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Consistent Associations between Hepatic Lesions in English Sole (*Parophrys vetulus*) and Polycyclic Aromatic Hydrocarbons in Bottom Sediment

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A consistent and statistically significant association between prevalence of hepatic neoplasms in free-living sole (*Parophrys vetulus*) and levels of anthropogenic polycyclic aromatic hydrocarbons in bottom sediment from sites of fish capture was documented in a series of studies conducted over a period of 7 years in Puget Sound, Washington. This result strengthens the evidence supporting a causal relationship between exposure to sediment-associated hydrocarbons and development of hepatic neoplasms in this bottom-dwelling marine fish species. Prevalences of two other distinct categories of idiopathic hepatic lesions—megalocytic hepatosis and steatosis/hemosiderosis—also showed consistent, statistically significant associations with polycyclic aromatic hydrocarbon concentrations in bottom sediment, and association with prevalence of a third category (putatively preneoplastic foci of cellular alteration) approached statistical significance. On the basis of other studies, megalocytic hepatosis and foci of cellular alteration are both considered to be important precursor lesions in the stepwise histogenesis of hepatic neoplasms.

Introduction

Do sediment-associated contaminants actually cause disease in bottom-dwelling fish, as certain studies (*1*) have suggested? Eight field studies that bear on this question have been conducted in recent years by our laboratory (*1–7*). These individual studies (some limited in terms of sites sampled, other encompassing as many as 32 sites) have provided data on liver lesion prevalence in English sole (*Parophrys vetulus*) and sediment contaminant levels in Puget Sound, Washington. The most recent of these studies also add a small amount of information from California, Oregon, and Alaska. In this paper we analyze data from 49 different sites sampled between 1979 and 1986. Using suitable statistical methods, we assess the consistency of the associations between five categories of hepatic disease (including neoplasms) and levels of polycyclic aromatic hydrocarbons (PAHs) in bottom sediment. The consistency of an association between a disease and its suspected cause is one of the epidemiological criteria used to establish causal relationships on the basis of observational data.

The eight field surveys have revealed that hepatic neoplasms and other related idiopathic hepatic lesions are present in genetically heterogeneous populations of a species of fish that have been exposed to various levels of xenobiotic chemical agents in nature. These surveys have also provided information which can be used to investigate the etiologies (not yet fully understood) of these lesions. It would be difficult to obtain comparable types and amounts of toxicological information with experimental carcinogenesis studies, which are usually long-term, expensive, require genetically similar animals, and generally employ high doses of xenobiotic agents, often administered via injection. Indeed, Gilbertson (*8*) has argued that more studies of chemically induced diseases in fish in the natural environment (as opposed to laboratory studies) are needed to provide a basis for regulatory action.

Fortunately, special methods have been developed in the field of human cancer epidemiology to address the question of causal relationships on the basis of observational as well as experimental data. These methods, which involve testing statistical hypotheses, fall under the heading of analytical epidemiology. Analytical epidemiology is contrasted with descriptive epidemiology, which is largely nonstatistical [rather involving the study of disease rates and patterns of occurrence, e.g., by geographical area; see Colton and Greenberg (*9*) for an excellent summary of basic epidemiological methods].

Various sets of criteria for establishing causal relationships from observational studies using the methods of analytical

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epidemiology have been devised. Such criteria typically include the strength, consistency, and specificity of a putative association; the biologic credibility of the postulated effect; the time sequence of exposure and onset of the disease; and the demonstration of a dose-response gradient in the relationship between a disease and its suspected cause (*9,10*). Such criteria have been used to demonstrate relationships between exposure to various risk factors and the onset of certain diseases in human populations, including the relationships between ionizing radiation and leukemia and between smoking and lung cancer (*9*). Although all of these criteria must ultimately be evaluated before any statements can be put forward as to causal relationships between toxicological agents and neoplasia in free-living English sole, for reasons of space, the scope of this contribution will be limited to a single criterion, namely, consistency of association. For purposes of our analysis, we will define consistency of association as based simply on the outcome (positive relationship, negative relationship, or no relationship) of studies of the putative association. Obviously if there is no consistent association between a disease and its suspected cause, it is pointless to address the remaining criteria.

Colton and Greenberg (*9*) give three subcriteria for evaluating consistency of association, all of them involving the similarity of estimates of the strength of an association. These estimates should be similar in different studies, in different populations, and when using different methods of investigation. Basically, these subcriteria concern whether or not the existence of the association of interest can be confirmed and generalized beyond the original study reporting it. If a putative association is robust with respect to minor variations in experimental methodology, the argument for its existence is strengthened.

The strongest relationships between disease in fish and contaminants in bottom sediment found in previous Puget Sound investigations (*1*) were between prevalences of four separate categories of hepatic lesions in English sole and concentrations of PAHs in sediment from the site where the sole were captured. Although certain other classes of chemical contaminants (e.g., trace metals) have been measured in some studies, they have been omitted in others, complicating the consideration of possible associations between categories of liver disease and classes of chemicals other than PAHs. Thus, it is logical to confine the scope of the present analysis to relationships with PAHs and to English sole initially, although there are indications that similar relationships also exist in another bottom-dwelling fish species (rock sole), and that other contaminants (e.g., polychlorinated biphenyls) are also associated with certain of these hepatic diseases in English sole (*1*). In this context, it is noteworthy that the PAHs considered here include a known fish hepatocarcinogen (see "Discussion").

After conducting a basic epidemiological analysis of hepatic diseases in English sole in Puget Sound, Rhodes et al. (*11*) reported that most hepatic diseases tend to occur with greater frequency in older fish and at certain sites, and that these diseases occur with roughly equal frequency in both male and female English sole. Some types of lesions have been shown to co-occur and to be associated statistically with one another in patterns of co-occurrence, with the implication that certain lesion types are necessary precursors of others (*12*). In particular, foci of cellular alteration are considered necessary precursors to the development of hepatocellular neoplasms. Johnson et al. (*13*) have suggested that exposure to sediment-associated contaminants may impair ovarian development in female English sole at some sites in Puget Sound.

Methods

Field Surveys

Major aspects of the eight Puget Sound field surveys of fish lesion prevalence and sediment contaminants considered here are summarized in Table 1 (*14*). Puget Sound is part of a fjord system connected to the northeastern Pacific Ocean by the Strait of Juan de Fuca (Fig. 1). Some portions of the shores of Puget Sound are heavily urbanized, while others are rural in character. Each study was conducted using methods similar to those described in Malins et al. (*1*). The most extensive study encompassed 32 sites, and the most restricted involved only 4 sites. In each study, both urban and nonurban sites were selected so as to include sediments with at least potentially substantial differences in concentrations of xenobiotic chemicals. Early surveys concerned primarily the central and southern basins of Puget Sound, while later ones often focused on the northern basin (Fig. 1). Linear distance from the northernmost site sampled to the southernmost was approximately 120 km (70 miles). Detailed findings of the studies have been reported in separate publications (Table 1). The last two studies also included two sites from California, one from Oregon, and one from Alaska as well as sites from Puget Sound. One relevant study (*15*) has been excluded because it involved only two sites. Likewise, 1984 data from Varanasi et al. (*6,7*) have been excluded because English sole were collected only at three sites that year.

Table 1. List of studies conducted between 1979 and 1986 providing data on concentrations of xenobiotic compounds in bottom sediment and hepatic lesion prevalence in English sole in Puget Sound, Washington.

Study	Period	Number of sites	Number of fish	Reference
1	1979–80	32	1887	(*1*)
2	1982	4	157	(*2*)
3	1983	11	249	(*4*)
4	1984	4	115	(*3*)
5	1984	9	265	(*5*)
6	1985	8	248	(*5*)
7	1985	6	175	(*6,7*)
8	1986	6	166	(*6,7*)
Total	1979–86	80[a]	3180[b]	

[a] A total of 49 sites were sampled at least once during the eight studies; some sites were sampled in more than one study.

[b] A sample from one site (President Point) was used as a reference sample in several studies.

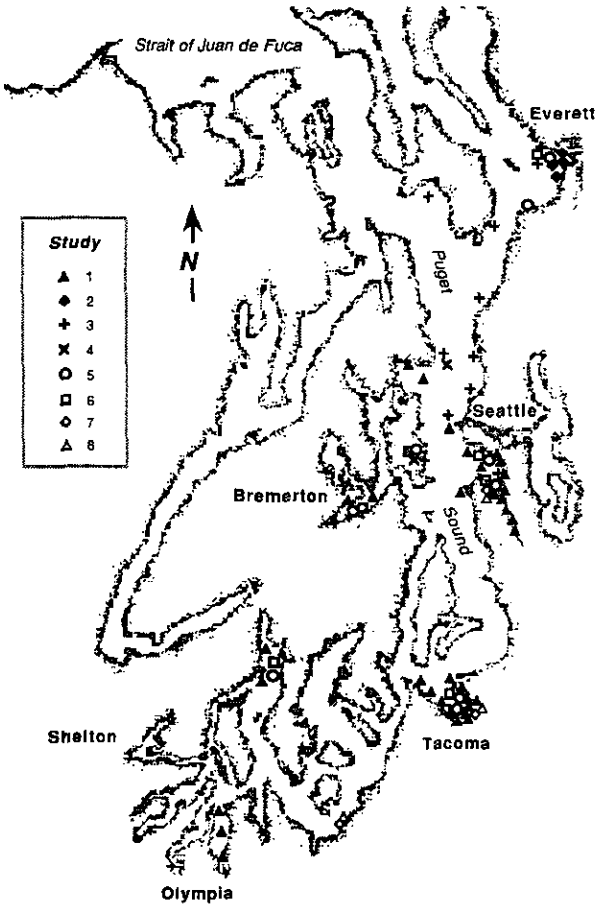

FIGURE 1. Map of Puget Sound, Washington, showing geographic distribution of 45 of the 49 sites sampled between 1979 and 1986 to collect data on hepatic lesion prevalence in English sole in relation to sediment contaminant levels; sites not shown are Bodega Bay and Monterey Bay, California, Coos Bay, Oregon, and Boca de Quadra, Alaska. Area shown by map spans from 122° 10′ to 123° 10′W and 46° N to 48° 10′W; distance from Everett to Olympia is about 120 km (70 miles).

Sediment Chemistry

In each of the eight field studies, samples of bottom sediment were analyzed for at least 40 (and sometimes as many as 100) xenobiotic chemicals, always including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), and sometimes including pesticides, chlorinated butadienes (CBDs), and trace metals as well. Sediment samples were collected using a modified Van Veen grab (0.1 m^2). Three stations were sampled per site during the first study. However, in subsequent studies typically only one station was sampled at each site. An exception was the fourth study, in which two to eight stations were sampled per site. To circumvent problems of small-scale patchiness in contaminant distribution, two grab samples were collected at each station, and a subsample was taken from the top 2 cm of the collected sediment. Subsamples from each station were composited in the lab, thoroughly mixed, and stored frozen prior to analysis.

Chemical analyses of xenobiotic organic compounds were performed using solvent extraction, column chromatography, and capillary column gas chromatography with mass spectrometry, flame ionization, and electron capture detectors (*16*). In the first study, 48 elements (e.g., zinc, lead, mercury) in addition to the organic compounds were analyzed either by atomic absorption spectrophotometry or by inductively coupled argon plasma emission spectroscopy (*1*), but elements were not usually analyzed in subsequent studies.

Because the concentrations of many xenobiotic compounds in bottom sediment covary (*1*), chemical concentration data collected during the first study were analyzed using principal component analysis (*1*). These chemicals were grouped into four covarying classes: *a*) PAHs, *b*) most metals, *c*) PCBs and certain metals, and *d*) most chlorinated hydrocarbons (*1*). Sediment concentrations of major classes of contaminants are largely uncorrelated in Puget Sound (*1*), suggesting that different types of contaminants emanate from different point sources, or from nonpoint sources in some cases but from point sources in others. The strongest relationships between liver lesions and contaminants in the first study involved PAHs; therefore, for the present analysis, only relationships between diseases and PAHs have been chosen for examination. Because the concentrations of PAHs covary in Puget Sound sediments (*1*), making it impossible to link diseases to levels of individual compounds, the sum of the concentrations of PAHs in sediment samples has been used for analysis of relationships between lesion prevalence and sediment contaminant concentrations. The PAHs selected for analysis are considered primarily to represent products of human activity, as opposed to naturally occurring compounds. The exact list of PAHs analyzed in bottom sediment has varied somewhat from study to study (Table 2). For the present analysis only the 17 compounds analyzed in all eight of the studies have been included. Total PAH concentrations for all available analyses for each site (typically three analyses, but ranging from one to nine) have been averaged to obtain a value representative of the site as a whole.

Histopathology

Each study involved collecting data on the prevalences of idiopathic lesions falling into 12 general categories, 7 of them found in the liver and 5 in the kidney. The hepatic conditions have been described in detail by Myers et al. (*12*). In all studies, English sole were collected at each site using an otter trawl; individual tows lasted 5 min and covered about 0.1 nautical miles (200 m). In the first study, 10 English sole were collected at each site, and most sites were sampled six times for fish (but only once for sediment chemistry) over a period of 2 years, hence about 60 fish (range 10 to 95) were collected per site on the average. In subsequent studies, most sites were sampled only once, but sites sampled in previous studies were sometimes revisited. Sample size was typically 30, but ranged from 16 to 108, depending on availability of fish and purpose of the study. In the fifth through eighth studies, the number of fish collected at each site was also typically 30, but ranged only from 27 to 39 in the fifth and sixth studies, and from 16 to 60 in the seventh and eighth studies.

Table 2. Polycyclic aromatic hydrocarbons analyzed in bottom sediment during field surveys, listed in order of increasing modular weight.[a]

Compound	1	2	3	4	5	6	7	8
Isopropylbenzene	•	•	•					
n-Propylbenzene	•	•	•					
Indan	•	•	•					
1,2,3,4-Tetramethylbenzene	•	•	•					
Naphthalene	•	•	•	•	•	•	•	•
2-Methylnaphthalene	•	•	•	•	•	•	•	•
1-Methylnaphthalene	•	•	•	•	•	•	•	•
Biphenyl	•	•	•	•	•	•	•	•
2,6-Dimethylnaphthalene	•	•	•	•	•	•	•	•
2,3,5-Trimethylnaphthalene	•	•	•	•				
Acenaphthene	•	•	•	•	•	•	•	•
Fluorene	•	•	•	•	•	•	•	•
Phenanthrene	•	•	•	•	•	•	•	•
Anthracene	•	•	•	•	•	•	•	•
I-Methylphenanthrene	•	•	•	•	•	•	•	•
3,6-Dimethylphenanthrene	•	•	•	•				
Fluoranthene	•	•	•	•	•	•	•	•
Pyrene	•	•	•	•	•	•	•	•
Benz[*a*]anthracene	•	•	•	•	•	•	•	•
Chrysene	•	•	•	•	•	•	•	•
Benzofluoranthenes	•	•	•	•				
Benzo[*e*]pyrene	•	•	•	•	•	•	•	•
Benzo[*a*]pyrene	•	•	•	•	•	•	•	•
Perylene	•	•	•	•	•	•	•	•
Indeno[1,2,3-*cd*]pyrene		•	•	•				
Dibenz[*a,h*]anthracene		•	•	•	•	•	•	•
Benzo[*g,h,i*]perylene		•	•	•				
Total number of compounds analyzed	24	27	27	23	18	18	18	18

[a]Seventeen compounds were analyzed in all eight of the studies.

After collection, each fish was examined for external lesions, and its length, weight, and sex were determined. Fish were then killed by severing the spinal column, and the otoliths were removed for age determination. Selected tissues (including liver, gill, and kidney) were then excised and preserved in Dietrich's fixative (*17*).

Tissues were routinely processed for paraffin embedding, sectioned at 5 μm, and stained with Mayer's hematoxylin and eosin-phloxine (*18*). All tissue sections were examined without any knowledge of site of capture for a particular specimen. Histologic criteria employed in hepatic lesion classification were derived from accepted criterion established primarily in mammalian studies. Classification of neoplasms and foci of cellular alteration was based on morphologic criteria established for rats (*19,20*) and mice (*21*). Specific lesion types that possessed a histomorphology comparable to the conditions described by the previous authors were given similar names. The nomenclature used for other lesion types was either descriptive or based on standard terminology for histopathology (*22*). The morphology of most of the lesions included in the lesion categories considered in the present study has been described in detail for English sole by Myers et al. (*12*).

Previous studies (*1*) indicated that, of the 12 liver and kidney lesion categories, 4 classes of liver lesions [neoplasms, foci of cellular alteration (FCA), megalocytic hepatosis (MH), and steatosis/hemosiderosis (S/H)] and also a summary category termed "any liver lesion" display the strongest relationships with concentrations of sediment-associated contaminants. For that reason, the present analysis has focused on these lesion categories (those found to be urban-associated in previous studies) (Table 3).

The neoplasm category includes both benign and malignant-appearing hepatocellular, cholangiocellular, mixed hepatocellular/cholangiocellular, and mesenchymal neoplasms. Foci of cellular alteration include eosinophilic, basophilic, and clear-cell foci of cellular alteration, and also hyperplastic regenerative foci. Megalocytic hepatosis is a unique degenerative/necrotic condition characterized by hepatocytes with markedly enlarged, hyperchromatic nuclei and increased cell diameter (*12*). Steatosis/hemosiderosis includes two distinct types of storage disorders that have been combined for the purposes of this analysis.

The "any liver lesion" category is included in the analysis for two reasons. First, since detoxification is a major function of the liver, it is logical to ask if there is evidence that this organ is affected in a general way by levels of contaminants. Second, this category has already been reported in previous publications, and so is included here for reasons of consistency. However, in some earlier studies, this category was based only on the presence or absence of the four lesion categories judged urban-associated, while in later studies this category sometimes also included four additional lesion categories not judged urban-associated [nonspecific necrotic conditions, nonneoplastic proliferative disorders, vascular disorders, and inflammatory lesions (*12,23*)]. This discrepancy has been corrected in the present analysis in that all statistical analyses are based on the four urban-associated categories.

It would be desirable to provide detailed treatment of age in relation to levels of disease—a crucial consideration in studies of humans, and unquestionably important in the case of fish as well. Unfortunately, the age of free-living fish gen-

Table 3. Categories of hepatic lesions in English sole considered in the consonance analysis.[a]

Disease category	Description	Overall frequency, %	Maximum observed prevalence, %
Neoplasms	Hepatocellular and/or biliary, both benign (adenoma) and malignant (carcinoma)	4.3	32.0
Foci of cellular alteration	Putative preneoplastic conditions	9.2	52.0
Megalocytic hepatosis	Unique degenerative/necrotic condition	15.8	86.4
Steatosis/hemosiderosis	Storage disorders	10.6	64.0
Any liver lesion	Summary category based on four disease categories (see text)	36.1	96.0

[a]Minimum observed prevalence is 0% in each case. Maximum observed prevalence always occurred at Eagle Harbor, WA, a small bay with a high level of creosote pollution (see text).

erally cannot be established directly. Instead, age must be estimated indirectly, for example, from otolith rings, length, or weight. For the studies reported here, age information based on otoliths is available for only approximately half of the fish examined. While age could be estimated from length or weight for the remaining specimens, it is conceivable that contaminants stunt the growth of fish in polluted areas. If so, this would introduce a systematic bias into age estimates based on size. We can, however, present some evidence that our samples of fish are not biased with respect to age (see "Results").

Statistics

Two main statistical methods have been employed in this investigation: Spearman rank correlation (*24*) for within-study analyses of associations between lesion prevalences and contaminants and the sign test (*24*) for between-study comparisons (i.e., assessing the consistency of the association). Fisher's combined probability test [first published by Fisher (*25*); see also Sokal and Rohlf (*26*)] has also been used to combine the results of the individual studies because methodology differed somewhat from study to study (see "Discussion").

Spearman rank correlation was used to investigate the relationships between prevalences of the categories of hepatic lesions in English sole and sediment concentrations of PAHs. The Spearman rank correlation method is limited in that it can be used only for bivariate comparisons, but it is robust to departures from the assumptions of other methods (i.e., that the prevalences to be compared are normally distributed and have equal variances) because it is nonparametric. By the same token, it is relatively low in statistical power when the parametric assumptions listed above are met. However, since the overlying distribution of fish lesion prevalence is inherently binomial rather than normal (individual fish being classified either as affected or as unaffected in these studies), it is clear that the validity of results obtained using parametric statistical methods would be open to question.

The sign test is based on the binomial distribution. Its null hypothesis is that plus and minus signs (representing positive or negative association, in this case) are equally likely to occur in the population sampled (that is, the outcomes of the Spearman rank correlation tests for association between disease prevalence and sediment PAHs in studies two through eight).

Although other statistical tests suitable for combining the results of different scientific studies are also available, Fisher's combined probability test (FCPT) is simple to carry out and performs well (*27*). Because the first study included a large number of sites and because its results were used to select the lesion categories for analysis in the present study, the sign test and the FCPT have been applied only to studies two through eight.

When multiple comparison tests are performed on the same body of data, the probability of obtaining ostensibly significant relationships by chance alone increases with the number of tests performed (*28*). Therefore, the significance level for individual tests has been adjusted by dividing the overall significance level chosen (0.05 in the case of the present study) by the number of tests performed. This method, which utilizes a Bonferroni inequality, is simple but highly conservative (*28*); hence, a few of the results with significance levels greater than the adjusted significance value would likely be regarded as statistically significant by a less conservative (but more complex) adjustment method. Because the "any liver lesion" category is not independent of the others, it has not been counted as a separate analysis for the purpose of the multiple-comparison adjustment, hence the critical value of 0.05 value has been divided by 4 to give 0.0125 as the adjusted critical value for the later studies. In the first study, relationships between diseases and three classes of contaminants in addition to PAHs were initially evaluated (*1*), hence the 0.05 value has been divided by 16 to give 0.003 as the adjusted critical value.

Results

The analysis reported here is based on two primary statistical methods: Spearman rank correlation (used for investigating the relationship between lesion prevalence and sediment contaminant levels) and the sign test for assessing the consonance of the results of the field studies. Fisher's combined probability test has also been used to combine the results of studies two through eight. A brief summary of preliminary results of this consonance analysis and related work has been presented elsewhere (*29*), but this is first report of the final results giving full details of the methodology and incorporating information from the sixth, seventh, and eighth studies.

The overall results for the eight studies are based on 80 sediment samples and 3180 English sole collected at 49 different sites over a period of 7 years. Overall prevalences of lesions in the four hepatic categories ranged from 4% to 16% (Table 3) for all eight studies combined. The highest hepatic lesion prevalences were found in Eagle Harbor, WA, a small bay on the west side of central Puget Sound which has a high degree of creosote pollution (*4*). Concentrations of sediment-associated PAHs (Table 2) ranged from 0.005 ppm dry weight at an uncontaminated site (Nisqually Reach, north of Olympia, eighth study) to 540 ppm dry weight at Eagle Harbor in the sixth study, with an arithmetic mean of 16 ppm dry weight for all sites and all studies combined.

As previously indicated, we can present some evidence that our samples of fish are not biased with respect to age, namely, the fact that neither the mean length nor the mean weight of fish collected at the various sites is correlated with PAH levels in bottom sediment. Spearman rank correlation values for these relationships do not begin to approach statistical significance, nor is there any demonstrable relationship between age and PAH levels for the sites for which age data based on otolith rings are available.

Once the significance level has been adjusted for the number of tests performed (see "Methods"), correlations were significant in only one individual study (three tests out of five in the first study, which had the largest number of sites), and r_S values for individual lesion categories varied

Table 4. Correlations between prevalences of hepatic lesions in English sole and PAH concentrations in bottom sediment, and results of Fisher's combined probability test (FCPT) for two through eight studies.[a]

Lesion category	Individual study statistic	Study 1	2	3	4	5	6	7	8	Sign test significance	FCPT statistic
Neoplasms	No. of cases	49	11	29	29	16	14	2	5		
	r_S	0.48*	0.60	0.35	1.00	0.54	0.61	0.66	0.85		
	Significance	0.003	.250	0.148	0.100	0.066	0.058	0.110	0.040	0.004*	33.18*
Foci of alteration	No. of cases	128	14	44	33	24	32	13	5		
	r_S	0.47	0.00	0.00	0.80	0.55	0.78	0.64	0.66		
	Significance	0.004	0.500	0.500	0.170	0.066	0.020	0.120	0.110	0.016	28.23
Megalocytic hepatosis	No. of cases	161	41	777	58	53	78	20	14		
	r_S	0.54*	0.80	0.36	0.80	0.23	0.67	0.52	0.62		
	Significance	0.001	0.170	0.136	0.170	0.300	0.040	0.160	0.120	0.004*	27.83
Steatosis/hemosiderosis	No. of cases	138	17	57	46	38	30	4	6		
	r_S	0.49*	0.63	0.31	0.80	0.53	0.48	0.58	0.62		
	Significance	0.002	0.240	0.178	0.170	0.070	0.120	0.135	0.120	0.004*	27.65
Any liver lesion	No. of cases	470	74	157	81	134	133	56	43		
	r_S	0.36	0.20	0.42	0.80	0.30	0.54	0.37	0.55		
	Significance	0.023	0.3809	0.110	0.100	0.220	0.160	0.235	0.129	0.004*	24.64

[a] r_S is the value of the Spearman rank correlation coefficient; the adjusted significance level takes into account the number of individual statistical tests performed (see text).

*Individual result significant at $\alpha \leq 0.05$ based on adjusted significance level.

considerably from study to study (Table 4). Of the individual lesion categories, the range of r_S values for the relationship with sediment hydrocarbons was smallest for S/H (0.3–0.8) and greatest for FCA (0.0–0.8) (Fig. 2). Neoplasms had the highest median r_S value (0.58), followed by MH (0.53), FCA (0.51), S/H (0.51), and "any liver lesion" (0.37). The maximum r_S value for any categories was 1.0 for neoplasms in the fourth study, which involved only four sites.

On the basis of the FCPT, only neoplasms can be considered to show a significant association with sediment PAHs after adjustment for the number of statistical tests performed when studies two through eight are considered as a whole. However, on the basis of the sign test, all lesion categories except FCA showed consistent associations with sediment PAHs after adjustment for the number of tests performed in studies two through eight, and the result for FCA falls only slightly short of significance.

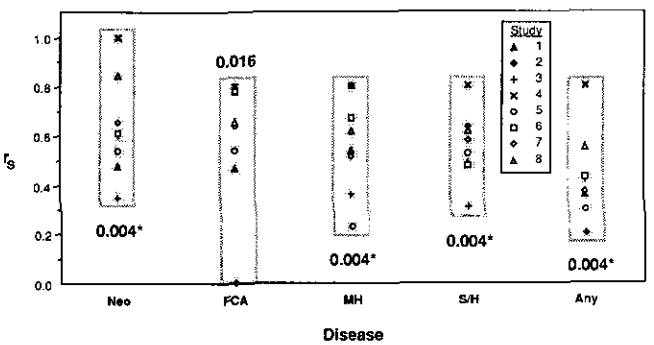

FIGURE 2. Consistency of association between categories of hepatic lesions in English sole and concentrations of PAHs in bottom sediment in eight Puget Sound field studies. r_S is the Spearman rank correlation coefficient evaluating the strength of an association; gray bars indicate ranges of coefficients obtained for different lesion categories; and p-values are for the sign test evaluating consistency of association for studies two through eight. FCA, foci of cellular alteration; MH, megalocytic hepatosis; S/H, steatosis/hemosiderosis; and Any, a summary category indicating the presence of any liver lesion. (*) Individual result significant at $p \leq 0.05$ based on adjusted significance level of 0.013.

Discussion

Four of the five lesion categories considered (neoplasms, MH, S/H, and "any liver lesion") show consistent associations with PAH concentrations in bottom sediment in the eight studies conducted at different Puget Sound locations over a 7-year period, and association with the fifth category (FCA) closely approaches statistical significance in this regard. Although these field surveys have great similarities in their methodology and have been conducted by essentially the same group of investigators, the studies in this series also differ in several respects (e.g., size and age range of fish collected, and season of the year conducted; see below). For this reason and because environmental samples typically exhibit high variability, one would not expect separate field studies to yield precisely the same results. However, it is of interest to determine the degree to which the results obtained in the individual studies are in agreement with each other (in other words, the consonance of the results), and fortunately, statistical methods are available for this purpose.

As discussed earlier, establishing the etiology of neoplastic diseases presents special problems. In particular, it is often emphasized that causal relationships cannot be established from correlational or observational studies (*30*), and certainly the importance of laboratory studies in establishing causation must not be underestimated (*31*). However, especially in the case of neoplasms, it is often difficult or impractical to perform laboratory experiments of sufficient duration to identify causal agents. According to one recent theory, the formation of a neoplasm involves a two-stage mutation process leading to homozygosity at an antioncogene locus within a cell and consequent loss of control over growth, with the second stage often beginning long after the first (*32*). A time lag between the initiation step and the formation of a histologically detectable focal lesion or neoplasm is also consistent with more conventional theories of hepatocarcinogenesis (*33*). Hepatic neoplasms do not

appear at appreciable prevalences in free-living English sole until about 3 years of age, and the mean age of sole with neoplasms is 5.6 years (*11*). In recently completed laboratory experiments in which English sole were exposed to known or suspected hepatocarcinogens, hepatic neoplasms were not induced within 18 months of exposure, although some precursor lesions were produced (*34,35*). Consequently, conventional laboratory experiments to establish relationships between suspected toxicological agents and hepatic neoplasms in this species are estimated to require a minimum of 2 to 3 years for completion.

Although four of the five lesion categories show a consistent association with PAHs in bottom sediment when studies two through eight are considered as a whole, the r_S coefficients for the relationships between individual lesion categories and sediment-associated PAHs vary considerably from study to study in some cases. The variability in results among studies is not especially encouraging, but neither is it entirely surprising, considering the variability usually exhibited by environmental samples and the small number of sites included in some of the studies.

Differences among Studies

Although the eight field studies have many similarities, they also differ in certain respects. In particular, they have been conducted in different years, and not always during the same season. Forty-nine different sites have been sampled at least once; later studies generally did not overlap greatly with the first study or with each other in terms of the exact locations of the sites chosen, although most major Puget Sound embayments sampled during the first study were sampled again in at least one of the subsequent studies. The first study utilized a wide size range (hence age range) of fish, while later studies concentrated primarily on a narrow size range composed of larger, older adults [those more likely to exhibit neoplasms (*11*)]. Slight differences in the assignment of lesions to broader categories have arisen between early studies and later ones. However, the principal research staff (particularly the histopathology staff) changed relatively little during the period of these field studies, hence there is little reason to attribute any differences among the findings of the various studies to differences in histopathological diagnoses. It should be noted that although differences among the studies complicate an overall statistical analysis, they strengthen the case for meeting the consistency of association criterion (which has, as one subcriterion, consistency of association in studies using different methods of investigation) as evaluated by the sign test.

Related Research

Previous studies (*36–38*) have shown that sediment-associated xenobiotic compounds (including PAHs) are readily bioavailable to English sole, and that PAHs are extensively metabolized. Benzo[*a*]pyrene, one of the PAH compounds detected in bottom sediment at some sites, is known to cause liver cancer in rainbow trout (*39*). In recent studies, several of the disease conditions discussed here [including MH and FCA, which are considered to be important precursor lesions in the histogenesis of hepatic neoplasms in English sole (*12*)] have been produced in the laboratory in English sole receiving a series of parenteral injections of either benzo[*a*]pyrene or a PAH-enriched fraction of a sediment extract from Eagle Harbor, Washington (*34,35*). As stated earlier, frank neoplasms were not induced in this study, possibly due to insufficient duration of the experiments. Although the route of exposure, and likely the dose, in these experiments are both different than in nature, the results support those of the consonance analysis.

Three of the lesion categories showing consistent or frequent correlations with PAH concentrations in bottom sediment (neoplasms, FCA, and MH) were also closely associated with PAH metabolites in bile of free-living English sole in Puget Sound (*4*). This provides additional evidence that uptake of PAH compounds is associated with the development of hepatic diseases.

A more detailed analysis of sediment-associated PAHs in relation to other possible etiologic factors affecting the prevalence of neoplasms in the studies summarized here is presently in progress. The detailed analysis uses logistic regression, a method which permits the construction of a multivariate statistical model relating the prevalence of a particular lesion category to the combined levels of several different categories of sediment-associated contaminants (e.g., PCBs and CBDs) in addition to PAHs (see "Methods"). Because not all classes of chemicals have been analyzed in all studies, the detailed analysis will be based on only a subset of the observations summarized here. Preliminary results of this analysis [briefly summarized in Malins et al. (*14*), final results to be reported in another contribution] are consistent with those of the consonance analysis which is presented here.

Conclusion

The overall conclusion from this analysis is that four of the five lesion categories considered (neoplasms, MH, S/H, and "any liver lesion") show consistent associations with PAH concentrations in bottom sediment in a series of studies conducted at different Puget Sound locations over a period of 7 years, and the fifth (FCA) shows an association closely approaching statistical significance. The fact that a consistent association between hepatic neoplasms in free-living English sole and levels of anthropogenic polycyclic aromatic hydrocarbons in bottom sediment was found in eight different studies encompassing 49 different sites (some separated by as much as 120 km within Puget Sound, and a few entirely outside Puget Sound) greatly strengthens the case for a causal relationship between sediment-associated PAHs and liver neoplasms in this bottom-dwelling fish species.

This conclusion regarding neoplasms is consistent both with toxicological knowledge and with recently completed experiments in our laboratory. It is further supported by the observation that prevalences of two other categories of hepatic lesions (FCA and MH), both thought to be lesion types essential to the eventual development of hepatic neoplasms, also showed consistent or frequent associations with PAH concentrations in bottom sediment.

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