of the Central Refuse Area (landfill) and immediately south of the south escarpment. It is bounded by

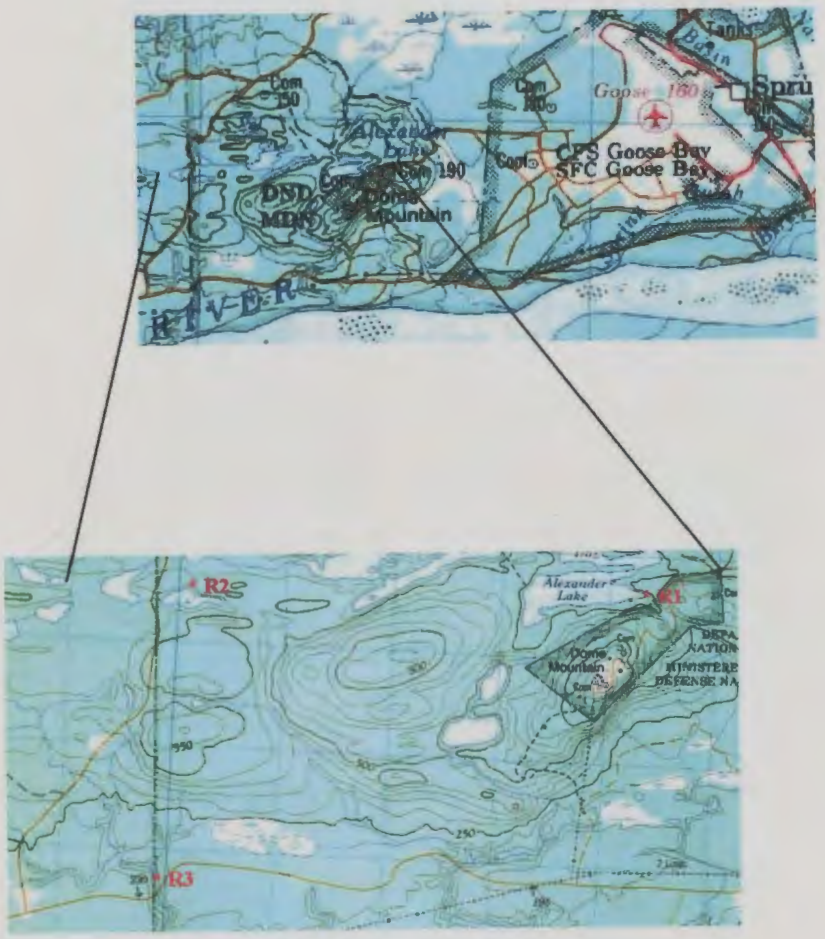

Figure 2.1: Topographic images of CFB Goose Bay and surrounding area identifying three (3) reference sample sites labeled R1-R3.

Figure 2.2: Topographic images of CFB Goose Bay and surrounding area identifying four stillwater sites (SW1 - 4).

the landfill site to the west and south and extends southeast, intersecting with the Churchill River Road (Trans Labrador Highway) until it reaches the Churchill River (Figure 2.3). SW1 is heavily polluted with automotive debris, free petroleum hydrocarbon products, garbage and metals (Figure 2.4 and 2.5). SW1 is approximately 15m wide by 600m long and between 0.5m and 3.0m deep. Despite the overwhelming stench of evaporating aromatic hydrocarbons and visible sheen of free petroleum products; there was a lush vegetation and many species of land and water fowl were present. The water was dark and murky with an orange tint (due to the high concentration of iron flocculent). Additionally, there was a high concentration of manganese (Mn) present on the surface water; which was identifiable by the way the slick broke apart leaving sharp corners and edges and did not congeal like a petroleum hydrocarbon sheen (Figure 2.6).

SW2 was one of the smaller stillwaters of the Spring Gulch Wetlands. It was approximately 10m wide by 150m long and between 0.5m and 2.0m deep. Located adjacent to the Central Refuse Area, it was not directly connected to the Churchill River. SW2 was isolated with very limited access from the direction of the Base. However, its southeastern end was accessible from the Trans Labrador Highway (Figure 2.7). Bordered along the northeast bank by the landfill site, SW2 was the smallest stillwater site sampled and was oriented along a northwest – southeast axis. The water was dark and murky with an orange tint.

Figure 2.3: SW1 sampling site looking southeast.

Figure 2.4: Upper northwest corner of SW1 adjacent to landfill site showing heavy pollution and silt fence.

Figure 2.5: Petroleum hydrocarbon sheen at SW1. Note the orange iron precipitate.

Figure 2.6: Manganese (Mn) slick at SW1. Note the sharp, jagged edges; evidence that once disturbed, the slick will not congeal again, unlike the petroleum hydrocarbon slick in Figure 2.5.

Figure 2.7: SW2 sampling site looking northwest from the Trans Labrador Highway. Note the orange/brown colour of the water and its opaqueness.

SW3 was located south of SW4 and only a small section of the northeast edge was associated with the landfill site. Completely isolated and surrounded by lush forest and vegetation, SW3 visually appeared to be the least impacted of the four study sites (Figure 2.8). This stillwater was approximately 75m wide by 300m long and between 0.5m and 3.0m deep.

SW4 was the most visually impacted of all the stillwater sites (Figure 2.9, 2.10, 2.11 and 2.12). At its widest point, SW4 was approximately 100m wide by 600m long and between 0.5m and 1.3m deep. Separated by the south escarpment (approximately 50m relief), SW4 was directly below the Upper Tank Farm and U.S. FFTA. SW4 was bounded to the east by the Central Refuse Area, oriented on an east – west axis, and was connected to the Churchill River approximately 3km to the southwest. The water was murky, completely opaque with suspended solids, orange in colour and, in a variety of places, a petroleum product sheen was clearly visible. The entire area had a strong odour of petroleum hydrocarbons. Discarded and rusting 45-gallon steel petroleum hydrocarbon drums, dating to 1952 and later, were littered everywhere. The high water mark was clearly visible on the shore and on dead trees within the middle of SW4 as it appeared as a black and orange ring. Visible impact clearly diminished as one moved westward from the end of the access road.

Three pristine reference sites, located at varying distance from CFB Goose Bay, were sampled for this study. Reference 1 (R1) was on Alexander Lake (Figure 2.13). The sampling location was at the southeastern end of the lake accessible only by a secondary

Figure 2.8: SW3 sampling site looking west. Note the relative clarity of the water and lack of visible pollution.

Figure 2.9: SW4 on top of the south escarpment looking southeast towards the Churchill River in the background. Note the relatively large size, orange colour and opaqueness of water.

Figure 2.10: SW4 sampling site showing discarded and rusting 45-gallon drum and electrical cable reel. Note the opaqueness and orange water colour.

Figure 2.11: SW4 shoreline. Note the high water contamination marks (red arrows) on the sand. Orange flags indicate sediment sampling sites from previous consultants.

Figure 2.12: Petroleum hydrocarbon sheen at SW4.

Figure 2.13: Sampling site, Reference 1, at Alexander Lake.

wood road from the north side of CFB Goose Bay. Reference site 2 (R2) was 17.8km west of Happy Valley-Goose Bay along the Trans Labrador Highway and then 3.5km north in a secondary wood road, at a pond on east side of the secondary road (Figure 2.14). R2 was sampled in the most northern section of the pond. Reference site 3 (R3) was 16km west of Happy Valley-Goose Bay on the north side along the Trans Labrador Highway in a small river-pool (Figure 2.15). R3 was sampled in both 1995 and 1996.

The three reference sites were chosen based upon close visual and scent inspection of the surface water and sediment. At all three reference sites there were no indications of petroleum hydrocarbon impact, discard of refuse or excessive human impact.

Figure 2.14: Sampling site, Reference 2, 17.8km west of Happy Valley-Goose Bay on Trans Labrador Highway.

Figure 2.15: Sampling site, Reference 3, 16km west of Happy Valley-Goose Bay on the north side of the Trans Labrador Highway.

2.2 Sample Collection Methodology

Pearl dace were collected from all stillwater and reference sites using minnow traps (48cm x 22cm with 11mm mesh) baited with boiled ham. Traps were set near shore in water depths ranging from 0.5m to 1.0m and were fished for a period of 48 hours. Three traps were baited and set per sampling area.

Captured live fish were transported by car in labeled, 5-gallon buckets to the field laboratory at CFB Goose Bay where an air supply was placed into each bucket to provide oxygen (Figure 2.16). Over the next 24-36 hours, all fish were measured, sexed and weighed. The spleen, head kidney and first, left gill arch of each sample were removed and prepared for histological processing initially by fixation in 10% neutral buffered formalin (Drury *et al.*, 1967). The gonads were weighed (for later calculation of gonadal somatic indices) and fixed in 10% NBF. A significant portion of the liver was removed from each fish, placed in a labeled "Ziplock™" bag and frozen on dry ice (sublimation point -78.5^0C; Canadian Liquid Air, personal communication). The frozen livers were then shipped via Air Nova Cargo to the Ocean Sciences Centre where they were immediately transferred to the Department of Fisheries and Oceans toxicology facility and placed in an -80^0C industrial ultra freezer until MFO activity could be assayed. The remaining liver was subsequently prepared for histological processing. All morphological parameters were recorded on standardized necropsy sheets.

Figure 2.16: Field laboratory, setup on top of the south escarpment overlooking SW4 with the UTF adjacent on the right.

2.3 Histological Preparation and Processing of Tissues

Fixed tissue sections were processed and embedded in Tissue Prep (Fisher^{TM}) wax following conventional methods of dehydration and embedding and cut to a thickness of seven (7) micrometers (μm) on an Optical 820 "Spencer Microtome" (see Appendix 1). Triplicate sections of tissue were heat-fixed for one (1) hour at 60^{0}C. One slide per tissue section was stained with hematoxylin and eosin (Drury *et al.*, 1967) for histological assessment. Additional spleen sections were stained according to Drury *et al.* (1967) using Perl's Prussian blue (potassium ferrocyanide) method for hemosiderin. Gill sections were also stained with periodic acid-Schiff (PAS) for carbohydrates in mucous cells. All slides were viewed on a random basis, in no fixed order thereby further reducing any potential bias.

2.3.1 Liver

Sample hepatic sections from each study site were microscopically examined randomly at 400X magnification for the presence or absence of melanomacrophage aggregates, cellular necrosis, clear cell foci and/or vacuolation, as well as other cytoarchitectural anomalies such as bile ductule hyperplasia. Histological examination using oil immersion magnification (1000X) was used to confirm or negate anomalies. Degree of lipid vacuolation was quantified using the following ordinal values:

\+ Normal to few vacuoles;

++ Sparse;

+++ Abundant but not widespread; and

++++ Widespread.

2.3.2 Gill

Sections of branchial tissue collected from the left, first gill arch were surveyed at 400X magnification for the presence or absence of histological abnormalities and any morphological changes of the gill filaments were noted (i.e. necrosis, branching). Histopathological gill lesions were recorded for changes in the gill epithelium (the degree of hyperplasia, epithelial lifting, necrosis, hypertrophy and rupture), bulging (telangiectasis) or fusion of gill lamellae, hypersecretion and proliferation of mucocytes, and any other architectural differences.

The degree of hyperplasia (i.e. lamellar epithelial proliferation) of the secondary gill lamellae was expressed on a numeric scale, ranging from 1 through 3 (based on the number of hyperplastic cell nuclei). Three classes of hyperplasia were identified:

1 = slight (number of nuclei < 2);

2 = moderate (2 < number of nuclei < 4); and

3 = excessive (number of nuclei $\geq$ 4).

Epithelial lifting was defined as separation of basal epithelium from the lamellar capillary and interlamellar thickening was recognized as trough hyperplasia between secondary lamellae. The interlamellar thickening was quantified on a fractional basis of the length on the secondary gill lamellae.

2.3.3 Splenic hemosiderin quantification

Splenic section samples of both the stillwater and reference sites were stained with Perl's Prussian blue for potassium ferrocyanide oxidation and quantitatively assessed for hemosiderin deposition by digital image analysis (Khan and Nag, 1993).

Images originating from slides were viewed under a Zeiss photomicroscope, captured using a Truevision Targa frame grabber board and stored onto a VHS videocassette using a digital VHS recorder (JVC, HR-D700V).

The frame grabber converts the videotaped images into a set of digitized values called pixels (picture elements). Viewing the image in black and white, the hemosiderin deposits (within melanomacrophage aggregates) appear as aggregates of black pixels against a white pixel background. Using commercially available software, the area of each deposit is determined (mm^2) and compared to the total viewable area ($\%/mm^2$).

Enumeration of macrophage aggregates in the spleen was conducted. Some macrophage aggregates were devoid of pigment and not detected by the Perl's Prussian blue method. Consequently, the number of macrophage aggregates (20-80μm in diameter) were enumerated (100X magnification) and expressed as the number / mm^2 from hematoxylin and eosin stained sections.

2.3.4 Gonads

Sample gonadal sections from each study site were examined at 400X magnification. Histological examination using oil immersion magnification (1000X) was additionally used to confirm or negate anomalies.

2.4 MFO Determination

2.4.1 Pearl Dace Liver Homogenate Preparation

Liver samples collected from SW4 (1995 & 1996) were thawed slightly from cryogenic (-80°C) storage to facilitate MFO determination. Liver from five (5) same sex

pearl dace were pooled to make approximately 0.4g of tissue. Ten samples (5 pooled female and 5 pooled male) of pearl dace liver homogenate were prepared using a 1:4 ratio of liver tissue (g) to ice-cold 50mM Tris-sucrose (pH 7.5) buffer (ml) and then making ten passes of a glass Ten Broeck hand tissue grinder to homogenize the sample. Pearl dace liver homogenates were centrifuged at 9000 x g (centrifugal force) for 10 minutes at 4°C. Precipitated pellets were discarded and each S9 supernatant was pipetted equally into three capped disposable (polypropylene) Eppendorf 1.5ml microcentrifuge tubes maintained at -80°C. Characteristic of the pearl dace liver, a top fatty layer was also removed after centrifugation and discarded.

2.4.2 7 Ethoxyresorufin-O-deethylase (EROD) Assay

EROD activity was assayed fluorimetrically using a Perkin-Elmer LS-5 fluorescence spectrophotometer (Porter *et al.*, 1989). The reaction mixture, with a final volume of 1.25ml, consisted of 1.01ml of 53nmol Tris-sucrose buffer (50mM, pH 7.5), 100µl of S9 liver homogenate and 15µl of 2.25nmol 7-ERF (150µM). The reaction mixture was started by addition of 125µl of 0.16mg NADPH (1.25mg/ml). After a 15-minute incubation at 27°C in a temperature controlled water bath, the reaction was terminated by the addition of 2.5ml of ice-cold HPLC grade methanol. Methanol blanks for each assay sequence contained the same reagent components as the sample tubes except the addition of methanol occurred before the addition of NADPH. An internal pooled sample was run with every incubation trial to maintain accuracy. Assay tubes were vortexed and the protein precipitate removed from suspension by centrifugation at 3600 x g for 5 minutes. The resorufin fluorescence formed in the supernatants was

measured using acrylic sets of fluorimetric curvettes (1cm path length) at 585nm using an excitation wavelength of 550nm (slit width of 0.5mm). Enzyme activity was linear with time (based on standard curve generation) and protein concentration – using the Lowry Protein Method (Lowry *et al.*, 1951). The rate of enzyme activity in pmol/min/mg protein was obtained from a regression graph of fluorescence against the standard concentrations of resorufin.

2.4.3 Protein Determination

Protein was determined using the Lowry *et al.* (1951) procedure and a Perkin-Elmer UV-Visible scanning spectrophotometer. Lowry reagent consisted of 20g of Na_2CO_3 (anhydrous) and 4g of NaOH dissolved in 950ml of double distilled water and diluted to 1L. Five (5) milliliters of a solution consisting of 1% $CuSO_4$, 2% Na-K tartrate and 100ml of the Lowry reagent was added to 0.5ml of the S9 suspension (20µl of S9 to 480µl of double distilled water). A series of bovine serum albumin (BSA) concentrations, ranging from 50µg/ml to 400µg/ml, were used as standards and blanked against 0.5ml of double distilled water (without protein) (Appendix 3). Following a 15-minute incubation at room temperature, 0.5ml of 2N (normal) Folin-Phenol Ciocalteau reagent was added to the mixture, which was immediately vortexed and incubated for 30 minutes, again at room temperature. Absorbance was recorded at 620nm. A linear standard curve for protein concentration versus absorbance was produced from the BSA standards and used to calculate S9 protein concentrations in mg/ml and mg/g liver.

2.5 Statistical Analysis and Methodology

Condition factor and gonadosomatic indices were transformed from raw necropsy morphological data by the following methods:

i. Condition (K) Factor = dressed (eviscerated) weight / (total body length)^3 x 100

ii. Gonadosomatic Index = gonad weight / dressed (eviscerated) weight x 100

Normal distribution of data was not assumed in this study. Prior to any statistical analysis all data were tested for normality using both the Kolmogorov-Smirnov test for goodness of fit (including Lilliefors Significance Correction) and the Shapiro-Wilk test. If the distribution of data was normal, then, using the Levene Statistic, homogeneity of variances was determined. Non-normal or heterogeneous data were log-transformed and tested again for normality and homogeneity prior to statistical tests. If parametric assumptions were not satisfied by data transformation, then non-parametric statistical tests were used. Such was the case for all but the MFO activity data.

Length, dressed weight data and condition factor were tested by Wilcoxon Signed Ranks test. Histopathology results of the spleen, liver and gonads (male and female gonadosomatic indices) were analyzed for significant differences using the Wilcoxon Signed Ranks test, while gill results were analyzed using a Kruskal-Wallis nonparametric test for differences in variance. MFO activity (7-EROD induction) for the stillwater sites and the reference sites was analyzed using an approximate one sample t-test, due to unavoidable small sample sizes. Nonparametric statistical tests that ranked data, as used in all other statistical analysis, were not useful here. Data sets were considered to differ significantly from each other when $p < 0.05$. All statistical analysis was completed using SPSS Base 10.0 statistics software.

3.0 Results

3.1 Population Analysis

Capture results are summarized in Figure 3.1. In 1995 only one petroleum impacted location (SW2) did not yield any pearl dace. Again in 1996, no pearl dace were captured from SW2 and in addition SW1 and 3 yielded no fish. The only petroleum impacted location in which fish were captured in both 1995 and 1996 field seasons was SW4. One reference site was sampled in 1995 (R3); in 1996 the same reference site (R3) was sampled again and two more reference sites (R1 and R2) were additionally sampled. The time required to capture the total numbers of fish was greatly lengthened between 1995 and 1996. It took only one weekend in 1995 to capture 119 fish while in 1996 it took almost three weeks to capture 253 fish. Figures 3.2 and 3.3 summarize the sexual maturity of 1995 and 1996 pearl dace populations. Figure 3.2 clearly illustrates the lack of juvenile fish and the prevalence of females in relation to males inhabiting the three petroleum hydrocarbon impacted locations in 1995 (SW1, 3 and 4) when compared to a reference location (R3). Figure 3.3 displays the higher, more equal prevalence of males in 1996. Especially important was the result from R1. Evident from the population sex percentages, males, for the first time, constituted a larger percentage of the population than females.

When the percentage of reproductively mature pearl dace from two populations over two successive years was compared (Figure 3.4), female pearl dace predominated over either males or juveniles for both locations over both years. Male pearl dace

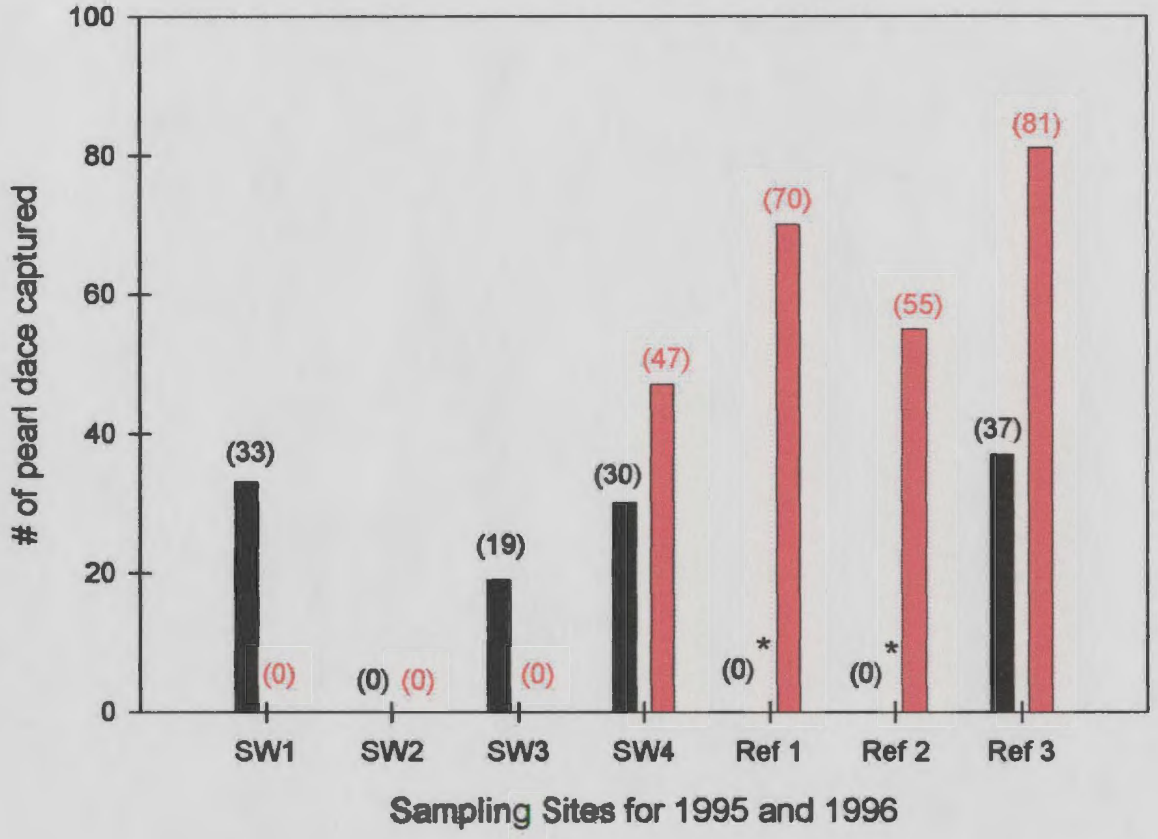

Figure 3.1: Sampling sites and numbers of pearl dace caught for 1995 (black, n = 119) and 1996 (red, n = 253) from four petroleum hydrocarbon impacted sites (SW1-4) and three reference sites (Ref 1-3). Note (*) that reference site 3 was the only one sampled both years.

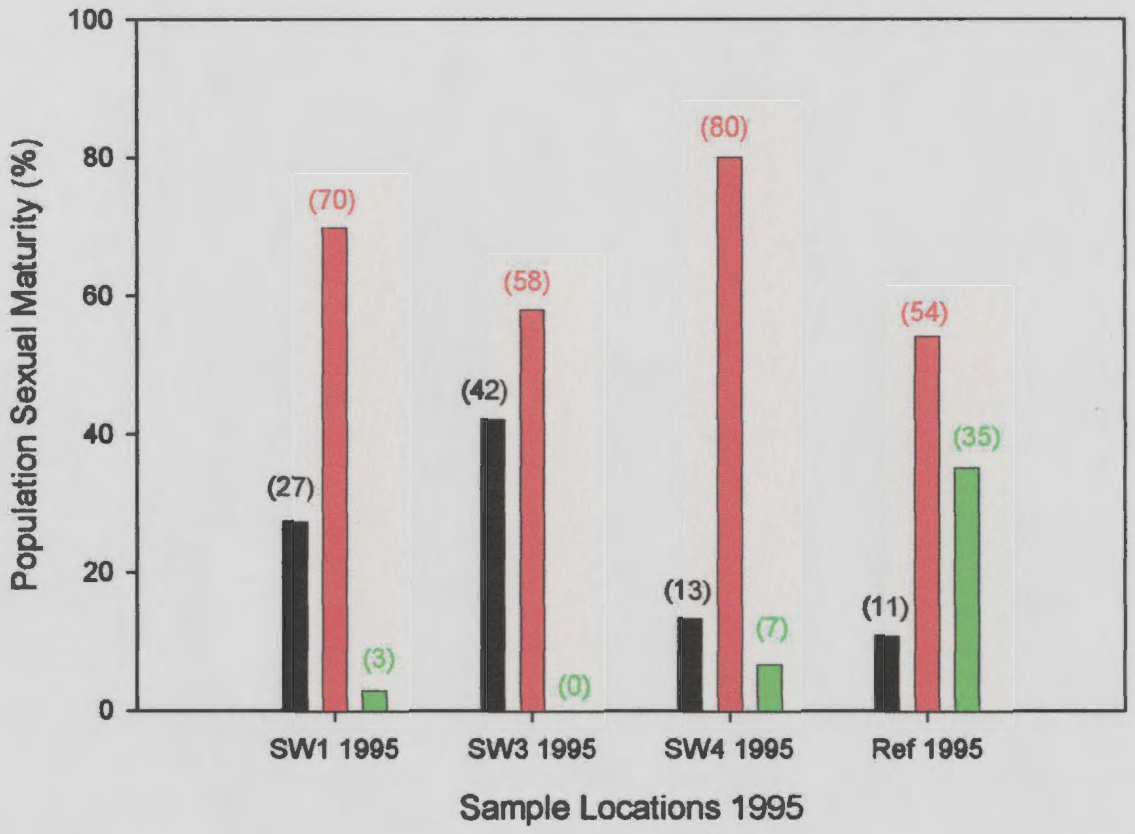

Figure 3.2: Population sexual maturity (%) of pearl dace (male = black; female = red; juvenile = green) from three petroleum hydrocarbon impacted locations (SW1, 3 and 4) and a reference location in 1995.

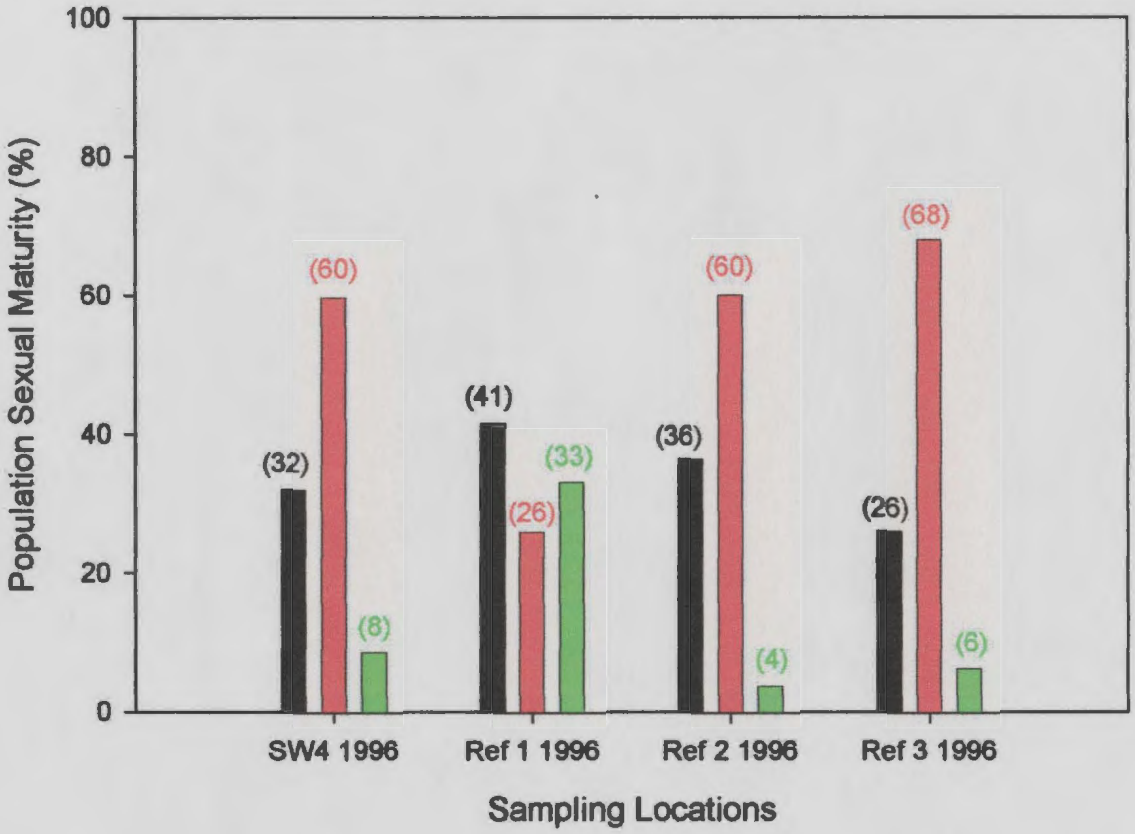

Figure 3.3: Population sexual maturity (%) of pearl dace (male = black; female = red; juvenile = green) from a petroleum hydrocarbon impacted location (SW4) and three reference locations in 1996.

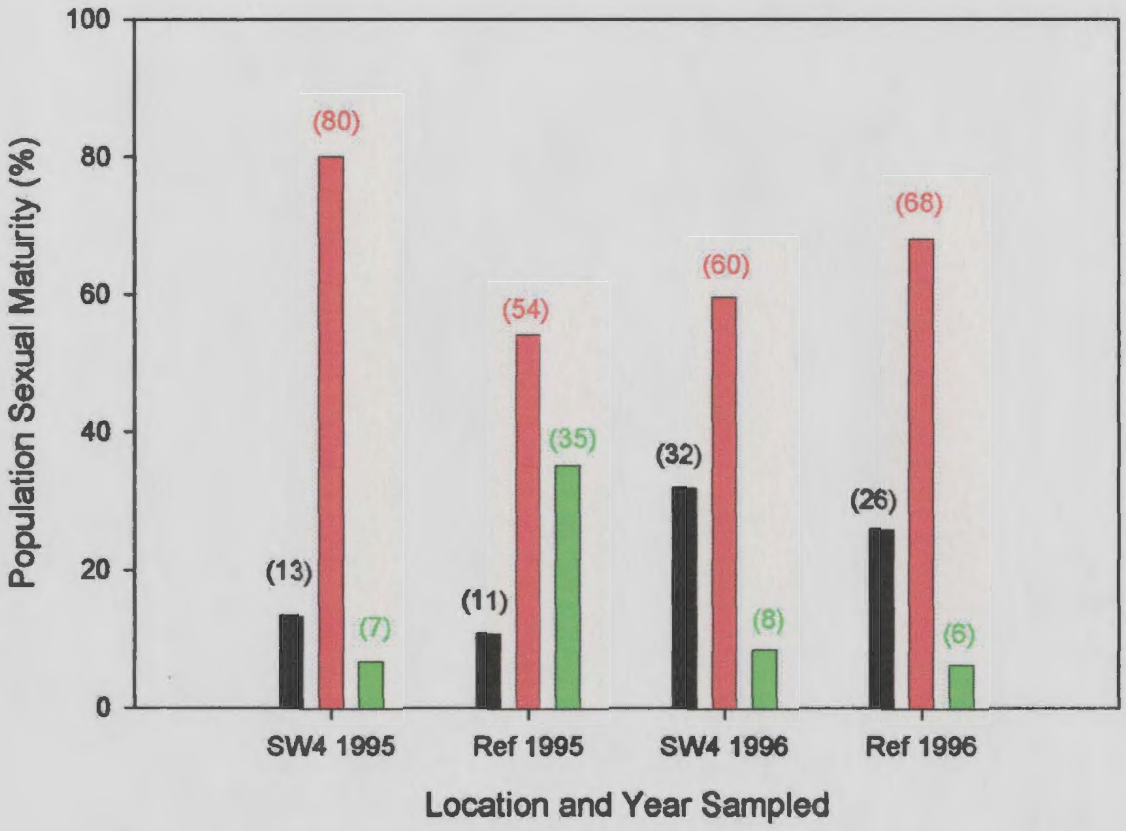

Figure 3.4: Population sexual maturity (%) of pearl dace (male = black; female = red; juvenile = green) from a petroleum hydrocarbon impacted location (SW4) and a reference location over a two-year sampling period.

were the next largest percentage of the sampled populations (by almost 2 to 5 times) over the juvenile class. This was the case except for R3 sampled in 1995 where the percentage of juvenile pearl dace in the population was more than three times the percentage of male pearl dace. There was a large decrease (20%) in the mature females at SW4 in 1996.

The 1995 pearl dace were captured the last weekend in August. At that time, female fish from SW1, 3 and 4 were producing mature eggs; however, none had spawned. From R3 in 1995, however, only spent females were captured. In 1996 pearl dace were captured in mid-July and the females from all reference locations (R1-3) had already spawned.

3.2 Biological Variables

Using the Wilcoxon Signed Ranks test, pearl dace collected from the petroleum hydrocarbon impacted sites were significantly ($p < 0.05$) heavier (g) and longer (cm) than fish sampled from reference sites for both years. Moreover, pearl dace sampled from petroleum impacted locations in 1995 had significantly higher body condition (K) factors (eviscerated mass/length^3) than fish sampled from R3 (Table 1). Conversely, 1996 fish sampled from SW4 had significantly reduced body condition factors when compared to R1, but, when compared to R2 and R3, there were no significant differences.

Table 1. Sample size (n) and comparison of three biological variables of pearl dace sampled at three petroleum hydrocarbon impacted sites (SW1, 3 and 4) and a reference site in 1995 and one petroleum hydrocarbon impacted site, SW4, and three reference sites in 1996.

Year	Site	n	Mass (g)	Length (cm)	K factor ($x10^{-3}$)
1995	SW1	33	8.82±0.67*	10.21±0.28*	7.73±0.13*
	SW3	19	10.13±0.76*	10.51±0.26*	17.90±4.66*
	SW4	30	8.19±0.64*	9.86±0.30*	7.84±0.07*
	Reference†	37	3.75±0.39	7.81±0.32	6.98±0.15
1996	SW4	47	7.82±0.64¥1,2,3	9.94±0.28¥1,2,3	126.28±34.90¥1
	Reference 1	70	5.07±0.40	8.43±0.23	374.60±61.70
	Reference 2	55	4.80±0.28	8.81±0.14	130.40±13.50
	Reference 3†	81	4.75±0.25	8.90±0.11	117.00±7.76

Note: Values are given as the mean ± S.E.

* Significantly different ($p < 0.05$) from the 1995 reference group.

¥1,2,3 Significantly different ($p < 0.05$) from 1996 reference 1, 2 and 3 groups.

¥1 Significantly different ($p < 0.05$) from 1996 reference 1 group.

† Same site, sampled two consecutive years

3.3 Histopathology

There were more histopathological lesions in fish sampled from the impacted sites when compared to the reference sites. When analyzed using various nonparametric statistical tests, most findings were significantly different from reference data. Histopathological lesions observed included: gill interlamellar hyperplasia, hepatic lipidosis, bile duct hyperplasia, large pigmented and non-pigmented splenic melanomacrophage aggregates and splenic hemosiderin deposits.

3.3.1 Spleen

Figure 3.5 shows the normal histology of splenic tissue in pearl dace. Table 2 summarizes the results after comparing mean hemosiderin concentrations, prevalence of melanomacrophage aggregates containing (MMA) and not containing (MMA NP) pigment, and the mean number of melanomacrophage aggregates per mm^2 localized in the spleen of pearl dace sampled from petroleum hydrocarbon impacted locations and reference sites in 1995 and 1996. Using Digital Image Analysis (Khan and Nag, 1993) and the Wilcoxon Signed Ranks test, it was determined that 1996 fish sampled from SW4 contained significantly higher concentrations of splenic hemosiderin (Figure 3.6) than fish sampled from the reference sites 1 (Figure 3.7) and 2. This was not the case for fish sampled in 1995. No significant statistical differences in concentrations of splenic hemosiderin were detected in 1995 pearl dace. Given this result, 1995 and 1996 petroleum contaminated fish were further compared with fish from reference locations for the prevalence of MMA and MMA NP. Secondly, the mean number of melanomacrophage

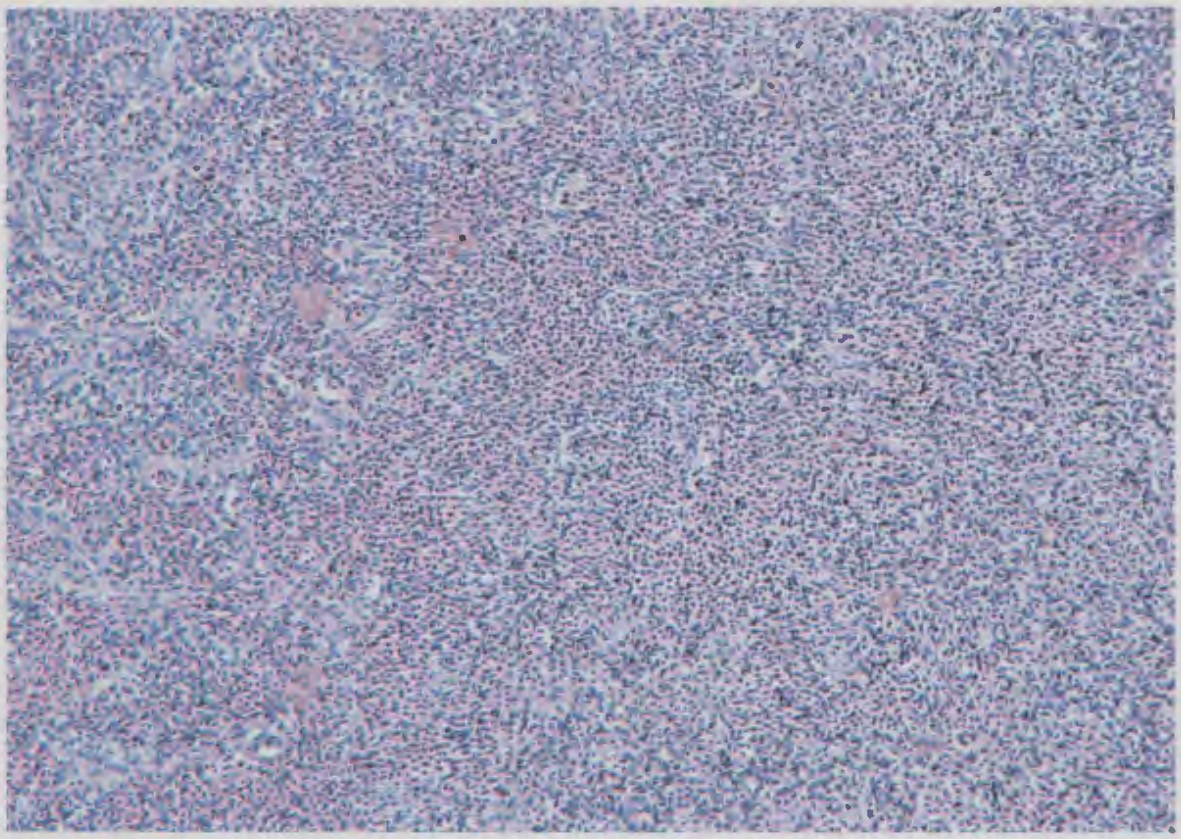

Figure 3.5: Normal splenic tissue histopathology from pearl dace. Stained with H&E and viewed at 100X.

Table 2. Sample sizes (n), comparison of mean splenic hemosiderin concentrations, prevalence (%) of melanomacrophage aggregates with hemosiderin pigment (MMA) and aggregates with little or no pigment (MMA NP), and mean number of melanomacrophage aggregates per mm^2 in the spleen of pearl dace sampled at three petroleum hydrocarbon impacted sites (SW1, 3 and 4) and a reference site in 1995 and one petroleum hydrocarbon impacted site, SW4, and three reference sites in 1996.

				Prevalence (mean number per mm^2 ± S.E.)		
Year	Site	n	Hemosiderin Conc. ± S.E. (%/mm^2)	n	MMA	MMA NP
1995	SW1	12	7.881±1.619	25	60 (4.8±0.5)*	36 (4.9±0.5)*
	SW3	11	7.577±2.469	10	30 (1.6±0.2)*	30 (3.7±0.4)*
	SW4	9	7.452±2.917	18	28 (1.8±0.3)*	39 (3.8±0.4)*
	Reference†	12	4.345±1.374	20	0 (0)	2 (<0.1)
1996	SW4	10	7.575±1.789¥	22	55 (11.8±2.1)	100 (13.2±2.1)
	Reference 1	10	0.184±0.090	25	24 (9.7±1.9)	12 (8.0±3.1)
	Reference 2	9	0.538±0.245	8	15 (5.0±0.0)	75 (6.8±3.7)
	Reference 3†	8	6.655±1.629	10	40 (6.8±2.2)	100 (7.1±2.3)

Note: ¥ Significantly different ($p < 0.05$) from the 1996 reference 1 and 2 groups.

* Significantly different ($p < 0.05$) from the 1995 reference group

† Same site, sampled two consecutive years.

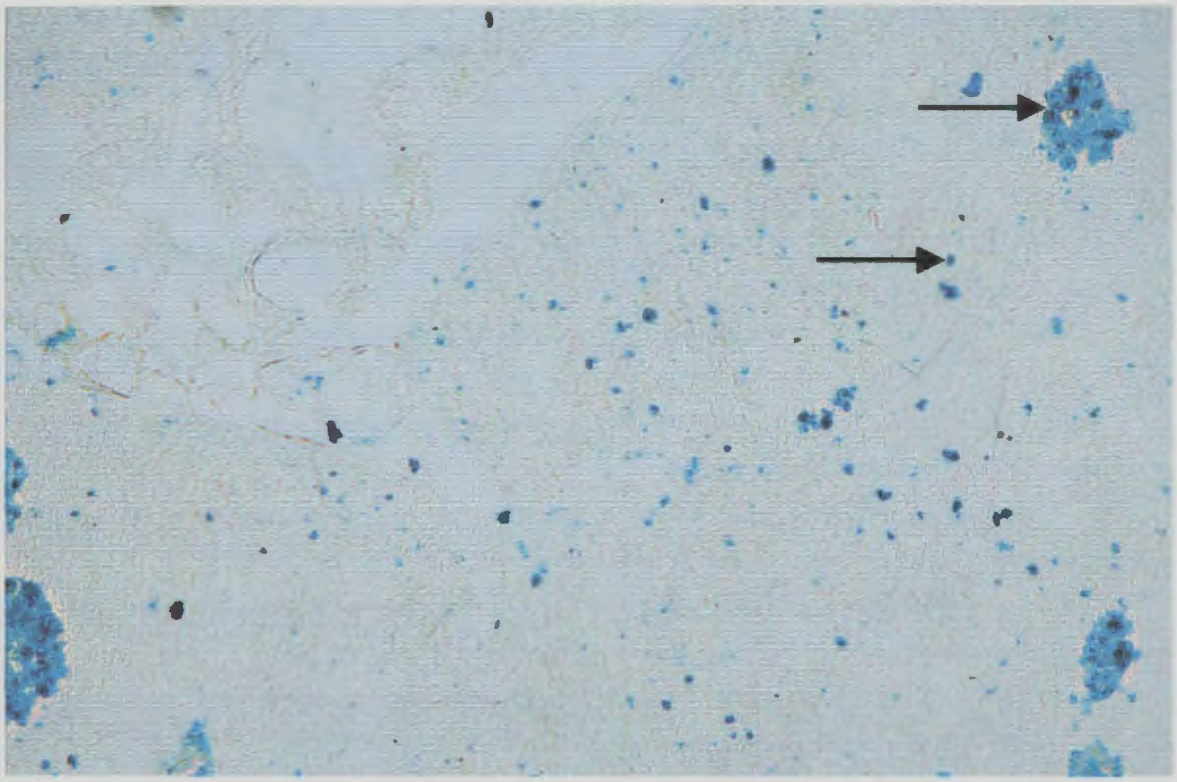

Figure 3.6: Splenic hemosiderin deposits (see arrows) in pearl dace sampled from a reference site appear as various shades of blue by staining with Perl's Prussian blue for ferric iron (100X).

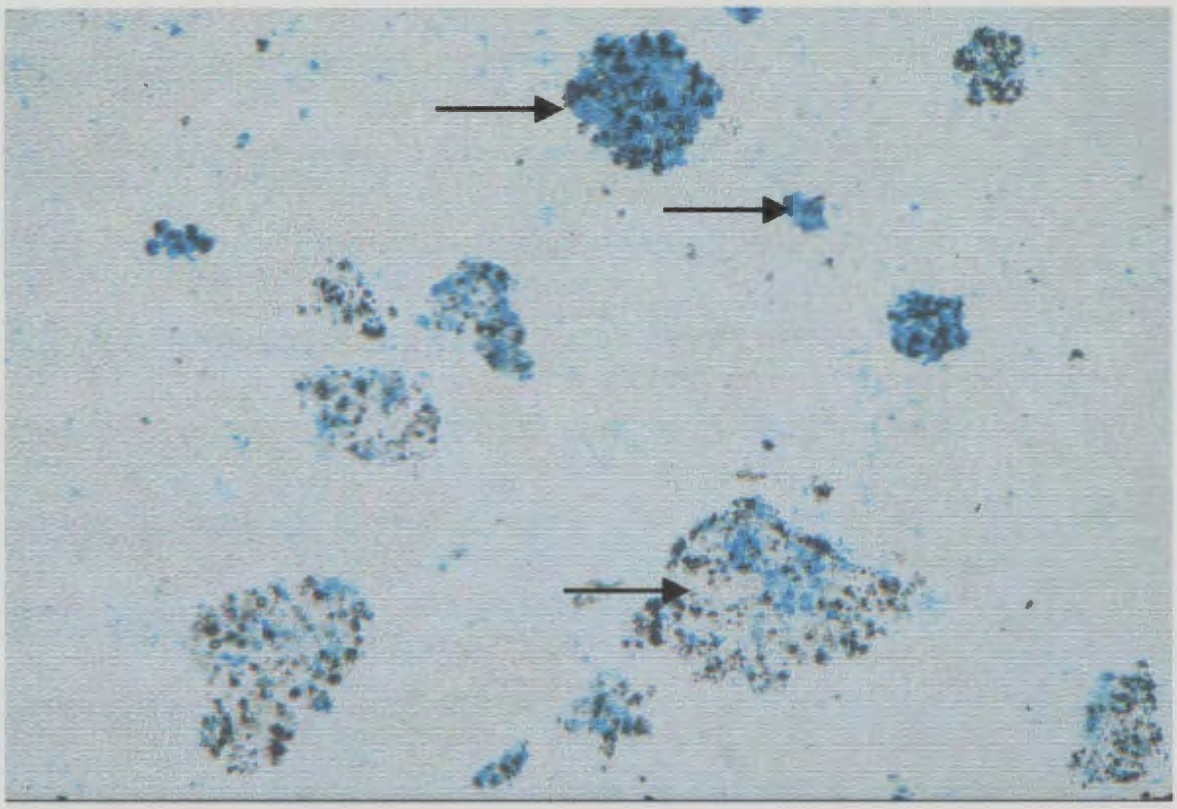

Figure 3.7: Splenic hemosiderin deposits (see arrows) in pearl dace sampled from petroleum impacted stillwater sites in CFB Goose Bay, Labrador appear as various shades of blue by staining with Perl's Prussian blue method for ferric iron (100X).

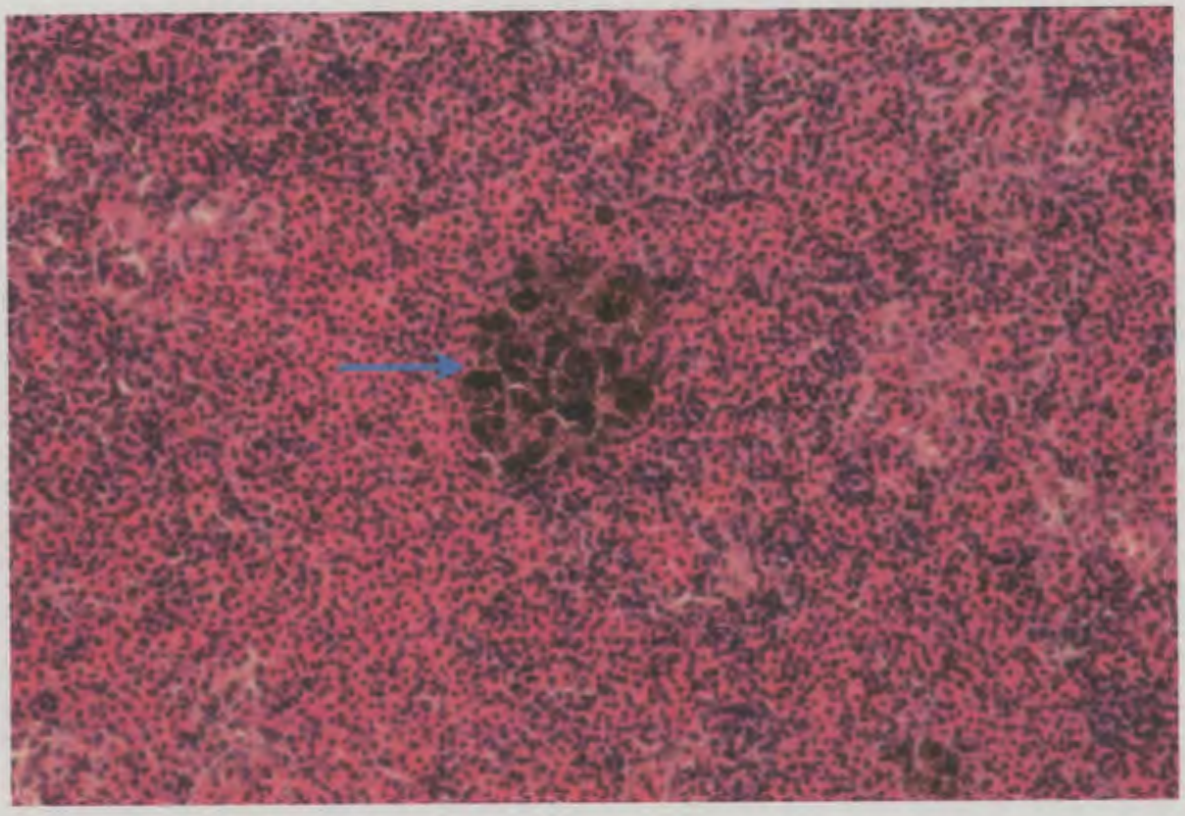

Figure 3.8: Splenic tissue of pearl dace exhibiting a pigmented melanomacrophage aggregate (see arrow), MMA, stained with H&E (100X).

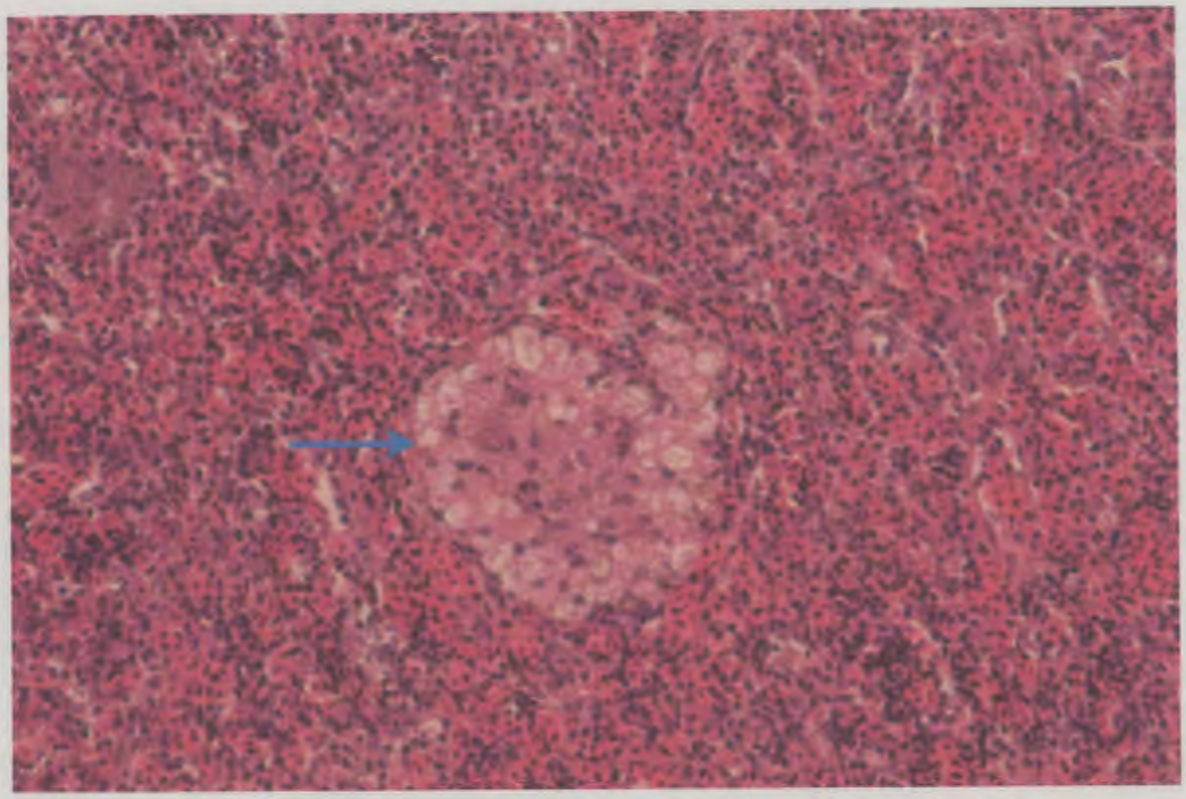

Figure 3.9: Splenic tissue of pearl dace stained with H&E. Note the non-pigmented melanomacrophage aggregate (see arrow), MMANP, (100X).

aggregates per square millimeter for impacted and reference fish were compared.

Using a Wilcoxon Signed Ranks test, it was apparent in 1995 fish that there were significantly higher prevalences of both MMA and MMA NP at petroleum hydrocarbon impacted locations (SW1, 3 and 4) then in fish from the reference site (R3). Furthermore, the aggregates, regardless of whether or not they contained pigment, were significantly larger in fish from the stillwater sites (SW1, 3 and 4) then at the reference site (R3).

In contrast to the results of 1995, pearl dace sampled from one petroleum hydrocarbon impacted site (SW4), when compared with fish from three reference locations (R1-3), had significantly higher hemosiderin concentrations than fish from two (R1 and R2) of three reference sites. Furthermore, fish from SW4 had a higher prevalence of MMA than at either of the three reference locations and also had a higher prevalence of MMA NP at two (R1 and R2) of the three reference sites. Unlike the results from 1995, the number of MMA and MMA NP observed in fish from SW4 were, however, not significantly different than in fish from three reference locations (R1-3).

3.3.2 Liver

Table 3 summarizes the prevalence of lipid accumulation in vacuoles within the liver, the mean degree of lipid accumulation and the prevalence of bile ductule hyperplasia (BDH) in pearl dace sampled from three petroleum hydrocarbon impacted sites (SW1, 3 and 4) and one reference site (R3) in 1995 and one petroleum hydrocarbon impacted site (SW4) and three reference sites (R1-3) in 1996. Fish from all the reference sites in 1995 and 1996 had a very high prevalence of normal (+) to only a few lipid vacuoles within

hepatic cells (Figure 3.10). There were no examples at all of sparse (++) or widespread (++++) distribution of lipid vacuoles in fish sampled from reference sites in 1995 (R3) or 1996 (R1-3) (Figures 3.11 and 3.13). Abundant lipid vacuolation (+++) was only seen in 5% of the reference population in 1995 and between 0-12% of the reference populations in 1996 (Figure 3.12).

The characteristics of reference fish (R3 in 1995; R1-3 in 1996) differ greatly with regards to the degree of lipid accumulation within vacuoles of pearl dace sampled from the stillwater locations over both years. Fish from the stillwater sites exhibited a much lower prevalence of the normal lipid accumulation condition (+) and were characterized by much higher percentages of sparse (++), abundant (+++) and widespread (++++) lipid vacuolation. This trend was statistically substantiated when the mean degree of lipid accumulation in fish from petroleum impacted locations was compared to that of fish in reference samples using a nonparametric Wilcoxon Signed Ranks test. All fish from stillwater sites (SW1, 3 and 4 in 1995; SW4 in 1996) contained a significantly greater degree of lipid vacuolation within hepatic cells than did fish sampled from a reference locations.

Bile ductule hyperplasia (Figure 3.14) was non-existent in liver examined from fish sampled at reference locations in 1995 and 1996. The prevalence of BDH in the liver of fish from petroleum impacted sites, however, ranged from a low of 6% in 1995 to 16% in1996 at SW4 to 20% in SW3 1995 and a high of 24% in SW1 in 1995. The development of BDH was closely associated with only pearl dace inhabiting ecosystems contaminated

Table 3. Prevalence (%) and degree of lipid accumulation in vacuoles and bile ductule hyperplasia (BDH) in the liver of pearl dace sampled at three petroleum hydrocarbon impacted sites (SW1, 3, 4) and a reference site at CFB Goose Bay, Labrador in 1995 and at SW4 and three reference sites in 1996.

			Prevalence (%) & Degree of Lipid Accumulation					
Year	Site	n	+	++	+++	++++	Mean (±S.E.)	BDH
1995	SW1	25	28	24	24	24	2.4±0.2*	24
	SW3	10	30	20	20	30	2.5±0.4*	20
	SW4	18	50	22	6	22	2.0±0.3*	6
	Reference	20	95	0	5	0	1.1±0.1	0
1996	SW4	22	4	14	32	50	3.3±0.2¥[1,2,3]	16
	Reference 1	25	96	4	0	0	1.0±0.0	0
	Reference 2	8	50	38	12	0	1.6±0.3	0
	Reference 3	10	90	0	10	0	1.2±0.2	0

Note:

Degree of lipid accumulation:

+	Normal to few vacuoles
++	Sparsely distributed
+++	Abundant but not widespread
++++	Abundant and widespread

* Significantly different ($p < 0.05$) from the reference group.

¥[1,2,3] Significantly different ($p < 0.05$) from all three reference groups.

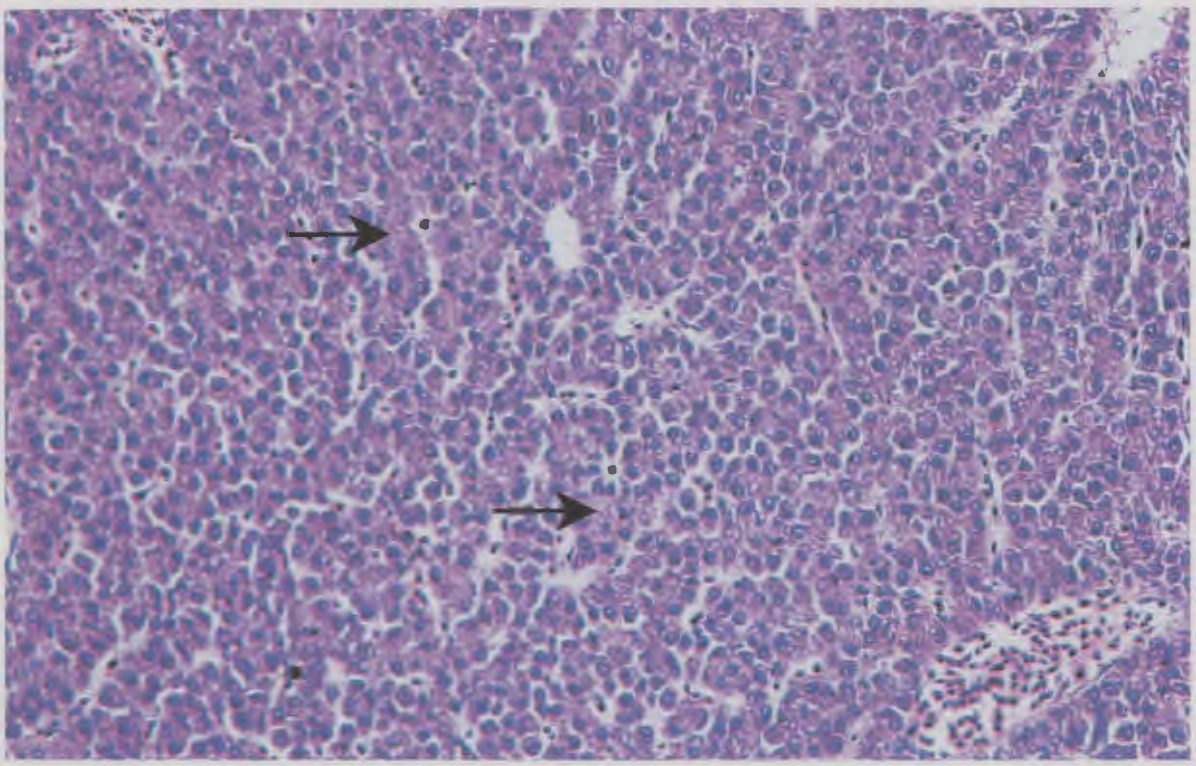

Figure 3.10: Hepatic tissue of pearl dace sampled from a reference site stained with H&E (400X). Note the lack of lipid vacuolation (arrows) that can be classified as normal (+).

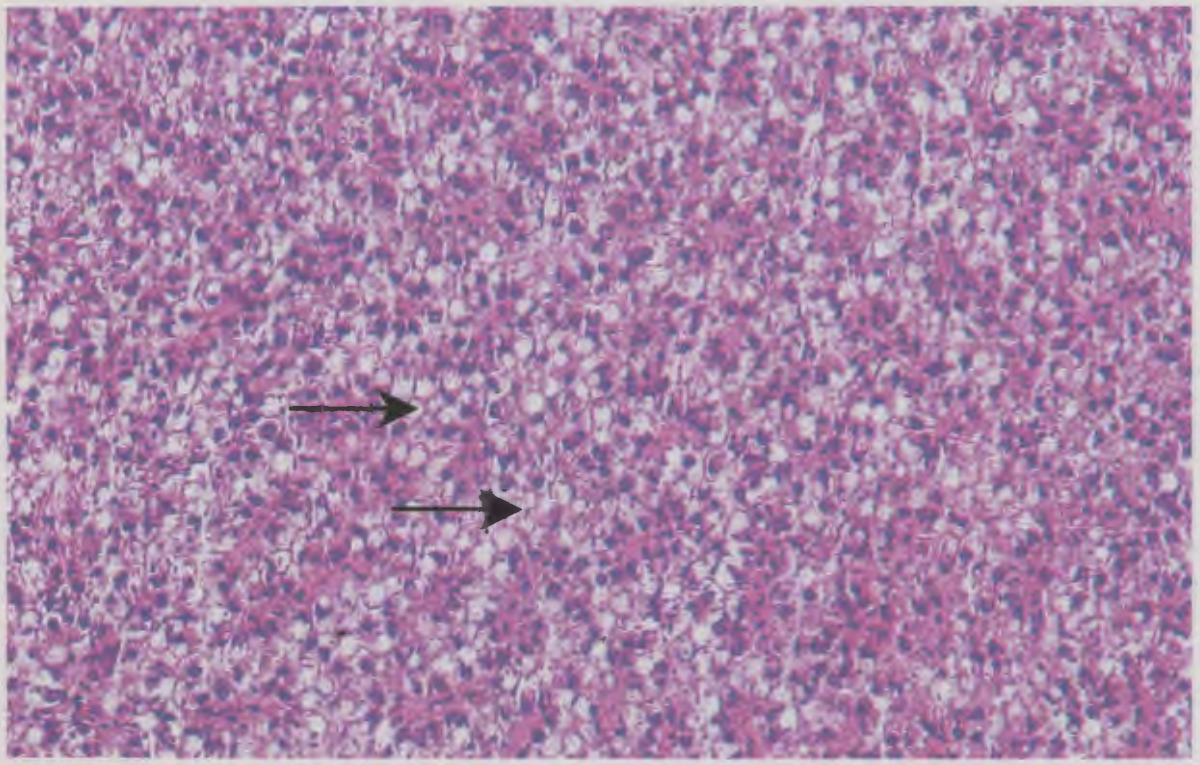

Figure 3.11: Hepatic tissue of pearl dace stained with H&E (400X). Note the sparse (++) degree of lipid vacuolation (arrows).

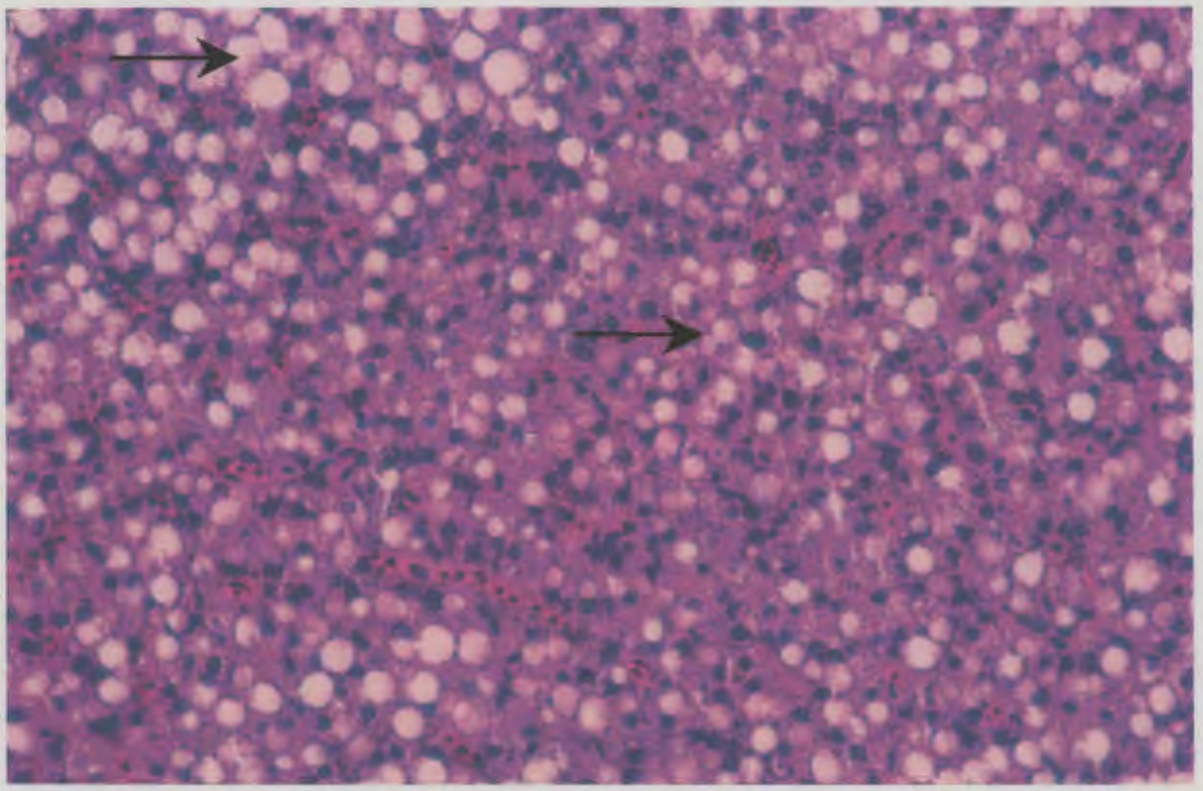

Figure 3.12: Hepatic tissue of pearl dace stained with H&E exhibiting an abundant (+++) degree of lipid (arrows) vacuolation (400X).

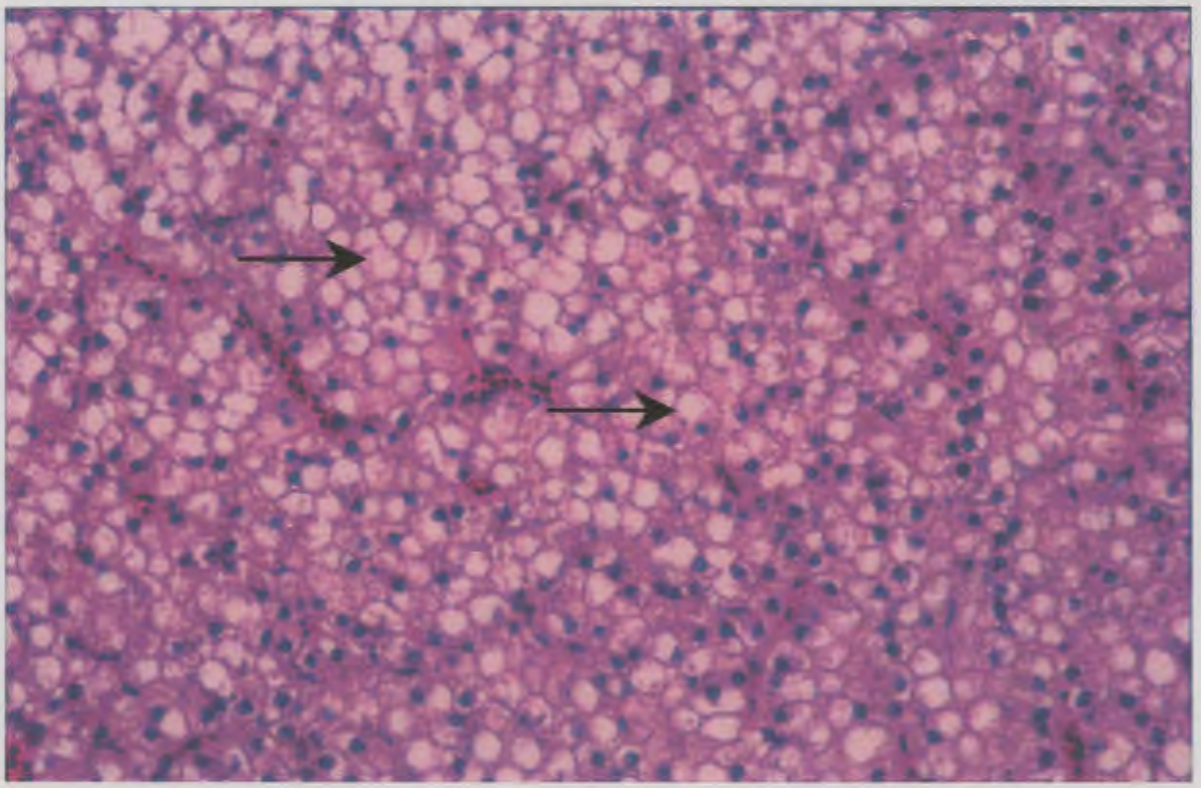

Figure 3.13: Hepatic tissue of pearl dace stained with H&E exhibiting a widespread (++++) degree of lipid vacuolation (arrows). Viewed at 400X.

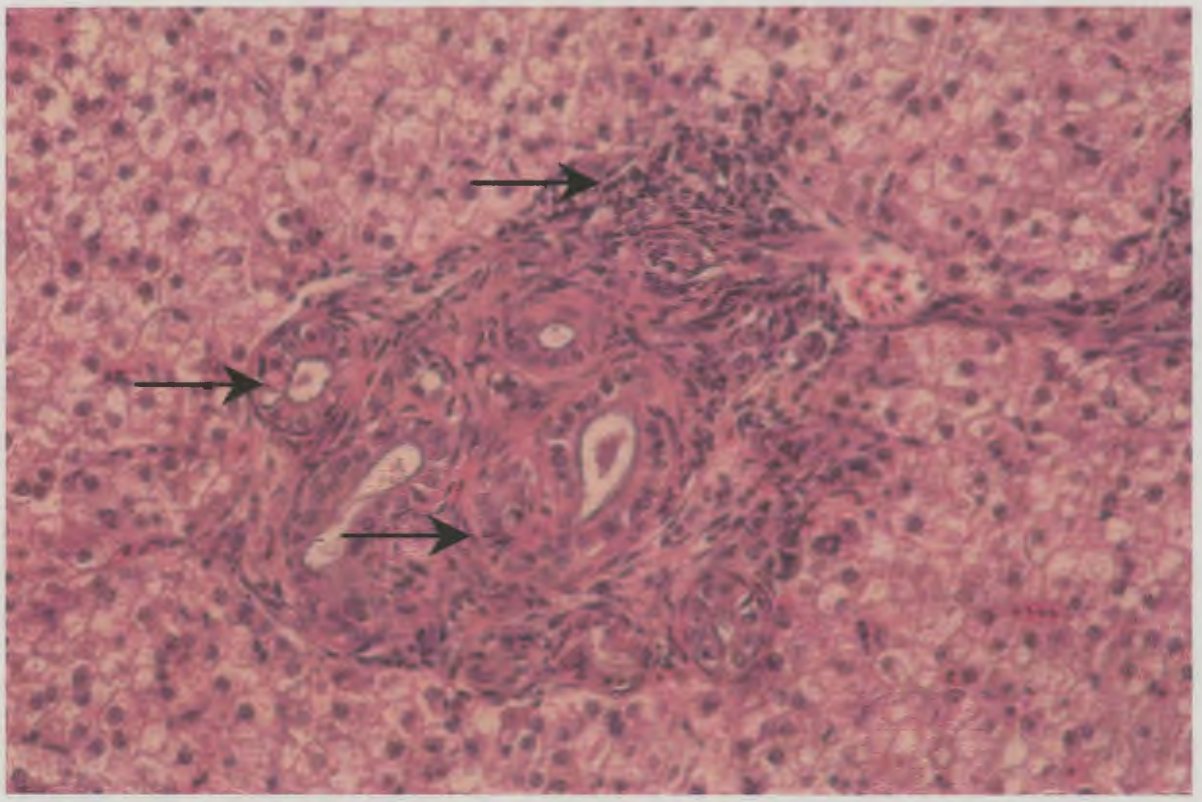

Figure 3.14: Hepatic bile ductule hyperplasia (arrows) found only in pearl dace sampled from petroleum hydrocarbon impacted sites. Tissue stained with H&E and viewed at 400X.

with petroleum hydrocarbons and, this lesion was not found in uncontaminated aquatic systems.

3.3.3 Gills

Pearl dace sampled in 1995 and 1996 from petroleum impacted sites did not differ significantly ($p < 0.05$) in the degree of gill interlamellar hyperplasia when compared to the degree of lamellar hyperplasia in fish sampled from the respective reference sites (Table 4). The prevalence of lamellar hyperplasia in fish sampled from SW1, 3 and 4 in 1995 and SW4 in 1996 when compared to reference samples was generally higher. Pearl dace from the reference sites displayed evidence of slight (Figure 3.15) hyperplasia (epithelial thickening of less than two nuclei) almost three times more often than in fish from the stillwater sites. Furthermore, fish from the reference population displayed a very low prevalence (11%) of excessive (Figure 3.17) hyperplasia (R3 in 1996) and lastly, the prevalence of moderate (Figure 3.16) hyperplasia was the lowest in fish from the reference populations.

SW4 fish sampled in 1996 also presented some unique gill histopathological anomalies. More notably seven of the ten fish exhibited fused secondary lamellar filaments (Figure 3.18). Eight out of the ten fish examined exhibited "clubbing" or telangiectasis of the distal gill extremities Figure 3.19). Lastly, with additional staining using the periodic acid-Schiff (PAS) technique, differences in the numbers and distributions of mucous cells in gill epithelial tissue of fish sampled from petroleum impacted locations (Figure 3.20) were clearly different then those of fish sampled from reference locations (Figure 3.21).

Table 4. Prevalence (%) and degree of gill interlamellar hyperplasia in pearl dace sampled from three petroleum impacted sites (SW1, 3 and 4) and one reference site in 1995, and one petroleum hydrocarbon impacted site (SW4) and three reference sites in 1996 from CFB Goose Bay.

			Prevalence (%) & Degree of Hyperplasia			
Year	Site	n	1	2	3	Mean Degree of Hyperplasia (±S.E.)
1995	SW1	20	20	50	30	2.1±0.2
	SW3	19	26	53	21	1.9±0.2
	SW4	33	19	42	39	2.2±0.1
	Reference	10	60	40	0	1.4±0.2
1996	SW4	10	20	70	10	1.9±0.2
	Reference 1	9	67	33	0	1.3±0.2
	Reference 2	9	56	44	0	1.4±0.2
	Reference 3	9	78	11	11	1.3±0.2

Note:

Degree of Hyperplasia: 1 Slight hyperplasia (number of nuclei < 2)
2 Moderate hyperplasia (2 ≤ number of nuclei < 4)
3 Excessive hyperplasia (number of nuclei ≥ 4)

* Significantly different ($p < 0.05$) from the 1995 reference group

Figure 3.15: Pearl dace gill exhibiting slight (degree 1) secondary lamellae hyperplasia (arrows) stained with H&E and viewed at 100X.

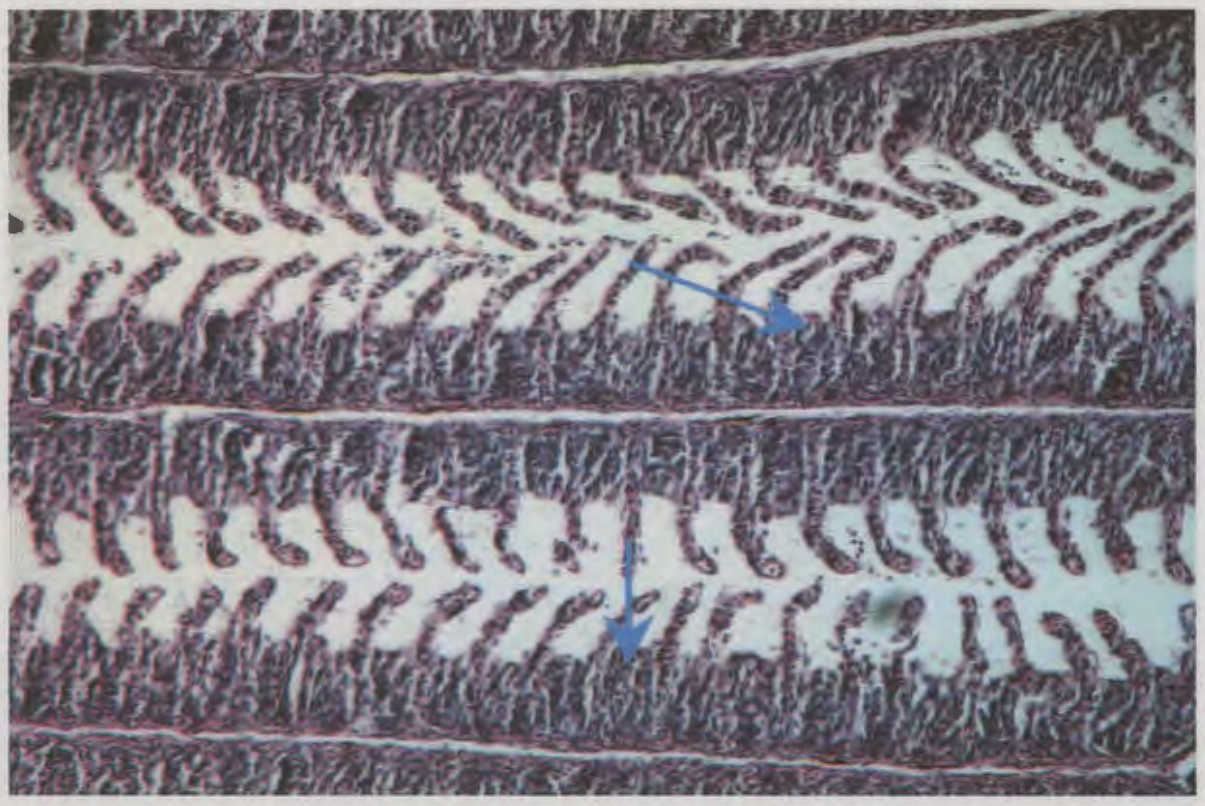

Figure 3.16: Pearl dace gill exhibiting moderate (degree 2) secondary lamellae hyperplasia (arrows) stained with H&E and viewed at 100X.

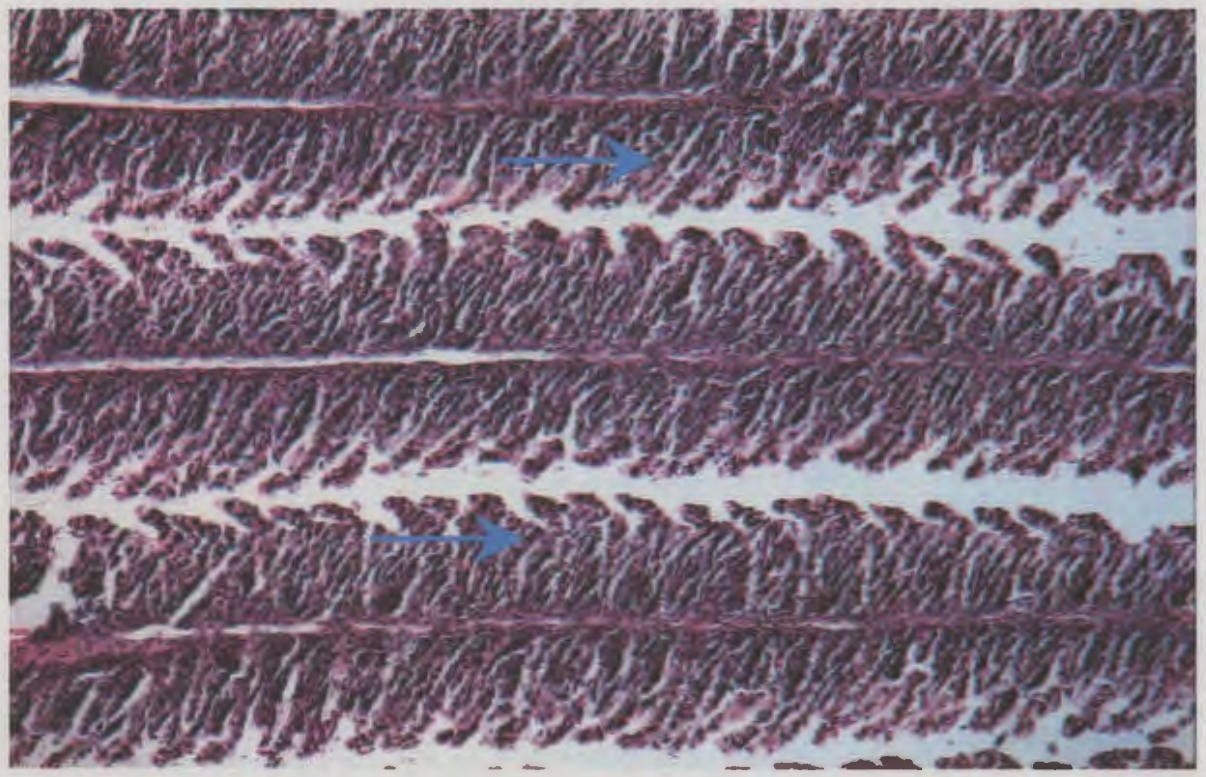

Figure 3.17: Pearl dace gill exhibiting severe (degree 3) secondary lamellae hyperplasia (arrows) stained with H&E and viewed at 100X.

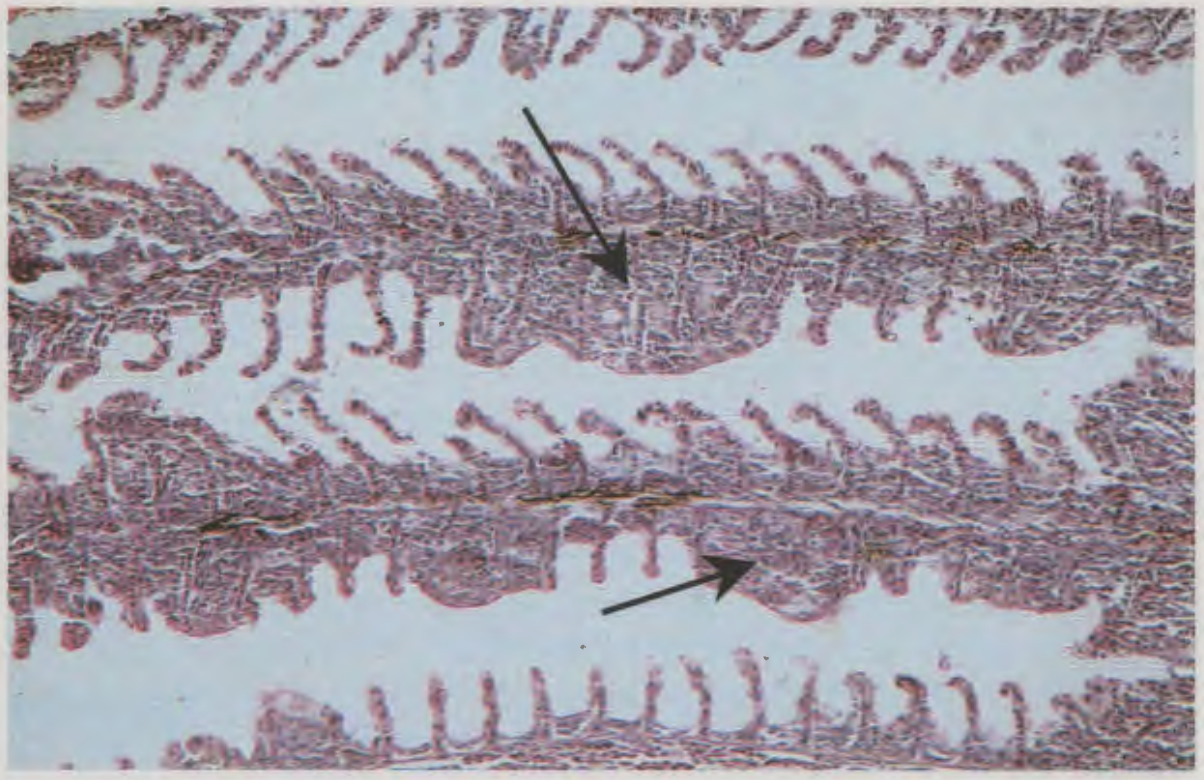

Figure 3.18: Pearl dace gill from petroleum impacted site. Note fusion of secondary gill lamellae (arrows). Stained with H&E and viewed at 100X.

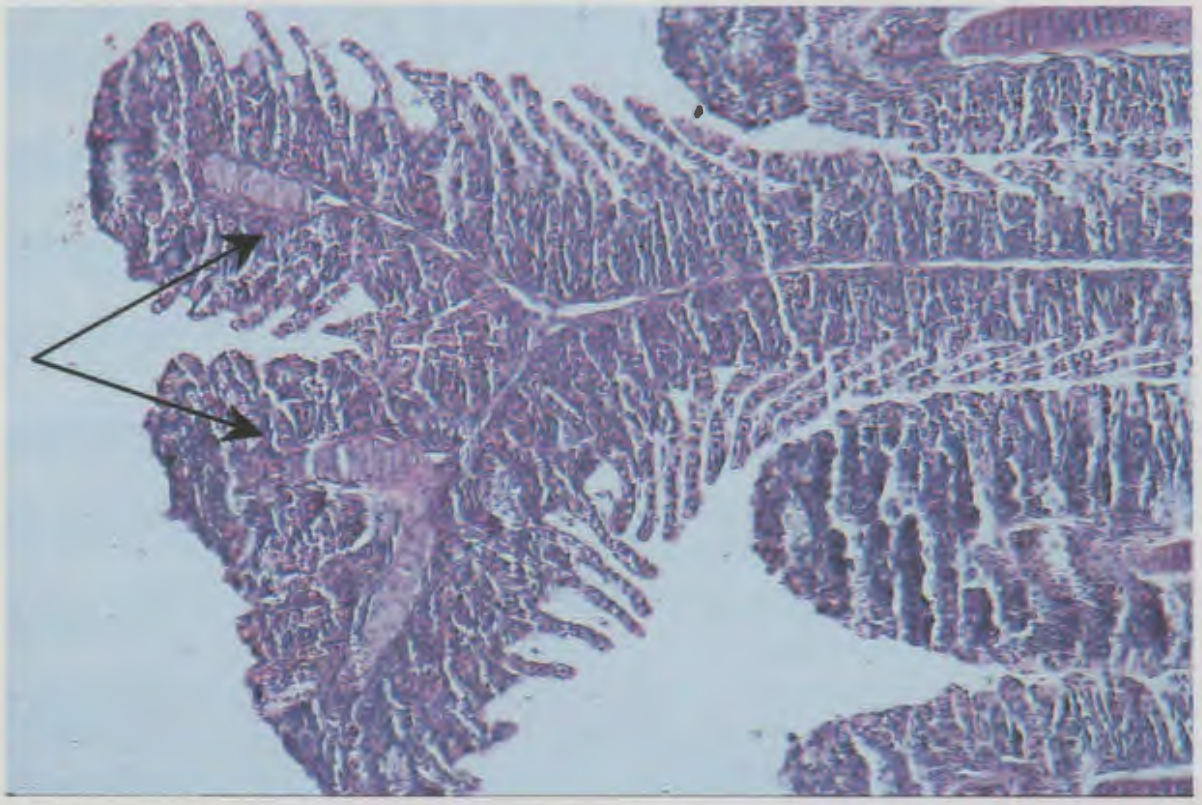

Figure 3.19: Gill of pearl dace sampled from petroleum impacted site exhibiting bifurcation of the distal end (arrows). Stained with H&E and viewed at 100X.

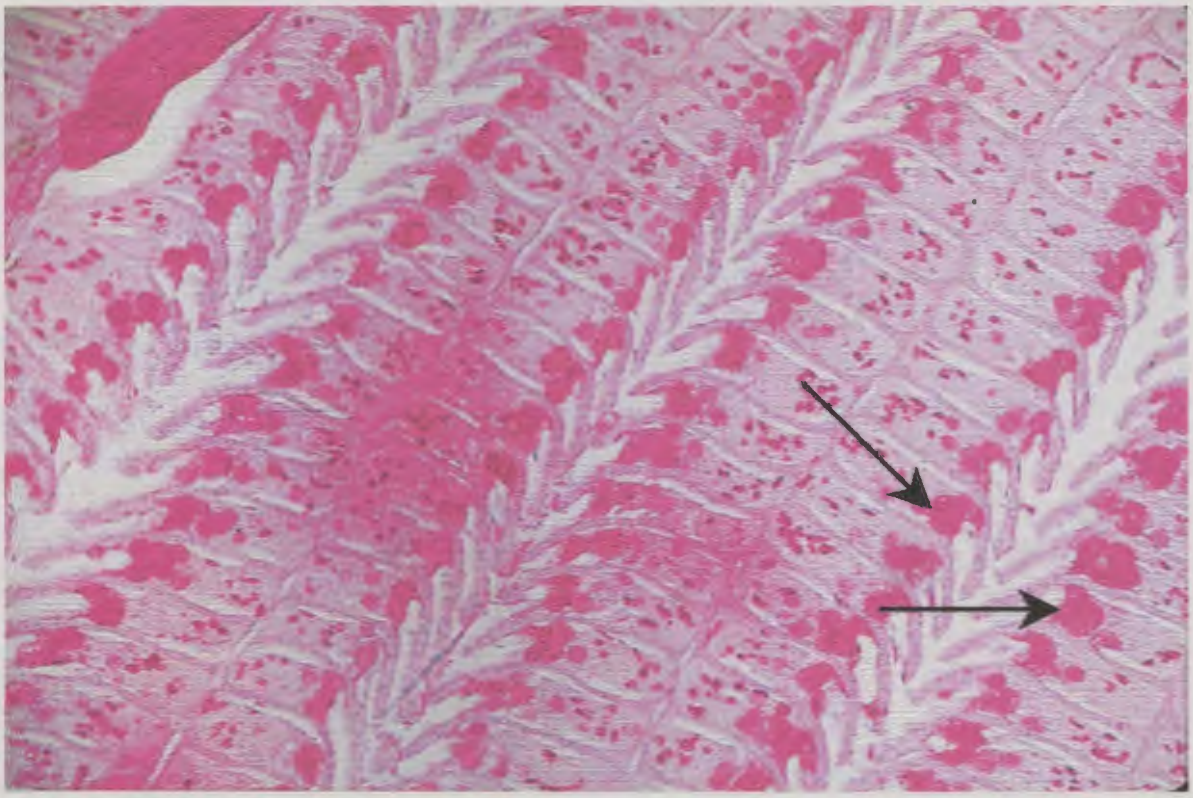

Figure 3.20: Gill of pearl dace sampled from a petroleum hydrocarbon impacted site at CFB Goose Bay, Labrador stained using the periodic acid-Schiff method for mucous cells (arrows) and viewed at 400X.

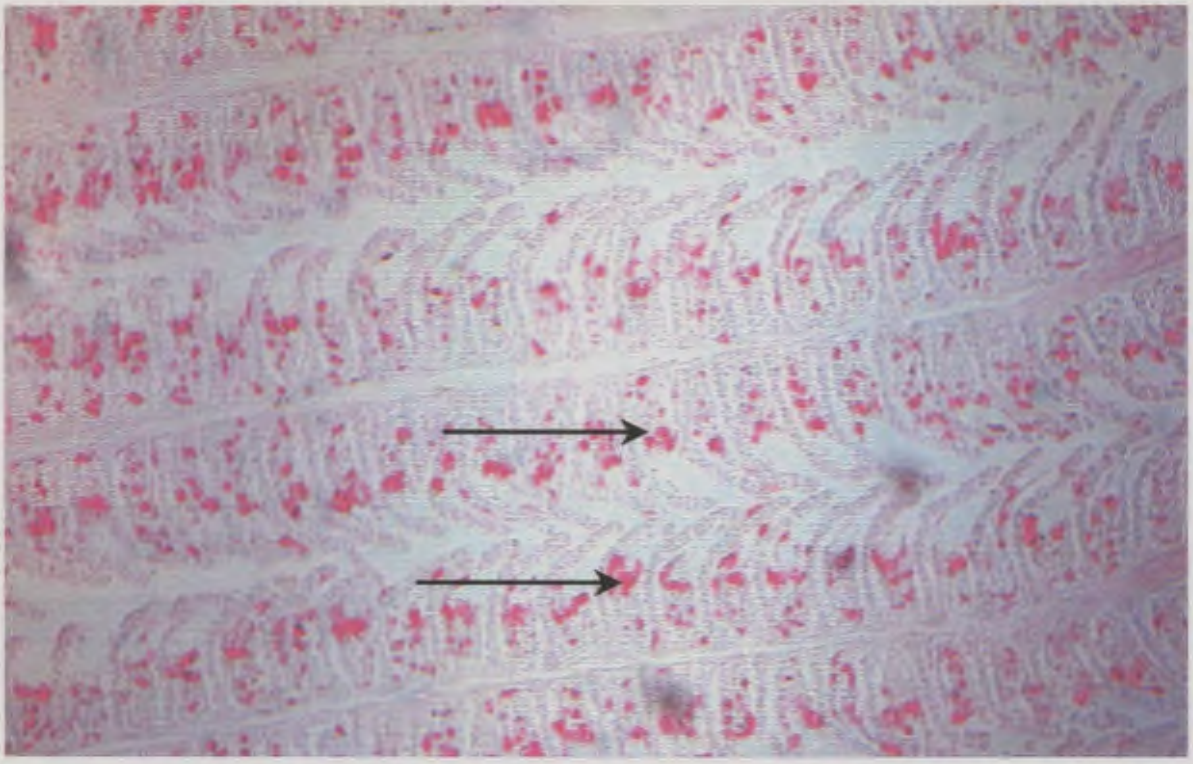

Figure 3.21: Gill of pearl dace sampled from a reference site stained using the periodic acid-Schiff method for mucous cells (arrows) and viewed at 400X.

3.3.4 Gonads

There were distinct differences in macro- and microscopic gonadal development between pearl dace sampled at the petroleum impacted and reference sites in both 1995 and 1996. In 1995, the gonads in both female (Figure 3.22) and male (Figure 3.23) fish from R3 were small and had regressed after spawning / spermiating whereas in fish from SW1, 3 and 4, the ovary contained mature eggs (Figure 3.24) and the testes (Figure 3.25) contained mature sperm. In 1996, females from the reference sites (R1-3) exhibited ovaries with fully developed eggs and males that were spermiating. These characteristics were not noted in female and male fish from SW4.

Gonadosomatic indices (GSI) were calculated for both male and female pearl dace captured from two petroleum impacted sites (SW1 and 4) and a reference site (R3) in 1995 and one petroleum impacted site (SW4) and three reference sites (R1-3) in 1996. A Wilcoxon Signed Ranks test was used to evaluate whether there were significant differences between mean GSI values for pearl dace from SW1 and 4 compared to R3 GSI values in 1995, and 1996, SW4 GSI means were compared with R1, R2 and R3 GSI values.

Male GSI values were only significantly different in 1996 between SW4 and R3 (Table 5). Female mean GSI values (Table 6) in 1995 at both SW1 and 4 were significantly greater when compared to R3. In 1996, there were no significant differences between female GSI values from SW4 when compared to R1, R2 or R3 values.

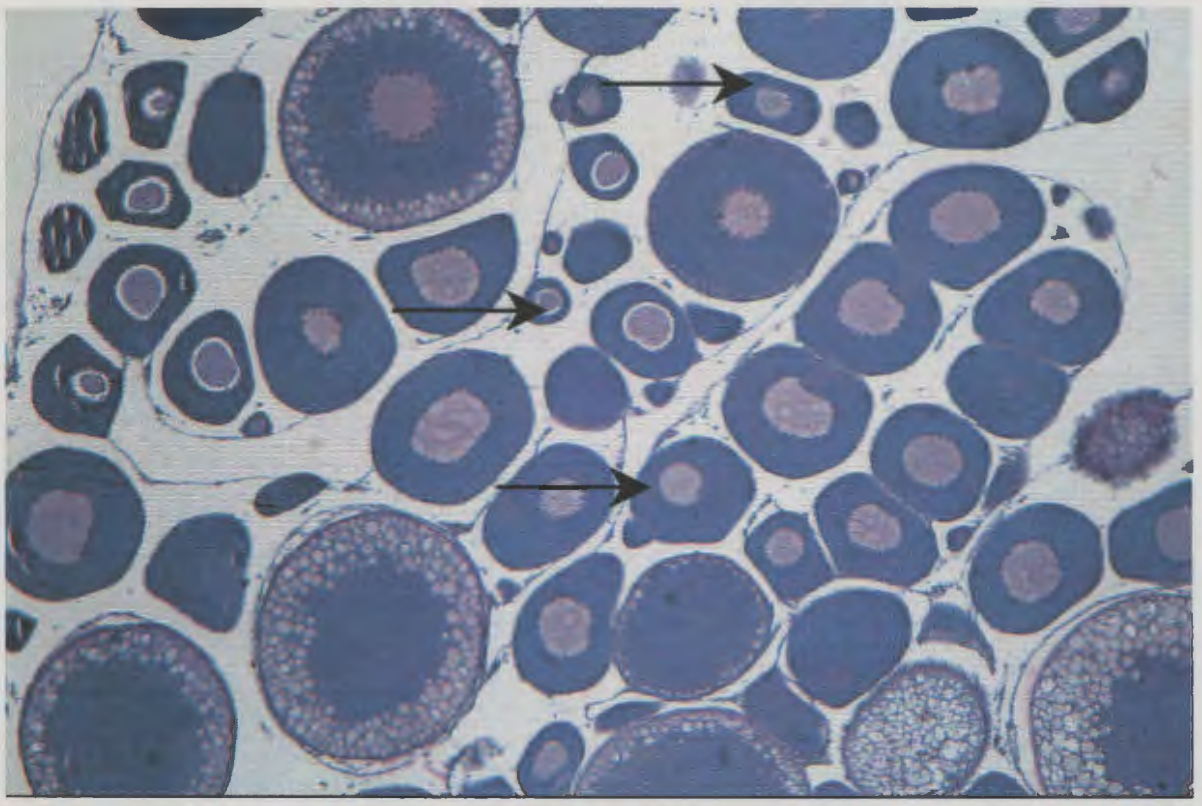

Figure 3.22: Spent (post-spawning) ovary from pearl dace sampled from a reference site in 1995. Note the abundance of small immature eggs (arrows). Stained with H&E and viewed at 400X.

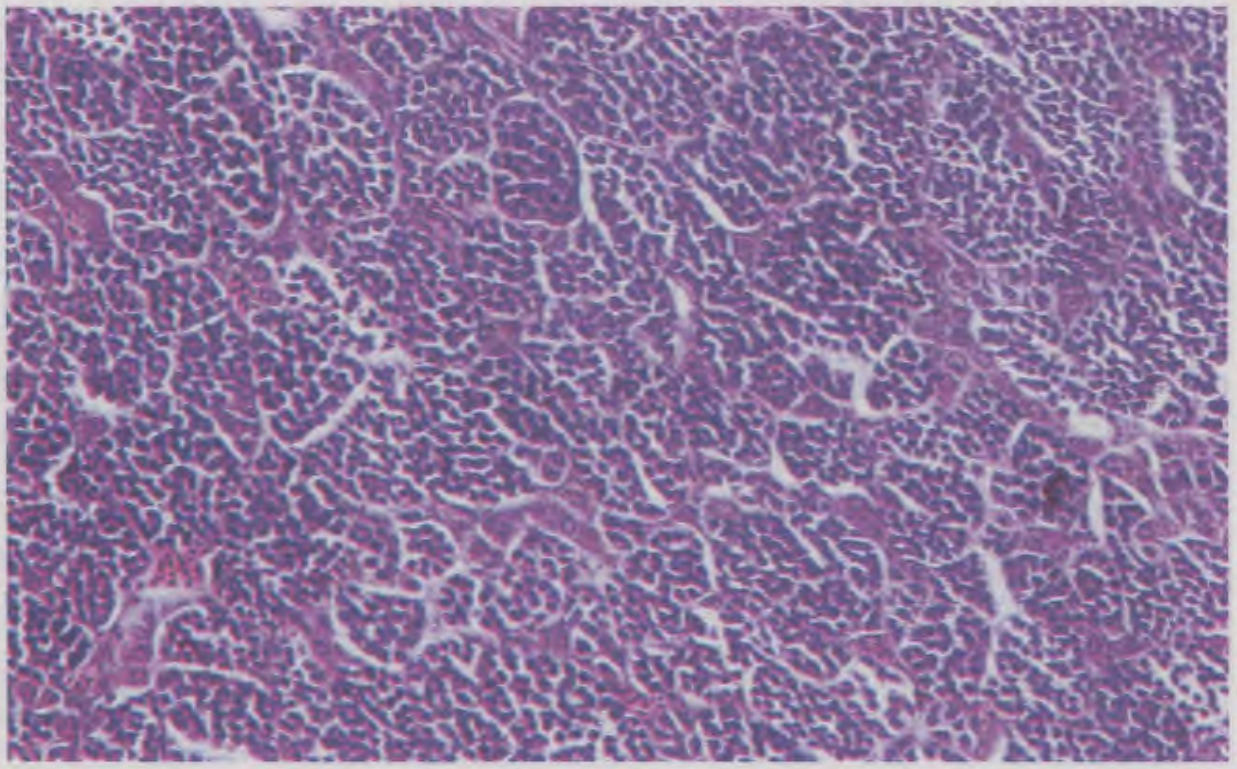

Figure 3.23: Spermiated testis of pearl dace sampled from the reference site in 1995. Stained with H&E and viewed at 400X.

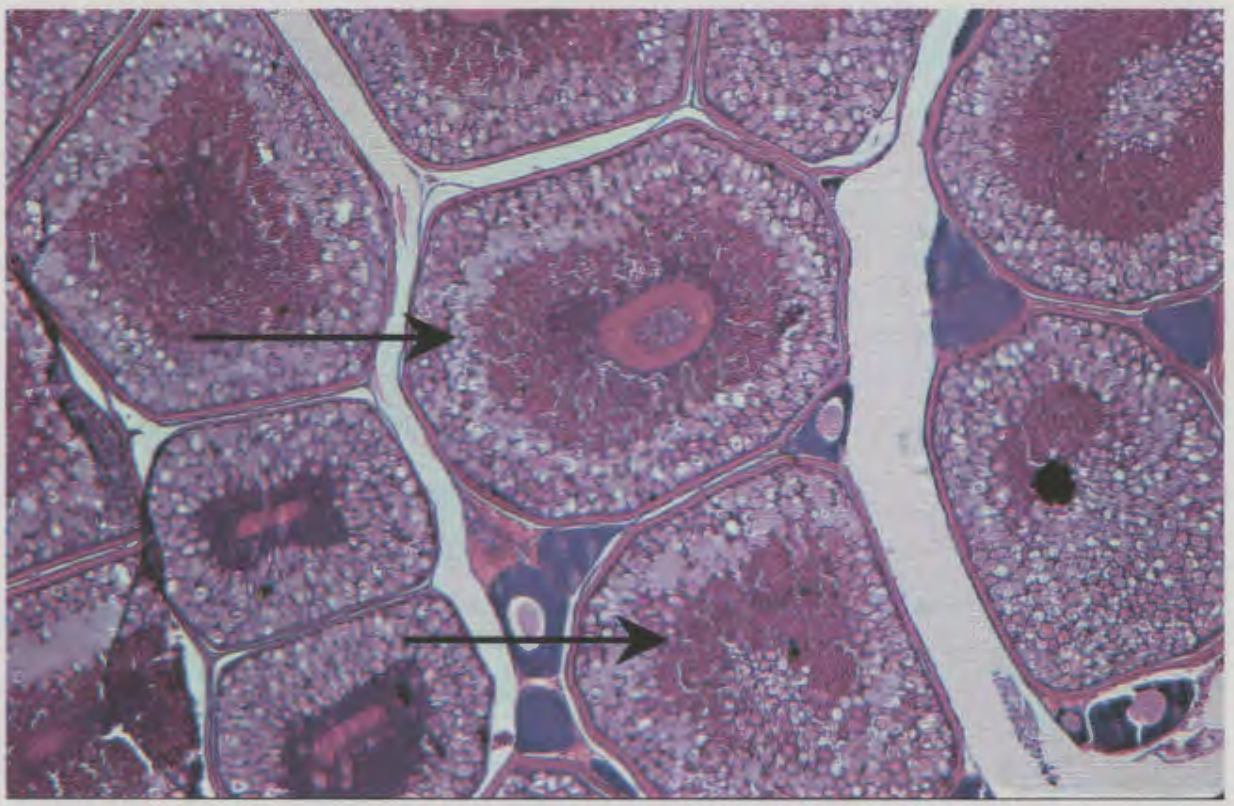

Figure 3.24: Delayed spawning ovary from pearl dace sampled from SW4 in 1995 containing mature eggs (arrows). Stained with H&E and viewed at 400X.

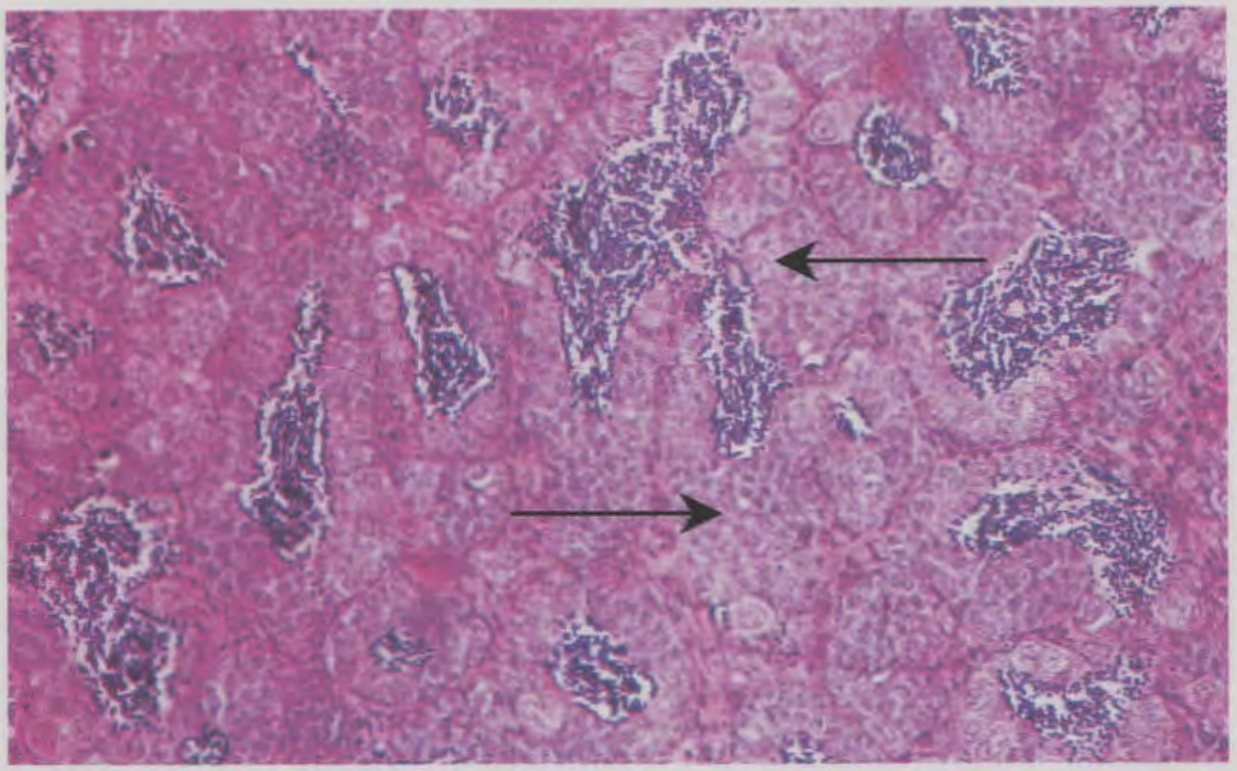

Figure 3.25: Mature testis of pearl dace sampled from SW4 in 1995 containing developed sperm (arrows). Stained with H&E and viewed at 400X.

Table 5. Sample size (n) and comparison of male gonadosomatic indices (GSI) of pearl dace sampled at two petroleum hydrocarbon impacted sites (SW1 and 4) and a reference site in 1995 and one petroleum hydrocarbon impacted site, SW4, and three reference sites in 1996.

Year	Site	n	GSI ($x10^{-2}$)
1995	SW1	10	1.06±0.10
	SW4	4	1.06±0.08
	Reference†	10	1.77±0.78
1996	SW4	10	0.88±0.08¥
	Reference 1	10	0.82±0.18
	Reference 2	10	0.83±0.14
	Reference 3†	7	2.80±0.99

Note: Values are given as the mean ± S.E.

¥ Significantly different ($p < 0.05$) from the reference 3 1996 group.

† Same site, sampled two consecutive years.

Table 6. Comparison of female gonadosomatic indices (GSI) of pearl dace sampled at two petroleum hydrocarbon impacted sites (SW1 and 4) and a reference site in 1995 and one petroleum hydrocarbon impacted site, SW4, and three reference sites in 1996 (n = 10 per site).

Year	Site	GSI ($x10^{-2}$)
1995	SW1	9.07±0.41*
	SW4	9.79±0.65*
	Reference†	5.77±0.57
1996	SW4	5.01±0.97
	Reference 1	3.57±0.51
	Reference 2	5.10±0.43
	Reference 3†	3.54±0.43

Note: Values are given as the mean ± S.E.

* Significantly different ($p < 0.05$) from the 1995 reference group.

† Same site, sampled two consecutive years

3.4 MFO Induction

Comparisons of detoxification enzyme activity were made between pearl dace sampled at the petroleum impacted and reference sites in 1995 and 1996. A number of factors led to pooling female livers (5 livers per sample) and thus to unavoidable small sample sizes. Consequently, nonparametric statistical tests that ranked data, as used in all other statistical analysis, were not useful here. According to Sokal and Rohlf (1981), when there are only two means to be tested at a time, as in this case, hypothesis testing of equality of means by way of a t-test may be carried out. Furthermore, when the assumption of equal variances is not met, also as in this case, an approximate one sample t-test can be performed. Table 7 summarizes EROD induction levels and significant differences as determined by one sample t-test, using the mean reference induction level as the expected test variable.

When 7-EROD values of both sample years were compared, pearl dace from petroleum impacted sites had significantly different levels of MFO induction compared to reference sites. In 1995, induction levels at three petroleum hydrocarbon impacted sites (SW1, 3 and 4) were significantly lower when compared against induction levels in fish from R3. In contrast, 1996 MFO induction levels in fish sampled from SW4 were significantly greater then those in fish sampled from R2 and R3. Given the biological interrelationships involved in the MFO system of fish, and its specificity, these results provide insight into the reproductive dynamics of the populations involved. Specifically, there was an inverse relationship between GSI values and EROD activity in

Table 7. Pooled sample size (n) and induction levels of the mixed function oxygenase enzyme, as determined by 7-ethoxyresorufin-O-deethylase (EROD) activity, in pearl dace liver sampled from three petroleum hydrocarbon impacted sites (SW1, 3 and 4) and one reference site in 1995 and one petroleum hydrocarbon impacted site (SW4) and three reference sites in 1996.

Year	Site	n	7-EROD Induction (pmol resorufin / mg protein / min)
1995	SW1	2	4.55±0.05*
	SW3	2	1.65±0.05*
	SW4	4	2.88±0.65*
	Reference†	2	29.45±0.05
1996	SW4	10	$37.17\pm5.40^{\text{¥2,3}}$
	Reference 2	2	3.30±2.00
	Reference 3†	3	4.23±3.93

Note: Values are given as the mean ± S.E.

* Significantly different ($p < 0.05$) from the 1995 reference group.

¥2,3 Significantly different ($p < 0.05$) from the 1996 reference groups 2 and 3.

† Same site, sampled two consecutive years.

pre-spawning (1995) females (Figure 3.26) but this was not apparent in the fish taken at the impacted and reference sites.

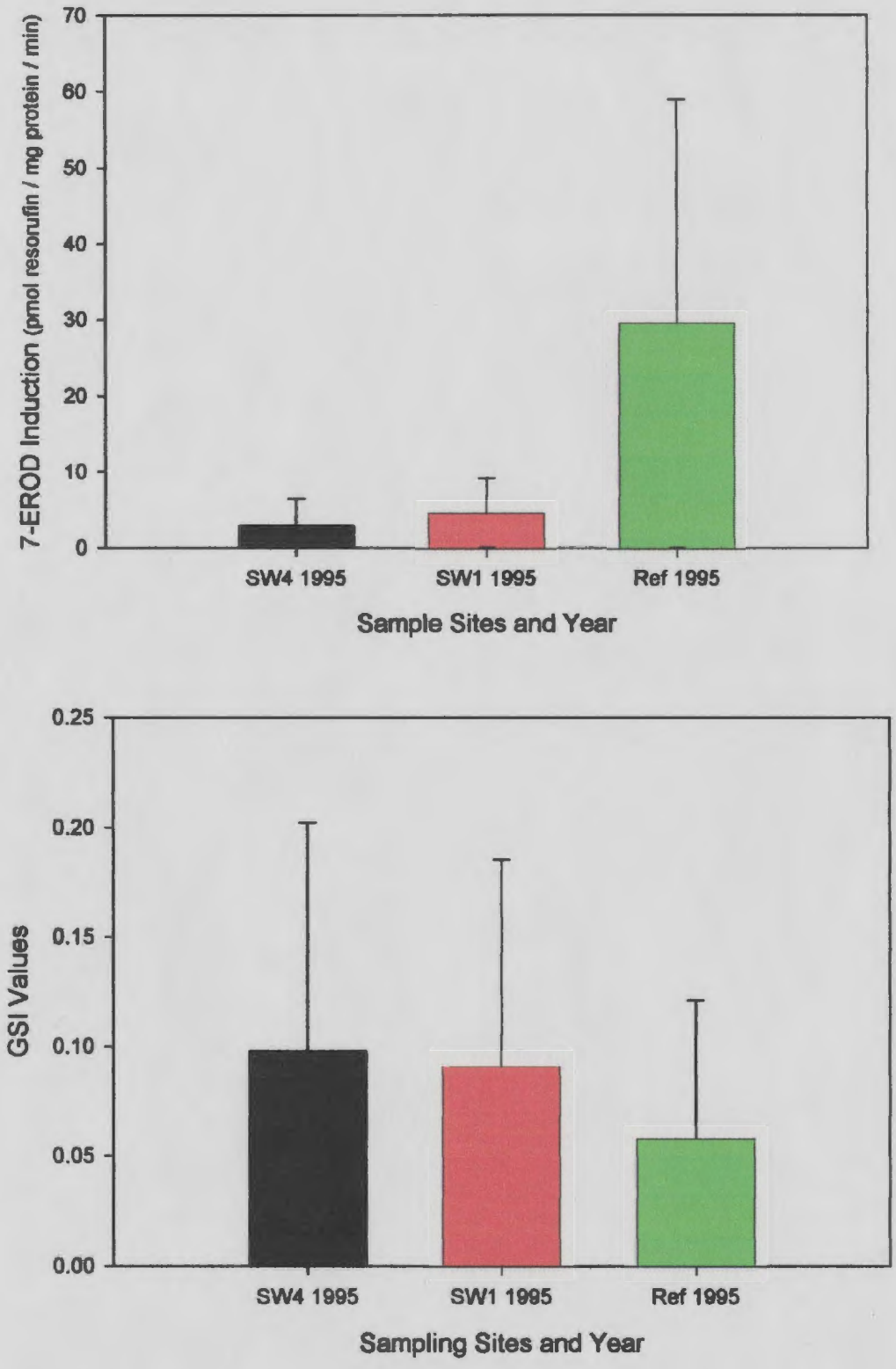

Figure 3.26: Female 1995 pearl dace 7-EROD induction and GSI means (± S.E.) for two petroleum hydrocarbon impacted sites (SW1 and 4) and one reference site.

4.0 Discussion

In a laboratory setting, evaluation of chronic toxicity using dose response tests can provide an initial approximation of the concentrations in the environment that can produce harmful acute effects (Ewald, 1995). Several field and laboratory studies have reported that chronic exposure to petroleum hydrocarbons can affect feeding, growth, and reproduction, and cause irreversible tissue damage (Haensly *et al.*, 1982; Khan and Kiceniuk, 1984; Kiceniuk and Khan, 1987). Using a number of biomarkers to measure chronic toxicity in the natural environment is an important and expanding area of environmental toxicology since the majority of contaminated sites do not exhibit acute effects. Furthermore, the predominance of chronic contamination is often just as expensive and, in some regards, more demanding to cleanup than acutely contaminated sites (delineation is often a problem). However, choosing biomarkers that will readily measure and thereby quantify exposure levels at different sites in the field can be an arduous task. Fortunately, the absolute effects revealed by biomarkers justify this difficult task and thus, significant findings can be more easily identified and solutions more readily applied to specific decision-making processes and environmental management in general.

Using *Margariscus (Semotilus) margarita* as a biological indicator species and a multifaceted approach using ten biomarkers (mass, length, body condition factors, digital image analysis of hemosiderin deposition, histopathology of the liver, spleen, and gills, gonadosomatic indices of male and female pearl dace, and levels of female hepatic MFO induction) the primary aim of this study was to ascertain the impact of chronic petroleum

hydrocarbon contamination on four stillwater locations at CFB Goose Bay. The second goal of this study was to investigate the potential of pearl dace as a reliable environmental indicator of the impact of petroleum hydrocarbon contamination on its aquatic environment.

Results from the present study, namely body condition factors, gonadosomatic indices, histopathology and MFO system induction, indicate that the health of pearl dace was impaired at SW1, 3 and 4 in contrast to the samples from reference locations. Atalay and Hwang (1996) reported that various incidents, such as, spills, leaks, or subsurface disposal of petroleum products are known to contaminate soils and groundwater with organic compounds including aromatic, chlorinated, and polycyclic aromatic hydrocarbons (PAHs). The three impacted sites (SW1, 3 and 4) were situated down gradient from facilities that were the main storage locations for petroleum hydrocarbons at CFB Goose Bay. It is documented that significant petroleum hydrocarbon contamination had occurred over the life history of the Base. Given these two factors, it is likely that the impairment of health evident in the pearl dace, and thus the Spring Gulch Wetlands, was associated with the xenobiotics.

4.1 Population Analysis

Pearl dace sampled from SW1, 3 and 4 in late August / early September 1995 had not spawned; however, female gonads were producing mature eggs. Pearl dace normally spawn in late May and early June (Scott and Crossman, 1973) when the water temperature

is above 13^{0}C (Smith and Smith, 1986). Gonadal maturation typically begins in late summer following an intense feeding period and continues until spawning the following season (Fava and Tsai, 1978). The observation that female pearl dace at SW 1, 3 and 4 had not spawned as late into the season as early September provides strong evidence to support the belief that female reproduction was disrupted and resulted in delayed gonadal development.

Closely associated with female reproductive delay, are high levels of circulating estradiol (Van Der Kraak *et al.*, 1990). According to Tyler and Sumpter (1996) vitellogenin is synthesized in the liver of fish in response to circulating estradiol (derived from the ovary). Vitellogenin is the principle, if not the only, precursor of yolk proteins. If the liver of pearl dace was damaged by toxicants (evident by the degree of lipid vacuolation) to the extent that it could not produce the required amount of vitellogenin, then adequate yolk protein synthesis and any subsequent development and maturation of oocytes would be delayed.

A high percentage of juvenile fish composed R3 in 1995. This was evidence of a growing population with reproductively active adults. The absence of juvenile fish, delay in seasonal maturity and overall poor characteristics of mature fish in SW1, 3 and 4, however, suggested that the population was aging, declining and possibly facing eventual extinction. In 1995, trapping in SW2 failed to reveal the presence of pearl dace or 3 spine sticklebacks (*Gasterosteus aculeatus*) which were present at SW1, 3 and 4 and the reference site (Khan, unpubl. data). Consequently, it can be hypothesized that SW2 may

have already faced a pearl dace population extinction and thus may have provided a glimpse into what could be expected for the pearl dace populations at SW1, 3 and 4.

Evidence to support this population extinction hypothesis may have been gathered in early July 1996. Despite numerous attempts at various locations to trap fish in SW1, 2, 3 and 4, pearl dace were captured only in SW4. The additional minor stress of a short sampling regime for this study in 1995 may have pushed the pearl dace populations of SW 1 and 3 beyond the point of population recovery, and extinction of these pearl dace populations might have occurred. In hindsight, the small sample size of SW3 (n = 19) in 1995 might have provided some indication as to the rate of failure of the population. Additional studies into these stillwater sites may provide for a more definitive explanation as to the reason(s) for the absence of pearl dace from SW2 in 1995 and SW1, 2 and 3 in 1996.

Stasiak (1977) reported that the sex ratio of pearl dace at Bone Creek, Nebraska in March was 1:1 but that this ratio changed during the spawning season when, in accordance with Langlois (1929), males comprised a greater percentage of the populations at the spawning sites. Comparing the 1996 population composition in SW4 to reference sites 2 and 3 did not highlight any significant differences. However, in comparing SW4 with reference site 1, it was observed that the population composition in mid-July 1996 was consistent with the reports of Langlois (1929) and Stasiak (1977), that males were the predominant gender (Figure 3.3; males: 41%; females: 26%; juveniles: 33%). In short, these data indicated that the population of pearl dace in reference site 1 was in a spawning

configuration.

This observation provided strong evidence that pearl dace in CFB Goose Bay spawned over a full month later (that is, in June-July) than shown in data published in the literature. Consequently, this evidence supported the premise that all pearl dace, sampled in late August / early September1995, at the petroleum-impacted sites were reproductively delayed. Lastly, considering the higher latitude (sub-arctic conditions) and correspondingly cooler water temperatures surrounding CFB Goose Bay, pearl dace were probably at the most northern limit of their distribution.

4.2 Biological Variables

The health of fish hinges on nutrition and environmental quality, and when these are impaired by chronic exposure to toxicants, such as petroleum hydrocarbons, negative immunomodulation mediated by the release of corticosteriods can affect resistance by suppressing lymphocyte proliferation and antibody response and culminate in disease (Barton, 1997). The biological variable data recorded in this study (mass, length and condition factor) of fish sampled from petroleum impacted sites in 1995 and 1996 differed from the results in the literature (Kiceniuk and Khan, 1987; Barker *et al.*, 1994; Khan, 1998; 1999b) as these biological variables were significantly greater in the impacted than reference samples. It is possible that disruption of gonadal development in the impacted groups culminated in increased somatic growth. Given that gonadal development, maturity and spawning deplete reserve resources and disruption of gonadal development implies a

reduced use of these resources, which contribute to somatic growth, then, somatic growth is probably greater in fish in which gonadal disruption occurs than in fish with normal spawning populations.

Tallman and Gee (1982) reported that lack of intraspecific partitioning of essential food resources and subsequent competition between different age classes of pearl dace at higher latitudes is most severe in populations that reside in environments that are harsh or marginal. This situation is applicable to the pearl dace that resided in SW1, 3 and 4 of CFB Goose Bay.

The pearl dace populations in SW1, 3 and 4 were located in a restricted and secure area of a military air force base. The only vehicular access to the area was via a small access road, which was gated and always locked. Consequently, in comparison to the reference locations, the SW1, 2, 3 and 4 were relatively protected from external human pressures, such as fishing. More important, the generally lower biological variable results for the reference populations obtained in this study were plausible given that in growing populations, there is stiff competition for food resources. In such an environment, it can be argued that the populace will be fitter and thus more able to compete for survival. The increased level of fitness is apparent in the lower condition factor of the reference fish. In the stillwater sites, the populations were small, decreasing (evident from the time it took to capture pearl dace in 1996 and that no dace were captured from SW1 or 2) and even though they were composed of mature adults, food resources were not considered "limited". Moreover, in SW1, 3 and 4 only one other fish species was captured over the

two sampling years, thus, reducing interspecific competition which occurred at the reference locations. In short, conditions at SW1, 3 and 4 permitted ample growth and survival to a greater age, for an already mature fish population, evident from the higher condition factors.

4.3 Histopathology

Histopathological changes in animal tissues are powerful bioindicators, as early warning signals, of prior exposure to environmental stressors, such as petroleum hydrocarbon contamination (Hinton and Laurén, 1990). Histopathological changes permit identification of specific target organs, cells, and organelles that have been affected *in vivo*. Furthermore, in the field, histopathology is a method of assessing both short- and long-term exposure to toxicants. The effects can be assessed at several potential tissue sites which incur injury. According to McKim (1985) and Meyers and Hendricks (1985), when histopathological changes are combined with other biomarkers, as in the present study, it may be feasible to predict the possible effects of a toxicant on processes or activities such as growth, reproduction and population stability.

The literature is replete with excellent references and successful examples of studies using histopathology to investigate the effects of toxicants on resident populations (Payne *et al.*, 1978; Fletcher *et al.*, 1982; Khan and Kiceniuk, 1984; Mallatt, 1985; Leino *et al.*, 1987; Khan and Nag, 1993; Barker *et al.*, 1994; Khan *et al.*, 1994; Rousseaux *et al.*, 1995; Clark *et al.*, 1997; Khan, 1998; Myers *et al.*, 1998; Khan, 1999a; 1999b;

George-Nascimento *et al.*, 2000).

4.3.1 Spleen

Hematopoietic tissue is one of the most metabolically active in fish and is an ideal site to examine for chronic contamination impact (Hinton and Laurén, 1990). The deposition of hemosiderin is most likely a byproduct resulting from hemoglobin catabolism as a consequence of increased erythrocyte destruction within the spleen (Agius and Agbede, 1984). Hemosiderosis is an abnormality characterized by excessive deposition of a yellow-brown pigment, hemosiderin, and is a symptom that toxic contaminants are having a detrimental impact upon a fish (Barker *et al.*, 1994; Mercer *et al.*, 1997; Khan, 1998; Khan, 1999a; 1999b; George-Nascimento *et al.*, 2000). Hemosiderosis was the major lesion observed in the spleen of pearl dace. In dace sampled from SW1, 3 and 4 in 1995 and SW4 in 1996, there was a significant increase in the concentration of hemosiderin, as determined by digital image analysis (Khan and Nag, 1993), in comparison with that in the spleen of fish sampled from reference locations in 1996.

Prevalence of melanomacrophage aggregates which contained pigment (MMA) and those which did not contain pigment (MMA NP) were also useful biomarkers (Khan and Kiceniuk, 1984) that indicated chronic splenic insult by contaminants. The pigment within the aggregates found in pearl dace was usually hemosiderin and thus MMAs were indicative of hemosiderosis. However, other pigments, namely, lipofuscin or melanin can also be found within melanomacrophage aggregates (Agius and Agbede, 1984). Lipofuscin appears to be derived from damaged cellular components, such as effete

mitochondria, through the peroxidation of their unsaturated lipids. Melanin may well be derived from phagocytosis of melanin granules or their precursor organelles from melanocytes. Because fish are poikilothermic, they require high levels of unsaturated fats in their tissues to maintain the membrane fluidity essential for normal metabolic processes even at low temperatures (Agius and Agbede, 1984). Unsaturated fats are particularly prone to peroxidation with the resultant possible formation of pigments.

Duress, such as exposure to chronic levels of petroleum hydrocarbons, induces pigments to accumulate in considerable quantities in fish species as a result of cellular damage. Before a sequence linking tissue atrophy with increasing numbers of pigmented cells can be established, it is important to resolve if damaged cell components were phagocytized immediately and transported to the hemopoietic organs within the macrophages or if they were transported by the blood stream and removed from circulation by the macrophage sheath of the splenic ellipoids (Agius and Agbede, 1984). The findings of this study, in accordance with Agius (1979), provided strong evidence in support of the second hypothesis that the spleen in pearl dace, possibly because of its metabolic rate, was the primary site for deposition of hemosiderin, over both the kidneys and liver.

Melanomacrophage aggregates containing no pigment (MMA NP) are also a telltale sign of tissue damage and the absence of pigment might indicate that the fish has recycled (i.e. hemosiderin into new hemoglobin) or catabolised the pigment and the aggregate is now non-pigmented (Agius and Agbede, 1984). In short, both MMA and

MMA NP are clear indicators of damage from xenobiotics. Additionally, the mean number of MMA and MMA NP observed could be quantified to determine splenic anomalies and test statistically for differences in distribution of hemosiderin and the number of deposits (Khan, 1999b). In this study, the concentration of hemosiderin between sites in 1995 was not significantly different but, both the prevalence and number of deposits were significantly greater in fish from the petroleum impacted sites than a reference site.

Combining both pigment quantification techniques, as done herein, provided strong evidence to support the view that chronic petroleum hydrocarbon contamination resulted in decreased health of pearl dace. Destruction of erythrocytes resulted in higher levels of hemoglobin catabolism, and consequently significantly more and greater distribution of hemosiderin in some tissues. Lastly, splenic melanomacrophage aggregates (containing and not containing pigment) and higher concentrations of hemosiderin in fish were characteristic of pearl dace from the impacted sites versus fish from reference locations.

4.3.2 Liver

The liver in fish is important in many aspects of nutrition and energy storage, including lipids and glycogen, and detoxification, during periods of reproduction (Scott and Scott, 1988). Histopathology of hepatic tissue is one of the most common methods for evaluating the impact of toxicants on fish. In this study, the degree of lipid vacuolation was used as a quantitative method to examine the effect of chronic petroleum hydrocarbon contamination on pearl dace. Lipid peroxidation has been receiving increased attention as

a mechanism of toxicity for a variety of organic and inorganic environmental pollutants (Palace and Klaverkamp, 1993) since it may lead to disruption of cellular and subcellular membranes, and to altered activities of enzymatic membrane proteins (Krinsky, 1988). The liver tissue from pearl dace taken at SW1, 3 and 4 in 1995 and SW4 in 1996 displayed significantly higher degrees of lipid accumulation. Khan (1999b) also found that the degree of lipid vacuolation was significantly greater in pearl dace from SW1, 3 and 4 over those of a reference site.

Accumulation of lipid in hepatic cells may cause injury to cellular components, impairing the catabolism of organic pollutants and their metabolites (Krinsky, 1988; Rousseaux *et al.*, 1995). Many researchers have found that exposure of fish to various toxicants, including petroleum hydrocarbons, induced the liver cells to display cytoplasmic vacuolation containing lipid (Fletcher *et al.*,1982; Solangi and Overstreet, 1982; Capuzzo, 1985; Meyers and Hendricks, 1985). Hence, the increase in the lipid accumulation within the hepatocytes of pearl dace from petroleum hydrocarbon impacted locations, compared to pearl dace sampled from non-impacted sites, is likely a result of bioaccumulation of petroleum hydrocarbons and/or its metabolites. This response, consequently, is a useful biomarker for exposure to petroleum hydrocarbon contamination.

Bile ductule hyperplasia (BDH), also called pericholangitis, was also a very significant lesion found in pearl dace from petroleum impacted locations over the course of this study. This lesion has been observed in many species of fish inhabiting contaminated locations (Rousseaux *et al.*, 1995; Khan, 1999a; 1999b). Indeed, Krahn *et*

al. (1986) reported that within five days of a large oil spill in the Columbia River, mean concentrations of metabolites of aromatic compounds in the bile of white sturgeon (*Acipenser transmontanus*) were significantly higher than those caught upriver of the spill. Hinton and Laurén (1990) supported this finding when they reported that hepatocytes found near bile ducts often showed toxicant-induced alteration. They speculated that bile containing PAH metabolites passing within the hierarchy of ductules and ducts within the liver was capable of inducing further toxic change in hepatocytes near those passageways. Furthermore, because of the accessibility of bile, and because the liver accumulates most toxicants and the hepatocytes anabolise and transport bile conjugates, analysis of biliary metabolites have the potential to become an important bioindicator (Krahn *et al.*, 1986).

4.3.3 Gills

The lack of significant differences in the degree of interlamellar hyperplasia found in the gills of pearl dace taken from SW1, 3 and 4 when compared to fish from the reference sites over 1995 and 1996 can not be explained with the available data. Further investigation into this area is required. However, prevalence of moderate and severe lamellar hyperplasia were higher in fish from SW1, 3 and 4 over both years when compared to pearl dace from R3 in 1995 and R1, 2 and 3 in 1996. Severe hyperplastic epithelium obliterated the spaces between adjacent respiratory lamellae and culminated in fusion of the lamellae. This lesion occurred also in samples from SW4 in 1995 (Khan, 1999b) and in 1996. Other lesions were also evident and they included, clubbing (telangiectasis) of the distal gill lamellae, bifurcation of the distal lamellae and differences

in the numbers and distributions of mucous cells.

Interlamellar hyperplasia is well known as an excellent biomarker for exposure to petroleum hydrocarbon contamination (Payne *et al.*, 1978; Woodward *et al.*, 1981; Solangi and Overstreet, 1982; Khan and Kiceniuk, 1984; Mallatt, 1985; Leino *et al.*, 1987; Barker *et al.*, 1994; Khan, 1998; 1999a; 1999b; George-Nascimento *et al.*, 2000). The gills of fish perform critical physiological functions including gas exchange, ionoregulation and excretion of nitrogenous wastes. Consequently, by partially filling the spaces between adjacent respiratory lamellae, interlamellar hyperplasia impairs respiratory functions by diminishing respiratory surface area, increasing the thickness of the respiratory blood-water barrier and lastly, reducing the fish's capability to maintain homeostasis especially with respect to ionoregulation and excretion of metabolic wastes. Excessive secretion of mucus, as demonstrated by the PAS positive deposits in the gills of dace from SW4, probably also impaired gaseous exchange.

4.3.4 Gonads

In this study, the gonadal mass of male and female pearl dace, as a ratio of eviscerated mass of the fish, was used to calculate gonadosomatic indices (GSI). This procedure is well documented and validated in the literature (Payne *et al.*, 1978; Stott *et al.*, 1980; Goede and Barton, 1990). Female GSI from fish in SW1 and 4 in 1995 was significantly higher than GSI values for the 1995 reference fish. Many studies have shown that exposure to contaminants causes a negative impact on reproductive processes in fish (Tuvikene, 1995). Numerous studies with petroleum hydrocarbons have shown decreased

gametogenesis, decreased gonad size and lowered egg production (Payne *et al.*, 1978; Fletcher *et al.*, 1982; Khan and Kiceniuk, 1984; Walton *et al.*, 1983; Kiceniuk and Khan, 1987). Collier *et al.* (1992) reported that the reproductive process in female English sole (*Paralichthys dentatus*) was disrupted by PAH exposure and that sublethal contaminant levels might exclude females from the spawning population. Kime (1995) reported that there is increasing evidence to support the view that sub-lethal pollution may decrease the fecundity of fish populations, leading to long-term decline and eventual extinction.

The observation that GSI values in 1995 fish from SW1 and 4 were significantly greater as late into the season as September 01, indicates that the females were reproductively delayed. By late August and early September, 1995, spawning should have occurred already in pearl dace, as evident from the significantly lower GSI of the reference fish and the spawning of 1996 reference fish. Barker *et al.* (1994), in a study of winter flounder (*Pleuronectes americanus*), reported similar findings. A plausible explanation for the greater female GSIs in SW1 and 4 than in reference populations was a delay in oocyte maturation. The maturation-inducing steroids such as 17β estradiol causes germinal vesicle migration to the periphery of the oocyte, which is usually followed by spawning. Unlike the prolonged period of vitellogenesis, oocyte maturation is of short duration, less than one day in cyprinids, and is very susceptible to pollution, especially in systems where it may correspond with a surge in pollutant concentration from a closely adjacent contaminated source (Kime, 1995). In SW1 and 4, it is possible that petroleum hydrocarbon contaminant levels were such that they impaired germinal vesicle migration

and consequently, the females retained mature eggs and did not spawn.

The significantly lower GSI for male fish taken from SW4 in 1996 than GSI calculated for fish from R3 is consistent with the literature. Payne *et al.* (1978), Fletcher *et al.* (1982) and Kime (1995) reported a consistent tendency for testes to be smaller in oil exposed fishes than in controls. Furthermore, significantly lower male GSIs, as a result of exposure to petroleum and various pollutants, were also reported herein. This observation supports the hypothesis that the GSIs of male pearl dace exposed to petroleum hydrocarbon contamination represent a beneficial and reliable bioindicator (Payne *et al.*, 1978).

The fact that no pearl dace were captured in SW2 in 1995 and that the number of locations with no pearl dace in 1996 increased three-fold (from SW2 in 1995 to SW1, 2 and 3 in 1996) provides strong evidence that indicates complete reproductive failure and demise of the pearl dace populations in the Spring Gulch Wetlands. That the male and female GSI values for both sample years did not corroborate each other only highlights the differences when sampling at different times in successive years. Moreover, this difference could have been a function of the limited availability of pearl dace for sampling in 1996 and, thus, further highlights the demise of the populations annually.

4.4 MFO Induction

Because environmental toxicants are present in sub-lethal concentrations, there is increasing use of sub-cellular biochemical indicators to predict population stresses arising

from chronic exposure to a given toxicant (Larsson *et al.*, 1985). The earliest biological indicators of toxicant-induced stress are usually shifts or changes in biochemical systems (Nriagu, 1988). In fish, the liver is important in many aspects of nutrition and energy storage (lipid and glycogen), but it also acts as the primary site for the detoxification of xenobiotics, through the induction of the MFO system. Validity of the MFO system to evaluate absolute contamination of a location has been documented (Jimenez and Stegeman, 1990). Since the mid-1970s, induction of the MFO system has been used as a method for determining whether xenobiotics, especially petroleum hydrocarbons, are affecting fish species inhabiting aquatic ecosystems (Payne, 1977; 1984; Payne *et al.*, 1984; 1987; 1995; Khan and Payne, 2001). With respect to petroleum hydrocarbon contamination, MFO enzymes have played a critical role in detoxification by conducting a series of oxidation reactions converting relatively insoluble organic compounds into forms which are substantially more water soluble and therefore more easily excreted (Payne, 1984).

In this present study, mixed function oxygenase activity (induction of 7-EROD) results partially corroborated the findings of other bioindicators, including population analysis, body condition factor and histopathological findings (liver, gills, spleen and gonads) of fish sampled at impacted sites in 1995 and 1996 when compared against reference samples. Specifically, hepatic biochemical analysis of female pearl dace indicated significant decreases in MFO activity (induction of 7-EROD) in pearl dace taken from SW1, 3 and 4 in 1995 and significant increases in fish from SW4 in 1996. In 1995 pearl

dace taken from SW1, 3 and 4, induction of 7-EROD was significantly lower than in fish sampled from the reference site.

These conflicting SW4 7-EROD results remain enigmatic but Khan (1999b) reported no differences in 7-EROD activities between fish sampled at SW4 and the reference site. It is evident, however, that 7-EROD activity was induced in pearl dace inhabiting the Spring Gulch Wetlands, especially samples originating from SW4 in 1996. It is likely that the level of MFO induction in the SW4 samples in 1995 was impaired by the levels of circulating estrogenic hormones as reported by Jimenez and Stegeman (1990). Additional studies on the induction of 7-EROD activity in both male and female pearl dace at impacted and reference sites are a prerequisite for clarifying these conflicting results.

Conclusions

While direct measurement of petroleum hydrocarbons by chemical analysis provides useful information on the extent of contamination, it gives no indication of the biological effects on living organisms. The integrated use of biomarkers in the present study provides a direct evidence of the impact of petroleum hydrocarbons on pearl dace inhabiting SW1, 3 and 4 of the Spring Gulch Wetlands at CFB Goose Bay, Labrador. The induction of 7-EROD activity in samples from SW4 in 1996 and the significant differences from the reference sites is indicative of exposure to organic compounds. Condition (K) factor, a measure of the nutritive status of fish (Saborowski and Buckholz, 1996) exhibited distinct trends depending on the stage of vitellogenesis, high until prior to reproduction and low after spawning. Gonadosomatic indices exhibited a relationship corresponding to the reproductive status of the various groups. Similarly, histopathological lesions were more pronounced in the impacted fish then samples from reference sites. Based on the population structure, which exhibited a predominance of females over males and an absence of juveniles, sampled at SW4, it is suggested that this population faces extinction.

Following analysis and discussion of the results the subsequent conclusions can be presented:

i. Fish were not present in SW2 over the two years of this study and the sudden disappearance of pearl dace from SW1 and 3 between September 1995 and July 1996, with further investigation, may provide significant

evidence of the extinction of pearl dace populations as a consequence of chronic petroleum hydrocarbon impact.

ii. Petroleum hydrocarbon contaminant levels in SW1, 3 and 4 impaired germinal vesicle migration. Females retained mature eggs and did not spawn; thus, GSI values were significantly greater as late into the season as September 01, which indicated that the females were reproductively delayed.

iii. The significant differences in the GSIs of male pearl dace exposed to petroleum hydrocarbon contamination and reference samples is evidence of disruption of gonadal development.

iv. The 1996 pearl dace population composition in reference site 1 indicated that the population was in a spawning configuration. This observation provided strong evidence that pearl dace in CFB Goose Bay spawned over a full month later than information is published in the literature.

v. Considering the higher latitude and correspondingly cooler water temperatures surrounding CFB Goose Bay, pearl dace were at the most northern limit of their distribution.

vi. The conditions at SW1, 3 and 4 permitted ample growth and survival to a greater age of an already mature fish population, resulting in higher condition factors.

vii. Destruction of erythrocytes probably resulted in higher levels of

hemoglobin catabolism, and consequently significantly more and greater distributions of hemosiderin in the spleen of pearl dace at SW1, 3 and 4.

viii. Splenic melanomacrophage aggregates (containing and not containing pigment) and higher concentrations of hemosiderin in fish were characteristic of pearl dace from the impacted sites versus fish from reference locations.

ix. An increase in the lipid accumulation within the hepatocytes of pearl dace from petroleum hydrocarbon impacted locations when compared to fish sampled from non-impacted sites is a result of bioaccumulation of petroleum hydrocarbons and/or its metabolites. This response is a useful biomarker for exposure to petroleum hydrocarbon contamination.

x. The higher prevalence of moderate and severe lamellar hyperplasia in fish from SW1, 3 and 4 over both years when compared to pearl dace from the reference locations is a useful biomarker for chronic exposure to contaminants.

xi. 7-ERCD activity was induced in pearl dace inhabiting SW4 in the Spring Gulch Wetlands in 1996.

Future study, over a longer time period, again using an integrated battery of biomarkers, is required to fully investigate the hypothesized demise of the pearl dace population in the Spring Gulch Wetlands of CFB Goose Bay, Labrador due to chronic, sub-lethal, effects of petroleum hydrocarbon contamination. Based on the absence of

juvenile fish, high prevalence of tissue lesions, delay in seasonal maturity and the increasing extinction of fish from SW1, 2 and 3 over a two-year period suggests that the pearl dace population at SW4 also faces eventual extinction.

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Appendix

Appendix 1: Conventional tissue section processing schedule to wax.

1. 70% ethanol for 1 hour
2. 70% ethanol for 1 hour
3. 95% ethanol for 1 hour
4. 95% ethanol for 1 hour
5. Absolute ethanol for 1 hour
6. Absolute ethanol for 1 hour
7. 50:50 Absolute ethanol : cedar wood oil for 2 hours
8. cedar wood oil 2 hours at 60^0C
9. 50:50 cedar wood oil : wax 2 hours at 60^0C
10. wax for 2 hours at 60^0C
11. wax for 2 hours at 60^0C
12. embed

Appendix 2: Chemicals

All reagents used in these studies were of standard chemical grade obtained from various suppliers. The following chemicals are not common and are therefore listed below for reference:

a. 7-Ethoxyresorufin (150μM): Pierce Chemical Co., Rockford Illinois

b. Resorufin, practical: Kodak Ltd., Rochester, New York

c. B-Nicotinamide Adenine Dinucleotide Phosphate (NADPH), reduced form

d. methanol (HPLC Grade): Fisher Scientific, Montreal, Quebec

e. 2% Na-K tartrate

f. 2N Folin-Phenol Ciocalteau reagent

Appendix 3: BSA Standard Curve

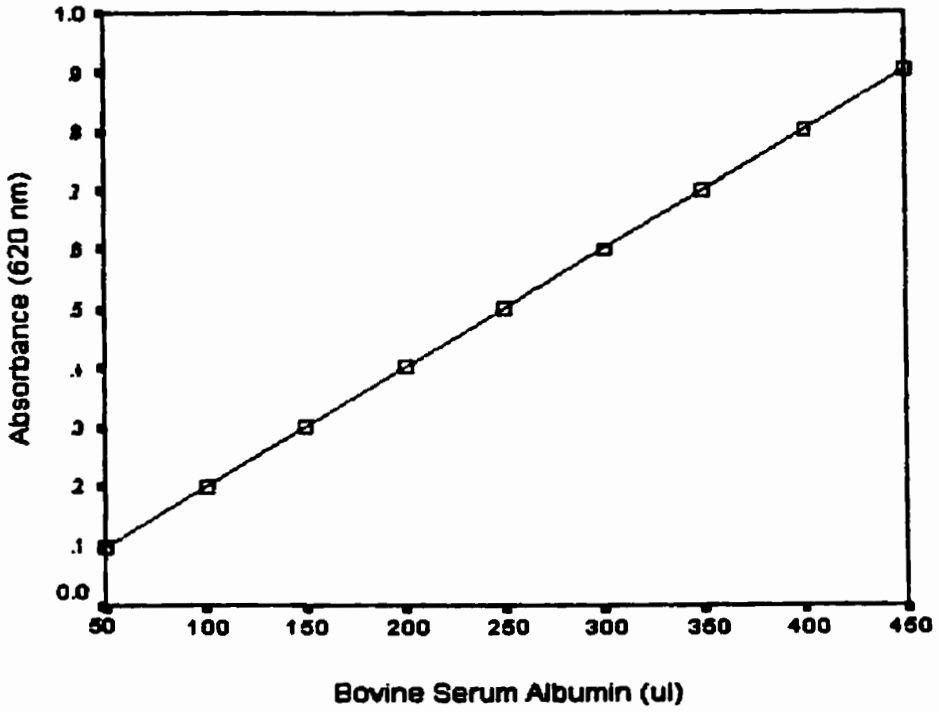

Figure A.1. Standard curve of Bovine Serum Albumin (BSA; Lowry Protein Determination) for 7-Ethoxy resorufin-O-deethylase (EROD) assay.