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Toxicological pathology of dab *Limanda limanda* along pollution gradients in the southern North Sea

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ABSTRACT: During the Bremerhaven Workshop non-overtly diseased female dab *Limanda limanda*, 17 to 27 cm in length, were sampled for detailed histopathology along a 200 km transect (Stns 3, 5, 6, 7, 8 & 9) extending out from the Elbe estuary region (Stn 3) to the northwestern region of the Dogger Bank (Stn 9) in the southern North Sea. During the period March 12 to 30, 1990, approximately 20 fish were examined from each of the above stations. Histopathological changes seen that were considered to be consistent with adverse exposure to xenobiotic compounds were confined to the heart, liver and kidney. In the heart, the prevalence of myocardial vacuolation, suggestive of fatty change, was significantly higher ($p < 0.05$) in fish from Stn 3 compared to fish from all other stations. In the liver, the most important lesions seen were well-developed foci of cellular alteration, high mitotic activity and high neutral lipid accumulation in livers of dab sampled from the most inshore station (Stn 3) compared to the reference station (Stn 7). Foci of cellular alteration and high neutral lipid accumulation were significantly greater ($p < 0.05$) at Stn 3 compared to Stn 7. In the kidney, the prevalance of proteinaceous/cellular debris in Bowman's space of renal glomeruli was significantly greater ($p < 0.01$) in fish from Stn 3 compared to fish from Stn 7 The high prevalence of foci of cellular alteration and high neutral lipid accumulation in hepatocytes in the liver of dab are consistent with the effects of adverse exposure to toxic xenobiotics. The other non-infectious changes seen in the liver, as well as those seen in the heart and kidney, are also consistent with xenobiotic exposure but other possible explanations are considered. The value of using detailed histopathology on small numbers of dab which appear grossly normal is clearly demonstrated.

INTRODUCTION

In Europe, investigations into fish diseases and their possible relationships with xenobiotic contaminants in the marine environment have tended to follow the protocols recommended by ICES (ICES 1989). These pathological methods of investigation, which were mainly limited to gross inspection, have revealed a variety of spatial and temporal trends in fish disease patterns in the North Sea (Bucke et al. 1984, Bucke 1988, 1991, Bucke & Waterman 1988, Dethlefson 1988). This epidemiological type of approach can be complemented by the application of techniques which operate at the molecular, cellular and tissue level of organisation (Köhler 1989, Moore 1990, 1992a, b, Moore & Simpson 1991, Stegeman & Lech 1991, Varanasi & Stein 1991, Cameron & Berg 1992, Köhler et al. 1992, Lowe et al. 1992, Moore & Evans 1992). The combined methodology coupled with the use of supportive laboratory bioassays should help to define causal links between some classes of contaminant and specific types of toxic injury in fish. In Europe, only a few field studies have traced links between fish disease and chemical exposure (see Köhler 1989) but in North America where xenobiotic contamination of the marine environment has been investigated more extensively, good data exists for linking hepatic neoplasms (and related lesions) with exposure to toxic chemicals in marine fish species (Mix 1986, Malins et al. 1988, Murchelano & Wolke 1991, Myers et al. 1991, Varanasi & Stein 1991).

Although the common dab *Limanda limanda* is the species recommended for offshore sampling in European fish disease surveys, little published information appears to be available on its general pathology. The objectives of this study were (1) to obtain a basic pathology profile of the major organs and tissues of dab, in specimens (females 17 to 27 cm in length) that appeared to be free from grossly visible signs of disease, (2) to highlight changes seen that are consistent with previous exposure to xenobiotics and (3) to link these with changes seen in similar specimens examined by techniques of cellular pathology. The rationale underlying the approach used in this study is defined by Moore & Simpson (1992) and by Moore (1992b). Preliminary data, obtained from this study and relating only to the liver, has been reported by Simpson (1992).

MATERIALS AND METHODS

Dab were collected using standardised methods from Stns 3, 5, 6, 7, 8 & 9. Captured fish were held in aerated tanks on board ship. At the dockside fish were allowed to settle for approximately 4 h before necropsy procedures began. Fish were killed by severing the spinal cord immediately posterior to the brain. The total length of each fish was recorded, as were the presence of both external and internal abnormalities. Fish with overt signs of external disease were excluded from this investigation. Fish were numbered individually and fixed in large containers of 10 % neutral formalin.

The following organs and tissues were submitted for histological processing and histopathological examination: skin, fins, myotomes, bone, cartilage, nares, eyes (including choroid gland), brain, spinal cord, notochord, dorsal root ganglia, peripheral nerve, pituitary gland, thyroid gland, oesophagus, stomach, anterior and posterior intestine, pyloric caeca, liver, pancreas, anterior and posterior kidney, gills, pseudobranch, heart and gonad. Portions of liver were stored in fresh formalin fixative for subsequent histochemical methods. Each tissue sample was trimmed in a standardised way, dehydrated through graded ethanols, cleared in toluene and embedded in paraplast wax at 58 to 60 °C. Transverse sections were cut and stained routinely with alum haematoxylin and eosin. Histochemical techniques were used to support histopathological diagnosis. These included periodic acid-Schiff (PAS) with/without diastase for glycogen and neutral mucosubstances, Alcian blue 2.5 for polyanionic groups, Lillie and Ashburn's Oil Red O for neutral lipid, Bromphenol blue for basic protein groups and various general triple staining techniques (Pearse 1968, 1970). Diagnostic data was entered into a computerised pathology system (ARTEMIS). Fisher's Exact Test was used to compare diagnostic data from the sites, using Stn 7 as the reference site against which all other sites were compared.

RESULTS

The complete spectrum of pathological changes recorded will be given elsewhere. Changes recorded and discussed here are those mostly considered to be relevant to contaminant impact. No microscopic neoplastic changes were seen, nor were there any major pathological findings seen in the nervous system, endocrine system and reproductive organs. No histopathological evidence of bacterial and fungal infections was seen. Histopathological evidence of viral infection was confined to lymphocystis disease in skin and occasionally kidney and spleen of dab from Stns 8 & 9. Over 26 % of dab from Stn 9 contained skin foci of mature lymphocystis cells with distinct capsules (this prevalence was significantly greater ($p < 0.05$) compared to dab from Stn 7).

Infection by various parasitic agents was seen at all sites along the transect. Parasitisation by helminths was most prevalent (over 52 %) in the anterior intestine of dab from Stn 9 but this was not statistically significant when compared to those recorded in the gut of dab from Stn 7 (35 %). Protozoan infection in the anterior intestine of dab from Stn 3 (25 %) was significantly different ($p < 0.05$) than that seen in the gut of dab from Stn 7 (5.6 %). On balance, parasitic infection tended to be most prevalent at Stns 3 & 9 and evoked a negligible host tissue response.

Findings considered to be of known or possible toxicological significance were restricted to the heart, liver and kidney. In the heart, the prevalance of changes in the myocardial wall consistent with lipid deposits was significantly higher in dab from Stn 3 (31.6 %) compared to that in the heart of the dab from Stn 7 (0 %). This type of myocardial vacuolation was present in just over 4 % of dab from Stn 9.

A variety of changes were seen in the liver and these are summarised in Table 1. Dab liver samples from Stns 3 & 9 had a greater degree of hepatocellular cytoplasmic vacuolation compared to other stations (Table 1). The prevalance of this hepatocyte cytoplasmic vacuolation was significantly greater at Stn 3 compared to that seen at Stn 7 ($p < 0.05$).

In paraffin sections, hepatocellular cytoplasmic vacuolation, which presents as irregular shaped vacuoles, crissed-crossed by strands of cytoplasm, is characteristic of glycogen, e.g in the glycogen secreting livers of salmonids and in male dab livers (Simpson

Table 1. *Limanda limanda*. Summary of histopathological findings in the liver of non-overtly diseased dab (17 to 27 cm)

Microscopic findings	Stn 3	Stn 5	Stn 6	Stn 7	Stn 8	Stn 9
No. livers examined	20	20	20	19	18	23
No. with:						
Hepatocellular cytoplasmic vacuolation (glycogen and lipid)	17*	10	10	8	10	17
Subcapsular sinusoidal dilatation	1	0	0	1	1	0
Scattered basophilic hepatocytes	3	1	1	1	0	0
Apoptosis	1	0	4	0	2	3
Haemorrhagic foci	1	0	0	0	0	0
Proliferation of melanomacrophage centres	2	2	0	0	1	0
Hepatocyte necrosis	3	1	0	0	0	0
Increased mitotic activity	4	1	0	0	0	0
Infiltration/fibrosis of melanomacrophage centres	0	0	0	0	1	0
Foci of cellular alteration	7*	3	2	0	2	4
Parasitic infection	0	1	1	0	0	5

* Significantly higher than the prevalence at Stn 7 by Fisher's Exact Test (*$p < 0.05$)

unpubl. obs.). PAS reactive material labile to prior treatment with amylase is indicative of glycogen. PAS staining of the livers of paraffin sections was generally minimal, though the irregular appearance of some of the vacuoles clearly indicated that glycogen had been present in life. This was consistent with the fact that glycogen is not particularly well retained in routine formalin fixed sections. In addition to vacuoles of glycogen type, sharp edged, regular shaped vacuoles were also seen in paraffin sections of dab liver sampled along the transect. These were considered to be consistent with neutral lipid. About 55 % of the dab from Stn 3 featured this hepatocellular cytoplasmic vacuolation of lipid type compared to almost 19 % in the liver of dab from Stn 7 (Fig. 1). This difference was significant ($p < 0.05$). That the vacuolation was consistent with neutral lipid was confirmed as such by Oil Red O staining of representative cryostat sections. Hepatocyte cytoplasmic vacuolation characteristic of 'hydropic change' was not seen.

A major finding of toxicological importance was the presence in the liver of foci of cellular alteration (altered cell foci). These phenotypically distinct lesions (Fig. 2) were classified according to the standardised criteria of Harada et al. (1989) and were most prevalent at Stn 3 (Table 1), especially altered cell foci of the basophilic type. The prevalence of altered cell foci was significantly greater at Stn 3 ($p < 0.05$) compared to dab from Stn 7 (Fig. 2). Other changes in the liver consisted of hepatocellular necrosis and prominent mitotic activity of hepatocytes only in dab from Stns 3 & 5 (Table 1). Morphological markers for programmed cell death (apoptotic bodies) were also seen in dab liver but the prevalence was lowest in livers from Stns 3, 5 & 7 (Table 1).

In the kidney, there was no evidence of significant tubular degeneration or repair in dab anywhere along the transect stations sampled (Table 2), but there was a

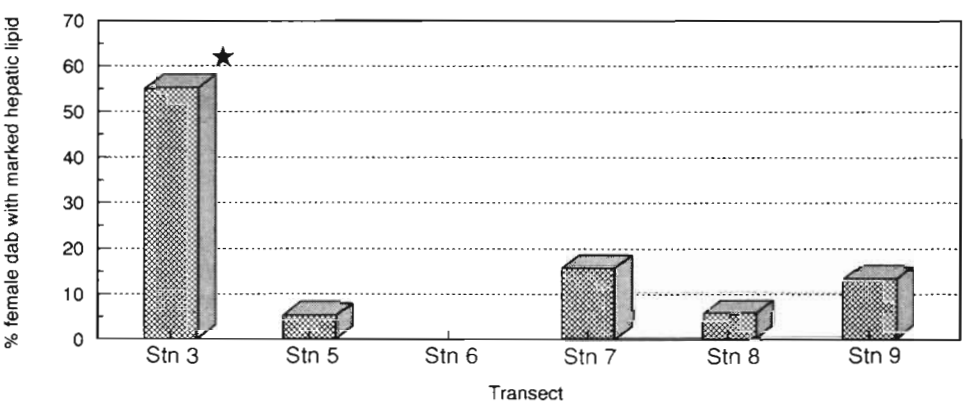

Fig. 1. *Limanda limanda*. Marked hepatocellular lipid vacuolation in non-overtly diseased dab (17 to 27 cm). * Significantly higher than the prevalence at Stn 7 by Fisher's Exact Test ($p < 0.05$). Vacuolation confirmed as neutral lipid by staining of representative sections with Lillie and Ashburn's Oil Red O

Table 2. *Limanda limanda*. Summary of histopathological changes in the posterior kidney of non overtly diseased dab (17 to 27 cm)

Microscopic findings No. fish examined:	Stn 3 20	Stn 5 20	Stn 6 20	Stn 7 19	Stn 8 18	Stn 9 23
No. with:						
Cellular debris/proteinaceous deposits in Bowman's space	11••	0	0	0	0	3
Glomerular fibrosis	1	0	0	0	0	0
Tubular fragments in ureter lumen	2	0	0	0	0	1
Interstitial granulomata	2	0	0	0	0	0
Lymphocystis disease	0	0	0	0	0	1
Tubular regeneration	1	0	0	0	0	0
Occasional necrotic tubule	0	0	0	0	0	0
Cystic dilatation	0	0	0	0	0	1
Interstitial protozoan infection	0	1	0	0	0	0
Unencapsulated helminth	0	0	2	0	0	0
Tubular vacuolation	1	0	0	0	0	0

•• Significantly higher than the prevalence at Stn 7 by Fisher's Exact Test (••$p < 0.01$, 1-sided)

significant accumulation of cellular debris/proteinaceous deposits in the Bowman's space of the renal glomeruli of dab from Stn 3 compared to those from Stn 7 ($p < 0.01$).

DISCUSSION

The most serious histopathological changes seen were in the liver. Foci of cellular alteration in the vertebrate liver are generally regarded as putative pre-neoplastic changes in hepatocytes. These occur spontaneously in laboratory rodents and increase in number as a function of age. Generally they do not progress to hepatic neoplasia, but do increase dramatically in young rodents exposed chronically to a wide variety of both genotoxic and non-genotoxic carcinogens (Bannasch et al. 1989, Harada et al. 1989). Foci of cellular alteration also occur in both laboratory-reared and wild fish (Hinton 1989, Hinton & Lauren 1990, Myers et al. 1990, Murchelano & Wolke 1991), can be induced by carcinogenic chemicals and contaminated sediments (Couch & Courtney 1987, Black 1988, Hinton et al. 1988, Myers et al. 1990, Schiewe et al. 1991) and show great similarities to foci induced in the liver of laboratory rodents (Couch & Courtney 1987). It is interesting that the prevalance of altered cell foci in the liver of dab sampled along the transect follows the trend of the contaminant levels established previously (Lohse 1990) and by Cofino et al. (1992). It has been established by Lohse (1990) that Stn 3 is the most contaminated station in terms of organochlorines, petroleum hydrocarbons and metals, decreasing out towards Stn 7 but rising again at Stn 9. In particular, lipophilic contaminants predominate at Stn 3. Unfortunately there are no data yet available on the pathogenesis of altered cell foci in the liver of dab either from field studies or from exposure to carcinogens in the laboratory.

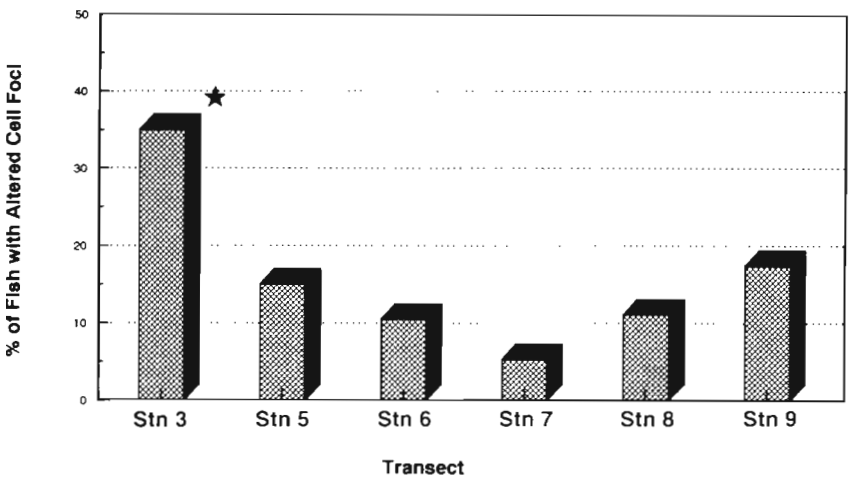

Fig. 2. *Limanda limanda*. Prevalence of altered cell foci in the liver of non-overtly diseased dab (17 to 27 cm). • Significantly higher than the prevalence at Stn 7 by Fisher's Exact Test (•$p < 0.05$)

The limited data from published studies on non-European species of flatfish indicate that the year class prevalance of altered cell foci is first shown to be above baseline at 5 yr of age and older (Rhodes et al. 1987). In this study the age composition of dab at all stations along the transect was dominated by 4

and 5 yr old fish (Rijnsdorp et al. 1992). Despite the dab along the transect being broadly of similar age, the prevalance rate for hepatic altered cell foci in dab from Stn 3 was significantly higher than at other sites. Conversely, the low prevalance seen in dab from Stn 7 is in keeping with similar low prevalance rates noted for dab taken from Lyme Bay, an area in the English Channel believed to be free from anthropogenic inputs (Simpson et al. unpubl. obs.). It is tempting to relate the relatively high prevalence rate of altered cell foci seen at Stn 3 to the presence of relatively high levels of lipophilic organics as established by Lohse (1990). However the situation is complicated by the recent findings of Rijnsdorp et al. (1992), where it has been shown that the dab populations over the transect are subject to some migratory movement.

It could be argued that altered hepatic cell foci could have been induced in the dab at locations other than of the transect, possibly as pelagic embryos inhabiting a xenobiotic-rich microlayer. Experimentally it has been established that pre-neoplastic and neoplastic hepatocellular changes can be induced in 0+ class fish by a single short pulse of carcinogen, with altered foci appearing within weeks of the initial exposure and hepatocellular neoplasms within months (Hendricks et al. 1984, Black 1988, Hawkins et al. 1988). This is seen against a very low spontaneous prevalance rate. Whether older fish already showing some background levels of altered cell foci can be similary induced is not known. Nor is it known whether the residence time of dab over particular stations along the transect is sufficiently long for foci to develop. Despite these limitations it is proposed that the age-related prevalance of hepatic altered cell foci in flatfish such as the dab be further investigated as potential medium term markers of xenobiotic exposure.

Currently, it is not known whether the presence of such hepatic foci relates to particular components of the sediments at stations where the fish were collected or merely reflects xenobiotic exposure from elsewhere. Be that as it may, the relatively high prevalence of foci of cellular alteration in the liver of dab from Stn 3, coupled with prominent mitotic activity and little evidence of apoptosis, collectively suggests a toxic effect with at least a reduction in those homeostatic mechanisms that regulate normal liver growth. Whether the environmental triggers are genotoxic or epigenetic carcinogens, promoters or enhancing factors, etc., remains to be established (see Weisburger & Williams 1991). In parallel studies to this one, Chipman et al. (1992) found no evidence of genotoxic liver damage in dab sampled along the transect. Therefore there is a possibility that the altered cell foci are being induced by a combination of factors which precipitate hepatocyte cell death (necrosis), induce cell proliferation (mitogenesis) and perhaps reduce programmed cell death (apoptosis). In the liver, necrogenic, mitogenic and apoptotic phenomena are important components in recent mechanistic approaches to experimental carcinogenesis (Cohen & Ellwein 1990, Kraupp-Grase et al. 1990, Schulte-Herman et al. 1990).

The fact that no evidence for station-related hepatic neoplasia was seen along the transect (Vethaak 1992, Vethaak et al. 1992) is not unexpected. It is argued that whilst it is feasible for a relatively rapid onset change such as foci of cellular alteration to be related to a particular station, the neoplastic progression that will occur only in a smaller number of individuals with foci will probably be expressed after the fish have left the area, or if the cumulative dose was inadequate to result in actual neoplasia. The tagging experiments of Rijnsdorp et al. (1992) have indicated that the dab population over the sampling stations along the transect reflects a temporary aggregation of fish originating from a large area. The tumour biology of no European species of flatfish is known. Experimental data is needed on the rapid induction of early onset changes, progression versus regression, tumour latency, and mortality of tumour bearing fish in order to help better interpret findings acquired by the application of epidemiological techniques in field surveys (see Köhler 1989).

In addition to the above changes, livers of dab presented with a storage type of hepatocellular vacuolation, which appeared to follow the trend of contamination established previously (Lohse 1990), i.e. livers of dab from Stn 3 were rich in neutral lipid, those from Stn 7 showed minimal lipid, and lipid was expressed again in the livers of dab from Stn 9. In the case of lipid, the annual reproductive cycle in female dab causes a mobilisation of body reserves which are partly used for the development and ripening of the eggs which coincides with a decrease in hepatic neutral lipid as the eggs develop (Htan-Han 1978, Kamman et al. 1990). In this study, all the females examined histologically appeared to be at the same stage of spawning, and therefore would have been expected to show a similar pattern of hepatic lipid utilisation. The oocytes in dab from all along the transect were histologically similar (stages IV to VI) according to the criteria of Htan-Han (1978). Assessing the data in isolation, a differential expression of hepatocyte neutral lipid along the transect could be explained by postulating dietary differences among the fish at different sites or general nutritional status. However, looking at the data within the context of other liver changes seen, then an alternative explanation is possible. The presence of the high levels of neutral lipid in livers of dab from Stn 3 coupled with the other hepatic changes described would suggest a toxic effect, such as fatty change, rather than a physiological over-production of normal storage lipid. Over-

all, the changes reported here are consistent with a toxic effect in the liver of dab from Stn 3 and are strengthened by the presence of lysosome impairment, reduced endocytosis, increased low density lipoprotein, increased oxyradical formation, increased endoplasmic reticulum, elevated EROD, elevated P4501A1 and the detection of *ras*-oncoprotein, also in livers of dab from Stn 3 (Köhler et al. 1992, Lowe et al. 1992, Moore 1992a, b, Moore & Evans 1992, Renton & Addison 1992). In total the data from these varied biomarkers suggest toxic liver injury in dab taken from the inshore stations. One final comment on the liver findings is that in the dab sampled there did not appear to be any evidence of hepatocellular vacuolation of the type described as hydropic change (M. J. Moore pers. comm.) and described for winter flounder, starry flounder, rock sole and white croaker from North American waters (see Moore et al. 1989, Bodammer & Murchelano 1990, Murchelano & Wolke 1991). The latter authors link the presence of such hydropic degeneration with exposure to xenobiotics. The apparent absence of this type of change in the dab will be the subject of further study.

Changes in the kidney were seen in dab sampled along the transect, namely proteinaceous, cellular debris in the Bowman's space of the renal glomeruli in fish from Stns 3 & 9. The prevalance of this renal finding was significantly greater with respect to controls only in dab from Stn 3. However, there was no significant evidence of renal tubular damage, consistent with exposure to nephrotoxic chemicals, nor was there histopathological evidence of regenerating tubules. It is possible that the proteinaceous material seen in Bowman's space represents unsalvaged protein, i.e. small molecular weight protein that normally undergoes reabsorption after passing through the renal glomeruli. This might occur if the pinocytotic activities of the renal epithelium was reduced in the kidneys of fish from Stns 3 & 9. Reduced pinocytotic activity was a feature of freshly isolated hepatocytes of fish from Stn 3 (Moore 1992b).

It is concluded that this first ever study of the major organs and tissues of the dab in non-overtly diseased specimens has provided a number of leads for further investigation and has confirmed the toxicological importance of the liver in such investigations. It has highlighted the need for further studies on the presence of foci of cellular alteration. The latter have already been used as biomarkers of liver injury in field studies in the USA (Murchelano & Wolke 1991, Myers et al. 1991). The pathology seen here represents a 'first pass' and it is possible that xenobiotic-linked changes in other organ systems may be revealed pending examination of further samples and generation of much larger data sets.

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