

**RECOMMENDED PROTOCOLS FOR  
FISH PATHOLOGY STUDIES  
IN PUGET SOUND**

For

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## INTRODUCTION

The kinds of pathological abnormalities measured most commonly in Puget Sound organisms are hepatic (i.e., liver) lesions in fishes, primarily English sole, *Parophrys vetulus*. These abnormalities are important for assessing the biological effects of chemical contamination, because numerous field and laboratory studies have established a putative cause/effect relationship between the prevalence of hepatic lesions and exposure of fishes to toxic chemicals. Although a variety of other pathological conditions has been found in Puget Sound organisms (e.g., fin erosion, skeletal anomalies, epidermal papillomas, parasites), none of these conditions has been linked to chemical contamination as strongly as hepatic lesions in fishes.

Given the importance of hepatic lesions in fishes for evaluating environmental contamination, the primary focus of this document is to recommend methods for conducting field surveys of fish liver histopathology in Puget Sound. These methods are based on a general review of the literature regarding hepatic lesions in feral fishes, as well as the results of a workshop and written reviews by representatives from most organizations that fund or conduct fish liver histopathology studies in the Sound (Table I). The purpose of developing these recommended protocols is to encourage all Puget Sound investigators conducting monitoring programs, baseline surveys, and intensive investigations to use standardized methods whenever possible. If this goal is achieved, most data collected in the Sound should be directly comparable, and thereby capable of being integrated into a Sound-wide database. Such a database is necessary for developing and maintaining a comprehensive water quality management program for Puget Sound.

Although the following protocols are recommended for most studies conducted in Puget Sound, departures from these methods may be necessary to meet the special requirements of individual projects. If such departures are made, however, the funding agency or investigator should be aware that the resulting data may not be comparable with most data of that kind. In some instances, data collected using different methods may be compared if the methods are intercalibrated adequately.

Because the use of fish pathology as a quantitative field assessment tool is a relatively new endeavor, the loose-leaf format of this chapter ensures that the recommended protocols can be modified as new information is collected. In addition, this format will allow protocols to be included in the future for pathological conditions other than hepatic lesions.

Before recommendations are made for field studies of fish liver histopathology in Puget Sound, historical information regarding the general subject is reviewed. Included are reviews of

hepatocarcinogenesis models for fishes and results of laboratory and field studies. The review of historical information will acquaint non-pathologists with the subject. In addition, it formed the basis for many of the recommended protocols for Puget Sound.

TABLE 1. CONTRIBUTORS TO THE FISH PATHOLOGY PROTOCOLS

Name	Organization
John Armstrong	U.S. EPA
Scott Becker	Tetra Tech
Ralph Elston	Battelle
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## REVIEW OF HISTORICAL DATA

In this section, historical laboratory and field studies of fish liver histopathology are reviewed. Many of the concepts and patterns described in these sections were used to develop the recommended protocols for field studies of fish liver histopathology.

### HEPATOGENESIS MODELS FOR FISHES

Two models of hepatocarcinogenesis in fishes have been proposed in the literature. The first is based on laboratory studies of rainbow trout, and the second is based on field studies of English sole. These two models are the most detailed ones available for fishes, and both were derived from extensive amounts of empirical data.

The most complete description of and nomenclature for the sequential cellular alterations involved in animal hepatocarcinogenesis are for rats and mice (e.g., Squire and Levitt 1975; Frith and Ward 1980; Stewart et al. 1980). By comparison, fish hepatocarcinogenesis studies are in their infancy (Hendricks 1982). Although many of the principles and much of the nomenclature used in rat studies have been applied to fish studies, the degree to which hepatocarcinogenic processes in rats are analogous to those in fishes is unknown.

#### Rainbow Trout

The species of fish most studied with respect to chemically induced hepatic neoplasms is the rainbow trout. The chemicals used most often to induce hepatic neoplasms in this species are aflatoxins (primary aflatoxin B<sub>1</sub> or AFB<sub>1</sub>), a group of potent carcinogens produced by the mold *Aspergillus flavus*. The relatively large amount of information available for this species has been synthesized by Sinnhuber et al. (1977), Hendricks (1982), and Hendricks et al. (1984). Because most studies have focused primarily on the mere presence of hepatic neoplasms rather than their developmental processes, the pathogenesis of liver cancer in rainbow trout is not well-documented. However, as more information is available for this species than for any other fish, it is instructive to review the available data.

In rainbow trout, the morphologic stages involved in hepatocarcinogenesis are as follows:

- Pale, swollen, individual cells with enlarged pleomorphic nuclei
- Eosinophilic foci

- Basophilic foci
- Hepatocellular carcinomas.

However, the sequential nature of these stages has not been confirmed (Sinnhuber et al. 1977).

The enlarged cells of the first stage undergo degeneration and necrosis, but do not form nodules of proliferating cells. Sinnhuber et al. (1977) suggest that the toxic influence of the carcinogen interferes with normal cell functions and division, thereby producing a polyploid, hypertrophic cell that eventually dies. The number of affected cells increases with increasing doses of aflatoxin. Islets of regenerating cells frequently are found in livers with degenerating cells, but their role in hepatocarcinogenesis is unknown.

Eosinophilic foci generally are small (i.e., <0.5-mm diameter). Cells within these foci have relatively normal nuclei, but are distinctly eosinophilic, hypertrophic, and devoid of glycogen. Mitotic figures are rare, and the cells do not compress surrounding tissue. The eosinophilia results primarily from extensive proliferation of SER. Although the role of eosinophilic foci in hepatocarcinogenesis is uncertain, there is evidence that these foci may give rise to basophilic foci. There is also evidence that one route to neoplastic transformation may begin with the eosinophilic stage progressing to the basophilic stage. Observations in more recent studies demonstrate that eosinophilic foci frequently are invaded by lymphocytes, resulting in varying degrees of cytotoxic effects. Because this apparent host-immune response presumably destroys the altered cells, Sinnhuber et al. (1977) believe that the contribution of eosinophilic foci to neoplasm development in rainbow trout is minimal. According to Hendricks et al. (1984), it is doubtful that eosinophilic foci contribute significantly to the carcinogenic process.

Basophilic foci vary in size from clusters of several cells to clusters several millimeters in diameter. The cells are small, unencapsulated, intensely basophilic, and deficient in glycogen. Nuclei are slightly enlarged and vesicular and nucleoli are prominent. Cells usually exhibit some mitotic activity, but do not compress surrounding tissue. The basophilia results from extensive granular endoplasmic reticulum and free ribosomes. Basophilic foci frequently are surrounded by normal cells and display no indication of a prior eosinophilic stage. Basophilic foci rarely elicit a host immune response. Although the role of basophilic foci in trout hepatocarcinogenesis is not firmly established, Sinnhuber et al. (1977) believe that the principal route of neoplastic transformation begins directly at the basophilic stage.

Hendricks et al. (1984) suggest that basophilic foci may be considered microcarcinomas or carcinomas in situ, because the only distinguishing characteristics between the two kinds of hepatic

lesion are size and degree of compression of surrounding tissue. As with basophilic foci, cells of hepatocellular carcinomas are intensely basophilic, mitotically active, devoid of glycogen, and grouped into cords several cells in thickness. In rats and trout, the appearance of the basophilic cell type signifies that neoplastic transformation is complete (Sinnhuber et al. 1977; Hendricks et al. 1984).

Although many authors distinguish adenomas from carcinomas on the basis of degree of differentiation and presence or absence of metastases, Sinnhuber et al. (1977) suggest that the potential for malignant behavior is present in all trout tumors, and may occur given sufficient time. They therefore recommend that all tumors induced by aflatoxin in rainbow trout be classified as hepatocellular carcinomas.

### English Sole

Myers et al. (1987) provide the first comprehensive documentation of close morphological similarities between idiopathic hepatic lesions in a feral fish and the established series of lesions induced in rodents following laboratory exposure to hepatocarcinogens. The study was conducted on English sole collected from Eagle Harbor, Washington. The sediments in Eagle Harbor are contaminated with a variety of hepatocarcinogens (particularly creosote-derived aromatic hydrocarbons), and prevalences of hepatic neoplasms and other liver abnormalities are among the highest found in English sole from any location in Puget Sound (Malins et al. 1985b; see section entitled Field Studies).

Myers et al. (1987) identified statistically significant associations between a variety of lesion types based on their patterns of co-occurrence. The authors assumed that co-occurring lesions may be caused by similar etiological agents and that these lesions may be temporally related to each other in terms of their development. A temporal relationship implies that the lesions may be induced in a sequence of progression that terminates with hepatic neoplasms. The authors also compared the lesions they observed in feral English sole, with similar lesions found by others in rodents and rainbow trout following controlled laboratory exposure to hepatocarcinogens. Myers et al. (1987) caution that although their results are based on strong circumstantial evidence, conclusive proof of the hepatocarcinogenesis model for English sole must await carefully controlled field or laboratory experiments.

Myers et al. (1987) identified the following major hepatic lesions that are thought to be related to or associated with the histogenesis of liver neoplasms in English sole:

- Nonspecific necrotic lesions
  - Hepatocellular coagulation necrosis
  - Liquefactive necrosis
  - Hydropic degeneration
  - Pyknosis
  - Hyalinization
  - Cystic parenchymal degeneration
  
- Specific degenerative conditions
  - Nuclear pleomorphism
  - Megalocytic hepatitis
  
- Nonneoplastic proliferative conditions
  - Nonhyperplastic hepatocellular regeneration
  
- Foci of cellular alteration
  - Eosinophilic foci
  - Basophilic foci
  - Clear cell or vacuolated cell foci
  - Hyperplastic regenerative foci
  
- Neoplasms
  - Liver cell adenomas
  - Hepatocellular carcinomas
  - Cholangiomas
  - Cholangiocellular carcinomas
  - Mixed carcinomas.

Although, nonspecific necrotic lesions are known to be caused by a variety of agents, Myers et al. (1987) excluded those lesions closely associated with visible infectious agents. The necrotic lesions reported by Myers et al. (1987) generally exhibited focal or multifocal distributions and rarely were found in a large proportion of any liver. These lesions frequently were accompanied by hemorrhage, fibrinization, mononuclear infiltrates, fibroplasia, and increased density of melanomacrophage centers.

The two specific degenerative conditions affected only hepatocytes, were diffusely distributed in nonzonal patterns, and occurred in the absence of cellular infiltrate. Nuclear pleomorphism was characterized by nuclei of various size and chromatin distribution/content. Aside from those aber

rations, hepatocytes with nuclear pleomorphism exhibited a normal appearance. Megalocytic hepatosis was characterized primarily by enlargement of both nuclear and cellular diameters and atypical distributions or densities of chromatin within vesicular nuclei.

Nonhyperplastic hepatocellular regeneration was the only nonneoplastic proliferative condition found that is thought to play a role in hepatocarcinogenesis in English sole. Although a second nonneoplastic proliferative condition (e.g., cholangiofibrosis) was found, Myers et al. (1987) concluded that it probably was not involved in the progression toward neoplasia. Nonneoplastic hepatocellular regeneration ranged in appearance from the undifferentiated morphology to the later stages of parenchymal replacement characterized by maturing, more differentiated hepatocytes.

Foci of cellular alteration were similar to the lesions in rats and mice that are thought to be precursors of neoplasms. Each type exhibited a distinct pattern of alteration, and was arranged in discrete micronodular foci. The borders of the foci blended indistinctly into the surrounding muralia and compression of adjacent parenchyma was minimal or absent.

Eosinophilic foci ranged from 0.1 to 0.9 mm in diameter, and were characterized primarily by slight to dramatic cellular hypertrophy, increased cytoplasmic eosinophilia with a granular texture, and varying degrees of nuclear pleomorphism. Basophilic foci ranged from 0.1 to 0.8 mm and were characterized primarily by hyperbasophilic cytoplasm in normal-sized hepatocytes with pleomorphic nuclei. Clear cell or vacuolated cell foci were smaller than the former two lesions (i.e., <0.4 mm) and were characterized by hepatocytes with either a vacuolated cytoplasm or a lacy, flocculent, poorly stained cytoplasm. Alterations of nuclei were minimal. Hyperplastic regenerative foci also were relatively small (i.e., 0.05-0.3 mm). In addition, these foci were hyperplastic and characterized by regenerative hepatocytes that exhibited reduced size and increased basophilia. Prevalence of hyperplastic regenerative foci were rare compared to prevalences of the other three kinds of foci of cellular alteration.

Neoplasms included those of hepatocellular (i.e., liver cell adenoma, hepatocellular carcinoma) and biliary (i.e., cholangioma, cholangiocellular carcinoma) origin. One kind of neoplasm included both hepatocellular and cholangiocellular elements (i.e., mixed carcinoma). Of these five kinds of neoplasm, liver cell adenomas and cholangiomas are considered benign, whereas the remaining three neoplasms are considered malignant.

As mentioned previously, the hepatocarcinogenesis model proposed by Myers et al. (1987) for English sole was based primarily on statistical associations among lesions and comparisons with similar lesions founds in laboratory studies of rodents and rainbow trout. Myers et al. (1987) propose the following sequence of events for the histogenesis of hepatocellular neoplasms in English sole:

- Nonspecific necrotic lesions and specific degenerative conditions appear as the initial, subchronic to chronic hepatocellular lesions manifesting the cytotoxic effects of exposure to hepatocarcinogens. These conditions provide the proper stimulus for a compensatory, regenerative, proliferative response.
- In the above environment favoring proliferation, foci of cellular alteration can develop. Because these foci are selectively resistant to the cytotoxic effects of carcinogens, they have a growth advantage over normal hepatocytes.
- Autonomous, neoplastic hepatocytes arise from some of the nonautonomous foci of cellular alteration. This transformation may occur by a complex multistep process of mutation followed by selection.

Myers et al. (1987) note that the pattern of histogenesis of biliary neoplasms in English sole presently is unclear.

## LABORATORY STUDIES

A relatively large number of chemicals has been found to induce hepatic lesions in various fishes following controlled laboratory exposure. As part of this project, historical information regarding laboratory induction of hepatic lesions in fishes was reviewed. Information was collected from review articles by Matsushima and Sugimura (1976), Myers and Hendricks (1982), and Couch and Harshbarger (1985), and from the results of additional laboratory studies conducted between 1981 and 1985 (Arrillo et al. 1982; Dalich et al. 1982; Goodman et al. 1982; Larsson and Haux 1982; Schultz and Schultz 1982a,b; Solangi and Overstreet 1982; Katti and Sathyanesan 1984; Kumar and Pant 1984; Park and Kim 1984; Lowe-Jinde and Niimi 1984; Rhodes et al. 1985; Wester et al. 1985).

The 87 chemicals considered in the literature review are summarized in Table 2. The chemicals are grouped according to the general scheme of Meyers and Hendricks (1982), to facilitate interpretation by environmental managers. As noted previously, all of the chemicals have induced hepatic lesions in fishes. These chemicals represent a wide variety of natural and anthropogenic products, including pesticides, fossil-fuel related compounds, chemotherapeutic agents, mycotoxins, plant derivatives, nitrogenous compounds, and inorganic compounds. Twenty-six (30 percent) of these chemicals have induced hepatic neoplasms in one or more species of fish. The major groups of chemicals having the highest percentages of hepatocarcinogens include mycotoxins (100 percent), nitroso- compounds (100 percent), miscellaneous nitrogenous compounds (75 percent), and plant derivatives (60 percent). Sixty-one (70 percent) of the chemicals listed in Table 2

have not induced hepatic neoplasms in fishes. Major groups having no apparent hepatocarcinogens include organochlorine herbicides, organophosphate insecticides, carbamate insecticides, miscellaneous herbicides, miscellaneous organic compounds, and inorganic compounds. Although these latter 61 chemicals have not induced neoplasms, they have induced other kinds of hepatic lesions in fishes and may be capable of inducing lesions under different sets of test conditions (e.g., different test species, different exposure routes, higher chemical concentrations, longer test durations).

Most of the fish species in which hepatic lesions (i.e., neoplasms and other kinds) have been induced by laboratory exposure to chemicals are listed in Table 3. This list represents a broad taxonomic spectrum, and includes 39 species from 20 families. The family Salmonidae is represented by the largest number of species (i.e., seven). The species used most frequently in laboratory tests have been rainbow trout, guppy, coho salmon, and zebra fish.

TABLE 2. CHEMICALS THAT HAVE INDUCED HEPATIC LESIONS  
IN FISHES FOLLOWING LABORATORY EXPOSURE<sup>a</sup>

	Number of Species Affected <sup>b</sup>	
	Neoplasms <sup>c</sup>	Other Lesions <sup>d</sup>
<u>Organochlorine insecticides</u>		
Chlordane	-	1
DDT	1	7
Dieldrin	-	>5
Endosulfan	-	1
Endrin	-	6
Heptachlor	-	3
Hexachlorocyclohexane (beta isomer, lindane byproduct)	-	1
Kepone	-	1
Lindane	-	3
Methoxychlor	-	3
Toxaphene	-	1
<u>Organochlorine herbicides</u>		
Dichlobenil	-	1
Dowicide G	-	1
2,4-D	-	3
Kuron (silvex)	-	1
Tordon 101 (picloram and 2,4-D as amine salts)	-	1
Tordon 22K (picloram, potassium salt)	-	1
<u>Industrial organochlorine compounds</u>		
PCB-Aroclor 1248	-	1
PCB-Aroclor 1254	-	4
PCB-Miscellaneous	-	3
Carbon tetrachloride	1	3
Monochlorobenzene	-	1
<u>Organophosphate insecticides</u>		
Abate (temphos)	-	1
Diazinon (Spectracide)	-	1
Dimethoate (Cygon)	-	1
Dursban (chlorpyrifos)	-	1
Dylox (trichlorfon)	-	1
Malathion	-	3
Methyl parathion	-	2

TABLE 2. (Continued)

	Number of Species Affected	
	Neoplasms	Other Lesions
<u>Carbamate insecticides</u>		
Aldicarb (Temik)	-	1
Carbaryl (Sevin)	-	3
Propoxur (Baygon)	-	1
<u>Miscellaneous herbicides</u>		
Acrolein	-	1
Amitrole-T	-	1
Dinoseb	-	1
Diquat	-	1
Hydrothol 191	-	1
Paraquat-CL	-	1
<u>Fossil-fuel related compounds</u>		
Benzo(a)pyrene (BaP)	1	1
Crude oil-whole	-	3
Crude oil-water soluble fraction	-	2
7-12 Dimethylbenz(a)anthracene (DMBA)	2	2
Oiled sediments	-	1
<u>Chemotherapeutic agents</u>		
Copper sulfate	-	3
Diethylstilbestrol (DES)	1	1
Sulfamethazine	-	1
Thiabendazole	-	1
<u>Mycotoxins</u>		
Aflatoxin B <sub>1</sub> (AFB <sub>1</sub> )	5	5
Aflatoxin G <sub>1</sub> (AFG <sub>1</sub> )	2	2
Aflatoxin M <sub>1</sub> (AFM <sub>1</sub> )	1	1
Aflatoxin Q <sub>1</sub> (AFQ <sub>1</sub> )	1	1
Aflatoxicol (AFL)	1	1
Ochratoxin A + B	1	1
Sterigmatocystine	3	3
Versicolorin A	1	1
<u>Plant derivatives</u>		
Cycad nut meal	3	3
Cycasin	-	1
Cyclopropenoid fatty acids (CPFA)	1	1
Gossypol	-	1
Methylazoxymethanol acetate (MAMA)	2	2

TABLE 2. (Continued)

	Number of Species Affected	
	Neoplasms	Other Lesions
Pyrrolizidine alkaloids	-	1
Tannic acid	1	1
<u>Nitroso- compounds</u>		
N,N'-dinitrosopiperazine (DNP)	1	1
N-nitrosodiethylamine (DEN)	7	7
N-nitrosodimethylamine (DMN)	3	3
N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)	1	1
N-nitrosomorpholine (NM)	2	2
<u>Miscellaneous nitrogenous compounds</u>		
2-Acetylaminofluorene (2-AAF)	3	3
o-Aminoazotoluene (o-AAT)	4	4
Ammonia	-	2
Benzidine	-	1
Carbazone	1	1
p-Dimethylaminoazobenzene (DAAB)	3	5
Thiourea	1	1
Urethane	1	1
<u>Miscellaneous organic and organometallic compounds</u>		
Bis(tri-n-butyltin) oxide	-	1
Dimethylsulfoxide (DMSO)	-	4
Methylmercuric chloride	-	2
4-Nitro-3-(trifluoromethyl)phenol	-	1
Phenol	-	1
<u>Inorganic compounds</u>		
Cadmium chloride	-	8
Cupric chloride	-	1
Cupric sulfate	-	1
Disodium arsenate	-	1
Lead nitrate	-	1
Mercuric chloride	-	3
Sodium arsenite	-	1

TABLE 2. (Continued)

**a** Chemicals are grouped according to the general scheme used by Meyers and Hendricks (1982), to facilitate interpretation by environmental managers.

**b** Note that the values represent the number of unique species, not the number of laboratory studies conducted.

**c** Any kind of hepatic neoplasm.

**d** All kinds of hepatic lesions except neoplasms. In studies where neoplasms were induced, other kinds of lesions rarely were reported by the authors. For the purposes of this table, it was assumed that other kinds of lesions were present in all studies in which neoplasms were induced.

Hepatic neoplasms have been induced in 8 of the 39 species (20.5 percent) listed in Table 3 (each denoted by an asterisk). These species include all three poeciliids (i.e., guppy, two topminnows), two of three cyprinodontids (i.e., sheepshead minnow, rivulus), two of seven salmonids (i.e., sockeye salmon, rainbow trout), and one of five cyprinids (i.e., zebra fish).

Couch and Harshbarger (1985) summarized the various amounts of time required for initial formation of hepatic neoplasms in a variety of fishes exposed to a variety of carcinogenic chemicals. The times to first neoplasm for all 105 fish/chemical combinations included in Couch and Harshbarger (1985) are presented in Figure 1. Some of these times probably are overestimates, because fish were not examined until the experiments were terminated. In 59 cases (56.2 percent), hepatic neoplasms were induced within 6 mo of exposure to the carcinogen. In 98 cases (93.3 percent), hepatic neoplasms were induced within 1 yr of exposure.

Direct extrapolation of laboratory results to field conditions are difficult to make (e.g., Johnson and Bergman 1984). In many cases, the species used for laboratory tests are selected because they are known to be very sensitive to hepatocarcinogens. In addition, the contaminant concentrations to which fishes are exposed in many laboratory studies are much higher than most observed concentrations in the environment. Finally, the duration of contaminant exposure in laboratory studies often exceeds that which might be expected under natural conditions. Despite these limitations, laboratory results may be useful as estimates of the worst-case conditions that may be encountered in the environment.

With the above caveats in mind, several patterns identified in laboratory studies have implications for interpreting the results of field studies. First, controlled laboratory studies demonstrate unequivocally that many contaminants found in the environment can induce the same kinds of hepatic lesions as those found in feral fishes from polluted habitats. This demonstration is essential for supporting the hypothesis that lesions observed in feral fishes are the result of chemical contamination. It does not, however, discredit the alternative hypotheses that lesions are induced by other agents (e.g., nutritional imbalances, viruses).

A second laboratory result with field implications is the fact that similar kinds of hepatic lesions in fishes have been induced by a wide variety of chemical contaminants. Although many of these lesions are thought to be indicative of toxic effects, their general nonspecificity makes diagnosis of a single causative agent difficult, if not impossible (e.g., Meyers and Hendricks 1982). This nonspecificity is extended to field studies by the observation of Harshbarger (1977) that nearly every kind of neoplasm (i.e., hepatic and others) found in fishes currently was known prior to 1940. This lack of differences has been maintained despite the large increase in quantity and variety of toxic chemicals to which fishes have been exposed since 1940. It therefore is highly unlikely that specific types of hepatic neoplasms in feral fishes can be used to identify definitively their causative agents.

A third laboratory result with field implications is the fact that hepatic neoplasms have been induced in certain fishes in time intervals shorter than 6 mo. Thus, even if a particular fish visits a contaminated site once and for a relatively short period of time, there is the possibility that hepatic lesions, including neoplasms, could be induced if the fish is suitably sensitive and if the contaminant concentrations in the environment are suitably high.

TABLE 3. SPECIES IN WHICH HEPATIC LESIONS HAVE BEEN  
INDUCED FOLLOWING LABORATORY EXPOSURE TO CHEMICALS<sup>a</sup>

<b>Family</b>	<b>Scientific Name</b>	<b>Common Name<sup>b</sup></b>
Petromyzonidae	<i>Petromyzon marinus</i>	Lamprey
	<i>Oncorhynchus kisutch</i>	Coho salmon
Salmonidae	<i>Oncorhynchus nerka</i>	Sockeye salmon*
	<i>Oncorhynchus tshawytscha</i>	Chinook salmon
	<i>Salmo clarki</i>	Cutthroat trout
	<i>Salmo gairdneri</i>	Rainbow trout*
	<i>Salmo trutta</i>	Brown trout
	<i>Salvelinus namaycush</i>	Lake trout
Cyprinidae	<i>Barbus conchoniuis</i>	<b>c</b>
	<i>Carassius auratus</i>	Goldfish
	<i>Cyprinus carpio</i>	Carp
	<i>Danio (Brachydanio) rerio</i>	Zebra fish*
	<i>Rhodeus amarus</i>	Bitterling
Heteropneustidae	<i>Heteropneustes fossilis</i>	<b>c</b>
Ictaluridae	<i>Ictalurus punctatus</i>	Channel catfish
Clariidae	<i>Clarius batrachus</i>	Walking catfish
Batrachoididae	<i>Halobatrachus didactylus</i>	Sapo
Oryziidae	<i>Oryzias latipes</i>	Medaka
Cyprinodontidae	<i>Cyprindon variegatus</i>	Sheepshead minnow*
	<i>Fundulus heteroclitus</i>	Mummichog
	<i>Rivulus marmoratus</i>	Rivulus*
Poeciliidae	<i>Peocilia (Lebistes) reticulata</i>	Guppy*
	<i>Poeciliopsis lucida</i>	Topminnow*
	<i>Poeciliopsis monacha</i>	Topminnow*
Atherinidae	<i>Menidia beryllina</i>	Inland silverside
Gasterosteidae	<i>Gasterosteus aculetus</i>	Threespine stickleback

TABLE 3. (Continued)

Family	Scientific Name	Common Name <sup>b</sup>
Channidae	<i>Channa punctatus</i>	Asian catfish
Centropomidae	<i>Dicentrarchus labrax</i>	Robalo
Centrarchidae	<i>Lepomis cyanellus</i>	Green sunfish
	<i>Lepomis macrochirus</i>	Bluegill
	<i>Lepomis microlophus</i>	Redear sunfish
Sciaenidae	<i>Leiostomus xanthurus</i>	Spot
Mugilidae	<i>Mugil auratus</i>	Lisa
Anabantidae	<i>Trichogaster fasciatus</i>	<b>c</b>
Pleuronectidae	<i>Parophrys vetulus</i>	English sole
	<i>Platichthys flesus</i>	Flounder
	<i>Pseudopleuronectes americanus</i>	Winter flounder
Soleidae	<i>Trinectes maculatus</i>	Hogchoker

**a** This list is based on the studies reported in Table A-1 (Appendix A).

**b** Species in which some kind of hepatic neoplasm has been induced in a laboratory study are denoted by an asterisk(\*).

**c** Common name not found.

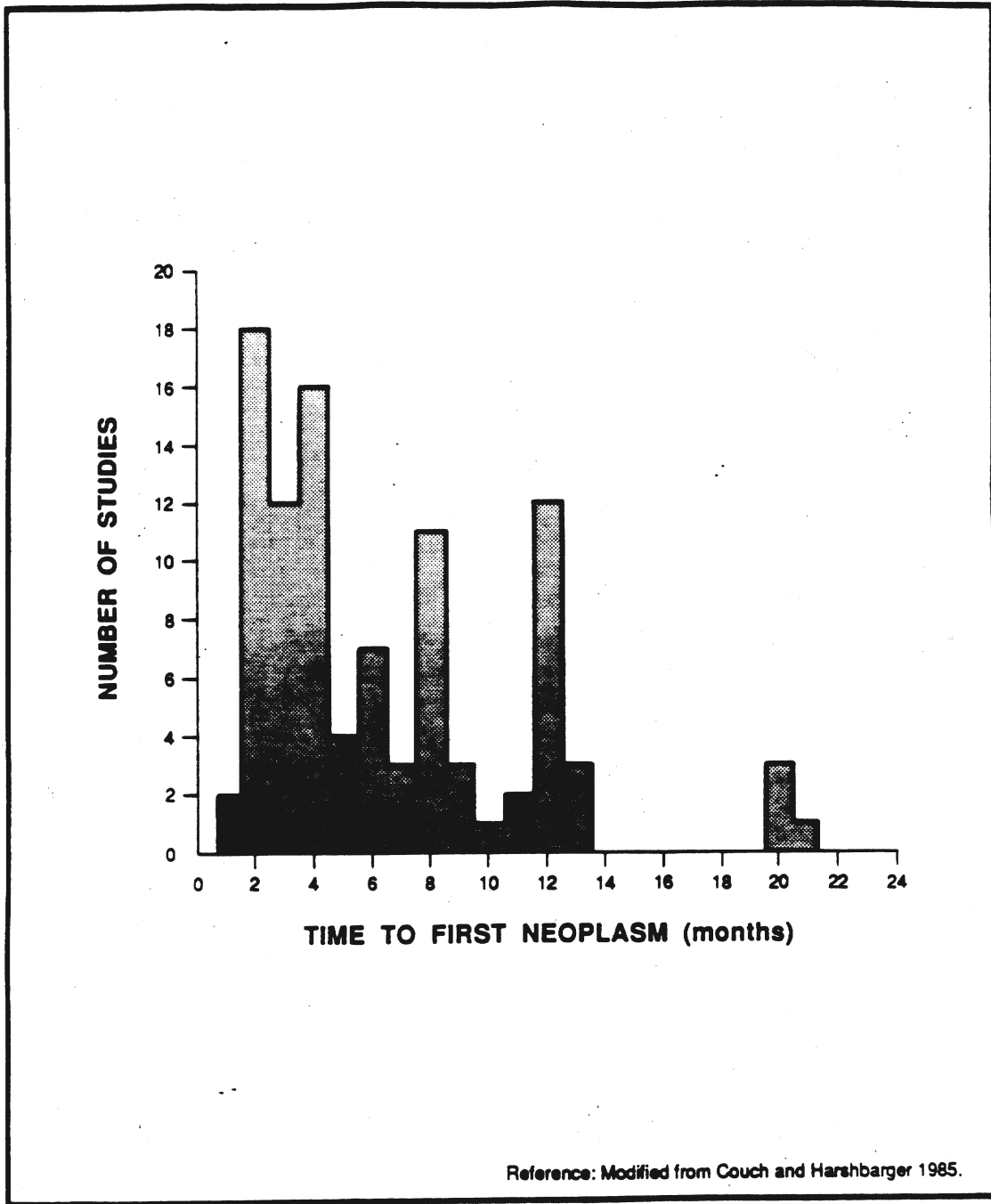


Figure 1. Distribution of times to first neoplasm for a variety of fishes exposed to a variety of chemicals in the laboratory

## FIELD STUDIES

Most field studies of hepatic lesions in fishes from contaminated environments have been conducted within the last 10 yr. However, this does not necessarily mean that these lesions were not present prior to the mid-1970s. The occurrence of hepatic lesions in many fishes initially was discovered inadvertently as specimens were being evaluated for other purposes (e.g., Falkmer et al. 1976; Pierce et al. 1978; Smith et al. 1979). In these cases, the presence of grossly visible nodules led to detailed microscopic evaluations of the affected livers. Once a putative relationship between environmental contamination and hepatic lesions in fishes had been established, many subsequent studies were designed specifically to evaluate microscopic hepatic lesions in fishes from unsurveyed, contaminated areas. Thus, the scarcity of data on hepatic lesions prior to the mid-1970s probably was due largely to the lack of studies designed specifically to evaluate these abnormalities.

This section reviews most of the field studies that have documented elevated prevalences of hepatic neoplasms and other liver abnormalities in feral fishes collected from chemically contaminated environments (Table 4). Mix (1986) also reviewed much of this information for studies conducted prior to 1985. The studies listed in Table 4 include nine geographic locations (seven in the U.S. and two in Europe), freshwater (five) and saltwater (four) habitats, and 12 species.

Most of the historical field studies (7 of 17, or 41 percent) have been conducted in Puget Sound, Washington (Table 4). The highest prevalence of neoplasms found in any field study was 100 percent (i.e., saugers in Torch Lake, Michigan). However, in all other cases, maximum neoplasm prevalence was less than 40 percent.

The following reviews describe the design of each field study, the observed prevalences of hepatic neoplasms and putative preneoplastic lesions, the microscopic characteristics of the observed liver abnormalities, any relationships between lesions and other variables (e.g., age, sex, chemical concentrations), and the major conclusions reached by the authors. Much of the information presented in this section was used to develop the recommended protocols for Puget Sound studies.

**TABLE 4. SUMMARY OF FIELD STUDIES IN WHICH ELEVATED PREVALENCES OF HEPATIC NEOPLASMS HAVE BEEN FOUND IN FERAL FISHES**

Location	Study <sup>a</sup>	Species <sup>b</sup>	Sample Size	Percent Neoplasms <sup>c</sup>
Puget Sound, WA	Pierce et al. 1978	English sole	62	32.3
	McCain et al. 1982	English sole	673	0-12.9
		Starry flounder	350	0-3.0
	Malins et al. 1984	English sole	2,190	0-16.2
		Rock sole	1,379	0-4.8
		Pacific staghorn sculpin	422	0-1.7
	Malins et al. 1985a	English sole	106	0-7.5
	Malins et al. 1985b	English sole	115	0-26.7
	Tetra Tech 1985	English sole	1,014	0-8.3
Krahn et al. 1986	English sole	249	0-20.7	
Fox River, IL	Brown et al. 1973	Brown bullhead	283	12.4
	Brown et al. 1977	Brown bullhead	284	13.8
Black River, OH	Baumann et al. 1982	Brown bullhead	?	1.2-33.0
	Baumann and Harshbarger 1985	Brown bullhead	125	38.4
Torch Lake, MI	Black et al. 1982	Sauger Walleye	23	100.0
			22	>27.3
Hudson River, NY	Smith et al. 1979	Atlantic tomcod	264	25.0
Boston Harbor, MA	Murchelano and Wolke 1985	Winter flounder	200	8.0
Deep Creek Lake, MD	Dawe et al. 1964	White sucker	12	25.0
Elbe Estuary, Germany	Kranz and Peters 1985	Ruffe	551	8.0
Gullmar Fjord, Sweden	Falkmer et al. 1976	Atlantic hagfish	23,600	0.6-5.8

<sup>a</sup> The details of all of these studies are presented in the text.

<sup>b</sup> Scientific names of species are presented in Table 4.

<sup>c</sup> Prevalence or range of prevalences found for any kind of hepatic neoplasm in the species of interest.

Puget Sound, Washington

Study 1--

Pierce et al. (1978) collected 62 English sole from the Duwamish River in Puget Sound, Washington from July 1975 to January 1976. For comparative purposes, 18 English sole were collected from Point Pully, a Puget Sound reference area. Microscopic examination revealed that 20 fish (32.3 percent) from the Duwamish River had hepatic neoplasms. None of the fish from Point Pully had neoplasms.

Most neoplasms were minimum-deviation basophilic nodules or eosinophilic nodules. The basophilic nodules frequently compressed surrounding tissue. In some cases, they appeared to have invaded surrounding tissue. The eosinophilic nodules, by contrast, frequently exhibited numerous areas of invasiveness.

A variety of nonneoplastic abnormalities were found in English sole from the Duwamish River. These included increased size and number of melanin macrophage centers, centrolobular fatty degeneration and necrosis, cord disarray, increased hepatocyte basophilia, and hepatocellular hypertrophy associated with bizarre nuclei and multiple nucleoli.

The authors conclude that chemical contaminants are the suspected cause of the observed liver abnormalities in English sole, but that other agents such as pathogens and nutritional deficiencies cannot be ruled out. They note that sediments of the Duwamish River are contaminated with DDT, PCBs, copper, and lead and that the liver damage observed in English sole resembles that induced in other fishes by PCBs and other chlorinated hydrocarbons.

Study 2--

McCain et al. (1982) collected 673 English sole and 350 starry flounder (*Platichthys stellatus*) from four areas of Puget Sound between October 1978 and October 1980. Three of the areas (Duwamish River, Snohomish River, Lake Washington Ship Canal) are chemically contaminated to various degrees. The fourth area (McAllister Creek) is an uncontaminated reference area. All four areas are influenced by fresh water to some extent.

In English sole, hepatic neoplasms (i.e., minimum deviation nodules, liver cell adenomas, hepatocellular carcinomas, cholangiocellular carcinomas, and mixed carcinomas) ranged from 0 percent in McAllister Creek and the Snohomish River to 8.2 percent and 12.9 percent in the Lake Washington Ship Canal and the Duwamish River, respectively. Prevalence of putative pre-neoplastic lesions [i.e., hepatocellular regeneration, hepatocellular eosinophilic hypertrophy (subsequently referred to as eosinophilic foci)] ranged from 0 percent in McAllister Creek and the Snohomish River to 9.4 percent and 10.2 percent in the Duwamish River and the Lake Washington

Ship Canal, respectively. A variety of nonneoplastic liver abnormalities were also found in higher prevalences in the Lake Washington Ship Canal and the Duwamish River compared with the Snohomish River and McAllister Creek. These included megalocytic hepatitis, cholangiofibrosis, necrosis, and hemosiderosis.

In starry flounder, adequate sample sizes were available only for the Duwamish River and McAllister Creek. Prevalence of hepatic neoplasms (i.e., minimum deviation nodules, liver cell adenomas, and cholangiocellular carcinomas) was 3.0 percent in the Duwamish River, compared to 0 percent in McAllister Creek. Prevalence of putative preneoplastic lesions (i.e., hepatocellular eosinophilic hypertrophy) was 1.4 percent in the Duwamish River, compared to 0 percent in McAllister Creek. Nonneoplastic liver abnormalities exhibiting elevated prevalences in the Duwamish River compared to McAllister Creek included megalocytic hepatitis, fatty change, and necrosis.

McCain et al. (1982) found that neither sex of English sole from the Duwamish River was affected disproportionately by any of the hepatic lesions evaluated. The authors did find, however, that prevalence of total hepatic lesions was positively related to fish length, and therefore indirectly to fish age.

### Study 3--

Malins et al. (1984) collected 2,190 English sole, 1,379 rock sole (*Lepidopsetta bilineata*), and 422 Pacific staghorn sculpin (*Leptocottus armatus*) from 19 urban and nonurban areas throughout Puget Sound. Hepatic neoplasms were found in all three species and included minimum-deviation basophilic nodules, liver cell adenomas, hepatocellular carcinomas, cholangiocellular carcinomas, and cholangiomas. Prevalences of neoplasms in English sole, rock sole, and Pacific staghorn sculpin exhibited the following ranges: 0-16.2 percent, 0-4.8 percent, and 0-1.7 percent, respectively.

Malins et al. (1984) also found a variety of putative preneoplastic lesions in fish livers, including nodular eosinophilic hypertrophy, hyperbasophilic foci, clear cell foci, and hyperplastic regenerative islands. Prevalences of preneoplastic lesions in English sole, rock sole, and Pacific staghorn sculpin exhibited the following ranges: 0-24.3 percent, 0-9.5 percent, and 0-3.4 percent, respectively.

Malins et al. (1984) also found a number of nonneoplastic abnormalities in fish livers. The most prevalent nonneoplastic abnormalities were megalocytic hepatitis, cholangiofibrosis, steatosis, and hemosiderosis.

In general, highest prevalences of most liver abnormalities were found in major urbanized areas for all three fishes. Lowest prevalences generally were found in nonurban areas. Using

multivariate and bivariate statistical analyses, Malins et al. (1984) found positive associations between sediment concentrations of aromatic hydrocarbons and certain liver lesions in English sole and Pacific staghorn sculpin, and between sediment concentrations of metals and certain liver lesions in English sole.

#### Study 4--

Malins et al. (1985a) collected 66 English sole from a contaminated area of Puget Sound near Mukilteo, Washington during June and July of 1983. For comparative purposes, 40 English sole were sampled from a Puget Sound reference area near President Point. Hepatic neoplasms (i.e., minimum-deviation nodules, liver cell adenomas, hepatocellular carcinomas, and cholangiocellular carcinomas) were identified microscopically in five fish (7.5 percent) from Mukilteo and in no fish from President Point. Putative preneoplastic lesions (i.e., eosinophilic foci and hyperbasophilic foci) were found in 11 fish (16.7 percent) from Mukilteo and in no fish from President Point.

Most nonneoplastic abnormalities found in fish livers were more prevalent at Mukilteo than at President Point. These included degeneration, necrosis, and regeneration. By contrast, steatosis and hemosiderosis were more prevalent at President Point.

Chemical analyses showed that sediment concentrations of aromatic hydrocarbons, chlorinated compounds, and carbazole were substantially higher at Mukilteo than at President Point. By contrast, sediment concentrations of toxic metals (except lead) were similar at both sites. In fish livers, PCB concentrations at Mukilteo were 17 times as high as those at President Point. Concentrations of hexachlorobenzene were also elevated in livers from Mukilteo. By contrast, aromatic hydrocarbons and carbazole generally were not detected in livers from either site. In fish bile, concentrations of benzo(a)pyrene-like and naphthalene-like metabolites in fish from Mukilteo were 6 times and 3 times, respectively, as high as those in fish from President Point. In fish stomach contents, concentrations of aromatic hydrocarbons and PCBs in fish from Mukilteo were 22 times and 3 times, respectively, as high as those in fish from President Point.

Malins et al. (1985a) concluded that their findings support the statistical relationships identified by Malins et al. (1984) between sediment concentrations of aromatic hydrocarbons and hepatic lesions in English sole. The authors note that they had documented for the first time the bioavailability of organic chemicals through the diet of English sole. They also note that the absence of detectable concentrations of aromatic hydrocarbons in the livers and the apparent presence of metabolites in the bile supports the hypothesis that biotransformation of aromatic hydrocarbons by English sole is both rapid and extensive.

#### Study 5--

Malins et al. (1985b) captured 75 English sole from Eagle Harbor, Washington between November 1983 and April 1984. Eagle Harbor is a small embayment in Puget Sound that is contaminated by creosote. For comparative purposes, the authors used the same 40 English sole from President Point as described in Malins et al. (1985a). Hepatic neoplasms (i.e., liver cell adenomas, hepatocellular carcinomas, cholangiocellular carcinomas, and mixed carcinomas) were identified microscopically in 20 fish (26.7 percent) from Eagle Harbor and in no fish from President Point. Putative preneoplasms (i.e., eosinophilic foci, basophilic foci, and clear cell foci) were found in 33 fish (44.0 percent) from Eagle Harbor and in no fish from President Point.

Most nonneoplastic abnormalities found in fish livers were substantially more prevalent at Eagle Harbor than at President Point. These abnormalities included degeneration, necrosis, regeneration, steatosis, and hemosiderosis.

Chemical analyses showed that sediment concentrations of aromatic hydrocarbons and the heterocycles carbazole and dibenzofuran were elevated substantially compared to President Point. By contrast, sediment concentrations of chlorinated hydrocarbons and toxic metals were not elevated substantially. In fish muscle and liver tissue, concentrations of aromatic hydrocarbons, carbazole, and chlorinated hydrocarbons generally were relatively low. Naphthalene and alkylated naphthalenes constituted the highest proportion of aromatic hydrocarbons found in livers. Although the concentration of PCBs was somewhat elevated (i.e., 1.1 ppm) in livers from Eagle Harbor, it did not differ substantially from that at President Point (i.e., 1.0 ppm). In fish bile, metabolites of aromatic hydrocarbons were substantially elevated in Eagle Harbor compared to President Point. In fish stomach contents, concentrations of aromatic hydrocarbons were substantially higher at Eagle Harbor than at President Point. By contrast, concentrations of chlorinated hydrocarbons and carbazole were similar in stomach contents from the two study sites.

Malins et al. (1985b) concluded that certain creosote components, acting individually or synergistically, were causally linked to the high prevalence of liver abnormalities observed in English sole from Eagle Harbor. The authors suggest that the diet is an important route of contaminant uptake. The authors also note that the presence of metabolites in bile demonstrates that English sole accumulated and actively metabolized creosote components.

#### Study 6--

Tetra Tech (1985) collected 896 English sole (age  $\geq 3$  yr) from chemically contaminated areas of Commencement Bay during June 1984. For comparative purposes, 118 English sole (age  $\geq 3$  yr) were collected from Carr Inlet, a nonurban reference embayment. Prevalences of hepatic neoplasms (i.e., liver cell adenomas, hepatocellular carcinomas, cholangiocellular carcinomas, and cholangiomas) ranged from 0 to 8.3 percent in Commencement Bay, and were absent from Carr

Inlet. Prevalences of putative preneoplastic lesions (i.e., eosinophilic foci, basophilic foci, and clear cell foci) ranged from 3.4 to 26.7 percent in Commencement Bay and was 5.1 percent in Carr Inlet. Prevalences of megalocytic hepatitis and nuclear pleomorphism were substantially higher in Commencement Bay than in Carr Inlet.

Tetra Tech (1985) found that prevalences of the four major kinds of lesions evaluated did not differ ( $P>0.05$ ) between the sexes of English sole. However, prevalences of neoplasms and putative preneoplasms were both positively correlated ( $P<0.05$ ) with fish age. Prevalences of megalocytic hepatitis and nuclear pleomorphism were not significantly correlated ( $P>0.05$ ) with fish age.

#### Study 7--

Krahn et al. (1986) collected 249 English sole from 11 areas throughout Puget Sound from November 1983 to January 1984. Stations were selected to represent a gradient of chemical contamination. Prevalence of hepatic neoplasms ranged from 0 to 20.7 percent. Prevalence of putative preneoplastic lesions ranged from 0 to 32.8 percent. Highest prevalences of both kinds of lesion were found in the Duwamish River. Prevalence of megalocytic hepatitis ranged from 0 to 86 percent, with the highest value found in Eagle Harbor. Prevalence of steatosis ranged from 0 to 41.4 percent, with the highest prevalence found in the Duwamish River.

In addition to fish liver lesions, Krahn et al. (1986) measured the bile concentrations of multi-ring aromatic compounds that fluoresce at the benzo(a)pyrene wavelength pair. English sole from Eagle Harbor had the highest concentrations of biliary metabolites. Significant ( $P<0.05$ ) positive correlations were found between the relative mean concentration of biliary metabolites at each study site and the prevalences of neoplasms, putative preneoplasms, megalocytic hepatitis, and total lesions (i.e., one or more of the four lesions considered). Correlations between lesion prevalences and sediment concentrations of selected aromatic hydrocarbons were not significant ( $P>0.05$ ). The correlation between sediment concentrations of selected aromatic hydrocarbons and relative mean concentrations of biliary metabolites also was not significant ( $P>0.05$ ).

Krahn et al. (1986) concluded that the significant correlations between biliary metabolites and hepatic lesions in English sole provide added evidence of the putative relationship between aromatic compounds and liver abnormalities.

Fox River, Illinois

Study 1--

Brown et al. (1973) collected 2,121 fishes from the highly polluted Fox River watershed near Chicago, Illinois between 1967 and 1972. Of the over 17 species sampled, only the brown bullhead (*Ictalurus nebulosus*) exhibited unusually high prevalences of hepatic neoplasms. Of the 283 bullheads examined, 35 (12.4 percent) had hepatic neoplasms. Brown et al. (1973) also sampled 4,639 fishes from reference sites in Canada and found that of the 101 brown bullheads sampled in those uncontaminated areas, 2 (2.0 percent) had hepatic neoplasms.

Brown et al. (1973) conclude that increased levels of such pollutants as mercury, lead, arsenic, toluene, crude oil, gasoline, benzanthracene, chlorinated hydrocarbons, phosphates, sulfates, and coliform bacteria in the Fox River system may have been responsible for the observed neoplasms. Factors such as dissolved oxygen, temperature, and nutritional variation were considered similar in both the Fox River and the reference area.

Study 2--

Brown et al. (1977) sampled 284 additional brown bullheads from the Fox River watershed from 1972 to 1976 and found that 39 (13.8 percent) had hepatic neoplasms. Of the 87 brown bullheads sampled in the Canadian reference areas from 1972 to 1976, only 1 (1.2 percent) had hepatic neoplasms. These results were very similar to those found by Brown et al. (1973) from 1967 to 1972, suggesting that the observed patterns were temporally stable.

Microscopic examination of the livers evaluated by Brown et al. (1977) revealed that neoplastic cells generally were pleomorphic and frequently multinucleate. The cytoplasm of neoplastic cells was sometimes vacuolated, and sometimes granular and acidophilic. Some neoplasms tended to invade surrounding tissue, but widespread metastasis rarely was observed.

Black River, Ohio

Study 1--

Baumann et al. (1982) collected brown bullheads from the industrialized Black River near Lorain, Ohio from April to June of 1980. For comparative purposes, 329 brown bullheads were collected from Buckeye Lake, Ohio, a less contaminated water body, from July to August of 1980. Hepatic neoplasms in fish from the Black River were grossly visible as small white nodules on the surface of the liver. These neoplasms were thought to be cholangiomas.

Microscopic examination revealed a large number of mitotic figures throughout the

neoplasms, and invasion of surrounding tissue. The central regions of the neoplasms contained acidophilic cells and large areas of necrosis.

The prevalence of grossly visible hepatic neoplasms in Black River fish  $\geq 3$  yr old (33.0 percent) was significantly higher ( $P < 0.01$ ) than the prevalence in fish  $< 3$  yr old (1.2 percent). None of the bullheads from Buckeye Lake had grossly visible hepatic neoplasms.

Baumann et al. (1982) noted that the Black River is contaminated by a wide range of organic contaminants, but that the basic difference between that waterway and Buckeye Lake is the presence of industrial effluents containing PAH. Chemical analyses conducted in conjunction with the pathology study documented high levels of PAH in Black River bottom sediments and elevated levels (relative to Buckeye Lake) in tissue of Black River bullheads. The authors concluded that PAH were the most likely cause of the hepatic neoplasms observed in the Black River bullheads.

#### Study 2--

Baumann and Harshbarger (1985) collected 125 brown bullheads from the Black River in 1982. Microscopic examination revealed that 48 fish (38.4 percent) had hepatic neoplasms. Cholangiocellular carcinomas (28.8 percent) were more common than hepatocellular carcinomas (19.2 percent). Neoplasms were equally common in 3- and 4-yr-old fish. Chemical analyses showed that sediment concentrations of PAH in the Black River were 1,000 times greater than those in Buckeye Lake. In addition, tissue concentrations in Black River bullhead were elevated relative to those of Buckeye Lake fish. Dioxins, dibenzofurans, DDT, PCBs, arsenic, and cadmium were not unusually elevated in Black River bullheads relative to Buckeye Lake fish. The authors concluded that the elevated prevalence of hepatic neoplasms in Black River bullheads was chemically induced and the result of exposure to PAH.

#### Torch Lake, Michigan

Black et al. (1982) collected 23 saugers (*Stizostedion canadense*) and 22 walleye (*Stizostedion vitreum*) from Torch Lake, Michigan in September 1979 and July 1980. Hepatic neoplasms (diagnosed microscopically as hepatocellular carcinomas) were grossly visible as nodules in all (100 percent) of the saugers and in at least six (27.3 percent) of the walleyes. Visible nodules ranged from 2 to 20 mm in diameter. Microscopically, neoplastic cells exhibited increased basophilia and moderate anaplasia. Cells had large nuclei and nucleoli, but only mild pleomorphism. Fibrosis was not common. Few mitoses were evident and neoplasm growth appeared to be slow. The neoplasms compressed and sometimes evoked atrophy in surrounding hepatocytes. Parasitic trematode cysts and melanin macrophage centers were present in most liver sections.

Black et al. (1982) noted that the saugers they evaluated were very old (i.e., probably  $> 12$  yr

old). They also noted that gonads frequently appeared atrophic in the saugers and less frequently so in the walleyes, suggesting that the populations of these species in Torch Lake may thereby be negatively affected (i.e., in terms of reproductive capacity).

Black et al. (1982) suggest that the copper mining wastes discharged to Torch Lake may be directly or indirectly responsible for the high prevalences of hepatic neoplasms in resident saugers and walleyes. Since 1900, over 20 percent of the lake has been filled with copper tailings. In addition, mine water pumpage and untreated municipal sewage were also discharged to the lake for many years. The authors suggest that some chemical component(s) of the mine wastes (e.g., copper, selenium, arsenic) may be carcinogenic. Alternatively, the mine wastes may be interacting with the sewage wastes to produce carcinogens (e.g., metal-catalyzed nitrosamines). The authors suggest there is no relationship between the parasitic trematodes and hepatic neoplasms.

#### Hudson River, New York

Smith et al. (1979) evaluated hepatic neoplasms in 264 adult Atlantic tomcod (*Microgadus tomcod*) collected from the Hudson River from December 1977 to February 1978. The presence of these neoplasms was noted incidentally as fish were being processed in the laboratory for growth, mortality, and reproduction studies. Fish were divided into three categories according to the gross characteristics of the liver abnormalities. Only four livers were examined microscopically: two from one group and one from each of the other two groups. Based solely on gross characteristics, Smith et al. (1979) estimated that approximately 25 percent of the 264 livers contained hepatic neoplasms. However, that figure may underestimate the true prevalence, as microscopic examination may have revealed neoplasms in livers that lacked grossly visible neoplasms. Based on gross examinations, none of the neoplasms exhibited metastasis.

Microscopic examination of the liver of the one fish from the group having the fewest number of gross abnormalities showed excessive vacuolation suggestive of fat deposition. Also observed were focal areas of subcapsular congestion and mild hemorrhage.

Microscopic examination of the single liver from the group characterized by small (1-3 mm) light grey pustule-like lesions revealed several small neoplasms. The neoplasms were not encapsulated and appeared to be invading surrounding normal tissue. Neoplastic cells generally were poorly differentiated and enlarged. Nuclei of neoplastic cells were pleomorphic, swollen, and vesicular. Nucleoli were also swollen and mitoses were uncommon. The cytoplasm of all neoplastic cells exhibited increased basophilia. Necrotic cells were scattered diffusely throughout the neoplasms.

Microscopic examination of two livers from the groups characterized by dark red or purple lesions of various sizes revealed numerous small neoplasms that were histologically similar to those described for the liver with light grey lesions. However, in one liver from the third group, a single

neoplasm involved approximately 60 percent of the liver. Focal areas of sinusoidal congestion and subcapsular hemorrhage were also found in one liver from the third group. In the more advanced neoplasms from the third group, cells were greatly enlarged (i.e., 5-6 times normal), highly pleomorphic, and often binucleate or multinucleate. The nuclear:cytoplasmic ratio appeared to be reduced, nucleoli were often swollen, and the cytoplasm was frequently vacuolated.

Smith et al. (1979) noted that livers of some of the Atlantic tomcod contained relatively high levels (i.e., 10.9-98.2 ppm) of PCBs (Aroclor 1016 and 1254), and suggested that those chemicals may have caused the observed neoplasms.

#### Boston Harbor, Massachusetts

Murchelano and Wolke (1985) collected 200 winter flounder (*Pseudopleuronectes americanus*) from Boston Harbor, Massachusetts in April and June 1984. Microscopic examination revealed that 16 fish (8.0 percent) had hepatic neoplasms and 20 fish (10.0 percent) had either neoplasms or putative preneoplastic lesions. Neoplasms included hepatocellular (2.5 percent) and cholangiocellular (7.0 percent) carcinomas, cholangiomas (0.5 percent), and adenomas (0.5 percent). Preneoplastic lesions included basophilic (3.5 percent) and vacuolar (4.5 percent) foci. The authors note that prevalences of preneoplastic lesions may have been higher had more liver sections been examined for each fish.

The most common nonneoplastic abnormalities observed in the Boston Harbor fishes were increased numbers of melanin macrophage centers (68 percent) and hepatocyte vacuolation (68 percent). Other nonneoplastic lesions included pericholangitis, vasculitis, focal necrosis, biliary hyperplasia, and cholangiofibrosis.

Murchelano and Wolke (1985) noted that only fish collected off Deer Island had grossly visible hepatic lesions. Deer Island is the discharge point for much of Boston's primary-treated municipal sewage. The authors also noted that the high incidence of vacuolated cells and increased numbers of melanin macrophage centers were consistent with the action of a hepatotoxin. However, they do not speculate as to what kind of hepatotoxin may have been responsible for the observed liver abnormalities.

#### Deep Creek Lake, Maryland

##### Study 1--

Dawe et al. (1964) performed gross necropsies on six fishes from Deep Creek Lake, Maryland during September 1963. Of 12 white suckers (*Catostomus commersoni*) evaluated, 3 (25 percent) had intrahepatic bile-duct neoplasms, none of which was detectable by external inspection or palpation. All of the fish with tumors were relatively old (i.e., 5-15 yr).

Microscopic evaluation of the three livers with neoplasms revealed that none of the neoplasms had metastasized. In all cases, parasitic protozoans (i.e., probably a haplosporidium species) were present within the neoplastic epithelium. However, similar protozoans were also found in the livers of fish without tumors.

Dawe et al. (1964) caution that the low sample size and relatively old age of many of the fish may bias the apparently high prevalence of neoplasms. The authors suggest that the neoplasms may have been caused by the parasitic protozoans, carcinogenic hydrocarbons (i.e., from boating activity), pesticides used to eradicate mosquitos, or rotenone used to sample fish in the lake at regular intervals.

#### Study 2--

Dawe et al. (1976) collected 74 white suckers from Deep Creek Lake between 1964 and 1974. Sixty-six of those fish were similar in length to those sampled by Dawe et al. (1964) in 1963. None of the 74 fish collected after 1963 had liver neoplasms. Dawe et al. (1976) also collected 3,134 white suckers from a wide variety of aquatic habitats throughout the U.S. and Canada and found only one fish with a liver neoplasm. That individual was taken from Pleasant Valley Lake, Maryland. Thus, the high prevalence of hepatic neoplasms found in 1963 may have represented an isolated case, rather than a general trend.

#### Elbe Estuary, Germany

Kranz and Peters (1985) collected 551 ruffe (*Gymnocephalus cernua*) from the Elbe Estuary near Hamburg, Germany from 1980 to 1982. Nodules suspected of being neoplastic were grossly visible in 8 percent of the livers. Microscopically, the initial stages of the nodules were seen as small groups of greatly enlarged basophilic cells. In the larger nodules, signs of necrosis were evident. The trabecular arrangement of the cells disintegrated and cells became increasingly pleomorphic. Vascular congestion sometimes occurred. Nuclear pleomorphism was slight. Melanin macrophage centers were large and numerous in the surrounding parenchyma.

Partial discolorations of the liver were grossly evident in 39 percent of the fish. Microscopic examination revealed that these discolorations were primarily areas of fatty vacuolation that resulted from excess storage of lipids. Glycogen also appeared to be accumulated in some of these areas. The authors noted that the observed excessive accumulation of lipid was probably pathological and similar to the kind of liver lipid degeneration that results from improper nutrition and reaction to certain pollutants.

Liver nodules were absent in small ruffe (i.e., <17 cm in length). However, prevalence of nodules showed a positive association with size for large ruffe. Because size often correlates with

age, nodule prevalence may have been a function of fish age. Condition (i.e., weight x 100/length<sup>3</sup>) was significantly lower for fish with nodules than for fish without gross liver abnormalities.

Kranz and Peters (1985) noted that the Elbe Estuary is affected by a variety of pollutants. They also noted that similar abnormalities were found in fishes following exposure to pesticides, PCBs, crude oil, and heavy metals. Finally, they suggested that fat-soluble hydrocarbons were possible causes of the observed abnormalities in the Elbe Estuary.

### Gullmar Fjord, Sweden

#### Study 1--

Falkmer et al. (1976) sampled 23,600 hagfish (*Myxine glutinosa*) from Gullmar Fjord, Sweden from 1972 to 1975. For comparative purposes, 1,183 hagfish were collected from the nearby open sea during 1972 and 1974. Many of the observed hepatic neoplasms were grossly visible as small white spots on the surface or within the parenchyma of the liver. Although liver color varied considerably among individuals, there was no association with neoplasms. No gross evidence of metastasis was observed.

Microscopic evaluation revealed two major kinds of neoplasms: hepatocellular and cholangiocellular. Both kinds of neoplasm frequently occurred in the same liver, but hepatocellular neoplasms generally were more common (i.e., 2-3 times) than cholangiocellular neoplasms. Hepatocellular neoplasms exhibited a range of characteristics. Some of these neoplasms were nodular hyperplasias of questionable neoplastic nature. They did not compress the adjacent liver parenchyma and formed boundaries that often were difficult to discern. A second group consisted of slightly larger nodules that were composed of highly differentiated hepatocytes that compressed or evoked atrophy in surrounding tissue. No cellular or nuclear pleomorphisms were exhibited in these neoplasms and the number of mitotic figures was low or absent. Falkmer et al. (1976) classified this second group as benign liver cell adenomas. A third group consisted of the largest hepatocellular neoplasms and was classified as carcinomas. Areas of necrosis and hemorrhage occurred frequently and invasive growth was evident. However, both the degree of cellular atypia and the number of mitotic figures were relatively low.

As with hepatocellular neoplasms, the characteristics of cholangiocellular neoplasms covered a wide range. The larger neoplasms were definite carcinomas, being either highly or poorly differentiated.

In 1972, prevalence of neoplasms in the Gullmar Fjord was 5.8 percent compared to 2.8 percent in the open sea. In 1974, prevalence of neoplasms in the fjord was 0.6 percent, compared to 0.9 percent in the open sea. Between 1972 and 1975, prevalence of neoplasms declined from 5.8

percent (1972) to 2.9 percent (1973) to 0.6 percent (1974 and 1975).

Falkmer et al. (1976) compared the body weight of hagfish with neoplasm prevalence and found a positive relationship. Because body weight generally correlated with age, these results suggest that neoplasm prevalence exhibits a positive association with age. Falkmer et al. (1976) also noted that neoplasms were absent in small hagfish (i.e., <25 g). Falkmer et al. (1976) concluded that because hagfish from the open sea generally were smaller than those from Gullmar Fjord, observed differences in neoplasm prevalence between the two areas may have been biased.

#### Study 2--

Falkmer et al. (1977) collected 3,700 hagfish from Gullmar Fjord in 1976 and found a neoplasm prevalence (i.e., 0.6 percent) identical to that found in 1974 and 1975. Preliminary chemical analyses showed that composites of livers (with and without neoplasms) from hagfish captured in the fjord contained PCBs at a concentration of 5 ppm (wet weight), whereas the concentration in composited livers from the open sea was approximately 0.2 ppm. Given that use of PCBs was prohibited in Sweden in 1971-72 and that neoplasm prevalence in hagfish from the Gullmar Fjord declined from 5.8 percent in 1972 to 2.9 percent in 1973 and to 0.6 percent in 1974-76, Falkmer et al. (1977) suggest that PCBs may have been the primary cause of the observed neoplasms.

## RECOMMENDED PROTOCOLS FOR FIELD STUDIES IN PUGET SOUND

This section presents recommended procedures for conducting field studies of fish liver histopathology in Puget Sound. Included are recommendations regarding study design, field sampling procedures, laboratory methods, and data analysis and interpretation. Recommendations were made as specific as possible without sacrificing their general applicability. Many of the recommendations are based on the information presented earlier in this report.

### STUDY DESIGN

#### Species Selection

Different fish species can exhibit markedly different sensitivities to toxic contaminants in the environment based on such factors as habitat, prey type, life span, migratory behavior, and genetic constitution. Many of these factors for the 12 species in which elevated prevalences of hepatic neoplasms were found in field studies are summarized in Table 5.

All of the species listed in Table 5 spend most of their time near the seafloor, in close proximity to any contaminants that may be present in bottom sediments. Seven of the species are known to sometimes bury themselves in sediment and thus further enhance possible contact with sediment contaminants. Ten of the species prey primarily upon benthic invertebrates, many of which are relatively stationary. In contaminated areas, there is a high probability that those invertebrates will also be contaminated and thereby transfer contaminants to their piscine predators. At least four of the fishes exhibit some degree of homing ability. This implies that although these species may migrate (e.g., seasonally), they may also have the ability to relocate contaminated areas and thereby be exposed repeatedly to contaminants. Finally, individuals from most of the 12 species commonly reach ages  $\geq 3$  yr. The potential therefore exists that some of these fishes may be exposed to contaminants for many years.

Based on Table 5, it appears that bottom-dwelling, bottom-feeding species in contaminated areas have a high potential for being affected by liver abnormalities. This is consistent with conclusions reached by Dawe et al. (1964) and Harshbarger (1977). However, a number of other species with characteristics similar to those of the fishes listed in Table 5 were sampled in contaminated areas and did not exhibit liver abnormalities (e.g., Brown et al. 1973, 1977; Falkmer et al. 1976; Kurelec et al. 1981; Sloof 1983).

TABLE 5. CHARACTERISTICS OF FISHES FOUND TO HAVE ELEVATED PREVALENCES OF HEPATIC NEOPLASMS IN FIELD STUDIES<sup>a</sup>

Family	Common Name	Scientific Name	Primary Habitat	Bury? <sup>b</sup>	Primary Prey <sup>c</sup>	Homing Ability? <sup>d</sup>
Myxinidae (hagfishes)	Atlantic hagfish	<i>Myxine glutinosa</i>	Bottom	Yes	F	
Catostomidae (suckers)	White sucker	<i>Catostomus commersoni</i>	Bottom	Yes	BI	Yes
Ictaluridae (bullhead catfishes)	Brown bullhead	<i>Ictalurus nebulosus</i>	Bottom		BI, P	
Gadidae (codfishes)	Atlantic tomcod	<i>Microgadus tomcod</i>	Bottom		BI, F	
Percidae (perches)	Ruffe	<i>Gymnocephalus cernua</i>	Bottom		BI	
	Sauger	<i>Stizostedion canadense</i>	Bottom		BI, F	
	Walleye	<i>Stizostedion vitreum</i>	Bottom		F	Yes
Cottidae (sculpins)	Pacific staghorn sculpin	<i>Leptocottus armatus</i>	Bottom	Yes	BI	
Pleuronectidae (righteye flounders)	Rock sole	<i>Lepidopsetta bilineata</i>	Bottom	Yes	BI	
	English sole	<i>Parophrys vetulus</i>	Bottom	Yes	BI	Yes
	Starry flounder	<i>Platichthys stellatus</i>	Bottom	Yes	BI	
	Winter flounder	<i>Pseudopleuronectes americanus</i>	Bottom	Yes	BI	Yes

<sup>a</sup> References: Bigelow and Schroeder (1953), Hart (1973), Scott and Crossman (1973), Day (1976).

<sup>b</sup> Partially bury themselves in sediment as part of normal behavior.

<sup>c</sup> BI = benthic invertebrates, F = fish, P = plant material.

<sup>d</sup> Evidence exists that fish can intentionally return to specific locations.

Interspecific differences in the presence or absence of liver lesions may largely be the result of interspecific differences in sensitivity to toxic chemicals. For example, such differences are evident in

the sensitivities of various salmonids to aflatoxins (Hendricks 1982). Rainbow trout is very sensitive to aflatoxin carcinogenicity, but brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*) are much less sensitive. In addition, coho salmon (*Oncorhynchus kisutch*) and sockeye salmon (*O. nerka*) are relatively insensitive to aflatoxins.

Based on the previous discussion, the most important requisite for a target species is sensitivity to toxic chemicals. That is, the species should have a high probability of developing hepatic lesions following exposure to chemical contaminants. It is likely that this species will be a bottom-dwelling, bottom-feeding fish, but all fishes having these characteristics cannot be expected to be sensitive. When selecting a target species for a fish liver histopathology study, historical information regarding the sensitivities of the species likely to be encountered in a contaminated area should be reviewed. In the absence of such information, preliminary field surveys or laboratory tests may be required to evaluate this characteristic. Preliminary field studies should evaluate candidate species at the most contaminated study sites. Laboratory tests should expose candidate species to chemical concentrations high enough to induce lesions in at least one species.

Once sensitive species have been identified, at least two other criteria should be met. First, the species must be present in both contaminated and uncontaminated areas so that statistical comparisons with reference conditions can be made. Second, the species should not be highly migratory, so that residence time in the contaminated area would be too short to induce liver lesions or that migration between contaminated and uncontaminated areas would destroy gradients in the prevalences of liver lesions and thereby confound interpretation of prevalence data from multiple sampling sites.

Other desirable characteristics of a target species are that it can be captured easily to provide desired sample sizes at reasonable cost and that it be either commercially or recreationally valuable.

Most of the recommended criteria for a target species require that considerable information be available regarding the characteristics of the species. Unfortunately, this kind of information is incomplete for many species. Based on the results of historical studies, the knowledge of which species are sensitive to chemical contamination is probably the most important information to have when designing a fish liver histopathology study.

For Puget Sound, English sole has been shown to be the best target species for fish liver histopathology studies. This species is sensitive, bottom-dwelling, bottom-feeding, widely distributed across contaminated and reference areas, and not highly migratory. In areas of Puget Sound where adequate sample sizes of English sole cannot be collected, an alternate species should be selected using the criteria discussed in this section.

### Age Limits

Several field studies have found a positive relationship between prevalence of hepatic neoplasms or putative preneoplasms and age, length, or weight of fish (Figure 2). Because length and weight generally increase with increasing age, it is presumed that age is the primary factor in all cases. In all of these studies, hepatic neoplasms were absent in the youngest fish. Elevated prevalence of hepatic neoplasms in older fish relative to younger individuals has also been noted by Baumann et al. (1982), Malins et al. (1982), and McCain et al. (1982).

The patterns in Figure 2 suggest that age may confound interpretation of the results of certain fish liver histopathology studies. For example, prevalence of hepatic lesions in fish from a contaminated area could be higher than prevalence in a reference area partly because fish in the former area may be older than fish in the latter area. To estimate the elevation in lesion prevalence that may be the result solely of chemical contamination, age differences between fish from different areas must be minimized.

Ideally, fish should be compared only within age classes. However, because this kind of stratification frequently reduces sample sizes below desirable levels, it may not always be practical. An alternative to making comparisons based on age classes is making comparisons based on samples having similar age frequency distributions.

In making comparisons based on age frequency distributions, strategies can vary from evaluating as broad an age range as possible to evaluating a specific component of the total population. If the objective is to evaluate lesion prevalence in the overall population of a species, the entire age spectrum should be considered. However, if the objective is to evaluate lesion prevalence in that component of the population most likely to be affected by lesions, age limits may be imposed on the comparisons. For example, because hepatic neoplasms were not found in the youngest hagfish (Falkmer et al. 1976), ruffe (Kranz and Peters 1985), and English sole (Tetra Tech 1985) from contaminated areas (Figure 2), future studies may elect to exclude fish younger than the age at which neoplasms begin to appear.

It generally is not practical to determine fish age in the field. Instead, some hard structure (e.g., otoliths, spines, scales, opercular bones, vertebrae) of each fish is retained and later analyzed for annual markings in the laboratory. If the study design calls for comparisons to be stratified by age, fish collected in the field can be stratified by an easily measured index of age (e.g., length), pending subsequent confirmation of actual age. For example, if only fish older than a certain age are to be evaluated histopathologically, a lower size limit corresponding to the minimum age can be imposed on the sample collected in the field. Because indirect measures of age are not totally accurate, the number of fish collected in the field should exceed the sample size desired for histopathological analysis.

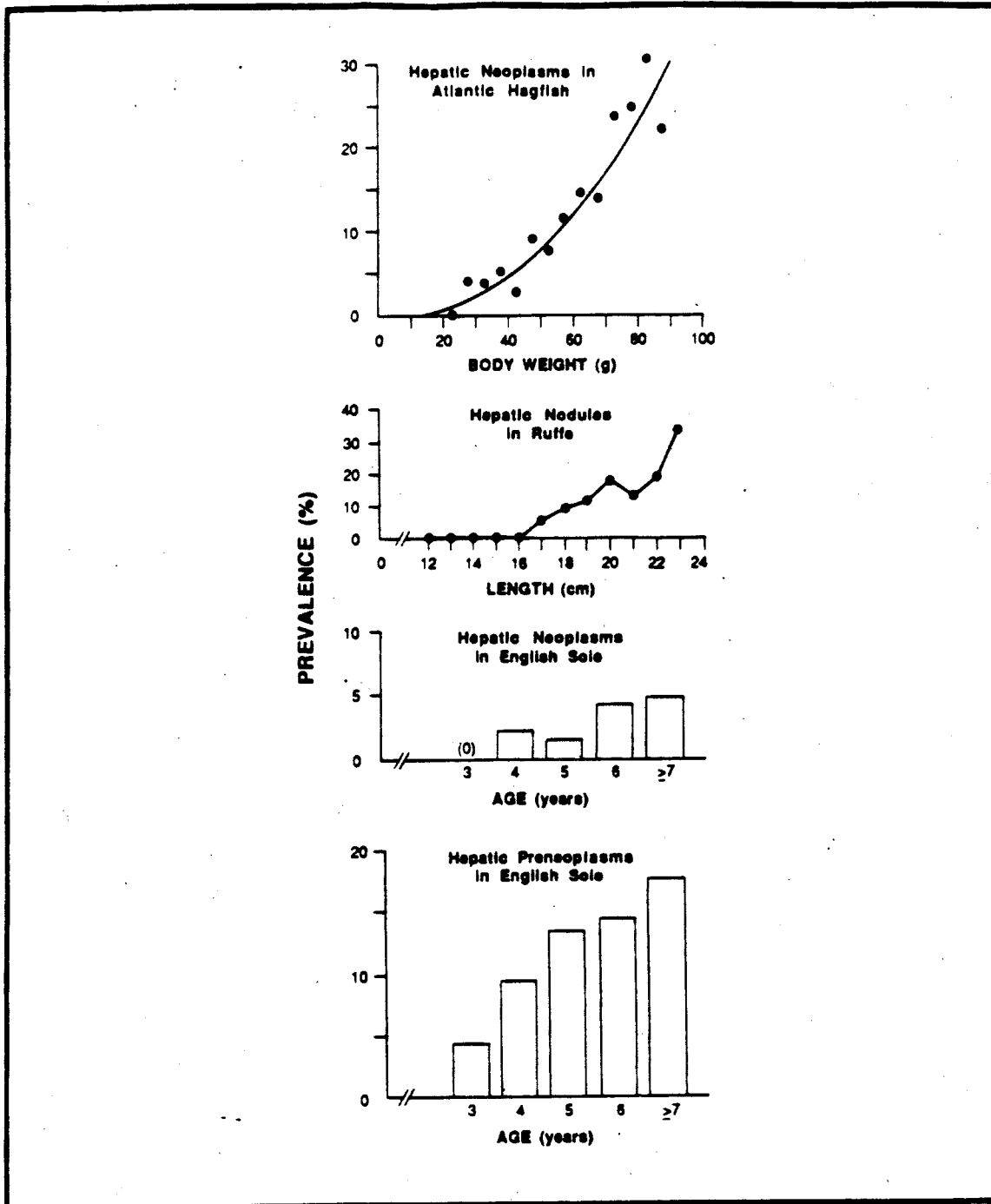


Figure 2. Relationship between hepatic lesions and size or age of Atlantic hagfish (Falkmer et al. 1976), ruffe (Kranz and Peters 1985), and English sole (Tetra Tech 1985).

The use of an indirect estimate of age (e.g., length, weight) for evaluating age differences among study areas is not recommended, because they generally are not suitably accurate, especially for older fish. The indirect measure of age used most commonly is length. However, the length frequency method of age estimation is useful only for young fish from populations in which spawning occurs during a single, short period and individuals grow at nearly the same rate (Royce 1972). Many species do not meet these criteria. Spawning may be protracted over a relatively long period, or individuals may grow at different rates depending on endogenous and exogenous factors. As fishes grow older, differential growth rates generally increase the observed range of lengths within an age group.

Several other factors may influence length/age relationships. Because fish from contaminated and reference areas may represent different populations with different growth rates (potentially due, in part, to contamination), length/age relationships may vary between these areas. Because some species exhibit sexual differences in growth rates (Royce 1972), failure to stratify length/age relationships by sex may confound length/age relationships.

Several of the problems associated with the length frequency method for estimating age of English sole are illustrated in Figure 3. All of the fish shown in Figure 3 were collected from a single embayment (i.e., Commencement Bay, Washington) and age was determined from otolith (sagitta) analysis. For both males and females, the observed length range increased as fish grew older. For example, the length range for females at age 3 was 5 cm (i.e., 22.5-27.5 cm), whereas the range at age 7 was 12 cm (25.5-37.5 cm). Thus, the ability to accurately estimate age from length declines with increasing age. As demonstrated in Figure 3, the median size of females was larger than that for males at ages greater than 3. Furthermore, this disparity between the sexes increased with increasing age.

In addition to stratifying samples prior to comparisons, age can be used to evaluate the growth of fish using a length-at-age analysis (see section entitled Data Analysis). This kind of analysis is valuable for determining whether hepatic lesions are associated with reduced growth.

Based on the previous discussion, it is recommended that age be determined directly for all fish evaluated histopathologically. The preferred method for direct age determination in fishes is the annual ring method, using some kind of hard body part. Many of these techniques are reviewed in Chilton and Beamish (1982) and Jearld (1983).

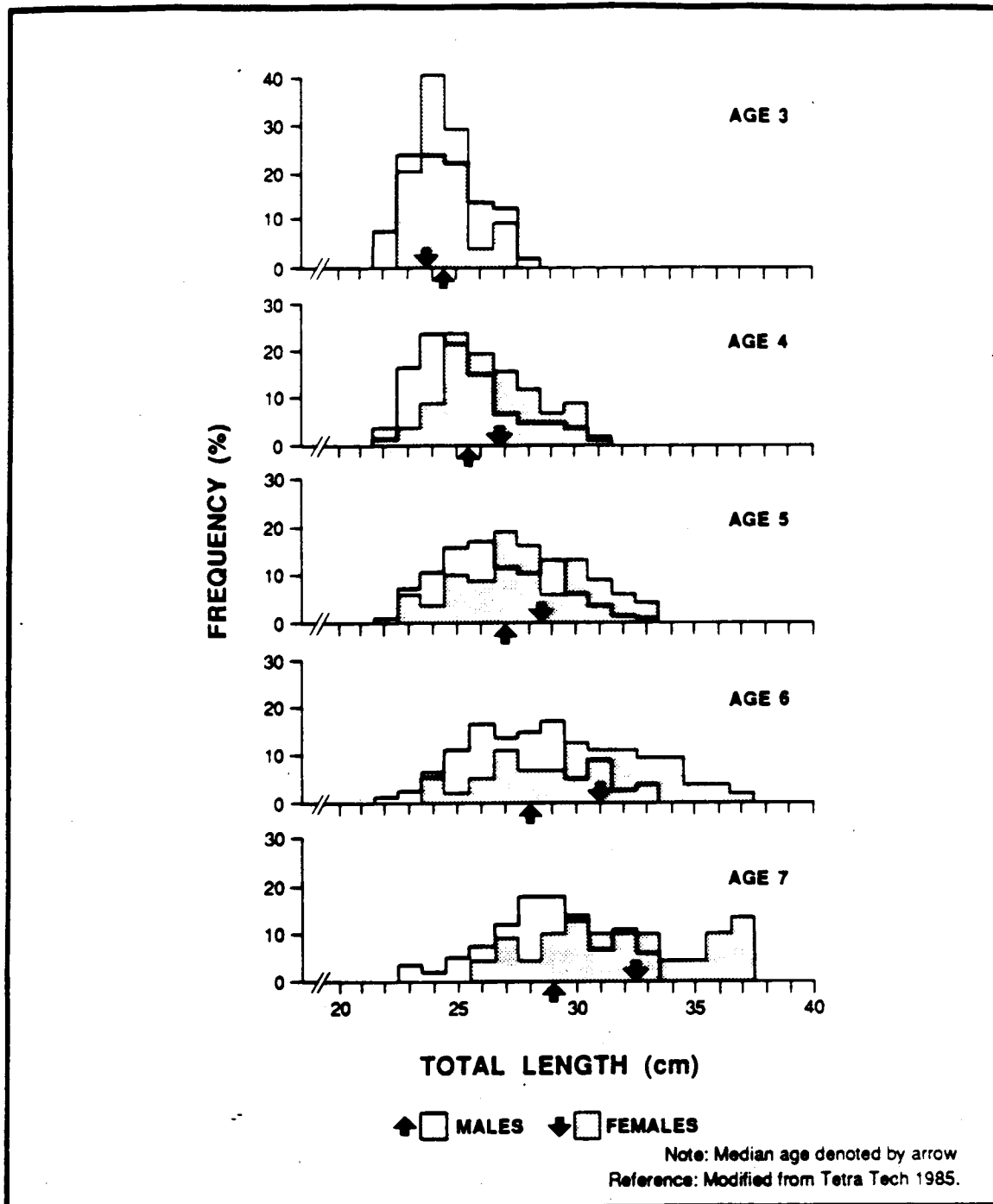


Figure 3. Length frequency distributions of various age groups of male and female English sole from Commencement Bay, WA.

## Sample Size

Most fish liver histopathology data collected in the field are expressed in the form of a proportion or percentage. The numbers represent the prevalence of a pathological condition in the sample evaluated. For example, if 10 of 50 fish were found to have hepatic neoplasms, the prevalence of hepatic neoplasms in that sample would be 0.20 (10/50) or 20 percent  $[(10/50) \times 100]$ . In an epidemiological context, prevalence is defined as the number of cases of a disease in a given population at a given time (Klontz 1984). Prevalence is distinct from incidence, another commonly used epidemiological measure, which is defined as the number of new cases of a disease in a population over a period of time (Klontz 1984). Prevalence therefore represents a static "snapshot" of the level of a disease in a population, whereas incidence is a dynamic property concerning the rate of introduction of a disease into a population.

One of the major considerations when designing a fish liver histopathology study is the sample size required to meet the objectives of the study. As objectives may vary widely among studies, it is not possible to make a single set of recommendations in the present report. Instead, two of the more common objectives that may be encountered during fish liver histopathology studies are evaluated. The principles identified as part of these evaluations apply to most kinds of objectives and can therefore be used to guide sample size determinations for specific studies.

### Objective 1--

One possible objective of a fish liver histopathology study is to determine whether a pathological condition (e.g., hepatic neoplasms) is present in a population of fish. This objective might be encountered during a reconnaissance study in an unsurveyed area or during a monitoring study of temporal changes of fish health in a previously uncontaminated area. The emphasis of these studies would be to collect a single individual having the pathological condition of interest.

A critical consideration in achieving Objective 1 is the minimum sample size required to detect a single occurrence of the pathological condition in the test population of fish. This minimum sample size is dependent primarily upon the following variables:

- Population size
- Prevalence of the condition within the population
- Level of desired confidence.

Simon and Schill (1984) present tables of required sample sizes in relation to a variety of specifications for the three variables listed above. Those tables are based largely on earlier work conducted by Ossiander and Wedemeyer (1973) and McDaniel (1979).

For the present study, the data presented by Simon and Schill (1984) are displayed graphically (Figure 4) for a variety of conditions that may be encountered during field surveys for a relatively rare (i.e.,  $\leq 10$  percent prevalence) pathological condition in a fish population. Prevalences of that magnitude might be expected for hepatic neoplasms in most environments. The desired confidence level was set at 95 percent; population prevalences were set at 1, 2, 3, 4, 5, and 10 percent; and population size ranged from 100 to 10,000 fish.

Above a population size of approximately 1,000 fish, the required sample size stabilizes for all population prevalences except 1 percent (Figure 4). For a population prevalence of 1 percent, the required sample size begins to stabilize substantially at population sizes greater than 3,000 fish. Because the fish populations surveyed by most field studies probably exceed 1,000 individuals, population size should have a negligible effect on required sample sizes when population prevalence is  $\geq 2$  percent.

At population sizes greater than 1,000 fish, the population prevalence has a substantial influence on the sample size required to detect a single affected fish. For example, approximate sample sizes of 30, 60, and 150 fish are required for population prevalences of 10, 5, and 2 percent, respectively. A sample size of between 260 and 300 fish is required for a population prevalence of 1 percent.

The results of Figure 4 can be used to determine the sample size required for a reconnaissance or monitoring study by specifying the minimum population prevalence that is desired to be detectable, based on the capture of a single fish having the pathological condition of interest. This assumes a confidence level of 95 percent and a population size greater than 1,000. For example, if 5 percent is the desired minimum detectable population prevalence, a sample size of 60 must be collected to be 95 percent confident that the survey would collect at least one affected individual. With a sample size of 60 fish, one could not be 95 percent confident that an affected individual would be collected if population prevalences were less than 5 percent. Thus, prevalences less than 5 percent would be considered undetectable at 95 percent confidence if 60 fish were sampled. If a sample size of 30 fish is used, population prevalences as high as 9 percent would not be detectable with 95 percent confidence. To be 95 percent confident of detecting a pathological condition at its earliest stages (i.e., prevalence  $< 1$  percent), sample sizes greater than 300 fish must be collected. Because sample sizes of that magnitude often are unaffordable, most researchers will have to accept the fact that very low prevalences of a pathological condition will not be detectable with 95 percent confidence.

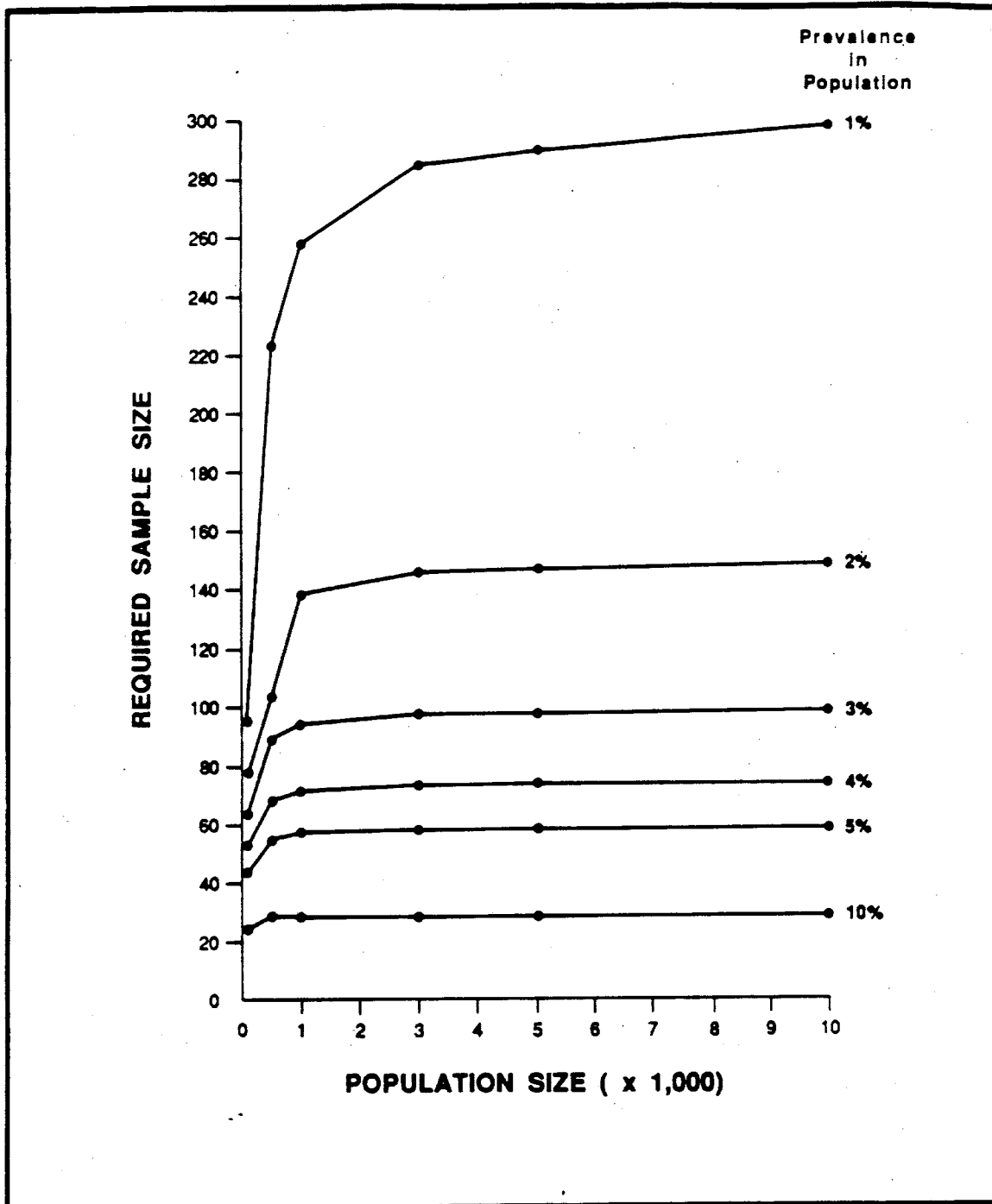


Figure 4. Sample sizes required to detect one individual affected with a lesion with 95 percent confidence, given various population sizes and prevalences.

## Objective 2--

A second possible objective that may be specified for a fish liver histopathology study is to determine whether prevalence of a particular lesion at a test site differs significantly from that at a reference site. This objective may be encountered in a study designed to test whether prevalence in a contaminated area is elevated above the level that would be expected in the absence of contamination.

A common method of comparing prevalences between two areas is the test of independence using 2x2 (i.e., two-way) contingency tables (cf. Sokal and Rohlf 1981). The significance of these comparisons can be made using either the chi-square statistic or G-statistic, the latter of which is recommended by Sokal and Rohlf (1981).

As part of the present study, the G-test of independence was evaluated at various sample sizes using the 2x2 case. The goal was to determine the statistical power of this test at the various sample sizes that may be used during most fish liver histopathology studies (i.e., 0-300 fish). The power of a statistical test is the probability of correctly rejecting the null hypothesis when, in fact, it is false. Power analyses were conducted over the range of prevalences that might be expected for most hepatic lesions in contaminated and reference areas (i.e., 0-25 percent). Results are presented graphically to provide quick reference to the approximate levels of statistical power that can be achieved for various study designs and various environmental conditions.

The general layout of a 2x2 contingency table is presented in Figure 5. The table is divided into two classes based on the kind of study area (i.e., rows) and two classes based upon the presence or absence of hepatic lesions in sampled fish (i.e., columns). Multiway contingency tables with more than two classes can also be used to summarize pathology results from more than two study areas.

In Figure 5, the expected prevalence (i.e., that at the reference site) and the observed prevalence (i.e., that at the test site) can be computed and compared to provide a statistical test of the null hypothesis of independence between study site and lesion prevalence. In most fish liver histopathology studies, a fixed number of fish are collected at each study site. Thus, the totals (i.e., the marginal sums  $N_{11} + N_{12}$  and  $N_{21} + N_{22}$ ) in the third column are fixed in each analysis. The test of independence therefore consists of computing the probability of obtaining the observed (or greater) departures from independence of lesion prevalences (i.e., the numbers that can vary), out of all possible two-way tables with the same marginal totals for study sites.

	NUMBER OF FISH WITH LESIONS	NUMBER OF FISH WITHOUT LESIONS	MARGINAL SUMS
REFERENCE SITE	$N_{11}$	$N_{12}$	$N_{1\cdot}$
TEST SITE	$N_{21}$	$N_{22}$	$N_{2\cdot}$
MARGINAL SUMS	$N_{\cdot 1}$	$N_{\cdot 2}$	TOTAL SAMPLE SIZE = $N$

NOTE: SUBSCRIPTS ARE DEFINED IN EQUATION 1.

Figure 5. Example of a 2 x 2 contingency table.

The G-test of independence is a likelihood ratio test (Neyman and Pearson 1928; Neyman 1950). The likelihood ratio criterion (expressed as G) for testing the null hypothesis of independence is:

$$G = \frac{N^N \prod_{i=1}^r \prod_{j=1}^s N_{ij}}{\prod_{i=1}^r N_{i\cdot} N_{\cdot j}}$$

(1)

where:

N = Total number of samples collected

$N_{ij}$  = Number of observations in the  $i, j^{\text{th}}$  cell of the  $r \times s$  contingency table

$N_{i\cdot}$  = Marginal sum of observations in the  $i^{\text{th}}$  row of the  $r \times s$  contingency table

$N_{\cdot j}$  = Marginal sum of the observations in the  $j^{\text{th}}$  column of the table

r = Number of rows in the  $r \times s$  contingency table (r=2 in a 2x2 table)

s = Number of columns in the  $r \times s$  contingency table (s=2 in a 2x2 table).

Under the null hypothesis of independence ( $H_0$ ), the distribution of  $2 \ln(G)$  tends to a  $\chi^2$  distribution as  $n \rightarrow \infty$ , where  $f$  is the degrees of freedom ( $f=1$  for a 2x2 test). For small sample sizes, it cannot be assumed that this approximation is close. As a result of deviations from the asymptotic distribution of the test statistic, the actual Type I error of the G-test tends to be higher than the nominal level. The approximation is also poorest when  $r$  and  $s$  are small and when  $p_{i\cdot} = N_{i\cdot}/N$  and  $p_{\cdot j} = N_{\cdot j}/N$  are near 0 or 1. Therefore, in applying the G-test in the analysis of 2x2 contingency tables with small sample sizes (i.e.,  $N \leq 200$ ), the use of correction factors has been recommended (e.g., Sokal and Rohlf 1981). This subject is treated in detail in Section 3.4.3. Because different studies may use different correction factors, the power analyses conducted in the present section did not employ correction factors. They therefore represent a more generalized evaluation of the G-test.

Two kinds of power analyses were conducted. In the first set of analyses, the probability of detecting selected differences in lesion prevalences between reference and test sites was calculated. In the second set of analyses, the minimum detectable difference in prevalence at the test site (i.e., compared to the reference site) was evaluated for different levels of prevalence at the reference site and at a fixed level of power.

Determination of the power of the G-test involves the calculation of the area under the curve in the critical region on the noncentral chi-square probability density (C\*). Thus, the power of the test can be found by evaluating the integral:

$$P(C^*|f, \lambda) = \sum_{k=0}^{\infty} e^{-\lambda/2} \frac{\lambda^k}{k! 2^{\frac{1}{2}f+2k-1} \Gamma\left(\frac{f}{2} + k\right)} \int_{\chi_f^2(\alpha)}^{\infty} x^{f+2k-1} e^{-\frac{1}{2}x^2} dx \quad (2)$$

where:

$\lambda$  = The noncentrality parameter

$f$  = Degrees of freedom.

The value of the noncentrality parameter ( $\lambda$ ) may be obtained from the following general rule. If under the null hypothesis ( $H_0$ ), the test statistic,  $T(X_1, X_2 \dots X_n)$  is asymptotically distributed as central  $\chi^2$ , then for  $n$  finite, the approximating noncentrality parameter ( $\lambda$ ) under an alternative hypothesis ( $H^*$ ) is simply the value of the test statistic,  $T(X_1, X_2 \dots X_n)$  with the expected value of  $X_i$  under  $H^*$  ( $E_{H^*}X_i$ ) substituted everywhere for  $X_i$ . Therefore, the noncentrality parameter to be used in determining the power of the G-test is given by:

$$\lambda = 2 \ln \frac{N^N \prod_{i=1}^r \prod_{j=1}^s (N_{i \cdot} p_{ij}^*)^{N_{i \cdot} p_{ij}^*}}{\prod_{i=1}^r (N p_{i \cdot}^*)^{N p_{i \cdot}^*} \prod_{j=1}^s (N p_{\cdot j}^*)^{N p_{\cdot j}^*}} \quad (3)$$

where:

$N$  = Total number of samples collected

$N_{i\cdot}$  = Marginal sum of observations in the  $i^{\text{th}}$  row of the  $r \times s$  contingency table

$N_{\cdot j}$  = Marginal sum of the observations in the  $j^{\text{th}}$  column of the table

$r$  = Number of rows in the  $r \times s$  contingency table ( $r=2$  in a  $2 \times 2$  table)

$s$  = Number of columns in the  $r \times s$  contingency table ( $s=2$  in a  $2 \times 2$  table)

$p^*$  = Sample proportions under the alternative hypothesis  $H^*$ .

The results of the first set of power analyses are summarized in Figures 6 and 7. These figures show the power of the G-test in relation to the number of samples collected at each location for selected prevalence levels at both the reference and test sites. These analyses were conducted for equal sample sizes at each study site, and the sample sizes (i.e., marginal sums) in Figures 6 and 7 represent the number of samples collected at each site.

Several patterns are apparent in Figures 6 and 7. First, at a fixed power, larger sample sizes are required to detect smaller elevations in lesion prevalence. For example, if lesion prevalence in the reference area is 0.1 percent (Figure 6) and power is fixed at 0.9, the approximate sample sizes required to detect elevations in lesion prevalences at the test site of 20, 15, 10, and 5 percent are 35, 50, 75, and 160 fish, respectively.

A second pattern identified by the power curves is that at a fixed sample size, power increases as the elevation in lesion prevalence at the test site increases. For example, if lesion prevalence in the reference site is 0.1 percent (Figure 6) and sample size is fixed at 40 fish, the approximate values of power to detect elevations in lesion prevalences at the test site of 5, 10, 15, and 20 percent are 0.35, 0.65, 0.85, and 0.95, respectively.

A third pattern identified by the power curves is that at a fixed sample size and elevation of lesion prevalence above reference levels, power declines as reference prevalence increases. For example, at a sample size of 40 and elevation in prevalence of 10 percent, the approximate values of power to detect the elevated prevalence when reference prevalences are 0.1 percent (Figure 6) and 5 percent (Figure 7) are 0.65 and 0.30, respectively. This suggests that every effort should be made during a fish liver histopathology study to locate reference stations in as uncontaminated an area as possible to enhance the probability that prevalence of chemically induced hepatic lesions will be very low (i.e., as close to 0 percent as possible).

The power curves presented in Figures 6 and 7 can be used to guide the selection of sample sizes for planned studies. If preliminary information exists regarding lesion prevalences in reference and test areas, these values can be applied to Figures 6 and 7 to determine the sample sizes needed to detect specific elevations in lesion prevalence with various degrees of statistical power. The power curves can also be used in an a posteriori analysis in which the focus is on the evaluation and interpretation of statistical analyses. For example, if lesion prevalence in the reference area was known (or assumed) to be close to 0 percent, and the study objective was to have an 80 percent probability of detecting a lesion prevalence of 10 percent at a test site, Figure 6 indicates that approximately 60 fish should be collected at each site. In instances where the null hypothesis has been accepted, the information provided in these plots also can be used to evaluate the probability of the corresponding type II error (i.e., the probability of accepting a null hypothesis when it is false).

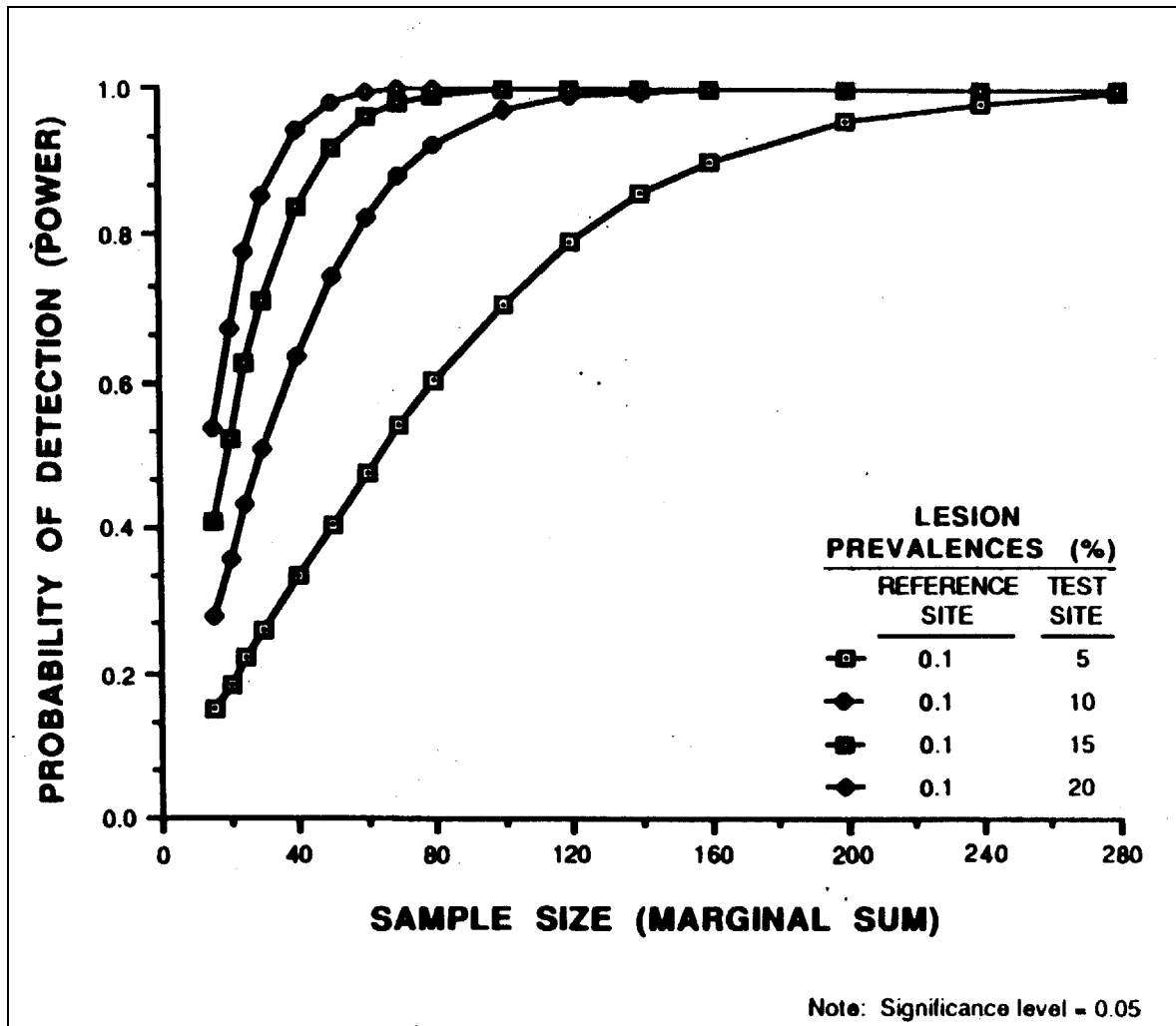


Figure 6. Power of the G-test vs. sample size when lesion prevalence at the reference site is 0.1 percent.

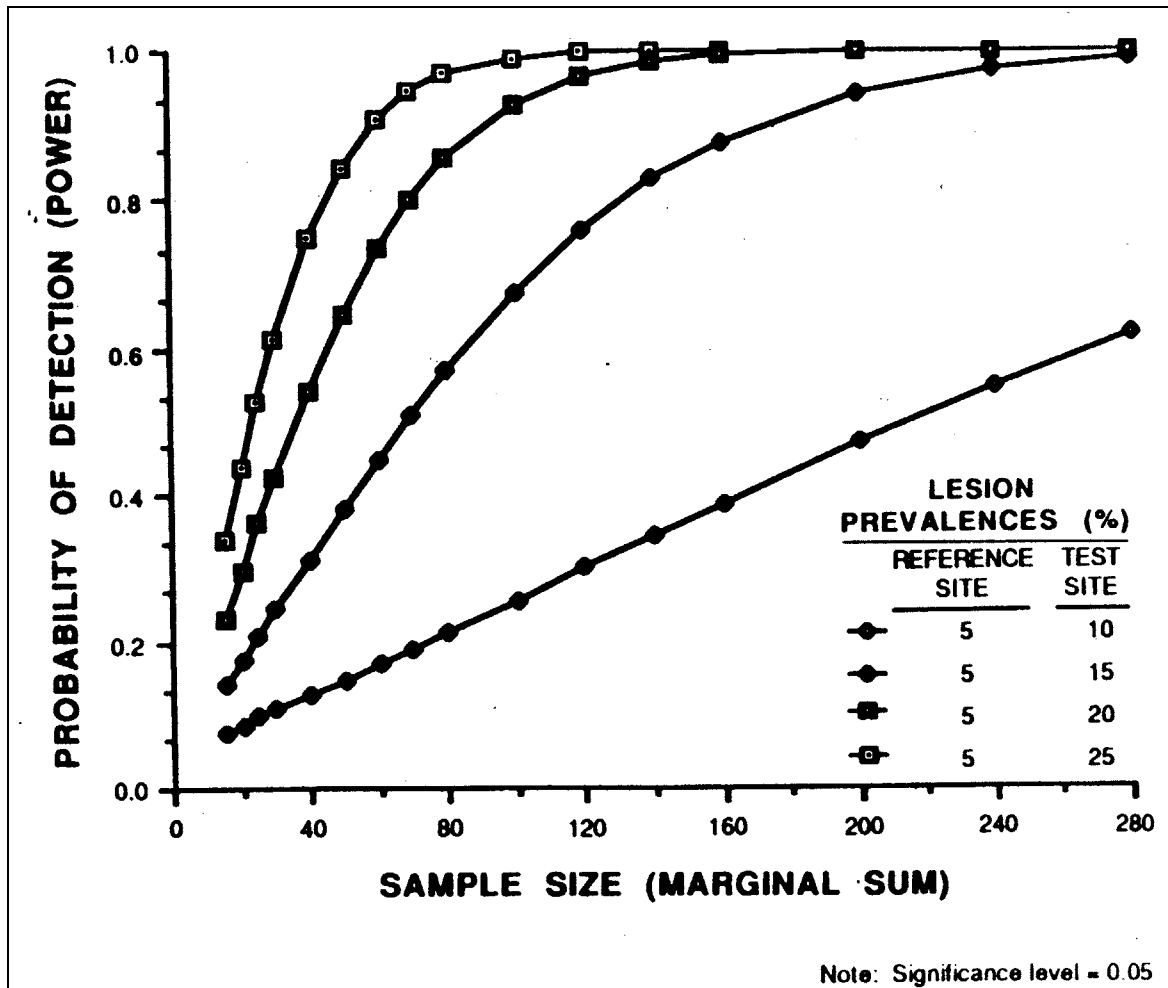


Figure 7. Power of the G-test vs. sample size when lesion prevalence at the reference site is 5.0 percent.

A second set of power analyses was conducted to provide a different view of the power of the G-test in specific applications. These analyses provide information concerning the relative benefits in terms of increased test sensitivity that can be obtained for corresponding increases in sample size. These analyses were conducted at a fixed power of 0.80. The minimum detectable prevalence at a test site that could be discriminated statistically ( $P < 0.05$ ) from that at the reference site was calculated for reference site prevalences between 0.1 and 20 percent. The analyses were conducted by fixing the noncentrality parameter ( $\lambda$ ) in Equation 3 for a power of 0.80 and solving the resulting equation for the number of lesions at the test site ( $N_{21}$ , see Figure 5). This is possible because the total numbers of samples at both the reference and test sites are equal in these evaluations, and the marginal sums for the reference site corresponding to the selected prevalence levels are fixed. The values of  $N_{21}$  were obtained by setting the resulting equation equal to zero and using the Newton-Raphson method to solve the single unknown ( $N_{21}$ ).

Results of the second set of power analyses are presented in Figure 8. They demonstrate that if prevalence at the reference site is constant, the minimum detectable prevalence at the test site decreases with increasing sample size. However, the rate of decrease is not linear. For example, when lesion prevalences at the reference site are near 0 percent, the approximate minimum detectable prevalences at the test site at sample sizes of 20, 40, 60, 100, and 200 fish are 26, 14, 10, 6, and 3 percent, respectively. Thus, by increasing sample size by 20 fish from  $N=20$  to  $N=40$ , the minimum detectable prevalence declines by 12 percentage points. By adding another 20 fish from  $N=40$  to  $N=60$ , the minimum detectable prevalence declines by only 4 percentage points. To realize an additional decline of 4 percentage points, 40 fish must be added from  $N=60$  to  $N=100$ . Finally, the addition of 100 fish from  $N=100$  to  $N=200$  reduces the minimum detectable prevalence by only 3 percentage points. Thus, the value of adding additional replicate samples declines as sample size increases.

Results of the second set of power analyses (Figure 8) also demonstrate that as prevalence at the reference site increases, the margin (or difference) between that value and the minimum detectable prevalence at the test site also increases. For example, if  $N=60$  and reference site prevalences are 0, 5, and 10 percent, the differences between those prevalences and the corresponding minimum detectable prevalences at the test site are approximately 10, 15, and 20 percent, respectively. Thus, as prevalence at the reference site increased within this range, the minimum detectable elevation in prevalence above reference levels doubled. These results support the recommendation made earlier in this section that every effort should be made to ensure that prevalences at the reference site are as low as possible.

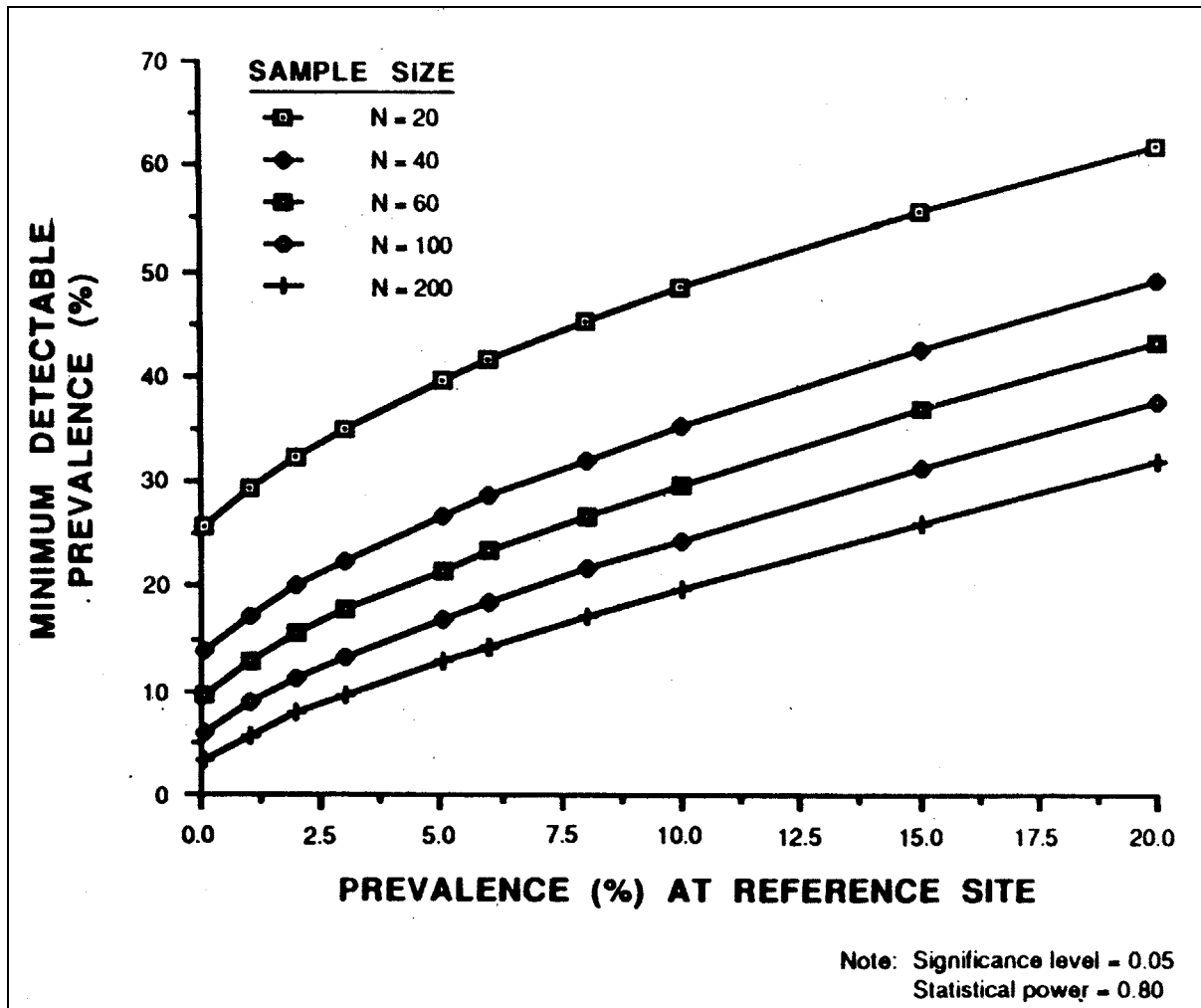


Figure 8. Effects of sample size on the minimum detectable prevalence at a test site relative to the prevalence at the reference site.

### Sampling Season

Little information is available regarding seasonal variation in prevalences of hepatic lesions in fishes. McCain et al. (1982) evaluated seasonal variation in the prevalences of neoplasms and putative preneoplasms in livers of 551 English sole from the Duwamish River, Washington (Figure 9). No significant difference among seasons ( $P > 0.05$ ; G-test of heterogeneity) was found for either neoplasms or preneoplasms.

Seasonal variation in the prevalence of hepatic neoplasms could result from the seasonal migrations exhibited by many fishes if fish with lesions behave differently than fish without lesions. For example, if fish with lesions do not migrate, lesion prevalence would be at a minimum when fish without lesions migrate into a contaminated area and would peak when fish without lesions leave the area. Seasonal variation in the prevalence of rapidly induced lesions also may vary if fish are more sensitive to lesion induction during particular times of the year.

Ideally, fish liver histopathology surveys should be conducted during the times of year when lesion prevalences are expected to peak (Sindermann et al. 1980). This strategy allows the worst-case conditions to be evaluated. It also increases the likelihood that the observed prevalences can be discriminated statistically from reference conditions. In the absence of information on seasonal variation in lesion prevalences, interannual comparisons should be made only between surveys conducted during the same season.

### Station Location

Appropriate locations of sampling stations depend upon the objectives of different studies. To evaluate the elevation of lesion prevalences above an expected level as a possible consequence of chemical contamination, stations frequently are located in contaminated and uncontaminated (i.e., reference) areas. This pairwise approach allows the observed prevalence in the contaminated area to be compared statistically with the prevalence that would be expected in the absence of contamination (i.e., the observed prevalence in a reference area). A more convincing case can be made for the association between lesion prevalences and contamination if stations are located along a gradient of contamination (i.e., from highly contaminated to moderately contaminated to uncontaminated).

In all of the above circumstances, it is recommended that chemical analyses of sediments be conducted in conjunction with fish histopathology to confirm the degrees of sediment contamination. It is also recommended that stations be located in areas where the spatial extent of contamination is large enough to reasonably expect that the sampled fish may have spent a considerable amount of time within the influence of the measured contamination.

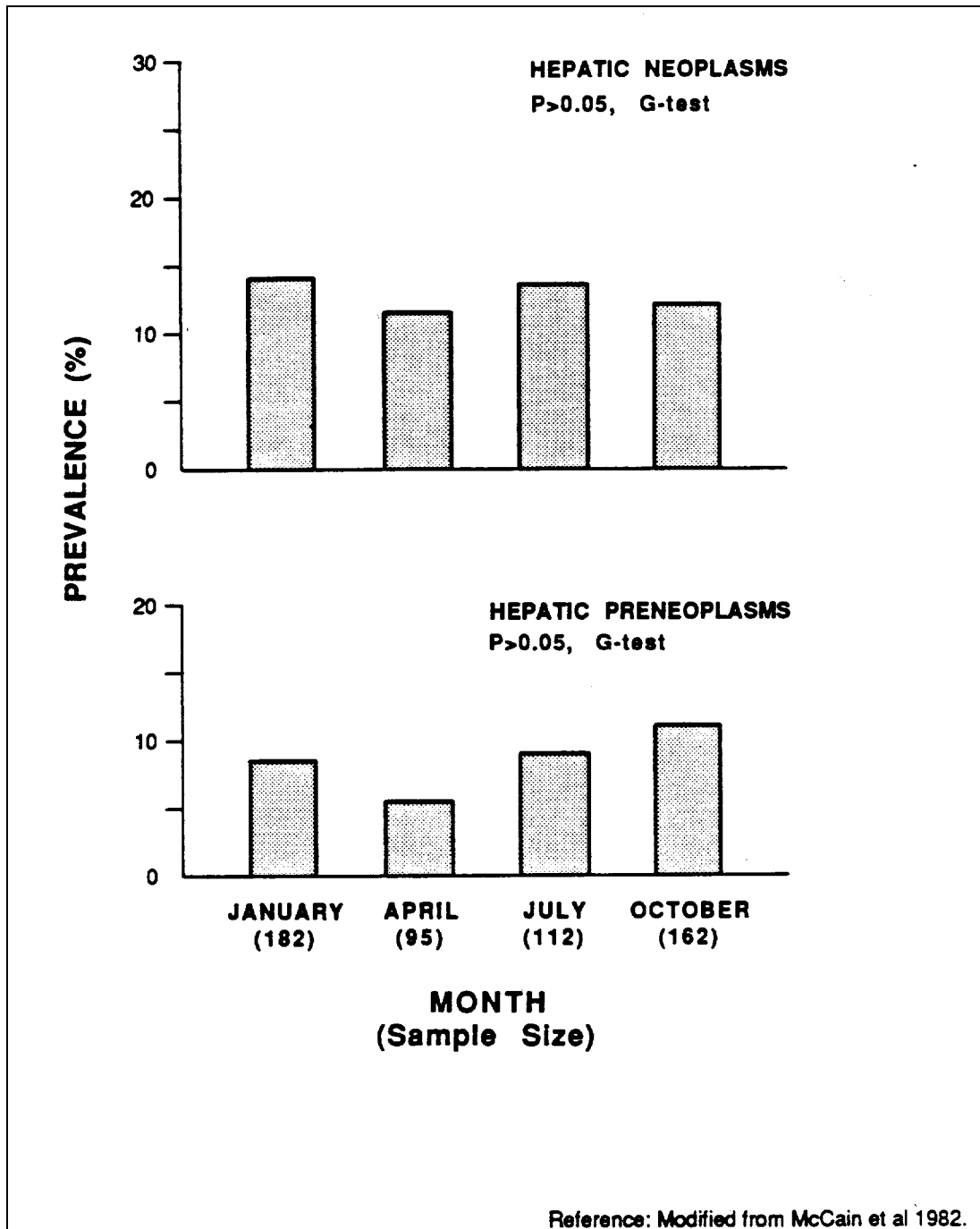


Figure 9. Seasonal variation of hepatic lesions in English sole from the Duwamish River, WA.

## FIELD SAMPLING PROCEDURES

### Fish Acquisition

A major concern when determining prevalences of hepatic lesions in fishes is that the collection technique does not bias the results. Bias will occur if fish with lesions are sampled differently than fish without lesions (Sindermann et al. 1980). For example, a passive collection technique (cf. Hubert 1983) that relies on fish feeding (e.g., hook-and-line, long-line) or fish movement (e.g., gill nets, traps) may undersample fish with lesions if their desire or ability to feed or move is reduced. By contrast, an active capture technique (cf. Hayes 1983) such as otter trawling may oversample fish with lesions if their swimming ability is reduced to the point that they would be less likely to escape the oncoming net than would fish without lesions.

At least one potential instance of sampling bias has been reported in the literature. Dawe et al. (1976) found high prevalences of hepatic neoplasms in white suckers from Deep Creek Lake, Maryland, using rotenone poisoning, but failed to find similar lesions in suckers from other localities by gill-netting the fish during spawning runs. The authors suggest that the suckers with neoplasms may not have taken part in spawning runs and therefore could not be sampled by the gill-netting technique.

Given the possible influence of collection technique on observed lesion prevalences, it is recommended that the technique used in each study of fish liver histopathology be selected to account for any known behavioral differences between fish with and without lesions. Unfortunately, little information is available regarding this topic. However, if some behavioral information exists, or if reasonable speculations can be made, this information should be used to evaluate the collection technique.

A second kind of collection bias can occur when fish are supposed to be randomly subsampled to represent a larger sample of captured individuals. This subsampling should not be biased by such factors as fish size, sex, condition, or gross abnormalities. If, however, the study design specifies that a particular component of the population (e.g., adult, juveniles, males, females) be selected, subsampling should focus on that component (e.g., using size limits or sexual characteristics), but be randomly employed within the target component. In many cases, an unconscious tendency may exist to collect larger individuals or individuals that appear to be unhealthy. Also, if fish are combined in a container prior to subsampling, large or active individuals tend to rise to the surface, whereas small or inactive fish tend to sink to the bottom of the container. It is therefore important to avoid selecting individuals from only one location in the container when subsampling.

### Holding Time and Conditions

Because some kinds of cellular alterations may begin immediately after fish are collected (e.g., due to stress, injury, death), it is recommended that hauls be relatively short in duration (i.e., 5-10 min) and that target fish be necropsied as soon as possible after collection (i.e., preferably within 15 min). If fish cannot be necropsied immediately, they should be held alive in a flow-through seawater tank.

### Labeling and Coding

At a minimum, labels should be used for liver samples and the hard body structures used for aging (e.g., otoliths, scales, spines). In some cases, the whole fish or part of the fish may be retained, and aging structures removed after sampling has been completed.

All labels should be made of waterproof paper, and all labeling should be conducted using indelible ink. Each sample container should be labeled both internally and externally (i.e., double-labeled). The external label can be gummed on one side to facilitate attachment to the container.

Each specimen should be given a unique code number. The code number should be used to label all samples that will be analyzed in the laboratory (e.g., liver samples, otoliths). The coding system can be simple, but must prevent the laboratory personnel from knowing any of the characteristics of the fish from which each sample was taken, including age, sex, health, and location of capture. This lack of knowledge will ensure that the analysis is conducted objectively. The process of ensuring sample anonymity at the time of laboratory analysis is called a "blind" system.

### Liver Subsampling

Before being necropsied, each fish should be weighed (nearest gram, wet weight) and measured (nearest millimeter, total length). The fish should then be scanned for grossly visible external abnormalities by a person trained to recognize those conditions. The fish should then be sacrificed by severing the spinal cord at the brain stem in a manner that poses no risk of damage to the liver or to the body parts used for aging.

Following severance of the spinal cord, the abdominal cavity should be opened, ensuring that the liver is not damaged in the process. Following primary incision, the entire liver should be removed gently from the abdominal cavity to provide a full view of the organ. When removing the liver, extreme care should be taken to avoid puncturing the gall bladder, as the bile stored within that organ is extremely caustic to liver tissue (Hendricks et al. 1976). If a liver is damaged by contact with bile, it should not be used for histological analysis.

Following liver removal, the fish should be scanned for grossly visible internal abnormalities. The sex of each fish and its reproductive state should also be noted at this time.

Each liver should be scanned for grossly visible abnormalities. The color and texture of the organ should also be noted. Color charts can be used to help standardize color descriptions. Particular attention should be paid to describing any abnormal foci or nodules. It may be useful to weigh each liver, photograph each anomaly, and identify on diagrams where subsamples were removed.

The process of tissue collection should be guided by the presence or absence of grossly visible abnormalities. In the absence of abnormalities, a tissue subsample (i.e., section) should be resected from the entire depth of the liver along its longest axis. When visible abnormalities are present, the tissue section should be taken so that the entire depth of the anomaly is sampled. The section should contain both normal and abnormal tissue, so that the pathologist can see the border between the two kinds of tissue. If more than one kind of abnormality is visible within a liver, each kind should be described and subsampled. Multiple sections within a single liver should be coded separately, so that histological preparations can be related to gross observations. To ensure proper fixation, each tissue section should not exceed 4 mm in thickness (Luna 1968).

### Tissue Fixation

Adequate fixation is essential for accurate histological determinations (Luna 1968). The goals of fixation are to:

- Preserve cells and their constituents in as lifelike a state as possible
- Prevent postmortem changes such as autolysis
- Protect and harden soft tissues to allow for easy manipulation during subsequent processing
- Convert the normal semi-fluid consistency of cells to an irreversible semi-solid consistency
- Aid visual differentiation of tissue structure when using stains.

To achieve these goals, fixation should be performed immediately after tissue removal, and as soon as possible after death of the organism. In addition, the thickness of the resected tissue should be  $\leq 4$  mm, and the volume of fixative should be at least 10 times that of the tissue subsample. In general, tissues should remain in the fixative for at least 48 h. For some fixatives, it may be

necessary to transfer the tissue to ethanol prior to infiltration and embedment. For most fixatives, it is advisable to preserve and store the tissues at room temperature (20° C) or below. Freshly prepared fixative should be used at all times.

The choice of fixative generally reflects the personal preferences of the pathologist, as well as the manner in which the tissue will be processed or evaluated after fixation. In a review of fixation procedures primarily for mammalian tissue, Hopwood (1969) concluded that no single fixative is ideal for all situations. Hinton et al. (1984) noted that, in contrast to mammalian studies, controlled fixation evaluations have not been undertaken with fish tissues.

The most common fixatives used to date for fish tissues are Bouin's fluid and 10 percent neutral buffered formalin (Hinton et al. 1984). Other fixatives used for fish tissue include Dietrich's fluid, Carnoy's fluid, Zenker's fixative, Helly's fixative, and Davidson's solution. None of the commonly used fixatives for light-microscopy studies of fish tissues yield fixation of high enough quality for electron microscopy (Hinton et al. 1984).

Given the above discussion, it is recommended that the method of fixation be evaluated carefully before a study begins. Because subsequent staining characteristics of fixed tissue vary with the kind of fixative, it is recommended that a single fixative be used in all surveys among which histopathological comparisons will be made.

#### Ancillary Data

When conducting fish liver histopathology surveys, a variety of ancillary data is helpful when evaluating histopathological results. Many of these kinds of data have been noted in earlier sections of this document. The following kinds of data should be included in most fish liver histopathology surveys:

- Fish age
- Fish sex
- Fish length
- Fish weight
- Gross pathological observations.

#### Age--

As described earlier, certain hepatic lesions in fishes are associated positively with increasing fish age. It is therefore critical that age dependence be evaluated for all lesions considered in a study. If age dependence is found, age differences among samples must be removed before statistical comparisons can be made. As recommended earlier, age should be determined directly using the annual ring method applied to an appropriate hard body structure.

A variety of hard body structures have been used for aging fish, including otoliths (primarily the sagittae), fin rays, scales, spines, and vertebrae (Jearld 1983). The method used for each kind of structure is different, but all require that they be performed by a well-trained and experienced individual. Also, different methods may be optimal for different species. Methods of fish aging are reviewed by Chilton and Beamish (1982) and Jearld (1983).

#### Sex--

Few field studies have examined whether hepatic lesions are found disproportionately in one sex. None of the studies evaluating sex dependence of hepatic lesions of English sole from Puget Sound found statistically significant relationships between lesion prevalence and fish sex (McCain et al. 1977, 1982; Malins et al. 1982; Tetra Tech 1985; Krahn et al. 1986). However, laboratory studies (Matsushima and Sugimura 1976; Hendricks 1982) have demonstrated that induction of hepatic neoplasms in fishes can differ between sexes. It is therefore recommended that sex differences in the prevalence of hepatic lesions be evaluated prior to comparing different sites. If sex-related differences are found, the sex ratios of the samples from the different sites should be adjusted so that they do not differ significantly.

Because patterns of length and weight of some species exhibit sex-related differences (Royce 1972), comparisons of variables based on length and/or weight (e.g., growth, condition) must be stratified by sex. It is therefore recommended that the sex of each fish selected for histopathological analysis be determined. In some cases, it may be necessary to evaluate the gonads histologically to verify the sex of an individual.

#### Length--

As described earlier, length of each fish can be used as a rough estimate of age when considered in conjunction with fish sex. This approximation is useful when fish are to be subsampled from the total catch on the basis of age. An approximation method generally is necessary during field sampling because exact ages usually are not determined until after sampling has been completed.

Length can be used to estimate the growth and condition of each fish when it is considered relative to age and weight, respectively. Thus, comparisons can be made between fish at reference and test sites or between fish with and without hepatic lesions to determine whether contamination or lesions are affecting fish growth and condition. Both of these characteristics have implications with respect to the health and behavior of individuals and the status of future populations.

It is recommended that total length (TL) be determined to the nearest millimeter for each individual of the target species, regardless of whether or not that individual will be used for histopathological analysis. Length should be measured prior to necropsy for those individuals selected for histopathological analysis. Total length is the length from the anterior-most part of the fish to the tip of the longest caudal fin rays. Two kinds of total length can be measured (Anderson and Gutreuter 1983). Maximum TL is determined when the lobes of the caudal fin are compressed dorso-ventrally, whereas natural TL is measured when the caudal fin is in its natural state. To be consistent with the convention used by most fishery investigations in the U.S., maximum TL should be measured (Anderson and Gutreuter 1983).

In some cases, erosion of the caudal fin in a substantial segment of a population may require that a measurement other than total length be used for affected individuals. If this occurs, it is recommended that maximum standard length (SL) be used as a substitute. Standard length is the length from the anterior-most part of the fish to the posterior end of the hypural bone. Anderson and Gutreuter (1983) state that in practice, SL may be measured to some external feature such as the last lateral line scale, the end of the fleshy caudal peduncle, or the midline of a crease that forms when the tail is bent sharply. Standard length can be related to total length by developing a regression relationship between these two measures for a sample that covers the complete length range observed in the population.

#### Weight--

As described earlier, weight generally is used in conjunction with length to evaluate fish condition. However, Falkmer et al. (1976) used weight as an index of fish size and, indirectly, of fish age. Generally, it is recommended that length, instead of weight, be used as a rough estimate of fish age. If weight is considered during a Puget Sound study, it is recommended that it be determined individually for each fish selected for histopathological analysis. Weight should be measured to the nearest gram (wet weight) of the whole body prior to necropsy.

#### Gross Pathological Observations--

Gross observations of external abnormalities in all fishes sampled (both target and nontarget species) are relatively inexpensive and should be performed routinely when conducting fish liver histopathology surveys. Gross observations of internal abnormalities of all individuals selected for

histopathological analysis also is recommended. Although gross observations generally are not definitive evaluations of fish health, they may be very useful for uncovering previously unknown pathological conditions in fishes from polluted areas. For example, liver abnormalities in Atlantic tomcod from the Hudson River, New York (Smith et al. 1979) and in English sole from the Duwamish River, Washington (Pierce et al. 1978) were discovered incidentally, as fishes were being evaluated for other purposes. In addition to uncovering previously unknown pathological conditions, gross observations can also be related to microscopic observations of the liver to investigate possible associations between different kinds of pathological conditions.

Gross external observations are relatively inexpensive because they do not require specialized equipment or preparation techniques and thus can be made as individuals are sorted from the catch. In addition, gross external observations generally do not require that a trained pathologist be aboard the sampling vessel. However, it is extremely important that at least one individual on board be trained by a qualified pathologist to identify the various kinds of pathological conditions that may be encountered. Sindermann et al. (1980) stress that pathological observations made by untrained personnel are usually useless and often misleading. For example, at least two pathological conditions (fin erosion and skin ulcers) can easily be confused with the external damage that fishes may suffer as they are dragged along the seafloor in an otter trawl.

Given the potential usefulness of gross observations and the need for accurate and verifiable determinations, it is recommended that representative fishes having each kind of pathological condition be archived for each major survey, and that the conditions be confirmed by a qualified pathologist. This verification step is especially important if different personnel make the gross observations during different surveys. For all suspected pathological conditions that cannot be identified in the field, representative specimens should be archived for later evaluation by a qualified pathologist.

Sindermann et al. (1980) reviewed the literature on the relationship of fish pathology to pollution in marine and estuarine environments, and identified the following four grossly visible conditions as acceptable for immediate use in monitoring programs:

- Fin erosion
- Skin ulcers
- Skeletal anomalies
- Neoplasms (i.e., tumors).

Fin erosion is found in a variety of fishes from polluted habitats. It probably is the most frequently observed gross abnormality in polluted areas (Sindermann 1983). In demersal fishes, the dorsal and anal fins are the ones most frequently affected whereas in pelagic fishes, the caudal fin is the one primarily affected. The causes of fin erosion are unknown and likely complex. They may

include chemical contaminants, low dissolved oxygen, and pathogens. Fin erosion has been induced in fishes after laboratory exposure to petroleum and PCBs (Couch and Nimmo 1974; Minchew and Yarbrough 1977).

Skin ulcers have been found in a variety of fishes from polluted habitats. Next to fin erosion, they are the most frequently reported gross abnormalities in polluted areas (Sindermann 1983). Prevalence of ulcers generally varies with season, and is often associated with organic enrichment. The primary cause of skin ulcers may be pathogenic organisms (e.g., *Vibrio* spp.) associated with pollution.

Skeletal anomalies frequently are more prevalent in fishes from polluted areas than in fishes from uncontaminated areas. Most observed skeletal anomalies involve the spinal column and include fusions, flexures, and vertebral compressions. Skeletal anomalies also include abnormalities of the head, fins, and gills. Skeletal anomalies have been induced in fishes after laboratory exposure to kepone and heavy metals (Sindermann et al. 1980).

Neoplasms or tumors have been found in elevated prevalences in a variety of polluted areas throughout the world. The most frequently reported grossly visible tumors include liver tumors, skin tumors (i.e., epidermal papillomas and/or carcinomas), and neurilemmomas. Liver tumors have been induced in fishes after laboratory exposure to a variety of chemicals (see section entitled Laboratory Studies). Two kinds of growths have been described as epidermal "papillomas" and pseudobranchial "tumors" in the literature (Sindermann et al. 1980). The predominant and pathognomonic cell type in these growths is the presently unidentified X-cell. Available evidence suggests that this cell probably is a protozoan parasite, possibly an amoeba of the family Harmanellidae (Dawe 1981; Myers 1981). No relationship between the prevalence of these skin anomalies and pollution has been demonstrated conclusively.

It is recommended that any survey of fish liver histopathology examine fishes for fin erosion, skin ulcers, skeletal anomalies, and neoplasms, at a minimum. The occurrence of parasites should also be recorded. In addition to the five conditions listed above, any additional grossly visible pathological conditions that are suspected of occurring in a specific locality should be monitored.

#### Other Ancillary Data--

In addition to the kinds of ancillary data recommended for all fish liver histopathology studies (i.e., those discussed previously), several other kinds of data may prove useful when interpreting observed patterns of lesion prevalences, including:

- Contaminants in sediment
- Contaminants in tissue
- Contaminants in stomach contents
- Contaminant metabolites in bile
- Stomach contents
- Sediment toxicity
- Benthic infaunal assemblages
- Identities and abundances of nontarget species.

## LABORATORY PROCEDURES

### Tissue Processing

#### Embedding--

Before a fixed tissue can be sectioned (i.e., sliced into very thin sections for microscopic analysis), it must be embedded in a firm medium (Luna 1968). The medium ensures that thin, uniform sections can be cut. The most common embedding medium used for fish tissue being prepared for light microscopy is paraffin. Other media considered suitable for light microscopy include celloidin and carbowax, as well as the relatively new plastic materials (e.g., methacrylate, epoxies) developed for high-resolution light microscopy and electron microscopy (Johnson and Bergman 1984).

It is recommended that paraffin be used to embed tissues being prepared for routine histopathological evaluation of liver abnormalities in fish. Paraffin is readily available in commercial laboratories and is relatively inexpensive. It allows examination of much larger tissue sections than do many of the more specialized techniques (e.g., methacrylate embedment). However, other media may be used if the objectives of the study go beyond routine histopathological examination using light microscopy.

The paraffins commonly used to embed fish tissue include Paraplast, Paraplast Plus, and Paraplast Extra. Of these media, Paraplast Extra generally provides the best results in terms of ease of sectioning and degree of resolution.

It is recommended that embedding be conducted using an automated tissue embedding center. Automated methods usually are better at providing high quality, uniform, and reproducible

results than are manual methods. The automated center should provide a guaranteed uniform temperature during embedment. The use of vacuum infiltration during embedment is recommended. Tissues generally are embedded in plastic cassettes (marked with unique specimen numbers) for ease of sectioning and subsequent storage and retrieval.

When paraffin is used as an embedding medium, tissues must first be dehydrated and cleared in solutions miscible with paraffin. Dehydration entails removing all extractable water from the tissue by having a dehydrant diffuse through the tissue. This generally is accomplished by immersing the tissue in a graded series of increasing concentrations of the dehydrant. The dehydrant used most frequently is alcohol (e.g., ethanol).

Following dehydration, the tissue must be cleared using a reagent that is miscible with paraffin and the dehydrant. Clearing renders the tissue amenable to paraffin infiltration by removing the dehydrant. As the dehydrant is removed, the tissue clears. When the tissue becomes transparent, the clearing process is considered complete. Commonly used clearing agents include xylene, toluene, and chloroform.

Following clearing, the tissue is impregnated by paraffin. Impregnation is the complete removal of the clearing reagent by substitution with paraffin. Impregnation usually requires two or three baths in paraffin under a controlled temperature that keeps the paraffin above its melting point. The temperature of the bath should never rise more than 5° C above the melting point of the paraffin, as excessive shrinking and hardening of the tissue may result. When a vacuum is applied during impregnation, it helps remove air, gases, and any remaining clearing agent. The vacuum also draws the paraffin into all areas of the tissue, especially those areas left void by the evacuation of air.

Following impregnation, embedding of the tissue is completed by properly orienting it in melted paraffin. When the paraffin solidifies, it provides a firm medium for keeping intact all parts of the tissue when sections are cut.

#### Sectioning--

Following embedment, tissues are sectioned (i.e., cut) into very thin slices from the paraffin block using a microtome equipped with a very sharp stainless steel blade (Luna 1968). High quality sectioning facilitates the pathologist's task of accurately identifying tissue and cellular abnormalities.

The quality of sectioning depends greatly on the ability of the sectioning technician and the quality and condition of the sectioning equipment. The technician must have adequate manual

dexterity and must be well-trained. Quality of sectioning should be preferred over performance rate. The most critical component of the microtome is the knife. The knife should always be maintained at its highest degree of sharpness, so that sections ribbon off the paraffin block in a flat, unwrinkled manner. The knife should be cleaned after each use by removing accumulated paraffin with a piece of gauze saturated with xylene.

The ideal section should be of uniform thickness and free from compression, wrinkles, and knife marks. Unsatisfactory sections should always be discarded and new ones taken. For histopathological analysis of fish liver tissue, it is recommended that sections be 4-5 um in thickness. Sections of this thickness can be produced readily by most commercial laboratories.

#### Mounting--

Following sectioning, tissues are mounted onto glass microscope slides (Luna 1968). This procedure involves floating tissue sections in a warm-water bath (50° C) to fully expand the section, and then transferring the section onto a glass slide. The slide may be precoated with albumin to facilitate adhesion. The section must lie flat on the slide with no wrinkles, tears, or bubbles present. Slides sometimes are heated to ensure the firm adhesion of the section to the glass.

#### Staining--

After tissue sections are mounted on microscopic slides, they can be stained using dyes to differentiate various tissue and cellular elements (Luna 1968). Staining enhances the pathologist's ability to recognize individual tissues and cell types, and to detect pathological alterations.

A wide variety of stains and staining procedures are available, both for routine and specialized purposes. The most common staining procedure used for fish liver tissue is initial staining with hematoxylin, followed by counterstaining with eosin. The hematoxylin and eosin procedure is often abbreviated as H&E staining. Hematoxylin imparts a blue or purple tint to alkaline (basic) cellular elements. Eosin, by contrast, imparts a pink or red tint to acidic elements. Cellular elements stained by hematoxylin are termed basophilic, whereas those stained by eosin are termed eosinophilic. Because numerous methods of H&E staining are available, it is recommended that several be evaluated before a fish liver histopathology study begins, and that the one providing the best results for the species of interest be selected for use in the study.

Although H&E staining is suitable for most diagnostic purposes, it may be necessary to use more specialized staining techniques to identify accurately certain tissue and cellular elements. Some adjunct staining techniques used in fish pathology include Periodic Acid-Schiff (PAS), Masson's trichrome, Prussian blue reaction for hemosiderin, and Best's carmine for glycogen. The choice of suitable special stains will depend upon the kinds of conditions detected. The need for special stains should be determined by the pathologist who examines the tissues.

Following any staining procedure, the tissue sections must be covered with glass coverslips. The coverslips are attached to the slide by using mounting medium. Several mounting media are commercially available. The one that is chosen should provide good optical clarity and should protect the tissue for long-term storage. A commonly used mounting medium is Protex.

#### Slide Coding--

In general, slides should be given the same code number as that given to each specimen in the field. However, in some cases the pathologist may be capable of discerning the site of capture from this code number. For example, the same pathologist may have been involved with the field collection of tissue sections. In such cases, it is recommended that a second code number be substituted for the original code number on each slide to ensure complete objectivity of histopathological evaluations.

#### Histopathological Evaluations

##### Qualifications of the Pathologist--

Probably the most important factors for ensuring accurate histopathological evaluations are the qualifications of the pathologist making those evaluations. Pathology is a science that relies considerably on training and experience. It is therefore recommended that, at a minimum, the pathologist be formally trained in the fields of human, veterinary, or comparative pathology. In addition, it is recommended that the pathologist have demonstrated experience in the histologic examination of fish tissue. This second requirement is necessary because pathological conditions in fish tissue may not directly resemble similar conditions in other groups of organisms (e.g., mammals). Ideally, the pathologist should have experience with the species of interest, because interspecific differences exist in the appearance and structure of fish livers. If a pathologist who meets all of the above criteria is not available for a particular study, it is recommended that the pathologist chosen for the study work closely with an experienced fish pathologist, until adequate experience has been gained to work independently.

##### Equipment--

To adequately perform the tasks required of a diagnostic pathologist, it is essential that high quality optical equipment be employed. The microscope should be a modern instrument equipped with multiple objectives and the capability of magnifications up to a minimum of 500 X. Ideally, the microscope should also be equipped with a camera system, so that observed abnormalities can be documented photographically.

#### Examination of Sections--

For each fish, at least one section should be examined microscopically. During this examination it is imperative that the entire tissue area be evaluated at a minimum magnification of 100-200 X. The investigator should begin by scanning the entire section at 50-X power to obtain an overall impression of the section. Subsequently the pathologist should examine each field in the section at a magnification of 100-200 X, and increase magnification to 400-500 X when necessary to verify the presence and characteristics of subtle abnormalities.

#### Descriptions of Lesions--

The field of fish histopathology does not have the long history enjoyed by the fields of human and veterinary pathology. As a consequence, the level of knowledge concerning the clinical effects of many lesions in fishes is incomplete. It is possible that future field studies will evaluate species for which prior histopathological data or even data on normal histology are not available. To avoid assignment of unwarranted prognostic connotations, it is recommended that descriptive, rather than diagnostic, terms be employed when evaluating the new species. For species that have been studied extensively, the use of diagnostic terms may be appropriate. The nomenclature used in descriptive histopathology is contained in most basic pathology texts (e.g., Robbins et al. 1984; Smith et al. 1972).

#### Coding and Recording Abnormalities--

As each tissue section is examined, individual abnormalities should be described on a pathology record sheet. In studies for which there are multiple examiners (pathologists), all cases bearing significant abnormalities should be set aside for confirmation by the chief pathologist. After confirmation, the abnormalities may then be entered in an appropriate computer format for storage and analysis.

Presently, the only available coding system specifically designed for use in fish histopathology studies is that maintained by the National Ocean Data Center (NODC) in Washington, DC. The NODC Fish Histopathology Code (i.e., File Type 13) was developed for use in descriptive and diagnostic fish histopathology studies. The code was developed by L.D. Rhodes and M.S. Myers of the Northwest and Alaska Fisheries Center (National Marine Fisheries Service, NOAA) in Seattle, Washington. This coding system serves the following basic purposes:

- Permit the recording of unique histopathologically evinced disease entities (i.e., lesions), infectious conditions, parasitic conditions, and cellular alterations onto computer formats for convenience in later entry, storage, and analysis

- Provide a standardized nomenclature for lesions detected in tissue sections
- Permit an assessment of the distribution and relative severity of any lesions detected, including any host response to infectious or parasitic agents.

The basic organization of this coding system was adopted from the Systematized Nomenclature of Pathology (SNOP) system which has been used in various forms by hospitals and animal research institutes for over 10 yr. However, the NODC code is designed specifically for use in fish histopathology studies and does not provide for entry of the kind of clinical data that the SNOP system allows. The organizational scheme of the NODC Fish Histopathology Code allows for specific identification and description of the following features:

- The organ affected
- The suborgan or tissue involved
- The lesion itself
- The distribution of the lesion within the organ (e.g., focal, multifocal, or diffuse)
- The relative severity of the lesion
- Any host response resulting from reaction to an infectious or parasitic agent.

The NODC code also is designed to be interfaced, via the unique specimen identification (accession) number, to other data formats within File Type 13 that are capable of documenting other essential information such as site, method, time and date of fish capture, bottom and surface water temperature (station header record), sex, sexual maturity, age, weight, length, and gross pathology data (gross pathology record). This kind of information facilitates the epizootiological analysis of the histopathology data and intersite comparison of lesion prevalences.

Specifically relating to lesion descriptions, the NODC Fish Histopathology Code is organized into repeating units of 12 digits that describe a specific lesion according to organ affected (3 digit code), suborgan or tissue type (3 digits), lesion description (3 digits), distribution (1 digit), severity (1 digit), and degree of host response in the case of parasitic/infectious agents (1 digit). On a typical 80-column data format, this permits the description of five lesions. However, a much larger number of lesions can be described for a particular specimen as a result of the sequence number in Column 80 that permits entry of additional descriptions in subsequent rows.

The organ code permits entry of up to 999 different organ types for a particular specimen, and therefore is quite flexible. This code therefore permits expansion beyond the 97 organ types used currently. It generally is organized into broad anatomical groupings, such as elements of the gastrointestinal tract, other digestive organs (liver and exocrine pancreas), and excretory, circulatory, reproductive, endocrine, skeletal, immune, and nervous systems, along with specific identification of skin and fin anatomical entities (e.g., caudal fin).

The suborgan/tissue code is also highly flexible and permits expansion of the current code, because it permits up to 999 different identifiers. It also is generally organized into broad groupings of tissue types, including epithelial subtypes (e.g., hepatocellular epithelium); connective tissue and the cells and other elements composing connective and supportive tissues; hematopoietic (blood forming) tissues and blood cell types; elements of the cardiac and circulatory system; elements of the central and peripheral nervous system; and elements of the skin, excretory, and reproductive systems. Currently, 353 identifiers are available within this subcode.

The lesion code itself generally is organized according to broad categories characteristic of different pathological processes. Within the 3-digit format for this code, the first digit (001) is reserved for identification of normal tissue. Generally, codes up to 099 are reserved for protozoal infectious agents; 100-199 for metazoan parasites and bacterial, viral, and rickettsial infections; 200-299 for inflammatory disorders; 300-399 for degenerative and necrotic conditions; 400-499 for cellular organelle changes (i.e., generally applicable to observations made at the electron microscope level); 500-699 for miscellaneous cellular and extracellular alterations; 700-799 for growth disorders such as tissue atrophy, proliferation, regeneration, and hyperplasia; 800-899 for preneoplastic and neoplastic conditions; and 900-999 for vascular disorders such as thrombosis and congestion. Within these categories, there exist numerous available open codes should other descriptors be needed.

The distribution code (1 digit) assesses the involvement of a lesion within an organ or suborgan according to its distribution. It uses a scale of 1 to 5 to describe focal, focal to multifocal, multifocal, multifocal to diffuse, or diffuse distributions, respectively.

The severity code (1 digit) uses a scale of 1 to 7. It describes the relative severity of a condition from minimal (1) to severe (7).

The final subcode in the NODC Fish Histopathology Code is the host response code (1 digit). It is used exclusively to describe the severity of host reaction to an infectious/parasitic agent. This inflammatory response is coded on a scale of 1 to 8, describing no observable response (1) to a severe response (8).

The NODC Fish Histopathology Code utilizes a nomenclature for pathological description derived from several sources to properly and specifically describe any observed lesions. Most terms are derived from the pathology text of Robbins et al. (1984), which is a standard reference for human pathology, including morphologic descriptions of histologic lesions. However, because this text deals strictly with human pathology, specialized texts for fish pathology (e.g., Ribelin and Migaki 1975; Roberts 1978) and for veterinary pathology (e.g., Smith et al. 1972) have been used for specialized terms applicable to fishes. Identification of parasites in tissue sections follows the criteria set forth in the monograph of Chitwood and Lichtenfels (1972). The nomenclature for specific degenerative, proliferative, preneoplastic, and neoplastic conditions in the liver of fishes has been adopted from terms used to describe similar lesions in mice (Frith and Ward 1980), rats (Stewart et al. 1980), and rainbow trout (Hendricks et al. 1984).

#### Quality Assurance/Quality Control

##### Interspecific Considerations--

Some fish liver histopathology studies may involve a diverse array of species from numerous geographic locations. Compared with mammals, fish are a relatively primitive group of animals with a long period of phylogenetic development. Because of this relatively long evolutionary history, the anatomical and histological differences that exist between different species of fish (even closely related ones) are much more profound than are those that exist between different species of mammals. This diversity is illustrated by the fact that an experienced pathologist can readily distinguish three sympatric species of flatfish (pleuronectidae) from Puget Sound, Washington simply on the basis of liver architecture. The hepatic tissues of these three fishes are so distinct in terms of distribution of hepatopancreas and melanin macrophage centers, and hepatocellular morphology that pathologists can readily sort slides by species without having to refer to data sheets. Such interspecific differences make it necessary for pathologists to become intimately familiar with the target species before beginning a field study, so as to accurately recognize anatomical features and to correctly distinguish seasonal or maturational changes from pathological alterations. Such interspecific differences also make it almost impossible for a pathologist unfamiliar with a given species to interpret accurately verification samples received under the auspices of a QA/QC program.

##### Internal Verification of Identification--

For studies in which multiple pathologists in the same laboratory are used to read slides, all cases bearing significant lesions should be examined and verified by the senior pathologist. In addition, at least 5 percent of the slides read by one pathologist should be selected randomly and read by a second pathologist without knowledge of the diagnoses made by the initial reader.

#### External Verification of Identification--

At least 5 percent of the slides read within a laboratory should be submitted for independent diagnosis to a pathologist not involved with the laboratory. These slides should be chosen to represent the range of pathological conditions found during a study, and the external pathologist should not be aware of the diagnoses made by laboratory personnel. The external pathologist should have experience with fishes and, ideally, with the species of interest.

#### Reference Collection--

Each laboratory should build a reference collection of slides that represents every kind of pathological condition found in various studies conducted by laboratory personnel. Each of these slides should be verified by an external pathologist having experience with the species of interest. These slides can then be used to verify the diagnoses made in future studies to ensure intralaboratory consistency among studies. The slides also can be compared with those of other laboratories to ensure interlaboratory consistency. A reference collection of photographs also can be made, but should not be substituted for a slide collection.

#### Photographic Record--

The chief pathologist should develop a photographic record that documents the significant classes of lesions encountered during the course of each study. The photographs should be of sufficient quality to illustrate clearly the diagnostic features of each lesion. Where necessary, multiple photographs taken at increasing levels of magnification should be included. The photographs should bear a label that indicates the degree of magnification and the code number of the tissue photographed.

#### Slide Set--

The chief pathologist should prepare a set of microscope slides that bear representative examples of major lesions encountered during each study. The slide set should also contain representative normal slides that illustrate the range of physiological variation encountered over the course of the investigation. The slide set should be accompanied by written descriptions of each slide including the code number, critical diagnostic features, and final diagnosis.

#### DATA ANALYSIS

Some of the general considerations for analyzing data generated during fish liver histopathology surveys are described in this section. The details of data analysis may vary widely among studies, depending upon the kind of data collected and the study objectives. Although all of

those details are not specified in this section, the general directions that detailed analyses should follow are recommended.

For most studies, two major kinds of analysis generally are made. The first kind of analysis involves comparisons among stations during single time periods. The objective of this kind of analysis is to evaluate gradients in lesion prevalence away from a contaminated area or to compare prevalences at stations close to a contaminated area with prevalences in a reference area. The second kind of analysis involves comparisons among different time periods at single stations. The objective at this second kind of analysis is to evaluate temporal changes in lesion prevalences.

#### Age and Sex Effects

As recommended earlier, the sex and age of each fish selected for histopathological analysis should be determined. When data on lesion prevalences are ready to be analyzed, they should first be tested for statistically significant relationships with sex or age.

If the prevalence of a particular lesion is related to either sex or age, the sex ratio or age distribution at all stations that will be compared should be tested for significant differences among stations. If such differences are found, individuals should be removed from stations until the adjusted sex ratios or age distributions do not differ significantly among stations. Once these adjustments have been made, lesion prevalences of the remaining fish can be compared without interference from the effects of sex or age.

An alternative to adjusting samples when relationships between lesion prevalence and sex or age are found is to stratify comparisons among stations by sex or age class. In doing so, however, sample sizes may be reduced substantially and the statistical power to detect significant differences among stations also would decline.

If no relationships are found between lesion prevalence and sex or age, it is not necessary to evaluate sex and age differences among stations. Instead, comparisons of lesion prevalences among stations can be made directly.

#### Growth and Condition

In many fish liver histopathology studies, the question arises as to how contamination or the presence of hepatic lesions is affecting the overall health of each fish. Two general indices of fish health that are measured frequently in studies of fishes are growth and condition. To evaluate these indices, the weight (nearest gram), length (nearest millimeter), and age of each individual for histopathological analysis should be measured.

Growth can be estimated as the length of an individual fish at a given age. Use of growth as an index of fish health assumes that unhealthy fish grow less rapidly than their healthy counterparts. Growth might be considered a relatively long-term indicator of fish health, as it may require many months for differences in length between healthy and unhealthy fish to be large enough for statistical discrimination. Potential effects of pollution or hepatic lesions on the growth of fish can be evaluated by comparing the lengths of each age class between fish from contaminated and reference areas or between fish with and without hepatic lesions.

Condition is a measure of the "fatness" of a fish and can be estimated as the weight of an individual relative to that individual's length. Use of condition as an index of fish health assumes that the condition of unhealthy fish will be reduced relative to their healthy counterparts. Condition might be considered a relatively short-term index of fish health, as it may only require several weeks for differences between healthy and unhealthy fish to be large enough for statistical discrimination.

Condition can be expressed as a weight-length regression relationship (Ricker 1975), and then compared among stations or between fish with and without hepatic lesions by using analysis of covariance. Condition of each fish may also be expressed in the form of an index that incorporates the weight and length of each individual. Index values can then be compared statistically among stations or between fishes with and without lesions. Three indices of fish condition used frequently are Fulton's condition factor (the most common), the relative condition factor, and Relative Weight (Anderson and Gutreuter 1983).

#### Comparisons Among Stations

In many fish liver histopathology studies, the prevalences of hepatic lesions are compared statistically among stations having various degrees of contamination. The simplest case is a pairwise comparison between a contaminated site and a reference site. As noted earlier, the statistical test recommended for this kind of comparison is the G-test of independence, using a 2x2 contingency formulation. This test also can be used with multiway contingency tables to compare lesion prevalences among more than two stations.

As noted earlier, values of G should be adjusted when sample sizes are small ( $N \leq 200$ ). At least two correction factors have been recommended in the literature: Yates' correction for continuity and Williams' correction (Sokal and Rohlf 1981). Yates' correction requires that observed values in the 2x2 table be adjusted by adding or subtracting a value of 0.5. Williams' correction for a 2x2 table requires that the calculated value of G be divided by q, where:

$$q = 1 + \frac{\left( \frac{N}{N_{11}+N_{12}} + \frac{N}{N_{21}+N_{22}} - 1 \right) \left( \frac{N}{N_{11}+N_{21}} + \frac{N}{N_{12}+N_{22}} - 1 \right)}{6(N)} \quad (4)$$

Based on the results of simulation experiments, Grizzle (1967) showed that the application of Yates' correction to the chi-square test statistic ( $\chi^2$ ) produces a test that is unduly conservative. Grizzle (1967) also reported that the likelihood ratio test statistic (i.e., G-test statistic) behaved almost exactly like  $\chi^2$ . Similar sampling experiments to evaluate the performance of the Williams' correction have not been published. However, Sokal and Rohlf (1981) indicate a preference for the application of the Williams' correction factor to the G-test statistic for small sample sizes.

To evaluate the effect of Yates' and Williams' corrections on the performance of the G-test, a series of simulations was conducted as part of the present study. These simulations were conducted in the following sequential manner:

- Equal sample sizes (i.e.,  $N = 20-100$ ) were specified for each site, and a true null hypothesis was assumed for a lesion prevalence ( $p$ ) of 10 percent at the reference and test sites.
- For individual sampling conditions, random samples were generated from binomial distributions, with parameters  $n$  and  $p$  corresponding to the selected sample sizes and prevalences, respectively. The method used to generate the binomial variables employed the fact that a binomial random variable is the sum of  $n$  independent Bernoulli random variables.
- The procedure of sample generation and analysis was repeated 10,000 times for each set of sampling conditions. All calculated values of the G-test statistic were saved and subsequently analyzed to determine the proportion of values greater than or equal to the critical value corresponding to a significance (i.e., Type I error) level of 0.05. The observed level of Type I errors in each simulation experiment was used to evaluate the effect of the correction factors on test performance.

Each of the simulation experiments representing the selected sampling conditions was repeated three times: once with the Yates' correction applied in the calculation of the G-test statistic, once using the Williams' correction, and once with no correction applied to the value of  $2 \ln(G)$ . Three sets of experiments and a total of 24 individual simulation experiments were performed.

Results of the simulation experiments are summarized in Figure 10. This figure also shows the performance of the G-test with and without the application of the selected correction factors. The test results based on the use of the Yates' correction factor, for example, indicate that the proportion of tests in which the null hypothesis was falsely rejected (i.e., probability of Type I error) is substantially less than the nominal level (i.e., 0.05) over the range of sample sizes evaluated. The test statistic resulting from the application of Yates' correction is classified as conservative, because the frequency of rejecting a true null hypothesis (i.e., incorrectly concluding that differences in the prevalence of lesions exist) is decreased over the nominal level of the test.

The use of uncorrected values of the G-test statistic will lead to errors in the direction opposite to that described for use of Yates' correction (Figure 10). That is, the frequency of rejecting a true null hypothesis will be increased over the nominal level of the test when sample sizes are small. For example, when simulated sample sizes at each sampling location were less than 30, the actual probabilities of the Type I error obtained at the nominal 0.05 significance level were greater than 0.076. The actual probabilities of the Type I error obtained at the 0.05 significance level in the simulation experiments were greater than the nominal level for all sample sizes less than 80.

When the Williams' correction factor was applied to the value of the test statistic, the G-test performed very close to its expected chi-square distribution (Figure 10). Over the range of sample sizes evaluated (i.e., 20-100), the actual probability of a Type I error corresponding to the nominal 0.05 significance level ranged between 0.041 and 0.061. The efficacy of the Williams' correction factor was especially evident at the smaller sample sizes. For example, at sample sizes of 20, the actual probability of a Type I error obtained at the nominal 0.05 significance level was 0.081 for uncorrected values of the test statistic, 0.041 for values corrected with the Williams' factor, and 0.013 using the Yates' correction.

Based on the simulation experiments conducted as part of this study, it is recommended that the Williams' correction be applied to the G-test for independence when lesion prevalences are compared among study sites and sample sizes at each site are small (i.e.,  $N \leq 80$ ). The Williams' correction should also be applied when multiway contingency tables are used and sample sizes are small. The formula for Williams' correction for multiway tables is more complex than that used for 2x2 tables and is presented in Sokal and Rohlf (1981).

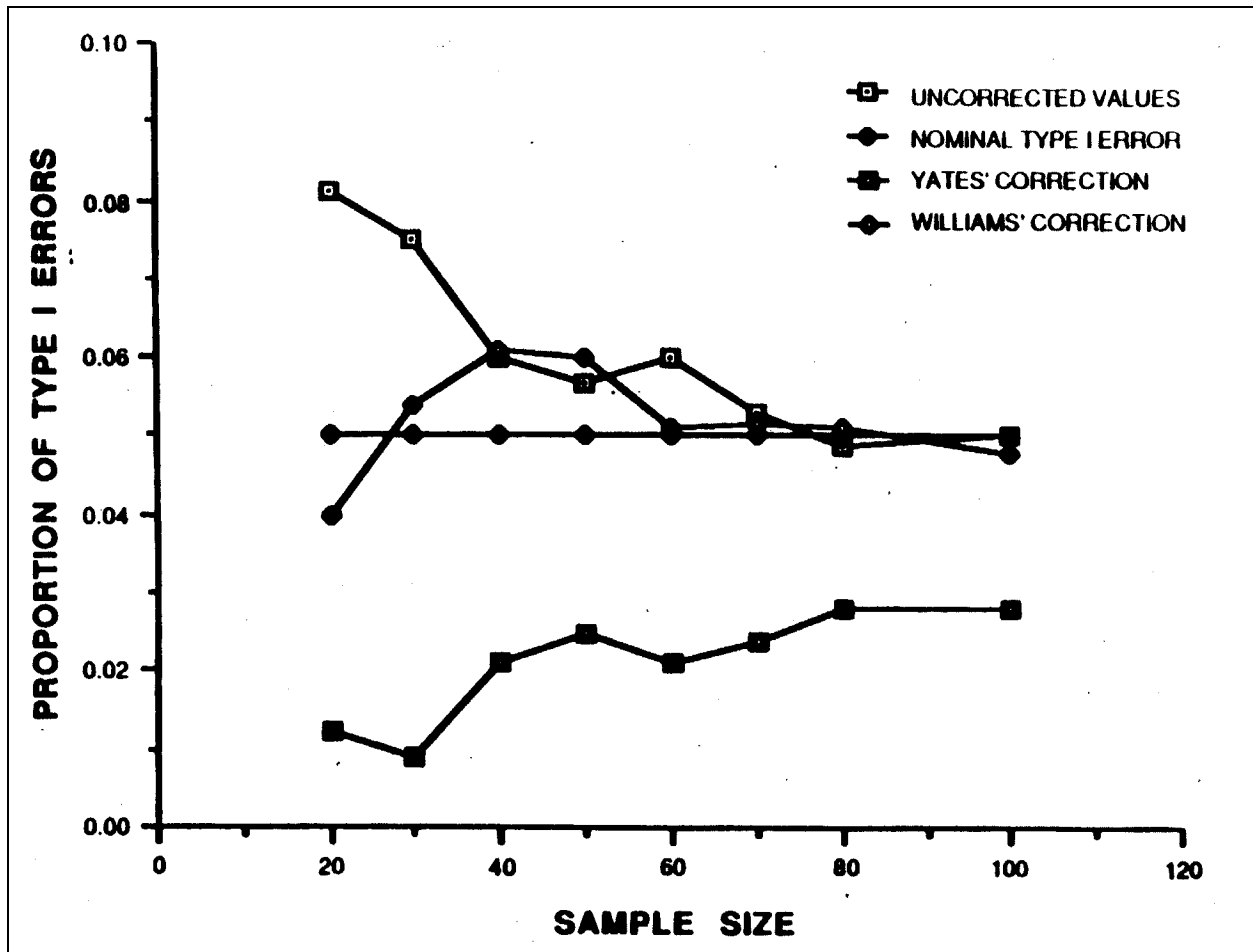


Figure 10. Results of simulation experiments showing the proportion of Type I errors in tests of the null hypothesis that lesion prevalence at both the reference and test sites equals 10 percent.

### Relationships with Ancillary Variables

Relationships between prevalences of hepatic lesions and a variety of ancillary variables can and have been evaluated in an attempt to determine potential causes of the observed lesions. A pairwise approach to evaluating potential causes is useful. For example, if lesion prevalences and the values of a variable are both high in a contaminated area and low in a reference area, a case can be made that the variable may be causally related to the hepatic lesions. However, a correlational approach is much more convincing than a pairwise approach. In such an instance, a gradient in lesion prevalence is related directly to a similar gradient (positive or negative) in the values of a variable.

The most common ancillary variable that has been related to prevalence of hepatic lesions in fishes has been chemical concentrations in bottom sediments. In most cases, a pairwise approach has been used. However, Malins et al. (1984) used a correlational approach.

Because a wide variety of chemicals generally is found in contaminated sediments, and because many of these chemicals covary across stations, it rarely is possible to test the effects of single chemicals, while holding others constant. The most common manner in which to analyze such data is to conduct a multivariate analysis that generates factors composed of covarying chemicals (e.g., Malins et al. 1984). The chemicals that load most strongly on each factor can then be considered the major characteristics of the factor. Factors can then be correlated with lesion prevalence. When a statistically significant positive correlation is found, the major characteristics of the factor are considered the putative causes of the lesions.

A second variable that commonly is measured in conjunction with lesion prevalence is chemical contamination of fish tissue. The tissues examined most frequently are muscle and liver tissue. The goal of these analyses is to relate tissue concentrations to lesion prevalences. The inference usually made is that the chemicals found in tissue may have been causally related to the observed hepatic lesions. However, this inference must be made with considerable caution, as many organic compounds (including potent carcinogens) are rapidly metabolized in the liver of fishes, and thus rarely are found in muscle or liver tissue (e.g., Malins et al. 1985a,b). Krahn et al. (1986) demonstrated that measuring metabolites in bile, rather than parent compounds in tissue, may be a more meaningful way of relating lesion prevalence to those compounds that are metabolized rapidly.

Several studies have measured chemical concentrations in the stomach contents of fish from contaminated and uncontaminated areas (e.g., Malins et al. 1985a,b). In general, stomach contents from polluted areas contain substantially higher concentrations of chemical contaminants than do

stomach contents from uncontaminated areas. The inference is that diet is a major route by which contaminants may enter the fish. Although this inference is correct, no quantitative measure of importance can be made because other potential routes (i.e., gills, skin) are not measured, and the fraction of chemicals that actually is absorbed from the stomach contents is unknown.

The stomach contents (i.e., prey composition) of fish from contaminated areas might be compared to the stomach contents of fish from reference areas to determine whether the diet in contaminated areas is reduced in quantity or quality relative to that in the reference area. The inference is that dietary deficiencies may facilitate or even cause lesion induction in fish livers. For example, a variety of studies have found that nutritional imbalances can induce hepatic abnormalities in fishes (e.g., Snieszko 1972) or enhance the toxicity of chemicals to fishes (e.g., Mehrle et al. 1977). In addition, outright starvation can induce such abnormalities (e.g., Segner and Moller 1984).

In addition to variables that may relate directly to induction of hepatic lesions in fishes, a variety of relatively independent biological indicators measured in conjunction with fish liver histopathology may assist the interpretation of observed patterns of lesion prevalence. Several kinds of parallel indicators measured in past studies of fish liver histopathology include sediment toxicity (i.e., using bioassays), alterations of benthic invertebrate assemblages, and diversity and abundance of nontarget fish species (e.g., Tetra Tech 1985).

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