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Liver anomalies in dab *Limanda limanda* from the southern North Sea with special consideration given to neoplastic lesions

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ABSTRACT: During 3 research cruises in the southern North Sea between 1986 and 1988 dab *Limanda limanda* L. were examined for liver anomalies. Macroscopic and microscopic descriptions include different color irregularities and neoplastic changes. The frequency of the various liver lesions observed were calculated, the influence of fish length, sex, and season on their occurrence studied, and their geographical distribution was documented. It is discussed whether examination of dab livers is a suitable additional method in biological effect monitoring programs.

INTRODUCTION

As the major organ of metabolism, the liver comes into intensive contact with toxic substances absorbed from the environment. Most of the xenobiotics are biologically transformed to less toxic substances and eliminated through the gall bladder. Some substances, however, are accumulated, and several are enzymatically transformed into even more reactive cytotoxic, teratogenic, mutagenic, or carcinogenic chemicals (Buhler & Williams 1988).

The liver, due to its complex functions, reacts to toxicant exposure by developing various diseases. Thus, a number of pathological anomalies have been described – e.g. lipoid liver degeneration and tumor formation caused by improper nutrition in aquaculture (Ashley 1970, Hille et al. 1980); different kinds of tissue lesions that have been experimentally produced by exposure to various toxic substances (Meyers & Hendricks 1982); and necrotic and neoplastic changes produced by mixtures of unspecified environmental pollutants (Peters et al. 1987).

The liver is a particularly important target organ for carcinogenic substances. Couch & Harshbarger (1985) reviewed the literature and compared the neoplastic changes in different organs caused by various carcinogenic substances, their latency periods, and other factors. They found that in over 90 % of cases, liver tumors were produced, and in only 10 % of cases were tumors produced exclusively in other organs.

Liver anomalies in North Sea fishes, and specifically in dab, were first described by Bucke et al. (1984), although this lesion was not classified as a tumor. The first epizootic occurrence of liver neoplasms in the North Sea was described by Vethaak (1987) among flounders in polluted regions off the coast of The Netherlands.

The piscine liver is unquestionably a sensitive indicator of harmful substances in the aquatic environment. Nevertheless, it has only been used to a very limited degree as a biological indicator of environmental contamination. The main objection to monitoring fish livers has been that it is too time consuming to examine them. Macroscopic examination of livers does not provide enough information for diagnoses, and supplementary histological investigations are necessary to determine the kind of pathological change and the degree of its malignancy.

Biological effects chosen for monitoring should also meet other criteria, including: (1) sufficient frequency of the condition to establish comparative percentages, especially when the number of samples is limited; and

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(2) either an independence of the condition from seasonal effects or a knowledge of the degree of such effects; as well as (3) independence of, or a knowledge of, differences in the prevalence between sexes and among age groups.

With the above considerations in mind, this investigation was designed to determine whether changes in the livers of North Sea fishes are suitable as criteria for monitoring biological effects caused by environmental factors.

The dab *Limanda limanda* is an important indicator fish for investigating diseases associated with gross external lesions, and since 1977, specimens from the southern North Sea have been regularly examined for such conditions (Dethlefsen et al. 1987). In comparison to other fishes, the dab displays remarkably high rates of affliction with lymphocystis, ulcerations, and epidermal papillomas. Furthermore, these fish do not show a strong tendency to wander out of their individual territories (Lozan 1988), making it possible to correlate the occurrence of the diseases and the presence of specific harmful substances.

The goal of this investigation was first to classify the spectrum of changes in the liver that can be macroscopically diagnosed and then, by analysing microscopic preparations, to correlate them with the various conditions observed. The findings can serve as a baseline for the description of the liver anomalies found in dab from the southern North Sea during the years 1986 to 1988, and to document their rates of occurrence.

MATERIAL AND METHODS

During three 2 wk research cruises in the southern North Sea in May and June 1986, June 1987, and January 1988, a total of 3376 dabs *Limanda limanda* L. were examined for liver anomalies. This corresponds to ca 20 fish per trawl. One trawl was made at each station. The fish were measured and their sex recorded before the livers were removed. Three color classes were established for this organ, and all externally visible anomalies, except parasites, were recorded. Macroscopically diagnosed conditions in 200 livers were investigated histologically. Samples fixed in Bouin's solution were embedded in paraffin; 5 µm sections were stained with haematoxylin and eosin (H & E), and parallel preparations were stained with Azan. Deep-frozen livers were sliced in a cryostat microtome to 10 µm, fixed in acetone or formalin, and stained with PAS and oil red O. All macroscopic liver data were recorded in a text and data processing system (DBase) in order to faciliate the correlation of individual liver conditions with fish length, sex, and the location at which it occurred within the network of sampling stations.

RESULTS

Macroscopic and microscopic descriptions of liver

Normal, homogeneous organs

Normal dab livers are light beige to dark reddish brown, according to the season and sex of the fish (Fig. 1a). In winter, livers in the majority of dabs of both sexes are more whitish, appearing light beige to a darker milky brown. These color ranges continue to predominate in the livers of males during May and June, while females lose the whitish shades and assume a general pale light brown to dark red coloration.

Histologically, dab liver consists predominantly of hepatocytes (Fig. 1c), which are arranged in trabeculae, each containing 2 cell layers. The trabeculae are bordered on both sides by sinusoids, which supply the hepatocytes with blood. On the side, between the cell layers, each hepatocyte is connected to the bile duct system.

Among the larger blood vessels, the afferent portal veins can be distinguished from the efferent vessels by their higher degree of connective tissue encapsulation. In regions of considerable connective tissue development, the portal veins are frequently associated with fairly large bile ducts.

Hepatic melanomacrophage centres are of variable abundance and size, and, in contrast to those in the spleen and kidneys, have a lower melanin content and are thus bright yellow.

Hepatocytes in H & E stained sections contained clearly defined nuclei with a centrally located, dark blue nucleolus and a peripheral rim of chromatin. The color of the cytoplasm ranges from colorless to dark blue.

Reserves of both fat and glycogen are dissolved during the histological embedding of samples in paraffin. The former locations of fat deposits are only rarely recognizable as large, circular 'holes' in the hepatocyte cytoplasm. It may occur that the entire cytoplasm remains unstained because reserve fat had been distributed in many small vacuoles. Sometimes, however, the nucleus is forced to the periphery of the cell by a large deposit of fat (Fig 1d). In such cases, the nucleus does not lose its spherical shape. The forcing of the nuclei from the center of the cells does not usually follow any regular pattern, and only rarely are regular 'fatty tracks' formed within the trabeculae (this would occur via a uniform displacement of the nuclei in the direction of the sinusoids and the accumulation of fat reserves on the side towards the bile caniculi in both cell layers).

In contrast, glycogen deposits (Fig. 1e) leave a

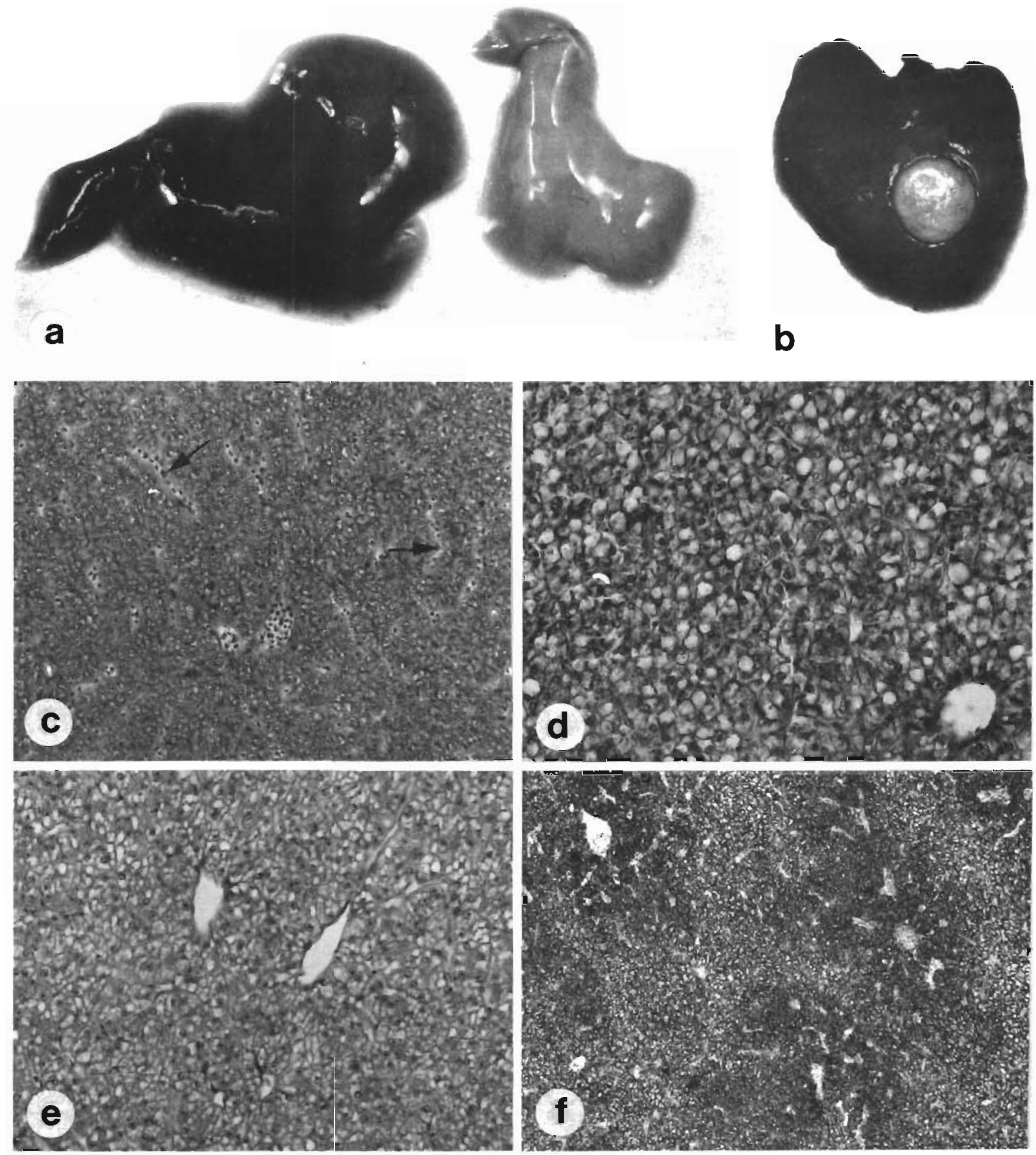

Fig. 1. *Limanda limanda.* (a) Gross appearance of healthy livers from a female (left) and a male (right) after spawning. From a catch in June 1986 (about natural size). (b) Gross appearance of a neoplastic liver nodule. The neoplasm is slightly raised from the surface and surrounded by a groove-like depression (natural size). (c) Normal histological structure of a dark red liver. Trabeculae of hepatocytes are separated by sinusoids containing blood cells (arrows); H & E, × 280. (d) Swollen hepatocytes with severe fatty vacuolation in a light beige liver. Nuclei are often forced to the periphery of the cell, the sinusoids are hardly recognizable; H & E, × 280. (e) Tissue of a dark red liver with moderate amounts of glycogen in hepatocytes. Sinusoids are only slightly compressed, nuclei remain centrally placed in the cytoplasm; H & E, × 280. (f) Histological appearance of liver tissue displaying marbling pattern on the surface. Areas with lower fat contents are mostly arranged around larger blood vessels; H & E, × 110

uniformly disrupted cytoplasmic structure in paraffin sections. The pale areas in the hepatocytes correspond to the locations of this freely distributed reserve material. As the glycogen content increases, the dark blue color, which is characteristic of cytoplasm in H & E stained sections of liver tissue poor in reserve material, becomes progressively lighter.

Cryostat sections revealed that the 'white' component of the liver (ranging in color from light, porcellain-beige to milky, coffee brown) results from various degrees of fat storage. Glycogen deposits cannot be recognized macroscopically from the coloration. Livers that are a pale red or light brown, but lack the white component, assume this paleness because the degree of vascular congestion is reduced.

Color anomalies

Frequently, livers did not display a single homogeneous color but rather a coarse or fine pattern of whitish marbling that was generally limited to a varying sized region. Only in the rarest cases was the whole organ involved.

Such marbling and whitish patches resulted from regional concentrations of increased fat storage (Fig. 1f). The marbling was frequently near larger blood vessels, but there was no notable distinction between regions of afferent and efferent vessels.

Certain fish had green livers. Nearly all of these fish originated from the same geographic location, where they occurred regularly. When this color appeared within light beige livers, the organ turned a pastel olive; when it appeared in a reddish-brown organ, a blackish-green coloration resulted. Such green colors did not always encompass the whole organ but more often appeared as flecks or patchy areas of various sizes in livers that otherwise displayed 'normal' coloration. In all such cases, an examination was made to determine whether the gall bladder had remained uninjured while liver was being dissected out and prepared for examination. Often, in the green region of the liver surface, or immediately adjacent to it, patterns of black dots just large enough to be detected by the unaided eye were displayed. These fine black dots were also observed on the dorsal parts of livers that had no green discoloration, but less often than in green livers.

Bile from fish lacking the green coloration ranged from yellowish to dark green, while that from fish with green livers was predominantly a very bright green or turquoise. Furthermore, in all fish from the region where green livers occurred, spleens were enlarged.

The green coloration, whether complete or patchy, could not be correlated with any histological peculiarity, either in paraffin sections stained with H & E or with Azan, or in kryostat sections stained with oil red O or PAS. Intrahepatic bile ducts of green livers could not be distinguished from those of other livers. However, special procedures for demonstrating storage of bile pigments in hepatocytes were not conducted.

The fine black dots (described for the areas bordering the green flecks or within the green patches) were produced by large aggregations of melanomacrophages. Unlike the melanomacrophage centers in normal livers, they were concentric and surrounded by thin connective tissue capsules. Furthermore, they contained an unusual gray pigmentation, and were sometimes surrounded by a cuff of small basophilic leucocytes. Staining with Prussian blue provided weak evidence for the presence of haemosiderin.

Another condition observed was the presence of dark red areas, especially on the periphery. These appeared to be produced by a seepage of blood, as the red material was soft, almost fluid in consistency. In most cases these blood-red discolorations seemed to have resulted from mechanical pressure, which was produced in the nets during the 1 h trawl. This symptom appeared at those stations where the catch was particularly large. In the affected areas, the tissue showed signs of rupture and hemorrhage. However, the hepatocyte structure in broken-off, isolated pieces of tissue was unchanged and intact. These blood-colored areas were distinct from the usually concentric ones which sometimes form during tumor development.

Neoplastic changes

A distinction must be made between the green, black, red, and whitish discolorations, which were all usually irregular or patchy, and lesions that were less discernible by color but which were distinguished by their round or spherical shape (Fig. 1b). These structures, called nodules, can be separated into 3 size groups: < 1.5 mm, 1.5 to 10 mm, and > 10 mm in diameter. Nodules of the smallest group were seldom raised above the surface of the organ but were detected primarily by their coloration, which was lighter than the surrounding tissue, occasionally having a glassy, hyaline appearance. Nodules of the middle-sized group were often raised above the surface or were surrounded by a slight groove-like depression (Fig. 1b). Their coloration differed only slightly from that of the surrounding tissue. Nodules in the largest category included most of those that deviated from a regular round shape; nonetheless, most were round and raised. Sometimes fluid-filled structures were visi-

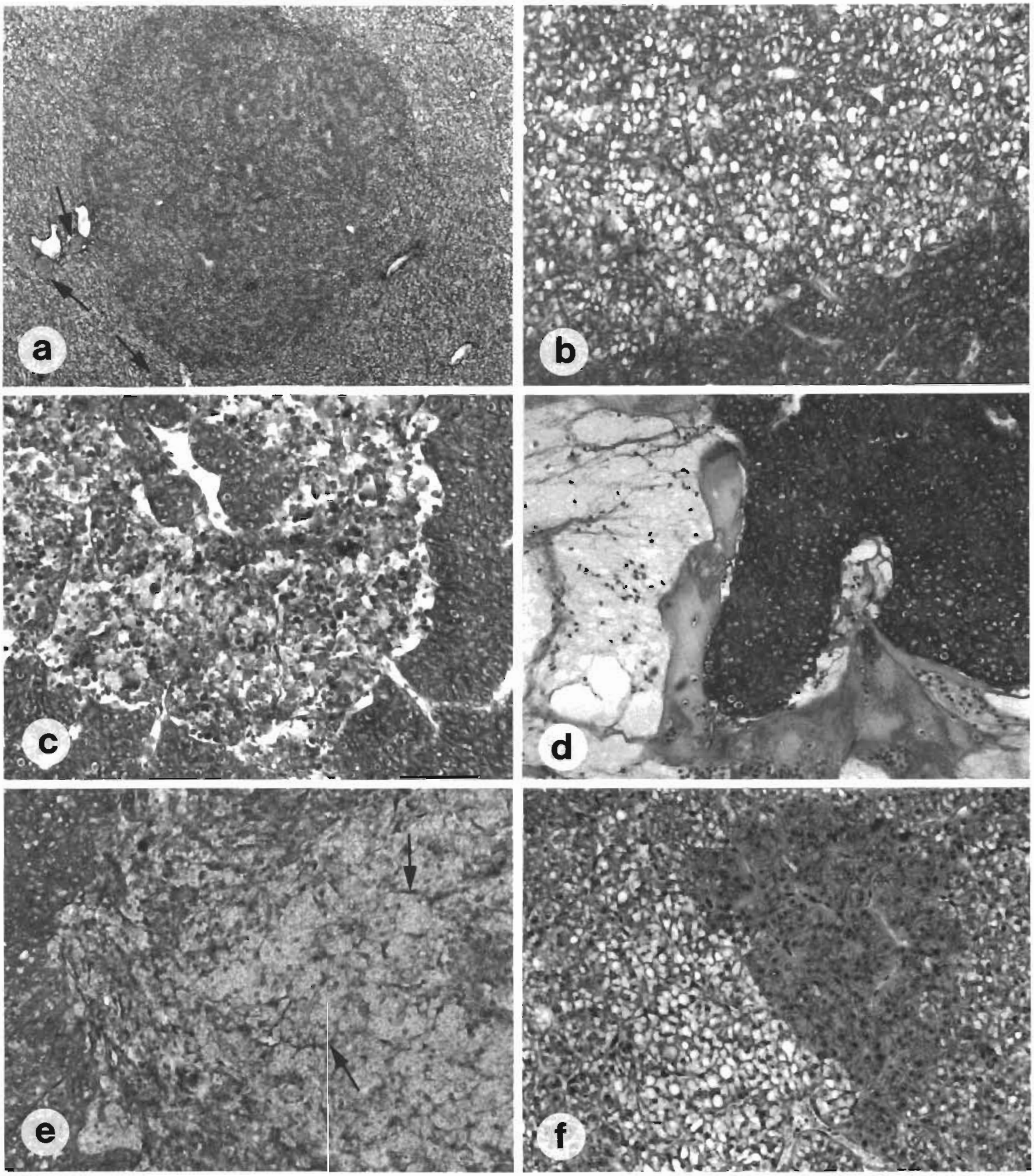

Fig. 2. *Limanda limanda*. H & E, × 280. (a) Basophilic nodule < 1.5 mm in diameter Note the slight compression of surrounding hepatic tissue at bottom of the photograph and several melanomacrophage centers (arrows). (b) Border between a vacuolated cell nodule and normal liver tissue. Vacuole sizes are very irregular. (c) Parenchymal degeneration within necrotic area of a large hepatic neoplasm. The tissue displays a high degree of cell desintegration. (d) Cystic region of same neoplasm as in (c) filled with fluid and scattered blood cells. (e) Haemangioma within a liver. Thin strands of vascular endothelium (arrows) extend between masses of red blood cells. (f) Early stage of neoplastic development in the liver. If this nodule is considered concentrical it consists half of basophilic, enlarged cells (top right) and half of vacuolated hepatocytes (bottom left)

ble within a nodule. In other cases, green or blood-red discolorations were observed in the nodular region.

Histologically, these varingly sized nodules were preliminary stages, associated signs, or advanced stages of neoplasias. Behind the glassy or white nodules < 1.5 mm in diameter were concentric areas of basophilic, eosinophilic, or vacuolated hepatocytes (Fig. 2a). These were clearly distinct from the surrounding liver tissue. The cells in the nodules of all 3 size groups, especially in those of the basophilic type, were slightly enlarged. These smallest nodules were usually multiple structures that may occur not only several times in a single organ but also in all three forms: eosinophilic, basophilic, and vacuolated cell nodules (Fig. 2b). A few cases even exhibited 2 cell types within a single nodule, half vacuolated cells and half basophilic hepatocytes (Fig. 2f).

The second size category (1.5 to 10 mm in diameter) included not only growths already described as basophilic, eosinophilic, or vacuolated cell nodules, among which the basophilic form showed a clear predominance, but also those that have characteristics of hepatomas. At the start of growth, neoplasias displayed a typical liver structure, i.e. trabecular in organization and often more uniform in appearance than normal surrounding tissue. This region frequently became compressed as the nodule increased in size, and the cells were flattened. Sinusoids within the nodules were somewhat expanded; large blood vessels and bile ducts were lacking. Melanomacrophage centers, which were especially abundant near nodules, were seldom formed in the actual neoplastic region.

In contrast to nodules displaying such an orderly structure, there were a few cases of growths in this size class which already exhibited the heterogeneous structures characteristic of more advanced hepatic neoplasias. Necrotic processes led to the development of cystic, fluid-filled regions containing accumulations of blood cells, concentrations of lymphocytes, and an invasion of connective tissue (Fig. 2c, d). Hepatocytes and their nuclei became pleomorphic, containing strongly basophilic pyknoses. Especially in those neoplasias displaying regions of surface green discolorations, apparently unstructured aggregates of macrophages appeared. Sometimes, less necrotic tumors were formed from various concentric layers of basophilic and eosinophilic cells. Histological examination usually showed tumors with dark red, blistered areas on the surface to be haemangiomas, in which thin strands of connective tissue extended between large chambers filled with erythrocytes (Fig. 2e).

Nodules in all 3 groups were more often identified on the convex, dorsal side of the liver, than on the concave, ventral side. The larger the fish, the more frequently did livers contain more than one nodule.

Frequency of anomalies

The frequency of liver anomalies that could be macroscopically diagnosed remained relatively constant among samples from all 3 cruises (Table 1). The percentages of livers classified as healthy (light beige to reddish brown with no distinctive alterations in surface color or structure) were 45, 41, and 46 % in 1986, 1987, and 1988, respectively. Also included as healthy are uniformly colored livers, which because of excess or deficient fat storage displayed shades of color that were atypical for the season or degree of gonad development. Atypical conditions were displayed in 20, 19, and 15 %, of dab in 1986, 1987 and 1988, respectively.

The most frequent gross hepatic lesions found were designated as nodules (39 % in 1986, 35 % in 1987, and 38 % in 1988). These were classified as pretumorous, tumorous, and tumor-associated conditions. Nodules in the smallest size class (< 1.5 mm) were 20 to 30 times more numerous than large tumors (> 1 cm in diameter).

The second most frequent gross lesion, found in 18 to 25 % of dab, was a mottled or patchy pattern of whitish discoloration while homogeneous or patchy green liver discolorations affected from 8 to 11 %.

The fine black dots produced by melanomacrophage centers on the surface of the liver only occurred half as frequently as the green discoloration, with which, however, this lesion appeared simultaneously in the majority of cases.

Haemangioma-like anomalies were found in fish from all 3 cruises but less than 1 % of the fishes examined were afflicted. Often haemangiomas were associated with other neoplastic structures originating in liver cells. Livers showing internal hemorrhages (generally peripherally), which were thought to have been caused by trauma during capture, accounted for a further 3 % of cases.

When the percentage of lesions from both summer cruises were compared with those from the winter catch, an extraordinary similarity was found (Table 1). Only minor differences in the frequency of nodules and the mottled or patchy pale discoloration pattern were noted. This produces a small divergence in the total percentage of livers not displaying pathological conditions.

If all liver lesions are classified by fish length, without regard for season or sex (Table 2), the percentage of fish with healthy livers decreased with increasing length and, possibly, age. Furthermore, there was a simultaneous increase in number of nodules in all 3 groups, their total number showing the most uniform increase. It was also obvious that the different-sized nodule types appeared successively with fish size. Nodules < 1.5 mm were not found in fish < 16.5 cm

Table 1. *Limanda limanda*. Frequency of occurrence of various gross liver lesions among all fishes examined during the 3 research cruises undertaken between June 1986 and January 1988. Males and females ranged from 12.5 to 38.5 cm in length. Values in parentheses are averages for the 2 summer investigations, conducted in 1986 and 1987

	June 1986 (n = 1339)	June 1987 (n =1024)	(Av.) (n = 1182)	Jan 1988 (n = 1013)
Livers appearing healthy				
Homogeneous, incl. atypical coloration (%)	45	41	(43)	46
Atypical color for the season or the sex (%)	20	19	(20)	15
Preneoplastic, neoplasia-associated, and neoplastic lesions				
Nodules < 1.5 mm ∅ (%)	29	20	(25)	25
Nodules from 1.5 to 10 mm ∅ (%)	9	13	(11)	13
Nodules > 10 mm ∅ (%)	1	1	(1)	1
Sum of all nodules (%)	39	35	(37)	38
Color anomalies				
Marbling or patchy beige discolorations (%)	18	25	(22)	19
Green discolorations (patchy and homogeneous) (%)	8	11	(10)	10
Melanomacrophage centres (MMC) on the organ surface (%)	3	6	(4)	4
Bloody discolorations (%)	4	4	(4)	4

Table 2. *Limanda limanda*. Frequency of liver lesions (% of n) as a function of fish length. Percentages for each length class were calculated from sums of data recorded for males and females during the years 1986, 1987, and 1988. The abbreviated names for the liver lesions correspond to the descriptions given in Table 1. –: no lesions

Fish length (cm)	No. of fishes (n)	Healthy livers (%)	Small nodules (%)	Medium nodules (%)	Large nodules (%)	Sum of nodules	Marbling/ patchy discolorations (%)	Green colors (%)	MMC (%)	Bloody colors (%)
< 16.5	58	86	3	–	–	3	14	7	–	–
17.5	51	71	12	–	–	12	16	6	–	–
18.5	189	60	8	1	–	10	30	15	4	3
19.5	286	57	15	3	–	18	22	14	3	4
20.5	372	53	18	4	0.3	23	24	13	5	2
21.5	395	50	23	6	0.3	29	22	10	6	4
22.5	389	49	28	9	0.8	38	16	6	4	4
23.5	344	45	26	12	0.3	39	18	8	4	4
24.5	283	37	34	11	1.4	46	21	7	3	2
25.5	239	36	29	20	1.3	51	16	4	5	6
26.5	242	31	33	21	2.5	57	20	5	4	4
27.5	171	27	33	17	3.5	54	23	8	3	5
28.5	112	25	30	26	1.8	58	21	10	5	8
29.5	97	14	34	32	4.1	70	19	9	2	6
> 29.5	148	17	33	26	6.1	66	29	18	3	16

long. Larger nodules first appeared in fish > 18.5 cm long, and only in fish > 20.5 cm could growths > 10 mm in diameter be found. In the cases of the other liver lesions, such clear relationships between occurrence and fish length were not encountered, with the possible exception of the haemangioma-like structures, for which a slight positive relationship could be detected when the data for more than one length class were pooled.

Regression curves for the frequency of nodules in all 3 size groups combined, as a function of fish length, were calculated for all 3 cruises. Those for 1986 and 1988 proved to be highly significant (Fig. 3).

Superficially comparing the distribution of lesions in male versus female dab (Table 3) gives the impression that males have fewer liver nodules. However, males have a shorter average length than females (Tables 4 and 5), and since the occurrence of liver nodules shows

Table 3. *Limanda limanda*. Distribution of data on livers according to sex. Values in parentheses refer to fishes lacking external features for sex determination (nd = not determined). Abbreviated names for the liver lesions correspond to the descriptions given in Table 1. – = no lesion

	1986			1987			1988		
	♂	♀	(nd)	♂	♀	(nd)	♂	♀	(nd)
No. of fishes (n)	398	909	(32)	333	670	(21)	137	871	(5)
Healthy livers (%)	49	40	(88)	33	44	(71)	57	44	(60)
Small nodules (%)	26	29	(9)	20	21	(19)	16	26	(40)
Medium nodules (%)	7	9	(9)	11	15	(5)	6	14	–
Large nodules (%)	–	2	–	1	2	–	1	1	–
Sum of nodules (%)	33	40	(19)	31	38	(24)	23	40	(40)
Marbling/patchy discolorations (%)	18	17	(6)	32	23	(10)	23	19	(20)
Green colors (%)	8	7	–	12	11	–	8	10	(20)
MMC (%)	7	1	–	11	3	–	4	4	–
Bloody colors (%)	3	5	–	1	5	–	3	4	–

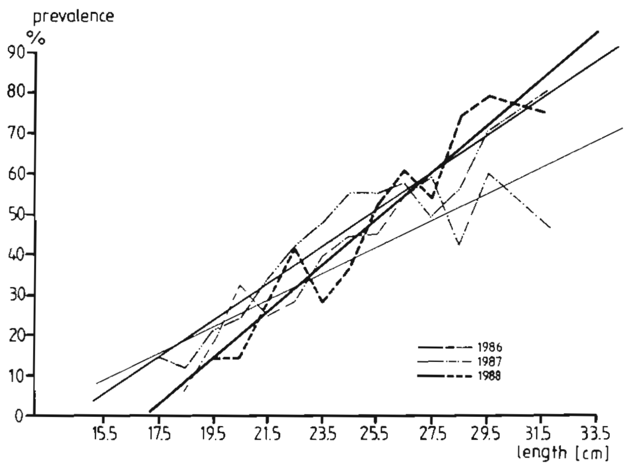

Fig. 3. *Limanda limanda*. Prevalence of liver nodules as percentage of sizes (from < 1.5 to > 10 mm) in each length class with corresponding regression curves for 1986 ($y = 4.6x - 66.0$), 1987 ($y = 3.3x - 42.4$), and 1988 ($y = 5.8x - 99.0$)

a linear increase with length, an interpolation of the slopes calculated for the regression curves, plotted as a function of length, demonstrates that males and females have almost identical nodule frequencies (Table 5). In contrast, for non-nodular liver conditions with rates of occurrence not depending on length, somewhat higher rates of affliction were recorded for males (Table 3). These conditions include 'marbling discolorations' and 'melanomacrophage centers on the surface'. Sorting liver coloration data according to sex revealed that discolorations occured at about the same rate in males and females (Table 6).

The data were also evaluated to determine whether there were regions within the southern North Sea where any of the dab, with the various liver lesions, were particularly concentrated and whether their geographical distribution remained constant from year-to-year. In Figs. 4, 5, and 6, the distribution patterns of nodular lesions for the 3 cruises are depicted. The

Table 4. *Limanda limanda*. Length distribution of all fishes according to sex. Absolute abundance is shown. Values in parentheses refer to fishes lacking external features for sex determination (nd = not determined); – = none found

Length (cm)	1986			1987			1988		
	♂	♀	(nd)	♂	♀	(nd)	♂	♀	(nd)
12.5	–	1	–	–	–	–	–	–	–
13.5	–	1	(1)	–	–	–	–	–	–
14.5	2	6	(3)	–	–	–	–	–	–
15.5	7	4	(5)	–	–	–	–	2	–
16.5	10	10	–	2	1	(1)	–	2	–
17.5	21	19	(1)	4	1	(1)	1	3	–
18.5	35	46	(2)	46	22	(2)	12	24	–
19.5	35	79	–	41	57	(3)	19	51	(1)
20.5	56	84	(1)	62	39	(2)	36	91	(1)
21.5	68	98	(4)	56	62	(2)	19	86	–
22.5	60	92	(3)	49	52	(4)	21	107	(1)
23.5	45	79	(2)	36	72	–	10	98	(2)
24.5	28	87	(3)	17	55	(2)	10	81	–
25.5	18	57	(3)	9	69	–	4	79	–
26.5	5	65	(3)	8	73	(2)	3	83	–
27.5	5	54	–	3	53	–	1	55	–
28.5	3	37	(1)	–	32	(1)	1	37	–
29.5	–	44	–	–	25	–	–	28	–
30.5	–	21	–	–	24	–	–	17	–
31.5	–	10	–	–	9	–	–	13	–
32.5	–	7	–	–	14	–	–	8	–
33.5	–	6	–	–	6	(1)	–	5	–
34.5	–	–	–	–	2	–	–	1	–
35.5	–	1	–	–	–	–	–	–	–
36.6	–	1	–	–	1	–	–	–	–
37.5	–	–	–	–	–	–	–	–	–
38.5	–	–	–	–	1	–	–	–	–

Table 5. *Limanda limanda*. Comparison of frequency of nodules actually observed (all size groups) with theoretical values calculated from the regression curves (Fig. 3). The average fish lengths which the calculated frequencies correspond to are estimated from the data given in Table 4. Values in parentheses refer to fishes lacking external features for sex determination (nd = not determined)

	1986			1987			1988		
	♂	♀	(nd)	♂	♀	(nd)	♂	♀	(nd)
Average length (cm)	21.4	23.6	(20.9)	21.4	24.6	(22.3)	21.5	24.1	(21.9)
Observed frequency (%)	33	40	(19)	31	38	(24)	23	40	(40)
Calculated frequency (%)	31.9	41.8	(29.6)	27.9	38.6	(30.8)	25.5	40.3	(27.8)

Table 6. *Limanda limanda*. Color of the livers in males, females, and fishes lacking external features for sex determination (nd) captured during different seasons. Colors considered normal are underlined. – = no samples

	Summer 1986 and 1987			Winter 1988		
	♂	♀	(nd)	♂	♀	(nd)
No. of fishes (n)	730	1581	(52)	137	871	(5)
Reddish brown (%)	17	<u>79</u>	(25)	22	14	–
Beige (%)	<u>82</u>	20	<u>(75)</u>	<u>77</u>	<u>86</u>	<u>(100)</u>
Green, homogeneous (%)	1	1	–	1	0.2	–

values shown are not absolute rates of occurrence, but rather slopes of the regressions (Fig. 3) correlated with the fish length.

Although, on average, only 20 fishes per station were examined, a trend was established suggesting that the centers for fish with hepatic nodular lesions exist in the German Bight, off the coast of The Netherlands, and along the southwestern coast of England. During all 3 cruises, the waters off the Danish coast north of Esbjerg and the region east of Edinburgh, at ca 56 °N, were the sites where few fish with lesions were observed.

Non-nodular hepatic lesions exhibited a completely different distribution pattern. 'Green' livers, both solid and patchy, had an especially evident center of occurrence off the British coast (Fig. 7; at the latitude of Edinburgh, 56 °N, and a short distance southward). On the other hand, especially low frequencies of hepatic neoplasms occurred here.

The 'melanomacrophage centers on the liver surface' were also especially common at 56 °N off the British coast (Fig. 8). The center for this symptom occurred farther south off the English coast during winter 1988. A similar pattern of distribution, with the same central area of occurrence, was found for 'marbling discolora-

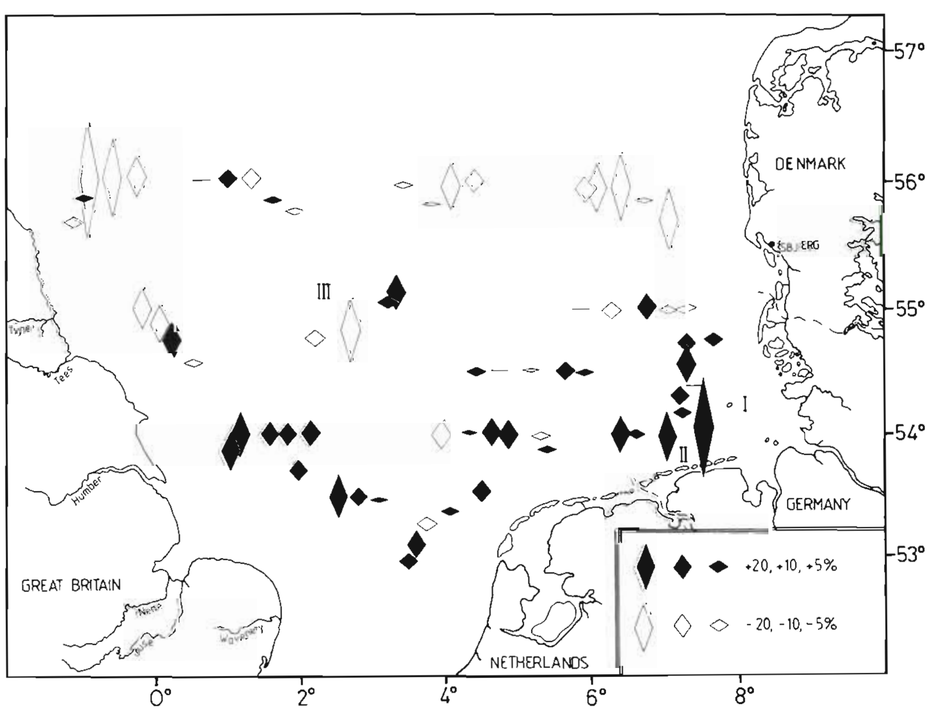

Fig. 4. *Limanda limanda*. Distribution of relative abundances of liver nodules < 1.5 mm to > 10 mm in diameter in males and females from the 1986 cruise. Values are corrected for length of fish by calculating average fish length at each station and correlating observed prevalence of liver nodules to the theoretically calculated value provided by the regression curve (Fig. 3). Positive divergences of observed values from theoretical prevalence are proportional to the height of the black diamonds, while negative divergences are similarly depicted by white diamonds. A line indicates no divergence. (I) German Bight; (II) East Friesian Islands; (III) Dogger Bank

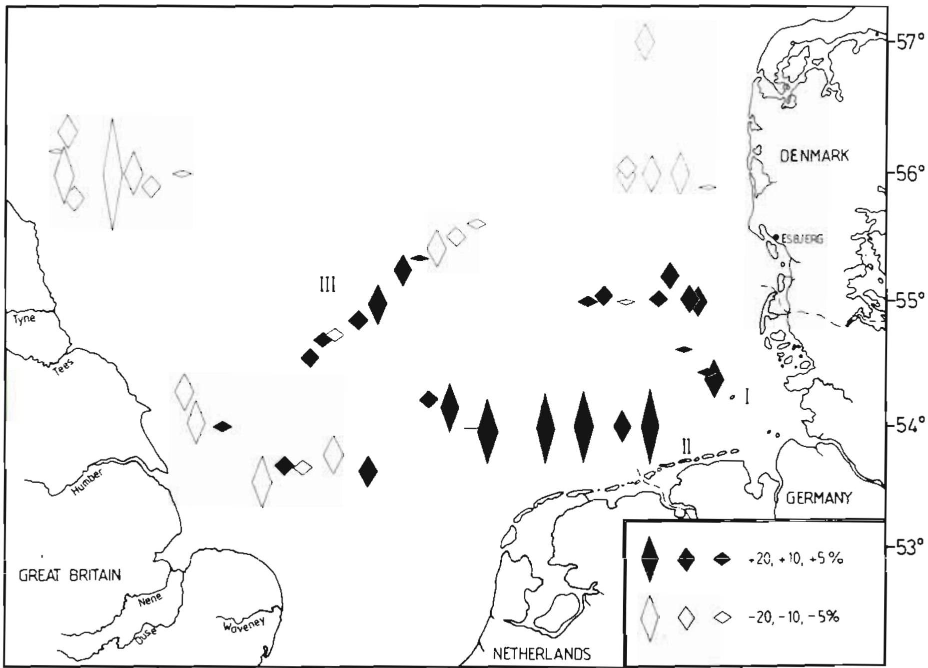

Fig. 5. *Limanda limanda*. Distribution of relative abundances of liver nodules from the 1987 cruise. For further details see legend Fig. 4

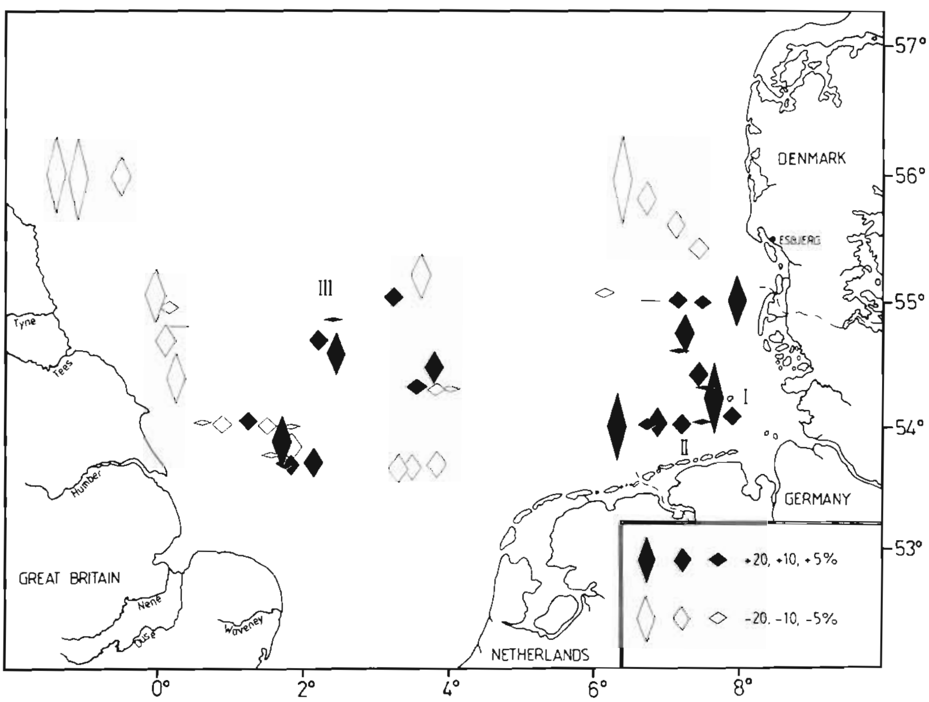

Fig. 6. *Limanda limanda*. Distribution of relative abundances of liver nodules from the 1988 cruise. For further details see legend Fig. 4

tions' (Fig. 9). Compared to the 'green' livers, however, these geographical correlations were not as strong.

DISCUSSION

The results show that, on average, the percentage of dab (*Limanda limanda*) from the sampling area displaying one or more types of liver lesions is greater than that of fully healthy individuals. The greatest proportion of liver lesions were those which are grouped together and designated as nodules and represented either various stages of neoplastic development or changes associated with neoplasms.

Liver tumors have been subjected to many studies on fish carcinogenesis since an epizootic of this disease

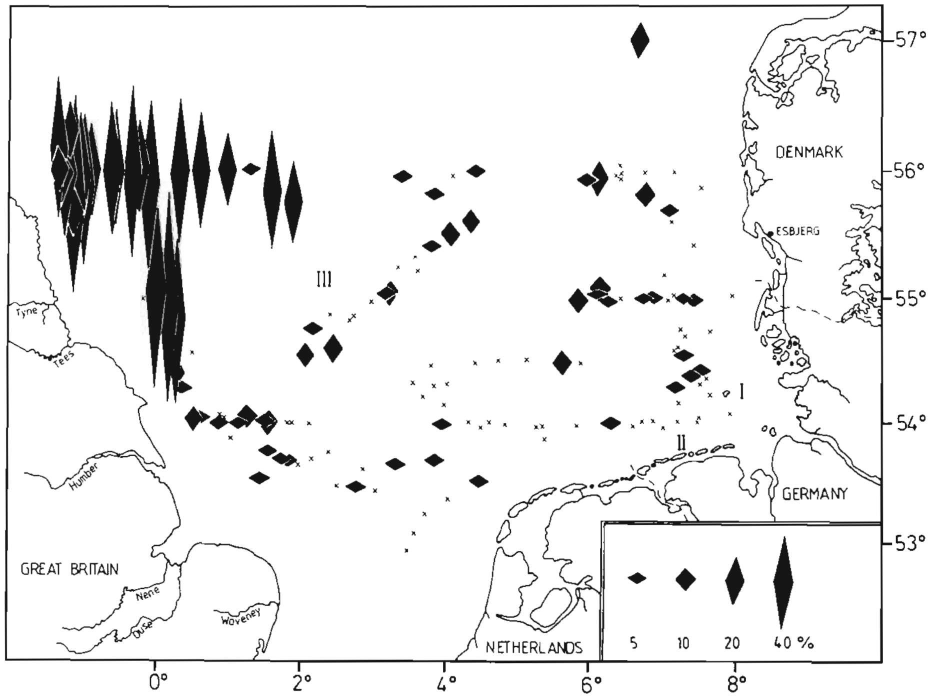

Fig. 7. *Limanda limanda.* Distribution of green liver discolorations for all sampling stations during 1986, 1987, and 1988. Values include patchy and homogeneous discolorations in both sexes of all length classes. Height of diamonds corresponds to absolute frequency of the condition at each station as a percentage of fish examined. An (x) designates a Station at which this condition was not observed. (I) German Bight; (II) East Friesian Islands; (III) Dogger Bank

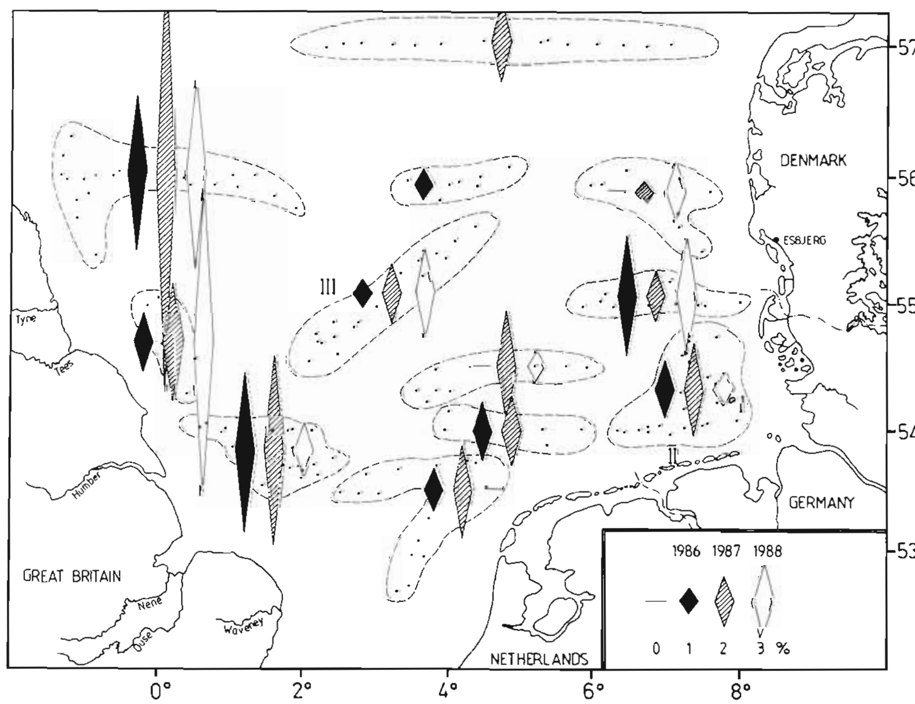

Fig. 8. *Limanda limanda.* Percentage of fish examined in which melanomacrophage centers were observed as fine black dots on the surface of livers. Each of the diamonds represents a summary of the data for all stations (•) sampled within the area surrounded by the broken line during one of the years of the investigation; (I) German Bight; (II) East Friesian Islands; (III) Dogger Bank

occurred in the 1960s and early 1970s among salmonids in aquaculture facilities all over the world. The formation of neoplasias due to exposure to aflatoxins and other carcinogenic substances was determined in relation to age of fish, method of application, duration of exposure, dosage, sex, and other parameters (Bailey et al. 1984, Black 1984, Hendricks et al. 1985, Nakazawa et al. 1985, Bailey & Hendricks 1988). In addition, the stages of neoplastic development, and its progress, were described in detail (Hendricks et al. 1984). Neoplasia of English sole *Parophrys vetulus* in Puget Sound have also been thoroughly investigated histologically (Myers et al. 1987).

If hepatic neoplasms of salmonids, English sole, and North Sea dab are compared, it is evident that the similarities among them are great. It is noteworthy that

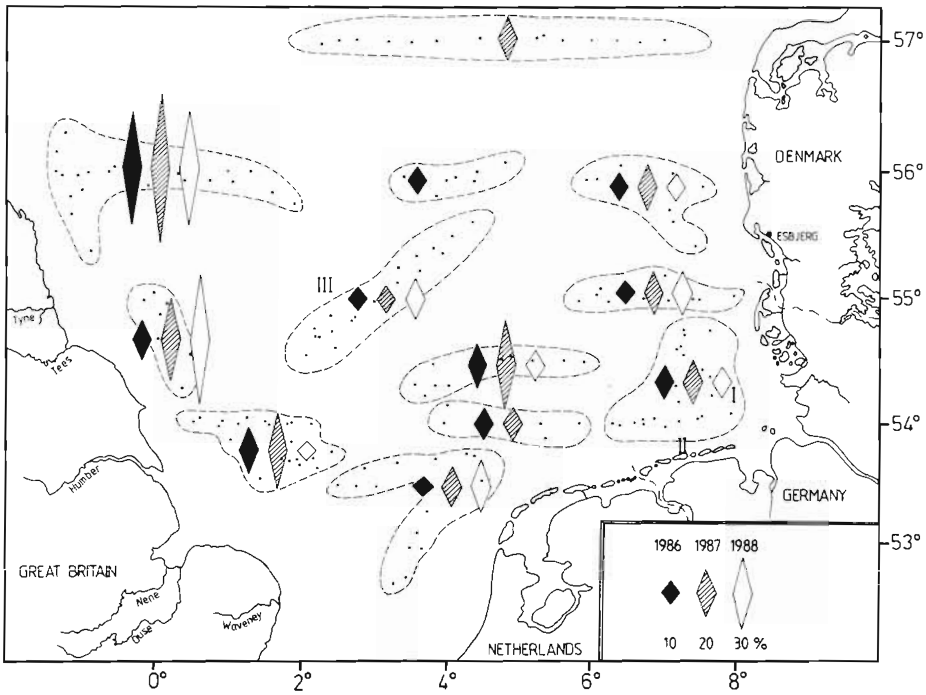

Fig. 9. *Limanda limanda*. Percentage of fish examined in which a marbling or patchy pattern of beige discoloration was observed on the liver. For further details see legend Fig. 8

there have been no observed cases where neoplastic changes have affected bile ducts of dab. In contrast, there have been several cases of haemangioma that seem to be identical to a single neoplasm (described as a haemangioendothelioma) in an English sole.

The early stages of neoplastic development and its associated lesions have been interpreted in different ways. Hendricks et al. (1984) thought that what he called glycogen storage nodules and foci of lipid-engorged hepatocytes in rainbow trout resulted from toxic effects, possibly evoked by the presence of the carcinogen, but having nothing directly to do with tumor formation. Myers et al. (1987), however, considered clear cell foci, eosinophilic, and basophilic foci in the English sole as putative preneoplastic liver lesions. Murchelano & Wolke (1985) classified eosinophilic, basophilic and vacuolar cell foci in windowpane and winter flounder as preneoplastic liver lesions. In any case, whether or not one of these interpretations is actually correct, the neoplasia-associated conditions mentioned here actually seem to represent criteria for determining the presence of carcinogenic substances in the aquatic environment.

No attempt was made to determine the malignancy of the dab liver neoplasms during this investigation. Data on the frequency of malignant tumors would not be representative because only a small proportion of the total fish samples examined were subjected to histological study. Furthermore, the stage of development of a neoplasia is not necessarily evident from its size, so the degree of malignancy cannot be judged macroscopically. If nodules < 1.5 mm are assumed to be early stages, and if their rate of occurrence is compared with those of the larger tumors, it can be concluded that ca 50 % of early stages (which are composed of basophilic, eosinophilic, and vacuolated cell clusters) are irreversible. Of course, for this to be true it must be assumed that even advanced stages of tumors do not cause death. Mortality of dab due to hepatic neoplasias has not been investigated. However, because (1) the liver is an organ with great capacity to regenerate, (2) the frequency of the disease increases with the size of the fish, and (3) metastasis of the tumors has never been observed, it can be assumed that the disease does not cause any increase in the mortality rate.

Data on the frequency of neoplasms in liver can only be reported as relative values. If routine examination is limited to macroscopic inspection of the organ surface, and histological examinations are made only of random samples, the distribution of neoplasias and their preliminary stages will be over-estimated because the lesions associated with them will also be counted. Such associations include the occurrence of eosinophilic and vacuolated cell nodules, which possibly develop in response to the presence of toxic substances. On the other hand, the frequency of occurrence will be underestimated by the number of tumors that develop in internal parts of the organs showing no signs of their presence on the surface (Fig. 2f). An investigation of liver neoplasias in fish from the Great Lakes, USA, revealed that by including both histological examinations of tissues cut at 5 levels and macroscopic inspection of the organ, the relative frequency of neoplastic lesions discovered could be increased from 33 to 80 % (Baumann & Harshbarger 1985).

Differences in the results recorded come about not only because of differences in method. The nature of the material itself limits comparison of data. In the study described here, the great dependence of the frequency of hepatic neoplasias on the size, and also apparently on the age of the fish became evident. For example, in trout, it was reported that not only does the latency period vary, but also the sensitivity of the various life stages to carcinogenic materials (Wales et al. 1978). If different sensitivities to the same carcinogenic substance are displayed even by different strains of trout (Hendricks 1982), it is not, therefore, reasonable to compare the frequency of neoplasms in different fish species. In both representatives of the family Pleuronectidae, i.e. plaice and dab, it is noteworthy that in spite of their similar distribution patterns in the North Sea, they exhibit differences not only in their susceptibility to hepatic neoplasias but also in the degree to which they develop diseases that can be diagnosed by external examination. During the 3 cruises, the livers of ca 500 plaice were examined macroscopically. In no case could any sign of a neoplastic liver lesion be found. In comparison to dab, the plaice rarely displayed signs of lymphocystis, ulcerations, and epidermal papillomas (van Banning 1987).

In spite of this, if others wished to use the data on the frequency of hepatic neoplasias in dab from the southern North Sea as a rough evaluation, they would find that the rate of occurrence (from 35 to 38 % for nodules < 1.5 to > 10 mm in diameter) is very high in comparison to the rates in international reports from other polluted regions of the world (Mix 1986).

While there is still limited information on liver lesions, a wide variety of data on the distribution of external diseases of the dab in the North Sea is available. The frequency of lymphocystis, epidermal papillomas, and ulcerations has been rather constant, within a certain range, for many years (Dethlefsen et al. 1987). The central occurrence areas of these external diseases are located: (1) in the center of the German Bight, with an extension of this area to the northwest as far as the 56 °N parallel; and (2) off the mouth of the Humber Estuary, UK, also with an extension to the 56 °N parallel, and to stations off the British coast, off the mouths of the Humber, Tyne and Tees, and areas off the coast near Edinburgh (56 °N). Newer, unpublished information from V. Dethlefsen revealed that there is also a high rate of occurrence of lymphocystis, epidermal papillomas, and ulcerations among dab in regions north of 56 °N. The distribution patterns of liver lesions, especially neoplastic conditions, deviate from that of these 3 external diseases. They occur especially frequently in the southern part of the German Bight and off the East Friesian Islands, and they are found somewhat less often along the southwestern coast of England. Lesions almost never occur north of 56 °N latitude. Because of the differences in the distribution patterns, it seems likely that external diseases have an etiology different from that of liver nodules, assuming that such infectious diseases as lymphocystis and ulcerations are induced by stress triggered by natural or anthropogenic influences (Wedemeyer 1970, Snieszko 1974). In contrast, anthropogenic pollutants are considered to be important factors in inducing liver tumors.

Definite causes for the formation of liver neoplasias in fishes have been identified only in the case of epizootics in aquaculture facilities resulting from the use of feeds containing aflatoxin. In natural waters, it is difficult to isolate substances which alone are capable of inducing neoplasia formation. For the epizootics of hepatic neoplasias in the Baltic Sea (Falkmer et al. 1977), Duwamish River (Washington, USA) (Pierce et al. 1978), and the Hudson River estuary (New York, USA) (Smith et al. 1979), PCBs (polychlorinated biphenyls) were the substances initially blamed for causing the disease. What seemed to be particularly strong evidence was provided by the case of the myxine hepatomas. After input of PCBs into Swedish waters was forbidden, the frequency of the disease decreased drastically. However, it has since been demonstrated that PCBs are not primary carcinogens for fishes, although it has been shown that these substances do function as promoters of neoplastic lesions (Shelton et al. 1986).

Lately, especially intensive investigations of neoplasias in the English sole in Puget Sound (Washington, USA) have brought much attention to PAHs (polycyclic aromatic hydrocarbons) as agents for inducing neoplastic liver formation (Krahn et al. 1986). In some respects, the work with the PAHs may explain the error concerning the importance of PCBs, since both substances frequently occur together.

If we consider the contamination of the dab livers, we note that the amounts of the metals Cd and Pb are elevated in regions far from the coast, especially on the Dogger Bank, while in the German Bight, only very small amounts of these elements are accumulated. Mercury, on the other hand, is more abundant near the coast (Claußen 1988). The greatest parallel trends are found for the frequency of the neoplastic liver lesions and the levels of PCBs, which are both elevated in fishes from the German Bight and depressed on the Dogger Bank and in regions far from the British coast, where the levels of other organochlorine compounds, including HCB (hexachlorobenzene), are elevated (Büther 1988).

Furthermore, the southern North Sea, the German Bight, and the sea lanes near the East Friesian islands are characterized by elevated concentrations of oil derivatives in the water column (Deutsches Hydrographisches Institut 1983, 1984, 1985). Considering the many substances introduced into the ambient water by human activities, it seems an oversimplification to assume that any individual substance, such as PCBs, PAHs or dioxin, could be the only one responsible for the development of hepatic neoplasias in fishes, especially since it has been learned that a great variety of substances may have an influence on the initiation and progress of the disease.

If we summarize the information needed to decide whether monitoring dab livers is a suitable method to judge the biological effects of environmental parameters, we find the following positive arguments. From diagnoses made by macroscopic examination, it is possible to distinguish with high probability the group of neoplastic diseases, including preneoplastic and neoplasia-associated conditions, from other idiopathic liver anomalies. Although it is not possible to distinguish the associated lesions from the various stages of neoplastic development or to determine the degree of malignancy of the neoplasms without histological analyses, it should be very useful to include this lesion complex, as diagnosed by macroscopic examination, in biological effect monitoring programs. The frequency of this condition strongly reflects the reaction to cancer-producing substances in the water.

The preneoplastic, neoplastic, and neoplasia-associated liver lesions have attained a rate of occurrence in the dab examined of 35 to 38 %. This far exceeds the rates for external diseases that are already included in biological effect monitoring programs. Because of the dependence of the occurrence rates on the length of the fishes, however, it is necessary to limit examinations to fishes of a specific length class and possibly also to one sex in order to provide comparable data.

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