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Skin and Liver Diseases Induced in Flounder (*Platichthys flesus*) after Long-term Exposure to Contaminated Sediments in Large-Scale Mesocosms

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Disease development in flounder (*Platichthys flesus*) was studied over a period of 3 years in three large mesocosms (40 m × 40 m × 3 m). Two of the mesocosms contained clean sand and the third, sharing a common water circulation with one of the clean-sand mesocosms, was stocked with contaminated dredged spoil. In this way, one of the clean-sand mesocosms was indirectly polluted via the water phase, and analysis of contaminant concentrations in sediments and flounder tissues showed that it had a status intermediate between the other two. Random samples of the flounder populations from the indirectly polluted and reference mesocosms were examined every 2 months for epidermal diseases (lymphocystis, skin ulcers, fin rot) and then released. In addition, every 6 months, random samples of fish from all three mesocosms were sacrificed for histological and chemical investigation. With regard to the development of epidermal disease, the results showed little difference between the reference mesocosm and the indirectly polluted mesocosm, with the exception that lymphocystis was significantly elevated in the indirectly polluted mesocosm. Although pollution may be a risk factor in the etiology of this disease, such a relationship would probably be obscured under field conditions due to variation arising from other factors. Histopathological analysis of the livers revealed in total four cases of hepatocellular adenoma (1.5% of sampled populations) in fish from the polluted mesocosms, the first occurring after 2.5 years of exposure in fish from the indirectly polluted mesocosm. Furthermore, several other liver lesions, including foci of cellular alteration and hydropic vacuolated lesions, developed during the course of the experiment before tumor formation was apparent. Prevalences of these conditions were very much lower in the reference mesocosm than in the two polluted mesocosms. Densities of melanomacrophage centers in the liver showed a similar trend. The findings clearly indicate that long-term exposure to chemically contaminated dredged spoil can induce liver neoplasia and other liver lesions in flounder at contaminant levels comparable to those found in the natural environment. *Key words:* biomarker, disease induction, fish disease, flounder (*Platichthys flesus*), liver neoplasia, marine pollution, mesocosm. *Environ Health Perspect* 104:1218–1229 (1996)

It is generally accepted that environmental stress, including pollution, predisposes fish to infectious and noninfectious diseases (1,2).

The possibility that marine pollution might contribute to the etiology of fish disease first gained interest in the United States in the 1960s when the discovery of high prevalences of tumors in fish led to attempts to correlate their presence with chemical pollution. Since that time, many studies have been carried out on both sides of the Atlantic, some having a histopathological focus and others, particularly in northern Europe, using an epidemiological approach to study externally visible gross lesions associated with diseases such as lymphocystis and ulceration (2).

To investigate the potential links between marine pollution and the occur-

rence of fish diseases in the North Sea, epizootiological surveys were conducted in the Netherlands from 1983 through 1989 (3–5). These surveys were directed towards grossly identifiable diseases (lymphocystis, skin ulcers, and liver nodules) and histologically identified liver lesions in flounder (*Platichthys flesus*) from areas subject to varying degrees of pollution. The flounder was chosen as an indicator species in these studies primarily because of its susceptibility to disease (4). Although the collection of field data led to a detailed description of the epizootiological characteristics of diseases in this species, interpretation of the results in relation to pollution proved difficult for two reasons: it was impossible to separate the effects of pollution on disease development from those of other natural and anthropogenic factors such as salinity

and the impact of fisheries and migration and dispersal patterns of flounder during the spawning period were believed to distort conclusions about long-term effects of pollution at specific locations.

Because of these limitations, consideration was given to the use of large mesocosms for studying flounder diseases. Exposure of fish to pollution in enclosed mesocosms would permit the study of long-term development of diseases within a single cohort, while interference from other stress factors and migration would be eliminated.

For such an approach to be successful, it was felt that an exposure period of at least 3 years was necessary. This conclusion was based on the field observation that liver tumors (a main target lesion of the study) were rarely found in flounder 2 years of age or younger (4).

A 3-year mesocosm experiment was started in 1990. The development of major epidermal diseases and histologically identified liver lesions (including neoplasms) was monitored in the mesocosms, while various analytical techniques provided direct information about the levels of pollution to which the fish were exposed and the resulting biochemical effects. The results were then interpreted in terms of a possible causal relationship between pollution and disease in the mesocosms. An attempt was also made to relate the experimental findings to the field observations described in related studies (3–5).

To date, only a limited number of investigations have dealt with the long-term induction of lesions in feral fish species by environmental pollutants. In most of these studies, fish were exposed in the laboratory to single compounds (6–8) or sediment extracts (8–13). In contrast, the present

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study deals with the induction of lesions in fish under semifield conditions at pollution levels similar to those occurring in a natural environment.

Methods

Experimental design. Three large concrete basins were available at the Netherlands Institute for Forestry and Natural Research on the island of Texel. Each basin measured 40 m × 40 m × 3 m and was filled with sea water to a depth of 1.5 m (Fig. 1).

Two of the three basins (the reference mesocosm, RM, and the indirectly polluted mesocosm, IM) contained a layer of sand approximately 40 cm deep collected from the nearby Marsdiep channel, the largest inflow to the Wadden Sea. Sand was chosen in preference to finer sediment because it would inevitably accumulate pollutants with time. Since the water supply also came from the Marsdiep channel, a certain degree of background pollution was unavoidable. Therefore, the use of sand from a completely unpolluted site was not justified. The third basin (the directly polluted mesocosm, DM) contained approximately 300 m³ of dredged spoil transported to Texel from Rotterdam Harbor. This spoil contained a complex mixture of organic micropollutants and heavy metals (14) and was classified as moderately toxic.

The system was set up in March 1988. Two pilot studies carried out in 1988–1989 established the suitability of the system for maintaining flounder alive for long periods but demonstrated that regular sampling of DM was impractical because of the muddy sediment (15). The main experiment began in May 1990 and was terminated in May 1993.

There was a continuous flow of Wadden Sea water into and out of the RM and the IM at a rate of 50 m³/day, leading to complete replacement every 2 months. The DM did not have such a flow; instead, it shared a common water circulation with the IM, exchanging water with it at a rate of about 650 m³/day. This pattern of water exchange began in November 1989, 6 months before the start of the experiment. In addition, pumps were used to circulate water in each basin from the corners to the centre. This was done to avoid stratification and patchiness of contaminants.

The RM and the IM were drained every 2 months to facilitate inspection of fish and sediments. To maintain a constant buffer of contaminants in the DM, it was drained only in November 1991, in November 1992, and at the end of the experiment in May 1993.

Each of the three basins had been stocked in March 1988 with 1000 lugworm (*Arenicola marina*), 1000 *Nereis diversicolor*, and 200 kg blue mussels (*Mytilus edulis*).

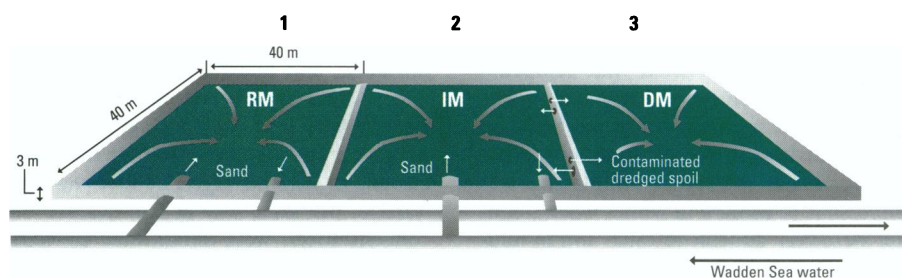


Figure 1. Schematic view of the experimental design used in the main study. Note the connection between Mesocosms 2 and 3. RM, reference mesocosm; IM, indirectly polluted mesocosm; DM, directly polluted mesocosm.

Table 1. Number of flounder released at the start of the experiment and size of samples examined for external disease on subsequent occasions

Sampling date	Month number	Reference mesocosm (no.) ^a	Indirectly polluted mesocosm (no.) ^a	Directly polluted mesocosm (no.) ^a
1990 May	0	1200 (9)	1200 (9)	400 (9)
July	2	—	—	—
Sept	4	—	—	—
Nov	6	50 (50)	50 (50)	—
1991 Jan	8	—	—	—
Mar	10	112	144	—
May	12	131 (40)	186 (40)	—
July	14	61	65	—
Sept	16	—	—	—
Nov	18	92 (40)	102 (41)	16 (16)
1992 Jan	20	53	89	—
Mar	22	86	108	—
May	24	88 (26)	80 (26)	—
July	26	60	71	—
Sept	28	60	94	—
Nov	30	60 (25)	83 (25)	25 (25)
1993 Feb	33	20	28	—
May	36	44 (37)	74 (45)	76 (45)

^aNumbers of fish in subsamples sacrificed for analysis on each occasion.

Settlement of larvae introduced via the incoming water subsequently led to a more diverse community including, by 1990, various bivalve and polychaete species and a rich meiobenthos. Since the flounder apparently remained in good condition, no additional feeding was carried out during the course of the experiment.

The RM and IM were both stocked with 1200 flounder at the start of the experiment (May 1990). At the same time, the DM was stocked with 400 flounder, primarily so that a restricted sampling regime could be carried out, but also for purposes of bioturbation to ensure exchange of contaminants with the water phase. The fish used for the experiment were all apparently healthy 1-year-old individuals, the youngest age group available at that time. They were collected in April 1990 from the nearby Wadden Sea (Balgzand) and kept in quarantine for 2 weeks before being introduced into the basins.

Target diseases and host parameters. Flounder were examined for signs of the external diseases lymphocystis, skin ulcers,

and fin rot in the same manner as in the field study that was carried out in the Dutch coastal waters (4). Scars, most of which represented healed fin rot and healed skin ulcers, were also recorded. The criteria for diagnosis and categorization are described by Vethaak and Jol (4). Random samples of fish were collected for internal examination. The livers were excised, inspected for gross lesions visible on the surface, and then prepared for histopathological and other analyses.

All sacrificed fish were measured. The viscera and gonads were removed, and somatic weight and liver weight recorded. Condition factor (CF) and somatic liver index (SLI) were then calculated, as follows:

$$CF = 100 \times (\text{somatic weight, mg}) \times (\text{total length, mm})^{-3}$$

$$SLI = 100 \times (\text{liver weight}) \times (\text{somatic weight})^{-1}$$

The fish were always examined by the same team of observers.

Sampling design. The development of epidermal diseases was routinely monitored

in the RM and the IM. Sampling was carried out while the basins were drained. Initially, it was planned that sampling should take place every 2 months, with a target sample size of 100 fish per basin on each occasion; however, it soon became apparent that the target sample size was impossible to achieve in winter when flounder were very difficult to catch and in mid-summer when high temperatures restricted the period during which the basins could be left empty. For these reasons, sampling was not carried out in months 2, 4, 8, and 16. Also, a single sampling occasion in month 33 replaced the two proposed samplings in months 32 and 34 (Table 1).

The fish examined for epidermal disease were subsequently returned alive to the basins. Because they were subjected only to an external examination, their sex could not be determined. Every 6 months (i.e., months 6, 12, 18, 24, 30, and 36), a randomly chosen subsample of these fish was retained and sacrificed for gross inspection of the liver followed by histological examination and measurement of tissue contaminant concentrations and other parameters. The sample size varied between 16 and 50 fish per basin depending on the catch and the amount of tissue required for analysis (Table 1). Prior to stocking (month 0), a sample of 150 fish was retained for similar analyses in order to provide a baseline. In addition, samples were taken from the DM on three occasions (months 18, 30, and 36) (Table 1).

Basic water quality parameters. Salinity, temperature, dissolved oxygen content, and relative light transmission of the water were measured twice a week at the same time of day (11 A.M. approximately). For salinity measurements, a portable electrodeless induction salinometer (Beckman Instruments, Mijdrecht, The Netherlands) was used. Oxygen content was determined by means of a Yellow Springs Type 57 meter (Tamson, Zoetermeer, The Netherlands), and light transmission was measured by means of a Type SN91 meter (Sea Tech Inc., Corvallis, OR). Light transmission was expressed in terms of relative visibility. Measurements of these routinely measured parameters were interrupted during months 8, 10, and 12 due to failure of the automatic recording system.

Analysis of chemical contaminants. Every 6 months, 10 sediment cores were taken from randomly selected locations within the RM and the IM. The <63 μm fraction was then isolated and stored at 4°C to await analysis. Similar samples were taken from the DM in months 0, 18, 24, 30, and 36. Methods for the analysis of sediments for the polychlorinated biphenyl PCB-153, the summed concentrations of three polycyclic aromatic hydrocarbons

(PAHs) known to have carcinogenic properties (benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, and benzo[*a*]pyrene; Σ 3PAH), Cd, Pb, and organic content are described elsewhere (4). In addition, the sampling program for the mesocosms included analysis of Zn and Hg. The first was analyzed in the same way as Cd and Pb. Hg concentrations were determined by cold-vapor atomic absorption spectrometry after digestion with nitric acid in a pressure bomb. Concentrations of organic contaminants were expressed as microgram per kilogram organic content.

Analytical methods used to determine concentrations of metals, PCBs, PAHs, and total lipid content were as described above and by Vethaak and Jol (4).

A proportion (approximately one-third by weight) of the liver of each flounder sacrificed at months 0, 6, 12, 18, 24, 30, and 36 (Table 1) was removed. On each sampling occasion, these subsamples were pooled into two samples per mesocosm: one comprising livers from male fish and one comprising livers from female fish. The samples were then frozen at -20°C and stored for analysis of heavy metals and PCB-153.

Preliminary analysis of the data indicated that there were no consistent differences between the two sexes and, therefore, no distinction was made in this study during further analysis of the results.

In addition, concentrations of the non-*ortho*-substituted PCBs CB-77 and CB-126 were measured in eggs released by fish, which were in spawning condition during sampling in month 18. The first 10 mature female fish sacrificed from each mesocosm were used to provide the egg samples, which were pooled into a single sample per mesocosm. For full details of the methods and a full set of results, see de Boer et al. (16).

It is assumed that the analysis of the two PCB congeners mentioned above provides accurate information on the expected toxic effect of PCBs because, in general, they are thought to be responsible for 85–90% of the toxicity of the total PCB load in Dutch fish based on an assessment using toxic equivalency factors (16).

Additional analysis of heavy metals, PCB-153, and Σ 3PAH was conducted of whole specimens of 100 (pooled) blue mussels from the RM and the IM in months 0, 6, 12, 18, 24, 30, and 36 and from the DR in months 0, 18, 30, 36. Analysis of heavy metals and PCB-153 was conducted of whole specimens of 50 (pooled) three-spined sticklebacks (a major food item of flounder) collected from each mesocosm in months 18 and 30, (Table 1).

Lugworms, benthic food items of flounder, have a low capability for the

transformation of PAHs (17). Therefore, these animals were considered to give a representative picture of the presence of PAHs in the environment as well as in the food. Samples of 25 lugworms were collected from the RM and the IM in months 0, 6, 12, 24, 30, and 36. Lugworms were not present in sufficient numbers in the DM. The animals were kept in sea water for 24 hr to allow emptying of the intestine and then stored at -20°C to await analysis for PAHs. Each sample of 25 lugworms was pooled into a single sample for analysis.

Biochemical indices. The exposure of fish to PAHs was also investigated directly by measurement of biliary concentrations of 1-OH-pyrene, a marker metabolite, using synchronous fluorescence spectrometry (SFS) as described elsewhere (18). Because the PAH profile in flounder remains roughly constant at different locations, it has been suggested that the concentration of 1-OH pyrene is a useful relative measure of total PAH uptake (especially of PAHs containing four or more rings) in this species. For example, concentrations of 1-OH pyrene have been found to correlate rather well with those of 3-OH-benzo[*a*]pyrene (3-OH-BaP) in flounder but are usually higher by a factor of 300–600 (19). BaP is a well-known model carcinogen.

Bile was collected from a subsample of the flounder sacrificed in months 18, 24, 30, and 36; the first 12–14 flounder sacrificed per mesocosm were used on each sampling occasion. Bile samples were stored temporarily in 2-ml Eppendorf microvessels on ice and then frozen at -20°C until they were analyzed.

The following *CYP1A* indices in the liver of flounder were measured to assess the presence of organic compounds with a relatively planar molecular structure: *CYP1A*-mRNA, *CYP1A* protein, and 7-ethoxyresorufin-*O*-deethylase (EROD).

A small part of the liver was taken from each fish sampled in month 30 (25 fish/mesocosm). Additional sampling was conducted in month 18 (RM, 38 fish; IM, 32 fish; DM, 9 fish) for EROD analysis only. Each liver was analyzed separately. Methods for the determination of *CYP1A* indices, together with comprehensive results, have been published elsewhere (20).

Liver homogenates were also analyzed for the occurrence of PAH-DNA adducts using a ³²P post-labeling method, as described by Baan et al. (21). In month 30, a small part of the livers of 6 fish were chosen at random out of the 25 fish sampled from each mesocosm. Each liver was analyzed separately. Comprehensive results are given by Baan et al. (21).

Histological techniques. On each sampling occasion, livers from most fish sacrificed were sampled for histopathological analysis (Table 1). Whenever possible, a random subsample was taken to obtain a target sample size of 25 livers per mesocosm, but on some sampling occasions, the need to retain tissue for chemical and other analyses meant that this target sample size was not met.

Since the liver tissue had to be divided among the various analyses, only a part of each liver was available for histopathology. A 5 mm thick slice from the middle part of the liver was cut and fixed in 10% saline formalin. Wax sections 5 μ m thick were stained with hematoxylin and eosin (H&E); sections of livers collected at the end of the experiment (month 36) were also stained with the Periodic Acid Schiff (PAS) reaction for the identification of melanomacrophage centers (MMCs).

Semiquantitative assessments of diffuse hepatic vacuolization and MMC density were made using the criteria given by Vethaak and Wester (5).

Statistical analysis. Data on epidermal disease were analyzed using a log-linear model (4), with exposure (RM vs. IM) and time (month) as the two factors. The full model included two main effect terms and the interaction between them, with a deviance of zero because there was no replication. Similar methods were used to compare prevalences of liver lesions in the three mesocosms. In the case of hepatocellular adenoma, focal hepatocellular hypervacuolization, and hydropic vacuolization of biliary epithelial cells, the statistical analysis was restricted to data from months 30 and 36; analysis of the complete data set did not provide a good fit due to the many empty cells.

The sex of the fish could not be determined during screening for epidermal diseases, while sample sizes were too small in the histological investigation to allow a sex-specific analysis. The sex of the fish was therefore ignored. Dependence between observations (because of repeated sampling from the same mesocosm) was also ignored.

Associations between the various diseases in individual fish were investigated by making pairwise comparisons using contingency table analysis and Yates' corrected chi-square test for small sample sizes. The critical chi-value was set at $p = 0.05$.

Results

Basic water quality parameters. Figure 2 shows monthly mean values of salinity, water temperature, dissolved oxygen, and relative light transmission in the three mesocosms. The differences between the mesocosms were

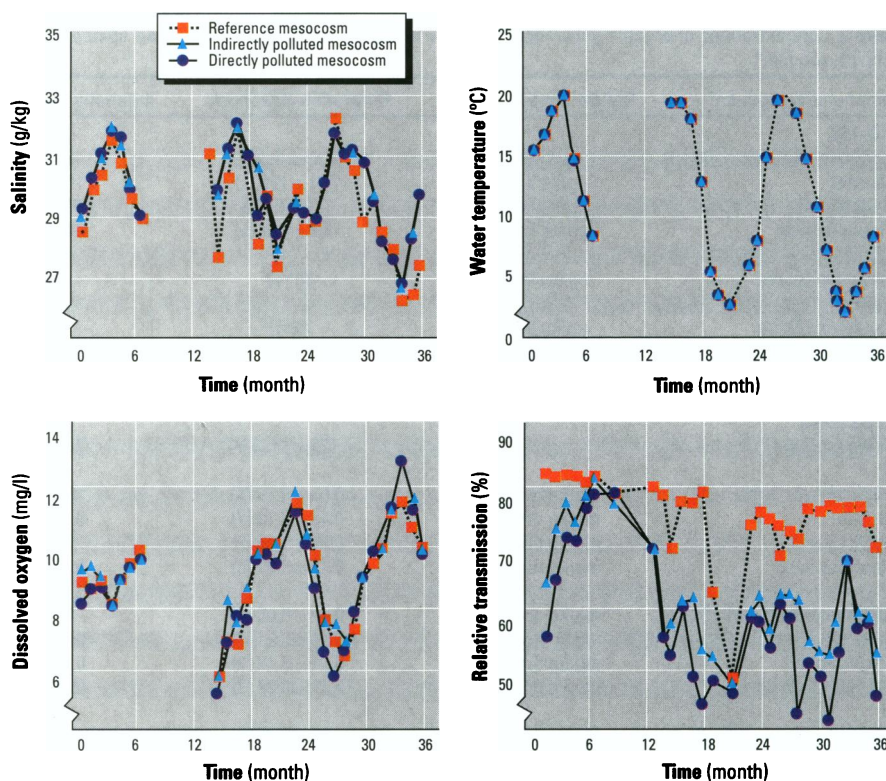


Figure 2. Monthly means of salinity, water temperature, dissolved oxygen, and relative light transmission in the three mesocosms derived from samples taken twice weekly. May 1990, month 0; May 1993, month 36.

small and insignificant, with the exception of relative light transmission. The lower light transmission in the DM and the IM is in accordance with the nature of the sediment.

Salinity ranged between 27 and 32 g/kg, reflecting natural fluctuations in the salinity of incoming water from the Wadden Sea, with a summer peak due to evaporation. Fluctuations in water temperature followed natural seasonal variation. Due to the mild winters, hardly any ice was formed in the mesocosms, and the lowest measured water temperature was 2°C.

Minimum values of dissolved oxygen were between 5 and 6 mg/l and occurred when water temperatures were highest. These concentrations are close to the lowest value (5 mg/l) recommended by Davis (22) for cold-water biota at 20°C. Lower values may have occurred during the night or at dawn because of diurnal cycling, but the possibility of hypoxia can be discounted. No signs of mortality related to low dissolved oxygen values were detected by visual observation during sampling, and catch sizes did not fall during periods of low dissolved oxygen.

Chemical contaminants. Mean values of organic contaminants and heavy metals in sediment and mussel, stickleback, and flounder tissues were calculated using data from all sampling occasions. In the case of Cd in sediment, some analyses were considered unreli-

able and the results were discarded; the same applies to PCB-153 and heavy metals in the flounder liver samples. Thus the number of samples was frequently less than the number of sampling occasions (Table 2).

For most organic contaminants, there was a clear pollution gradient with lowest values in the RM and highest values in the DM. For example, the ratio of concentrations of PCB-153 in the sediment of the three mesocosms was 1:4:18. Concentrations in mussels, sticklebacks, and flounder livers were higher, reflecting a higher lipid content of these animals or tissues, but the gradient was less pronounced, for example, in the case of flounder liver 1:3:6. Concentrations of two other PCB congeners in flounder spawn showed similar trends, but for CB-126 there was little difference between the two polluted mesocosms. Concentrations of PAHs in sediment and mussels and concentrations of 1-OH-pyrene in flounder bile also showed clear pollution gradients (1:2:5, 1:4:13, and 1:6:19, respectively) (Table 2). The biliary 1-OH pyrene concentrations showed similar trends but were consistently lower than those measured for flounder from a small-scale mesocosm experiment exposed to the same sediments (19). This effect is due to a different feeding pattern: in the small-scale experiment, the fish were starved for 2 days prior to analysis, resulting in more concentrated bile fluid.

Table 2. Concentrations of organic pollutants and heavy metals in sediment, mussel, three-spined stickleback, and flounder tissues, and biochemical indices from each mesocosm^a

Compound/component	Units of measurement	Reference mesocosm	Indirectly polluted mesocosm	Directly polluted mesocosm
PCB-153				
Sediment (0–10 cm) <63 μm	μg/kg OC	58 ± 7 (7)	242 ± 76 (7)	957 ± 125 (5)
Mussel	μg/kg lipid	311 ± 107 (7)	1092 ± 297 (7)	2050 ± 534 (4)
Stickleback	μg/kg lipid	140 ± 54 (2)	1463 ± 919 (2)	1859 ± 672 (2)
Flounder liver	μg/kg lipid	654 ± 491 (5)	1853 ± 1150 (5)	3394 ± 1612 (4)
PCB 77				
Flounder spawn	ng/kg lipid	98 (1) ^b	400 (1) ^b	745 (1) ^b
PCB 126				
Flounder spawn	ng/kg lipid	15 (1) ^b	50 (1) ^b	47 (1) ^b
Σ3PAH				
Sediment (0–10 cm) <63 μm	μg/kg OC	6318 ± 1583 (7)	12555 ± 1596 (7)	27665 ± 2452 (5)
Mussel	μg/kg lipid	16 ± 11 (7)	57 ± 22 (7)	211 ± 40 (4)
1-OH pyrene				
Flounder bile	ng/ml	128 ± 51 (52)	734 ± 227 (50)	2400 ± 236 (36)
Cd				
Sediment (0–10 cm) <63 μm	mg/kg dry wt	0.82 ± 0.15 (3)	3.2 ± 0.5 (5)	8.5 ± 1.5 (5)
Mussel	mg/kg dry wt	0.43 ± 0.13 (7)	0.70 ± 0.25 (7)	1.38 ± 0.52 (4)
Stickleback	mg/kg dry wt	0.04 ± 0.01 (2)	0.02 (2)	0.05 ± 0.04 (2)
Flounder liver	mg/kg dry wt	0.12 ± 0.09 (3)	0.15 ± 0.06 (3)	0.14 ± 0.10 (3)
Hg				
Sediment (0–10 cm) <63 μm	mg/kg dry wt	0.41 ± 0.06 (7)	1.0 ± 0.2 (7)	2.4 ± 0.4 (4)
Mussel	mg/kg dry wt	0.54 ± 0.27 (7)	0.23 ± 0.10 (7)	0.19 ± 0.05 (4)
Stickleback	mg/kg dry wt	0.30 ± 0.10 (2)	0.25 ± 0.01 (2)	0.47 ± 0.21 (2)
Flounder liver	mg/kg dry wt	0.41 ± 0.29 (3)	0.38 ± 0.09 (4)	0.26 ± 0.19 (4)
Pb				
Sediment (0–10 cm) <63 μm	mg/kg dry wt	84 ± 10 (7)	124 ± 13 (7)	175 ± 10 (4)
Mussel	mg/kg dry wt	2 (7)	3 ± 2 (7)	7 ± 3 (4)
Stickleback	mg/kg dry wt	<2 (2)	<2 (2)	<2 (2)
Flounder liver	mg/kg dry wt	<2 (3)	<2 (3)	<2 (3)
Zn				
Sediment (0–10 cm) <63 μm	mg/kg dry wt	268 ± 31 (7)	455 ± 43 (7)	680 ± 47 (4)
Mussel	mg/kg dry wt	108 ± 27 (7)	83 ± 17 (7)	88 ± 11 (4)
Stickleback	mg/kg dry wt	254 ± 98 (2)	101 ± 8 (2)	219 ± 21 (2)
Flounder liver	mg/kg dry wt	147 ± 48 (3)	167 ± 57 (3)	118 ± 14 (3)
CYP1A mRNA				
Flounder liver	Relative absorbance	2.1 ± 4.4 (25)	3.0 ± 3.8 (25)	6.6 ± 12.2 (25)
CYP1A				
Flounder liver	Absorbance 405 nm	0.10 ± 0.06 (25)	0.12 ± 0.09 (25)	0.09 ± 0.05 (25)
EROD				
Flounder liver	pmol/mg protein/min	115 ± 136 (63)	156 ± 203 (57)	104 ± 157 (34)
PAH–DNA adducts				
Flounder liver	fmol/mg	0.06 ± 0.05 (6)	0.16 ± 0.09 (6)	0.59 ± 0.21 (6)

OC, organic content; EROD, 7-ethoxyresorufin-*O*-deethylase.

^aValues are given as mean ± standard deviation; figures in parentheses represent the number of samples analyzed.

^bData derived from de Boer et al. (16).

Differences were less pronounced in the case of heavy metals, with the exception of Cd and Hg in sediment where a clear gradient was observed. Heavy metal concentrations in mussels, sticklebacks, and flounder livers showed no apparent pollution gradient at all, with the exception of Cd and Pb in mussels.

PCB-153 concentrations in the livers of flounder from the IM and the DM showed a pronounced increase over time (Fig. 3). The same applied to PCB-153 concentrations in sediment from the IM (Fig. 3) and concentrations of PAHs in lugworms from the IM (Fig. 4).

However, concentrations of PCB-153 and PAHs in the RM showed a small but steady decrease (Fig. 3), indicating that contaminant input from the incoming water

was outweighed by the effects of water replenishment, metabolism, and/or the production of organic matter.

Similar trends in the three mesocosms were observed for PCB-153 concentrations in mussels (not shown). The range of concentrations of PCB-153 in sediment and flounder livers in this experiment is somewhat wider than the range measured in sediments and flounder from Dutch coastal waters, overlapping at the higher and lower ends. PCB-153 concentrations in mussels and PAH concentrations in sediment and mussels were in general agreement with values encountered in the field, as were concentrations of 1-OH-pyrene in flounder bile. Concentrations of heavy metals in sediment, mussels, and flounder livers were comparable to those found in Dutch coastal waters (Table 3).

Biochemical indices. Surprisingly, CYP1A protein and EROD activity in flounder liver were not significantly different among the three mesocosms (Table 2). Measurements of CYP1A mRNA, however, revealed a significant induction in female fish from the DM as compared to the RM, by a factor of 2 (Table 2).

In association with most organic contaminants and biliary 1-OH-pyrene, CYP1A mRNA and PAH–DNA adducts in flounder livers showed concentration gradients with lowest values in the RM and highest values in the DM (Table 2). The ratios were 1:2:6 and 1:3:10, respectively.

Growth and survival of test fish. The food supply provided by the benthic fauna in the self-supporting ecosystems in the mesocosms allowed the fish to survive and

grow well. Visual inspection during sampling indicated that most of the fish had full stomachs. During the summer of 1990, food items in all three mesocosms consisted predominantly of polychaetes and bivalves. During the following two summers, large increases occurred in the populations of three-spined sticklebacks (*Gasterosteus aculeatus*) inhabiting the IM and especially the RM, resulting in differences in food availability among the different mesocosms.

Fish in all mesocosms increased in weight over the 3-year period of the experiment. During the winter, growth slowed down, accelerating again in early summer. Growth during the first 6 months was considerably reduced by comparison with field-derived values, probably due to a shortage of suitable food items, but it increased again at 2 and 3 years of age and exceeded values observed in the field. Overall growth rates during the whole experimental period were comparable to those occurring in the field (Table 4).

Using analysis of variance, no difference between the sexes could be found in CF or in the SLI. Data for the two sexes were therefore combined to make comparisons between mesocosms. The CF of fish from the three mesocosms revealed differences in months 12, 18, and 30 only. On the first occasions, CF was significantly higher in fish from the IM than in those from the RM, but the difference was later reversed (Fig. 5). The apparent trends shown by CF were largely in agreement with the observed growth rates (Table 4).

Significant differences in the SLI were observed only in month 18, when fish from the RM occupied an intermediate position between those from the other two mesocosms (Fig. 5). There appeared to be no relationship with the pollution gradient.

Despite the difficulties experienced in collecting sufficiently large samples, there was no evidence of extensive mortality in

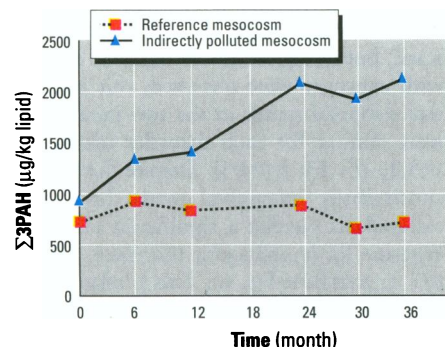


Figure 4. Concentrations of $\Sigma 3$ PAH (benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, and benzo[*a*]pyrene) in lugworms in the indirectly polluted and reference mesocosms on different sampling occasions.

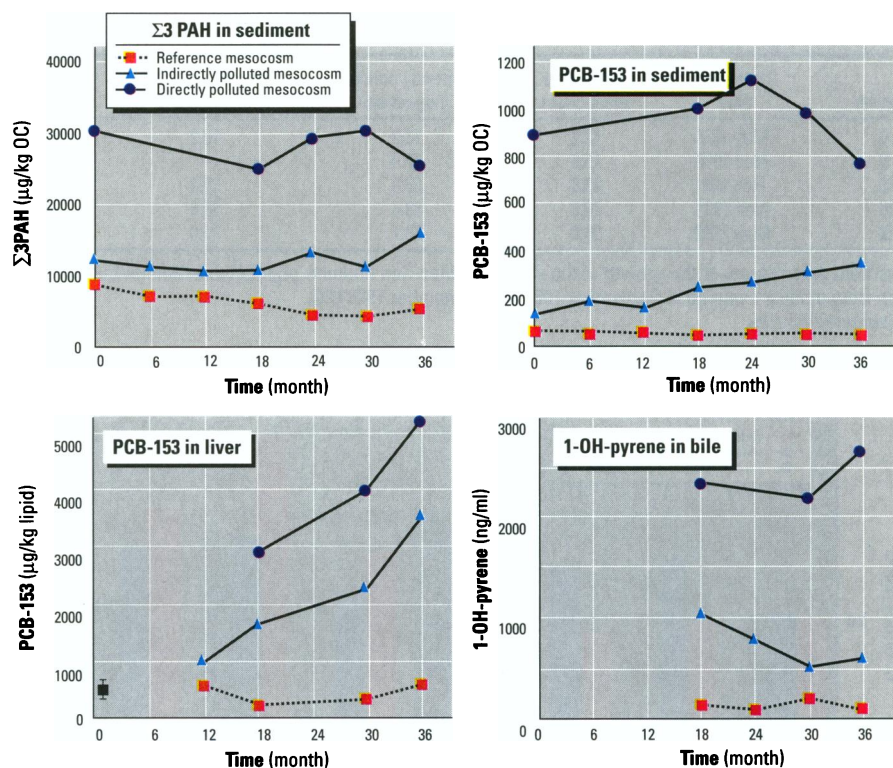


Figure 3. Concentrations of organic contaminants in the three mesocosms on different sampling occasions. For PCB-153 in liver, the mean concentrations (\pm standard deviation) from three pooled samples ($n = 50$ in each) in month 0, is presented. $\Sigma 3$ PAH includes benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, and benzo[*a*]pyrene.

Table 3. Comparison of the concentrations of organic pollutants and heavy metals in sediments, mussels, and flounder tissues from each mesocosm with the range observed in the field

Compound/component	Units of measurement	Reference mesocosm	Indirectly polluted mesocosm	Directly polluted mesocosm	Field
Sediment					
PCB-153	$\mu\text{g}/\text{kg OC}$	50–70	130–350	800–1100	60–700
$\Sigma 3$ PAH	$\mu\text{g}/\text{kg OC}$	4000–9000	11000–16000	26000–30000	4000–29000
Cd	$\text{mg}/\text{kg dry wt}$	0.5–1.0	2–4	7–10	0.2–6.0
Pb	$\text{mg}/\text{kg dry wt}$	70–100	100–150	160–190	40–230
Zn	$\text{mg}/\text{kg dry wt}$	200–300	400–500	600–750	120–700
Hg	$\text{mg}/\text{kg dry wt}$	0.3–0.5	0.6–1.3	2–3	0.1–2.1
Mussel tissue					
$\Sigma 3$ PAH	$\mu\text{g}/\text{kg lipid}$	10–40	40–90	90–260	20–270
PCB-153	$\mu\text{g}/\text{kg lipid}$	200–500	700–1500	1400–2500	300–2300
Cd	$\text{mg}/\text{kg dry wt}$	0.3–0.6	0.4–1.2	0.8–1.6	0.3–1.7
Pb	$\text{mg}/\text{kg dry wt}$	<3	2–6	5–10	2–7
Zn	$\text{mg}/\text{kg dry wt}$	70–140	60–100	80–100	60–180
Hg	$\text{mg}/\text{kg dry wt}$	0.1–0.9	0.2–0.4	0.1–0.2	0.1–0.4
Flounder liver					
PCB-153	$\mu\text{g}/\text{kg lipid}$	200–1000	1000–4000	3000–5000	300–3500
Cd	$\text{mg}/\text{kg dry wt}$	0.05–0.25	0.10–0.25	0.05–0.25	0.2–0.3
Pb	$\text{mg}/\text{kg dry wt}$	<2	<2	<2	<2
Zn	$\text{mg}/\text{kg dry wt}$	120–210	110–230	100–200	190–220
Hg	$\text{mg}/\text{kg dry wt}$	0.2–0.8	0.2–0.5	0.2–0.5	0.1–0.5
Flounder bile					
1-OH-pyrene	ng/ml	90–200	510–1030	2000–2640	30–1900 ^a

The field data are derived from samples collected in Dutch coastal waters observed in 1987–1991 during the month of September (4,23). The ranges represent the ranges of values of individual samples. OC, organic content.

^aData derived from Ariese (19).

Table 4. Average length (cm) of flounder in the three mesocosms, with comparable data from the field

Month	Calendar time	Reference mesocosm	Indirectly polluted mesocosm	Directly polluted mesocosm	Field
0	May 1990	13.2	13.2	13.2	12.5*
6	Nov 1990	15.6	17.0	—	20.0
18	Nov 1991	23.6	23.3	25.1	25.0
30	Nov 1992	29.5	28.4	30.4	29.0
36	May 1993	30.0	28.7	31.0	29.5*

The field data represent the growth of the Dutch Wadden Sea flounder population based on back calculation from otoliths sampled in September 1987 and September 1988 (24).

*Estimated figures.

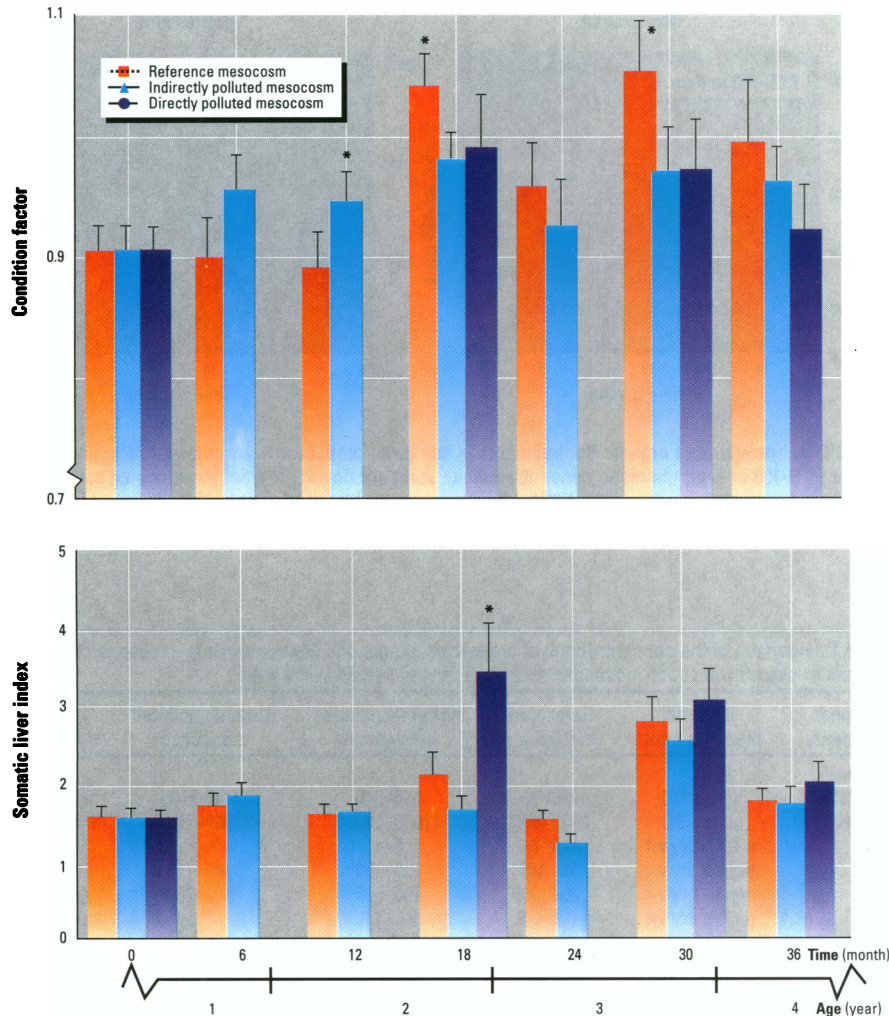


Figure 5. Mean values (+ 95% confidence interval) of condition factor and somatic liver index in flounder from the three mesocosms. Significant differences relative to the reference mesocosm are indicated with an asterisk.

any of the mesocosms. Dead fish were found only occasionally. Nevertheless, the data in Table 1 show that only approximately a one-fifth of the total number of fish (1200) in each of mesocosms 1 and 2 and a quarter (400) in mesocosm 3 were sacrificed during the trial or recaptured at the end (month 36). This suggests an apparently high mortality rate, as was also found in the pilot studies (16).

Two possible contributory factors were identified. First, predation by birds occurred during the third month of the experiment, but this was subsequently prevented by the installation of a net of wires in July 1990. Second, escape of young fish through the pipe system may have occurred during the first year of the experiment.

Despite the problems described above, the data in Table 1 also show that sampling

with constant effort produced more or less similar sample sizes in the RM and the IM on each occasion, suggesting similar population densities and mortality rates in these two mesocosms.

Most test fish reached maturity at 2 years of age in November 1991. This was apparent from the development of eggs and sperm in the gonads and from the stages of vitellogenesis visible in histological sections of the livers of sacrificed female fish.

Epidermal diseases. In the IM and the RM, outbreaks of lymphocystis, skin ulcers and fin rot were recorded from month 6 onward. Scars from healed ulcers, and fin rot gradually accumulated during the same period, indicating that a relatively large proportion of diseased fish had the capacity to recover and survive (Fig. 6).

Disease prevalences in the two mesocosms showed a strikingly similar pattern of temporal variation. Peaks in prevalences of the three diseases occurred in spring (months 12, 24, and 36). A consistent increase in prevalences of skin ulcers was observed, whereas the pattern for fin rot and perhaps lymphocystis appeared to indicate a stabilization of disease levels within the population.

Prevalences of lymphocystis, skin ulcers, and fin rot obtained during some sampling occasions (months 18, 30) in the DM were within the range of those observed in the RM and the IM. Prevalences of scars, healed ulcers, and fin rot from the DM were lower than those observed in the RM and the IM. At the end of the experiment (month 36) prevalences of the various disorders from the DR were lymphocystis (25/76; 33%), skin ulcers (12/76; 16%), fin rot (5/76; 7%), and scars from healed ulcers and fin rot (37/76; 49%). Data of epidermal diseases in the DR was excluded from the statistical analysis because of low sample sizes and infrequent sampling.

The statistical analysis of disease prevalences in the IM and the RM showed significant effects of time for all diseases (including scars), but no significant interactions of time with exposure were observed; this implies that temporal trends in the two mesocosms were similar for each disorder (deviance <19.2; $df = 13$; $p > 0.05$). Despite this apparent similarity in temporal trends, the statistical analysis yielded a significant effect of exposure for lymphocystis (deviance = 23.7; $df = 1$; $p < 0.001$; Fig. 6). This is related to a consistently higher prevalence of this disease in the IM from month 18 onward. The odds ratio for lymphocystis in the IM relative to the RM, based on the model time + exposure, was estimated as 1.60, with 95% confidence limits 1.32–1.94.

Liver histopathology. A total of 177 livers

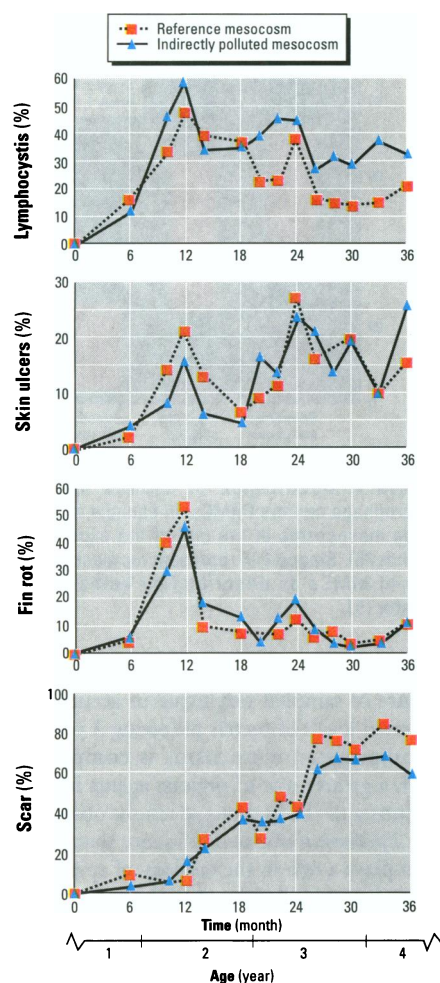


Figure 6. Observed prevalences of epidermal diseases (including scars) in flounder from the three mesocosms on different sampling occasions. Age of the cohorts is shown at the bottom of the figure.

from the RM, 174 from the IM, and 92 from the DM were examined (Table 5). The spectrum of histopathological lesions observed included the five major types of lesions as presented in Table 5.

Neoplasms developed only in the livers of fish from the two polluted mesocosms (Table 5). Two cases were observed in the IM and two in the DM; the diameter of the lesions ranged between 3 and 8 mm. All were detected during macroscopic screening and were histologically diagnosed as hepatocellular adenoma. The first case was detected at 3 years of age after 30 months of exposure, and the remaining three cases were detected after 36 months. Due to the low prevalence of this disease, no statistically significant differences could be detected (Table 6).

Foci of cellular alteration, which are putative preneoplastic lesions (5), were considerably more prevalent in fish from the DM (20.7%) and the IM (16.1%) than in those from the the RM (2.3%) (Table 5).

Table 5. Number of fish examined and number with hepatic lesions in the three mesocosms on different sampling occasions

Mesocosm	Fish age (month)	Sampling occasion (month)	Number of fish examined	HCA	FCA															
					CCF	BF	EOF	All	FHV	BDV	Other									
Reference	17	0 (May)	9	0	0	0	0	0	0	0	0	2								
	23	6 (Nov)	28	0	1	0	0	1	0	0	0	5								
	29	12 (May)	27	0	0	0	0	0	0	0	0	4								
	35	18 (Nov)	26	0	0	0	0	0	0	0	0	5								
	41	24 (May)	25	0	2	0	0	2	0	0	0	10								
	47	30 (Nov)	25	0	0	0	0	0	1	0	0	8								
	53	36 (May)	37	0	0	0	1	1	1	0	0	20								
Total			177	0	3	0	1	4	2	0	58	100%	0%	1.7%	0%	0.6%	2.3%	1.1%	0%	32.8%
Indirectly polluted	17	0 (May)	9	0	0	0	0	0	0	0	0	3								
	23	6 (Nov)	29	0	1	0	0	1	0	0	0	13								
	29	12 (May)	24	0	3	1	0	4	3	0	0	5								
	35	18 (Nov)	18	0	0	1	0	1	2	1	2	2								
	41	24 (May)	25	0	7	1	0	8	5	3	6	6								
	47	30 (Nov)	22	1	2	2	0	4	4	4	8	8								
	53	36 (May)	45	1	7	2	1	10	8	5	16	16								
Total			174	2	20	7	1	28	22	13	53	100%	1.1%	11.5%	4.0%	0.6%	16.1%	12.6%	7.5%	30.5%
Directly polluted	17	0 (May)	9	0	0	0	0	0	0	0	0	3								
	23	6 (Nov)	29	0	1	0	0	1	3	1	5	5								
	29	12 (May)	24	0	1	0	0	1	3	1	5	5								
	35	18 (Nov)	13	0	1	0	0	1	3	1	5	5								
	41	24 (May)	25	0	0	3	0	3	8	1	8	8								
	47	30 (Nov)	45	2	12	2	1	15	19	6	16	16								
	53	36 (May)	45	2	12	2	1	15	19	6	16	16								
Total			92	2	13	5	1	19	30	8	32	100%	2.2%	14.1%	5.4%	1.1%	20.7%	32.6%	8.7%	34.8%

HCA, hepatocellular adenoma; FCA, foci of cellular alteration; CCF, clear cell foci; BF, basophilic foci; EOF, eosinophilic foci; All, total foci of cellular alteration; FHV, focal hepatic hypervacuolization; BDV, hydropic vacuolization of biliary epithelial cells; Other, inflammatory lesions (hepatitis, pancreatitis), granulomas, parasitic cysts, and focal necrosis. Prevalences are given as percentage. Two other lesions were encountered: regenerative foci and fibrillar hepatocytes (see text). The characteristics of the various lesion types are described by Vethaak and Wester (5).

Table 6. Summary of the statistical analysis of histopathological liver lesions with deviances, residual degrees of freedom (*df*) and associated probabilities (* = $p < 0.01$)

	All months			Months 30 and 36 only			
	<i>df</i>	Deviance		<i>df</i>	Deviance		
		FCA	Other		HCA	FHV	BDV
Null model	17	75.4	49.1	5	5.1	29.2	15.3
Time + exposure	9	6.3	12.3	2	1.9	0.5	2.3
Without exposure	11	31.2*	13.3	4	4.9	28.8*	15.2*
Without time	15	47.0*	48.5*	3	2.1	0.8	3.4

FCA, foci of cellular alteration; Other, inflammatory lesions (hepatitis, pancreatitis), granulomas, parasitic cysts, and focal necrosis. HCA, hepatocellular adenoma; FHV, focal hepatic hypervacuolization; BDV, hydropic vacuolization of biliary epithelial cells.

The variation among mesocosms was statistically significant (Table 6). Most common were clear cell foci (overall prevalence 8.1%), followed by basophilic foci (2.7%), and eosinophilic foci (0.7%). No basophilic foci were observed in fish from the RM.

The occurrence of focal hepatocellular hypervacuolization, a hydropic vacuolated lesion, showed a clear relation with the pollution gradient (Table 5). Hydropic vac-

uolization of biliary epithelial cells, appeared to be about equally prevalent in fish from the DM and the IM, but was not observed at all in fish from the RM. Statistical analysis showed significant differences among mesocosms for both these types of lesions (Table 6). Differences among the three mesocosms in the prevalence of the category of lesions termed other were not significant (Table 6).

Regenerative foci were only occasionally found (fewer than five cases in total). Fibrillar hepatocytes, occasionally observed in field samples (5), were only detected at the end of the experiment (month 36) in the DM (two cases). These hepatocytes contain cytoplasmic fibrils oriented as parallel or converging basophilic stacks, probably representing diluted rough endoplasmic reticulum (RER) containing microfilamentous material (25); the significance of the lesion is unknown.

Diffuse hepatic vacuolization was a common feature among the livers examined, as in the field study (5). The degree of vacuolization correlated well with the SLI, indicating that it reflected nutritional status.

The density of MMCs in sections stained by PAS showed a clear positive relationship with exposure to pollution (Fig. 7).

In the case of the lesions in Table 5, only occasional combinations yielded sufficient numbers of cases for any association in individual fish to be tested. There was, however, a strong association between foci of cellular alteration and hydropic vacuolization of biliary epithelial cells ($p < 0.01$) and between foci of cellular alteration and focal hepatocellular hypervacuolization ($p < 0.05$), but not between the two types of vacuolated lesion ($p = 0.6$). The category termed other was not significantly associated with any of the above mentioned lesions. Out of four cases of hepatocellular adenoma, three also featured foci of cellular alteration, suggesting an association between these two types of lesions.

Discussion

Methodology. A basic assumption made in the experimental design is that differences in disease development in the mesocosms are due to differences among treatments (or pollution levels) and not to other sources of variation. Due to the lack of replication and various other confounding factors as discussed later, this assumption cannot be tested. Evidence for a causal relationship between pollution and disease in the present experiment depends basically on a comparison between the two sand basins (RM and IM), but pollution levels increased over time in one of these (IM). This provided an opportunity to investigate whether temporal changes in pollution levels and disease prevalence occurred in parallel. In the case of liver lesions, data were also collected from a third mesocosm (DM), giving the opportunity to study the effects of a pollution gradient.

Many potential sources of variation among the three mesocosms were controlled. These included several factors likely to confound the interpretation of field data, such as age, migration, and salinity fluctuations. The closely similar temporal

trends of disease prevalence in two of the mesocosms show that variation due to additional uncontrolled factors does not cause conditions to diverge substantially. There is, however, one other factor that deserves further consideration.

In the experiment, flounder diets were not the same in every mesocosm; in particular, the DM differed from the other two mesocosms. This would account for the somewhat different growth rates of the three groups of fish. It has recently been shown that the significance of food ingestion as a route of organic pollutant uptake increases with the increasingly hydrophobic nature of the pollutant (26). In the case of the PAHs and PCBs used as indicators in this study, uptake via food can be important. Differences in diets during the second and third year of the experiment may therefore have resulted in variation in the uptake of these pollutants and also, via an altered dietary lipid content, may have led to altered metabolism (27) and bioavailability of the same pollutants.

Contaminant concentrations. In terms of contaminant concentrations, conditions in the mesocosms appear to reflect quite well those found in Dutch coastal and inland waters (Table 3).

The source of pollutants used in the experiment was a complex natural mixture of environmental chemicals reflecting pollution of the Rhine. Although PAHs and PCBs have been identified as major components (15), the exact composition is complex and unknown. However, the advantage of using such a heterogeneous mixture of pollutants is that conditions then closely mimic those occurring in Dutch coastal waters and the Wadden Sea, areas that are strongly influenced by water outflow from the Rhine. Direct measurement of contaminant concentrations in the fine fraction ($< 63 \mu\text{m}$) of the Rotterdam Harbor-dredged spoil used in this experiment gave values varying between 332 and 547 $\mu\text{g}/\text{kg}$ dry weight for BaP and between 33 and 48 $\mu\text{g}/\text{kg}$ dry weight for PCB-153. Such concentrations are relatively low compared to those in other parts of Rotterdam Harbor, which may reach 3100 and 210 $\mu\text{g}/\text{kg}$ respectively (unpublished data).

Contaminant concentrations observed in the RM were lower than concentrations in the Wadden Sea near Texel and also lower than concentrations in the Eastern Scheldt, the reference site used for the field study (4,5).

The rather uniform concentrations of heavy metals across all three mesocosms (with the notable exception of Hg in sediments) suggested that any differences in disease prevalences were more likely to be asso-

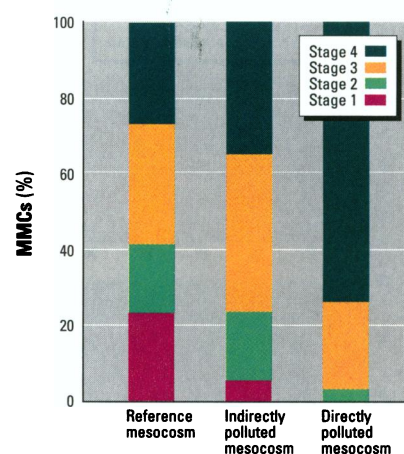


Figure 7. Occurrence of hepatic melanomacrophage centers (MMCs) in flounder from the three mesocosms at the end of the experiment (month 36). Stages 1–4 represent increasing density of MMCs as described by Vethaak and Wester (5).

ciated with organic contaminants. Also, the failure of sampled fish tissue to accumulate elevated levels of metals following 3 years in contaminated mesocosms is compatible with a greater role of organic agents in contributing to the pathologic lesions observed.

Epidermal diseases. Evidence that pollution plays a role in the etiology of epidermal disease is provided by the diverging prevalences of lymphocystis in the RM and the IM, with higher values in the latter (Fig. 6).

For fish of comparable age captured in the field, prevalences of lymphocystis varied from 10% to 30% and prevalences of skin ulcers varied from 2% to 35% (3,4). Once prevalences in the mesocosms had attained stable values when fish were 3 and 4 years of age (Fig. 6), they did not substantially exceed the field values, but these prevalences were at the higher end of the observed range. The patterns of seasonal variation in the mesocosms showed similarities to those found in the field, with the maximum occurring in the spring (4).

Comparison of prevalence values with those encountered in the field must take into account the fact that the populations in the mesocosms consisted of isolated cohorts of fish of similar ages, whereas field populations are highly dynamic and contain fish of a mixture of ages. The closed nature of the populations and the unusually high population density may explain the high disease prevalences attained in the mesocosms, especially at 2 years of age. However, population densities decreased considerably over time due to sampling and various possible sources of fish attrition in the mesocosms (including predation and escape, as previously mentioned) while prevalences of lymphocystis and skin ulcers remained high.

Another possible factor contributing to high disease prevalences is stress due to handling during bimonthly sampling, which may have produced effects comparable to those proposed for fishing gear in field studies (4). By damaging the protective mucous layer of the fish, handling could make them more vulnerable to infectious pathogens. Although handling stress might have resulted in a general increase in disease prevalences in the experiment, it could not explain differences between the RM and the IM because both groups of fish were treated similarly. It could, however, have obscured more subtle effects of pollution on prevalences of skin ulcers and fin rot.

One plausible explanation for the observed association between pollution and lymphocystis is pollution-induced immunosuppression, leading to activation of the lymphocystis virus. Recent studies have reported effects of this nature in laboratory experiments with fish (28). Environmental chemicals with immunomodifying effects include certain PCBs, PAHs, and organotin compounds (28,29). Some indication for immunomodulation by pollutants from the harbor-dredged spoil was recently provided. In a series of preliminary experiments, antibodies against lymphocystis virus were measured in flounder from the three mesocosms at months 30 and 36. The results obtained suggest a trend towards reduction in the numbers of immune fish in mesocosms containing the highest levels of pollutants (30).

Although an association with pollution was demonstrated for lymphocystis in the mesocosms, this does not necessarily mean that the disease is likely to be a useful pollution indicator in the field. Its relatively low sensitivity (odds ratio of 1.6 in the IM compared to the RM) might cause any relationship to be obscured in the highly variable marine environment. The lack of any association in the field appears to confirm this hypothesis (4).

Liver histopathology. The findings of the experiment are consistent with a pollution-associated progression of histological lesions, culminating in hepatic neoplasms. Foci of cellular alteration were first recorded after only 6 months, prevalences being lowest in the RM. Hepatocellular adenoma was detected after 30 months and occurred only in fish from the IM and the DM.

In general, the histopathologically identified lesions observed in the mesocosms were similar to those encountered in the field (5). The absence of neoplasms in fish younger than 3 years of age also agrees with field data (4), and prevalences of foci of cellular alteration were comparable to those recorded in the field for fish of similar ages

(5). Focal hepatocellular hypervacuolization, on the other hand, seemed much more prevalent in the mesocosms than in the field (5).

There was a suggestion of seasonal variation in prevalences of foci of cellular alteration, especially in the IM, with more livers affected in May than in November. This is in agreement with field data (5).

The two vacuolated cell lesions (hydropic vacuolization of biliary epithelial cells and focal hepatocellular hypervacuolization) showed an association with pollution. Moreover, in individual fish, both lesions showed a significant association with foci of cellular alteration, although not with each other. These findings support those of the field study (5).

Several authors have documented the association of hydropic vacuolization of biliary epithelial cells with contaminant exposure in several species (31,32), and it is generally considered a convenient histological biomarker of contaminant exposure. Similar nonneoplastic lesions were induced in fish exposed to various toxicants in the laboratory (33). The findings of the present study strongly supports the significance of this lesion as an indicator of exposure to toxicants, but it remains to be shown whether hydropic vacuolization plays a role in the pathogenesis of tumors.

The experimental findings also indicate a relationship between pollution and the density of melanomacrophage centers (MMCs). MMCs are considered to be part of the nonspecific defense system and accumulate residual poorly digested lipids and other materials including iron. The numbers and size of these MMCs are frequently used as a nonspecific indicator for stress (e.g., starvation, disease) and may thus indirectly reflect the status of the immune system. However, the precise meaning of such findings remains yet unclear (29).

Specific carcinogenic agents. Because the study used a complex mixture of anthropogenic chemicals as a source of pollution, it is difficult to draw firm conclusions about the specific agents responsible for the development of the observed lesions.

It has been proposed that PAHs are involved in the development of liver neoplasia in flatfish (7,8,11–13,32,34–37). In the present experiment, the number of fish developing neoplasms was too small to test whether a dose–effect response existed; however, the ratio of prevalence of foci of cellular alteration, considered to be preneoplastic lesions, did not exhibit such a pronounced gradient as did the ratio of concentration of PAHs in the three mesocosms. Thus a dose–effect response did not appear to exist in the case of these lesions. Given

the complexity of the pathway of chemical carcinogenesis (38–40) and the low prevalence of neoplasms, this is hardly surprising.

Other factors that could have caused the development of preneoplastic and neoplastic lesions to differ among mesocosms include differences in diet, as discussed above. Also, fish introduced into the mesocosms at 1 year of age might already have been exposed to carcinogens earlier in their lives. The observed high levels of planar PCBs in the spawn of flounder suggests maternal transfer as a potential source of exposure. The role of PAHs in the development of liver disease in this study is supported by the gradients observed for hepatic CYP1A mRNA, biliary 1-OH-pyrene and hepatic PAH–DNA adducts. These parameters represent critical steps in PAH-induced carcinogenesis, as widely reported for the model compound BaP in rodents exposed under laboratory conditions (41,42). The carcinogenic activation of BaP is suggested to be mediated by enzymatic transformation to the reactive metabolite 7,8-dihydroxy,9,10-epoxy,7,8,9,10-tetrahydro BaP (43). This metabolite is supposed to bind covalently to the DNA base guanosine, thus forming DNA-adducts (21). In mammals, this biotransformation pathway needs the cytochrome P450 1A (CYP1A) for the carcinogenic activation of BaP (44,45). Although in the present study a significant induction of hepatic CYP1A mRNA levels in flounder was observed along the pollution gradient, this was not the case for hepatic CYP1A protein level and its enzymatic activity (EROD). In contrast, gradients for hepatic PAH–DNA adducts in flounder were consistent with those for PAH sediment concentrations and biliary pyrene metabolite concentrations. It seems that these results do not fit into the commonly accepted hypothesis. Two conclusions can be drawn from these results: 1) the basic level of CYP1A present in flounder liver is enough to biotransform the different amounts of pyrene taken up in DM or 2) another isozyme is involved in the biotransformation of pyrene and the formation of PAH–DNA adducts. The present findings also indicate that hepatic EROD activity and CYP1A protein level in fish are not always dependable biomarkers for measurement of potential effects of environmental carcinogens. This viewpoint contrasts with that of other investigators (46).

PAHs are far from being the only possible carcinogenic agents in this study. For example, it is well known that mixtures of PCBs can induce preneoplastic lesions and hepatocellular carcinomas in animals when given at appropriate doses over extended periods of time (47). Furthermore, it has been shown that 80% of the mutagenicity—detected by the *Salmonella* mutagenicity

test—in the Rhine could not be explained by the presence of conventional compounds that are regularly measured; the results clearly indicated that the compounds responsible consist of nitro-substituted aromatics and aromatic amines (48). A 10-fold increase was detected in the bile of rainbow trout (*Salmo gairdneri*) exposed to water from the Rhine. More recently, it has been suggested that mutagenic compounds in isolates and fractions of Rhine water are primarily hydrophilic (49).

Conclusion

The present study is, to our knowledge, the first to demonstrate the induction of liver neoplasia in fish under simulated field conditions at pollution levels similar to those prevailing in a natural environment. There was compelling evidence that liver neoplasms, putative preneoplastic lesions, and associated vacuolated lesions similar to those occurring in wild flounder developed when flounder were exposed to a complex mixture of anthropogenic contaminants including PAHs. There also appeared to be an association between hepatic MMCs and pollution.

The high prevalence of epidermal disease observed in the mesocosms, particularly during the early part of the experiment, indicated that conditions did not exactly duplicate those found in the field. Nevertheless, there appeared to be a pollution-associated risk for lymphocystis. Pollution exposure did not alter lesion prevalence for fin rot or skin ulcers.

Since concentrations of pollutants in the present study were well within the range occurring in inland Dutch waters, it seems highly likely that such pollutants can influence the prevalence of certain lesions in feral fish populations.

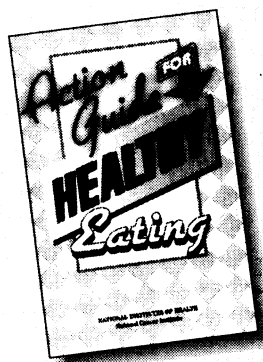
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