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ORIGINAL ARTICLE

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Assessment of some innate immune responses in dab (*Limanda limanda* L.) from the North Sea as part of an integrated biological effects monitoring

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Abstract The marine flatfish dab (*Limanda limanda*), which lives in direct contact with contaminated sediments, is frequently used as a sentinel species in international monitoring programmes on the biological effects of contaminants. In this study, immune responses were recorded as indicators of sublethal chronic effects of contaminants, in addition to measurement of the induction of mono-oxygenase ethoxyresorufin O-deethylase (EROD) in liver cells, the inhibition of acetylcholin esterase (AChE) in muscle and a quantification of grossly visible diseases and parasites. In total, 336 dab were analysed from five sampling areas in the North Sea, including the German Bight, the Dogger Bank, the Firth of Forth, and two locations close to oil and gas platforms (Ekofisk and Danfield). When considering plasma lysozyme levels, pinocytosis and respiratory burst activity of head kidney leucocytes, a clear gradient could be observed with decreased levels in individuals collected from the Firth of Forth and locations near the oil or gas platforms compared with dab from the Dogger Bank or the German Bight. Individuals with induced EROD activity displayed reduced lysozyme and respiratory burst activities. Lysozyme levels were also reduced in dab with lymphocystis or with nematodes. The data obtained indicate that the assessment of innate immune parameters in a monitoring programme provides supplementary information about immunomodulatory effects associated with the exposure of fish to contaminants. In particular, concentrations of plasma lysozyme, which can be analysed in an easy and inexpensive assay, are considered to be an appropriate parameter for use in a battery of other bioindicators.

Keywords Biological effect monitoring · Fish diseases · Innate immune response · North Sea · Pollution

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Introduction

The aquatic environment is being abused throughout the world by the introduction of a large number of xenobiotic compounds derived from human activities in industry and agriculture. Many of these substances have the potential to impact on the ecosystem at relatively low concentrations (Connell et al. 1999). In order to assess the risk for organisms posed by contaminant exposure and to classify the environmental health of an ecosystem under challenge, various monitoring techniques have been used (Van der Oost et al. 1997). These biomarkers are biochemical, physiological or histopathological indicators of either exposure to or early effects of anthropogenic substances, and they become apparent at exposures to concentrations less than those causing acute toxic effects. In various animals, the immune system appears to be particularly sensitive to toxic effects of chemicals of environmental concern. In the mammalian system, a battery of well-characterised immune assays to test for functional or histopathological parameters is available (Luster and Rosenthal 1993), and many of the same endpoints have been used in laboratory studies to demonstrate chemically-induced immunotoxicity in fish (see recent reviews by Zelikoff et al. 2000; Bols et al. 2001).

For field studies in "real-world" contaminated aquatic environments, international panels such as the Interna-

tional Council for the Exploration of the Sea (ICES) recommended monitoring of the biological effects of contaminants by means of, amongst other techniques, biochemical parameters, such as the induction of mono-oxygenase ethoxyresorufin O-deethylase (EROD) in liver cells or the inhibition of acetylcholin esterase (AChE) in muscle in addition to easily visible fish diseases and parasites (see Stebbing and Dethlefsen 1992; Diamant and Westernhagen 1999). As sentinel species, marine flatfish are frequently used in international monitoring programmes. In the North Sea and the Baltic Sea, these are mainly dab (*Limanda limanda*) and European flounder (*Platichthys flesus*) (Secombes et al. 1997; Broeg et al. 1999; Lang and Mellergard 1999; Lang et al. 1999; Grinwis et al. 2000; Lang 2002). For immune function assessment, studies reveal that contaminants modulate immune parameters in fish (Arkoosh et al. 1994; Secombes et al. 1995), but integrated studies, for instance correlating immune functions to measurements of biochemical biomarkers, are scarce.

In the present study, innate immune functions were assessed in dab collected at various locations in the North Sea along a contaminant gradient. From the same individuals, biochemical biomarkers and grossly visible disease symptoms were recorded according to published recommendations (see Stebbing and Dethlefsen 1992) and the implications of the different measurements were compared.

Methods

Sampling

Sampling was carried out on board RV "*Walther Herwig III*" during cruises conducted in August/September 1999, 2000 and 2001. Fishing was carried out by means of bottom trawling with standard gear and methods (GOV or 140 ft bottom trawl, towing time 1 h, towing speed 3–4 knots) at five different locations in the North Sea. The location of the sampling sites is shown in Fig. 1. In the North Sea, regions at P01 (Danfield) and P02 (Ekofisk) are characterised by oil and gas platforms, station N06 is located in the Firth of Forth, N04 at the Dogger Bank and JMP near Helgoland in the German Bight.

Fish were sorted from the catches immediately and kept alive in tanks with permanent seawater flow-through and aeration. Further processing took place within 1 h. In total, 336 female dab (*Limanda limanda* L.) of the size class 20–24 cm were used for this investigation. A maximum of 20 fish per site and campaign were collected and processed.

Sediment samples were taken only during the 1999 cruise at the six different sites and analysed for organic contaminants. Methods and results of this analysis are described in detail by Kammann et al. (2001).

Examination procedure

On board the research vessel, body length and weight were measured for each fish. Blood was drawn from the caudal vein into disposable syringes pre-filled with a lithium–heparin bead (Sarstedt, Germany). The haematocrit was determined from the blood according to standard procedures (Houston 1990). The remaining blood was then transferred to centrifugation tubes, centrifuged at 2,000 *g* for 15 min at 4°C, and the supernatant plasma was collected and frozen at −20°C. The fish were then inspected for obvious signs of diseases and parasites using standardised methodologies as described by Bucke et al. (1996). Briefly, the presence or absence of the following diseases and parasites was registered: lymphocystis, epidermal hyperplasia/papilloma, acute or healing skin ulcers, pigment anomalies, infections with nematodes and acanthocephalans on the liver surface. The otoliths of individual fish were taken for subsequent age determination.

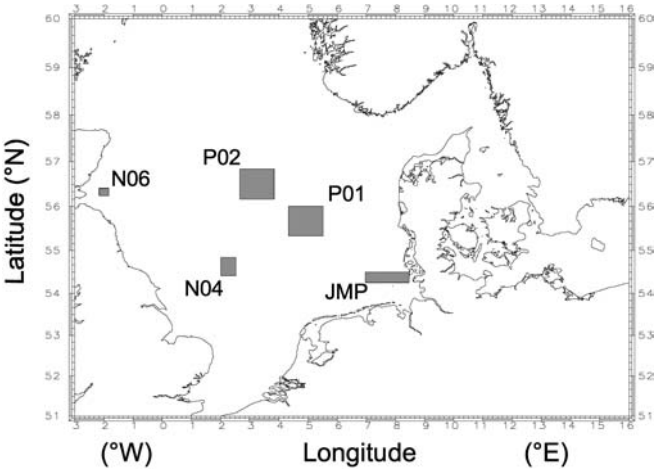

Fig. 1 Location of sampling sites for dab (*Limanda limanda*) in the North Sea. Danfield, oil and gas platforms (*P01*; 55°20–56°00′ N, 04°20′–05°30′ E); Ekofisk, oil platforms (*P02*; 56°10′–56°50′ N, 02°40′–03°50′ E), Dogger Bank (*N04*, 54°25′–54°50′ N, 02°00′–02°31′ E); Firth of Forth (*N06*; 56°15′–56°25′ N, 01°50′–02°10′ W); German Bight (*JMP*; 54°15′–54°30′ N, 06°58′–08°27′ E)

The fish were then dissected and the head kidney was removed and transferred into a centrifugation tube filled with wash medium (RPMI medium supplemented with 10,000 IU l^{-1} sodium heparin, medium; Biochrom, Berlin, Germany: heparin; Sigma, St. Louis, Mo., USA) and stored at 4°C for up to 24 h for further processing.

In addition, liver and muscle samples were collected from the same individuals for biochemical analysis.

Biochemical parameters (EROD, CYP, ACHE, GST)

The following biochemical parameters were measured in dab in the framework of a routine biological effects monitoring conducted by the German Federal Research Centre for Fishery: 7-ethoxyresorufin-O-deethylase (EROD) activity, total protein and levels of cytochrome P450 1A (CYP1A) protein were measured in dab liver according to standard methods [for EROD see Burke and Mayer (1974), protein content according to Lowry et al. (1951) and CYP 1A protein according to Goksoyr and Husoy (1992)]. From muscle tissue, cholinesterase activity (AChE) was measured colorimetrically according to Ellmann et al. (1961). Glutathion-4-S-transferase (GST) activity in liver was determined with the method described by Bressler et al. (1999). The total protein content of liver samples was measured according to Bradford et al. (1976).

Leucocyte isolation

Media and cells were kept on ice and washing procedures were performed at 4°C. Cell suspensions of head kidney leucocytes (HKL) were prepared by forcing the tissues through a 100 µm nylon screen (Swiss Silk Bolting Cloth Mfg, Zurich, Switzerland). Isolated HKL were washed three times with wash medium (10 min, 550 *g*) and resuspended in cell culture medium (RPMI-1640 supplemented with 100,000 IU l^{-1} penicillin, 100 mg l^{-1} streptomycin, 4 mM L-glutamine and 1% (v/v) carp serum

(chemicals; Biochrom, Berlin, Germany: carp serum; serum from 15 individual *Cyprinus carpio* L. was pooled, heat-inactivated for 30 min at 56°C, then 0.2 µm was filtered and stored at −20°C until use). Numbers of viable cells were determined by Trypan Blue exclusion in a Neubauer haemocytometer.

Production of reactive oxygen species by head kidney leucocytes

Generation of reactive oxygen species (ROS) by head kidney leucocytes was measured by means of the Nitro Blue tetrazolium salt (NBT) reduction assay. Cell suspensions were incubated in 96-well flat-bottom microtitre plates (10^6 cells in a final volume of 175 µl of cell culture medium) in triplicate and their ROS production was induced by adding 0.15 mg l^{-1} phorbol myristate acetate (PMA). The indicator NBT was added at 1 g l^{-1}. Wells without PMA served to determine the basal ROS generation of the cells. After incubation for 2 h at 18°C, the supernatants were removed and the cells were fixed by adding 125 µl of 100% methanol. Each well was washed twice with 125 µl of 70% (v/v) methanol. Methanol was removed and the fixed cells were air-dried overnight and stored in the dark for up to 2 weeks. The reduced NBT (formazan) was dissolved in 125 µl 2M KOH and 150 µl DMSO per well (all chemicals from Sigma-Aldrich, Germany). The optical densities were recorded with a spectrophotometer at 650 nm.

Endocytosis activity of head kidney phagocytes

Endocytosis activity of HKL was measured by means of Neutral Red retention, as described by Matthews et al. (1990). This assay was adapted to microtitre plates. Briefly, 10^6 cells were incubated in a final volume of 175 µl culture medium for 2.5 h at 18°C with 10 mg l^{-1} Neutral Red (NR; Sigma-Aldrich). All set-ups were made at least in triplicate. After incubation, each well was washed twice with 125 µl of phosphate-buffered saline (PBS). After removing the PBS, the cells were air-dried overnight and frozen at −20°C for up to 2 weeks. For spectrophotometric readings, the cells were lysed with 100 µl acid ethanol (3% HCl in 95% ethanol) and mixed with 100 µl PBS. The optical densities were recorded at 492 nm.

Lysozyme assay

Lysozyme activity of dab plasma was determined by means of a turbidimetric assay according to Parry et al. (1965). A suspension of 0.2 g l^{-1} *Micrococcus lysodeikticus* (Sigma-Aldrich) in 0.05 M sodium phosphate buffer (pH 6.2) was mixed with 25 µl of dab plasma to give a final volume of 200 µl per well. The optical density was read in a spectrophotometer at 530 nm immediately after mixing, after 0.5 min, and after 4.5 min at a temperature of 20±2°C. The decrease in absorbance was used to calculate the lysozyme activity. One unit of lysozyme activity is defined as the amount of sample causing a decrease in absorbance of 0.001 OD min^{-1}. Hen egg-white lysozyme (Sigma-Aldrich) was used as external standard, as described by Hutchinson and Manning (1996a).

Statistics

Normality of the data was tested with the Kolmogorov-Smirnov test. To determine the significance of differences between groups, data were compared using Student's *t* test, the Mann-Whitney rank sum test for not normally distributed data sets, or by a Kruskal-Wallis ANOVA and subsequent multiple comparison of means using the Student-Newman-Keuls method at a probability of error $P<0.05$. Correlations between data sets were tested with Pearson's product moment correlation test, or with the Spearman rank correlation test when the data were not normally distributed. Correlations were considered significant at a probability of error $P<0.05$. All calculations were done using the computer program Sigma Stat (SPSS Science).

Initially, the data were analysed for single campaigns separately. Because similar spatial differences were observed for all three campaigns, the measurements were combined and analysed as a pooled data set.

Results

Haematocrit

The haematocrit value of fish is considered to represent a simple, non-specific indicator of overall health (Blaxhall 1972; Anderson 1990). Decreased haematocrit values were found in fish with nutritional deficiencies, infections with microorganisms or other health problems (Blaxhall 1972). In the dab examined here, regional differences ($P<0.05$) were found between individuals from the North Sea locations: individuals from the German Bight (JMP) had higher haematocrit values than dab from the areas Ekofisk (P02), Dogger Bank (N04) and Firth of Forth (N06) ($P<0.05$), and individuals from Danfield (P01) had higher haematocrits than those from Dogger Bank (N04) and Firth of Forth (N06) ($P<0.05$, see Fig. 2). In both populations, length and age of the fish did not affect the haematocrit value, while a small, but significant ($R=0.29$; $P<0.05$) influence of weight was noted.

Lysozyme

The mean lysozyme activity in the plasma of dab examined here was not affected by weight, length or

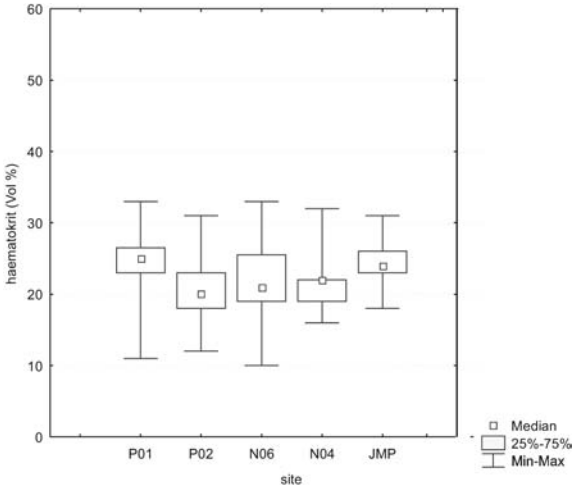

Fig. 2 Haematocrit in dab *Limanda limanda* collected at five different sampling sites in the North Sea during three consecutive sampling campaigns in August/September 1999–2001. Sampling locations: *P01* Danfield, *N04* Dogger bank, *P02* Ekofisk, *N06* Firth of Forth, *JMP* German Bight. For geographical locations see Fig. 1. In the North Sea, haematocrit was significantly higher in dab at P01 compared with individuals at P02, N06 and N04 ($P<0.05$). Dab from JMP also had significantly higher haematocrit values than fish from P02, N06 and N04 ($P<0.05$)

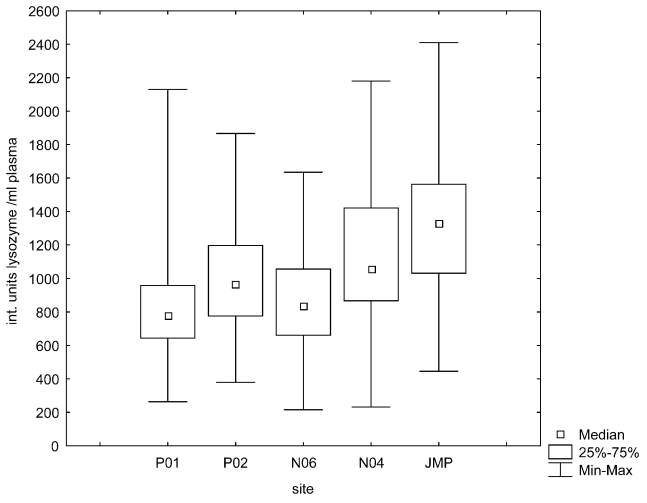

Fig. 3 Plasma lysozyme activity in dab *Limanda limanda* collected at five different sites in the North Sea during three consecutive sampling campaigns in August/September 1999 –2001. Sampling locations: *P01* Danfield, *N04* Dogger bank, *P02* Ekofisk, *N06* Firth of Forth, *JMP* German Bight. For geographical locations see Fig. 1. Individuals at JMP had significantly higher lysozyme levels than dab at P01, P02 and N06 ($P<0.05$). At N04, individuals had significantly higher lysozyme values than dab collected from P01 and N06 ($P<0.05$)

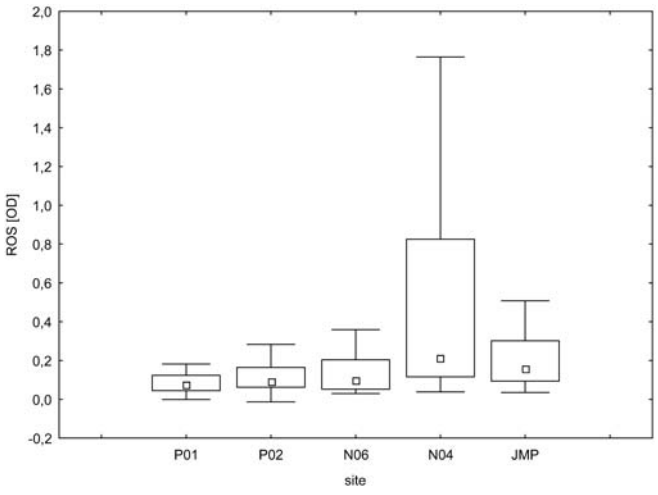

Fig. 4 Basal production of reactive oxygen species (ROS) by head kidney cells derived from dab (*Limanda limanda*) at five different locations in the North Sea. Sampling locations: Sampling locations: *P01* Danfield, *N04* Dogger bank, *P02* Ekofisk, *N06* Firth of Forth, *JMP* German Bight. For geographical locations see Fig. 1. Dab from N04 had a significantly higher ROS production than individuals from P01, P02 and N06 ($P<0.05$). Head kidney leucocytes from JMP dab also showed a significantly higher ROS production than cells from individuals at P01 ($P<0.05$)

age of the individuals. Significant differences ($P<0.05$) in activity, however, were found between dab from oil and gas platform areas (P01 and P02) and dab collected in the German Bight (JMP; see Fig. 3). Dab sampled at the Dogger Bank (N04) had significantly ($P<0.05$) higher plasma lysozyme levels than dab from Danfield (P01) and Firth of Forth (N06) (Fig. 3).

Endocytosis by head kidney phagocytes

The endocytosis activity of head kidney phagocytes was not affected by age or length of fish, but increased with increasing weight of the individuals ($P<0.05$). Head kidney phagocytes from dab collected in the German Bight (JMP) showed a significantly higher endocytosis activity than individuals from Ekofisk (P02) and Firth of Forth (N06) ($P<0.05$). In addition, cells from dab collected at N06 had a significantly higher endocytosis activity than individuals from the Dogger Bank (N04) and Danfield (P01) ($P<0.05$, data not shown).

Production of reactive oxygen species

Both basal and stimulated ROS production of head-kidney-derived leucocytes were significantly influenced by the age of fish (basal ROS production $R=0.19$, $P<0.05$; PMA-stimulated ROS production $R=0.40$, $P<0.01$). Weight or length had no effect on basal ROS production of HKL, while the PMA-stimulated ROS production of HKL was significantly ($R=-0.20$, $P<0.01$) influenced by fish weight.

Clear differences in ROS production were observed between dab from different areas in the North Sea. HKL from dab at the Dogger Bank (N04) had significantly higher ($P<0.05$) basal ROS production compared with individuals from the sites near gas or oil platforms (P01, P02) and at the Firth of Forth (N06). Cells obtained from dab collected near platforms at Danfield (P01) also had significantly ($P<0.05$) lower basal levels of ROS production than dab from the German Bight (JMP) (see Fig. 4).

When HKL were stimulated with PMA, the cells responded with increased ROS production and showed slightly higher values in dab collected in the German Bight (JMP) and at the Dogger Bank (N04) compared with fish from the oil and gas platforms. These differences, however, could not be confirmed as statistically significant (Fig. 5).

Correlation of innate immune responses with physiological biomarkers and grossly visible diseases

Measurements of innate immune responses and physiological biomarkers were carried out on the same individuals. When plasma lysozyme levels of individual North Sea dab were compared to endocytosis and ROS production of HKL from the same individual, a positive correlation was found for endocytosis and a negative correlation for PMA-stimulated ROS production (see Table 1). Dab with decreased haematocrits also had lower plasma lysozyme levels (Table 1).

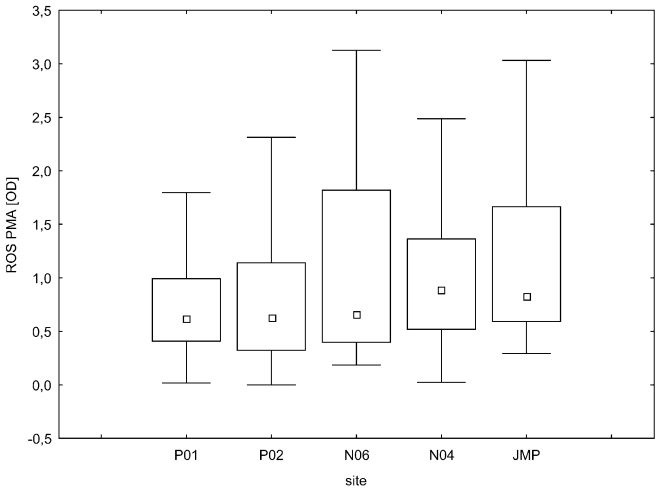

Fig. 5 Production of reactive oxygen species by head-kidney-derived dab (*Limanda limanda*) leucocytes upon stimulation with the phorbol ester PMA. The dab were collected at five different sampling sites in the North Sea during three consecutive sampling campaigns in August/September 1999–2001. Sampling locations: *P01* Danfield, *N04* Dogger bank, *P02* Ekofisk, *N06* Firth of Forth, *JMP* German Bight. For geographical locations see Fig. 1. Statistically significant differences were not found between the sampling locations

When results of the measurements of the physiological biomarkers of interest were compared with plasma lysozyme levels, pinocytosis activity and ROS production of HKL of the same individual, the following correlations were found: dab with induced EROD activity had decreased plasma lysozyme levels and decreased ROS production ($P<0.01$, Table 2) and individuals with impaired ROS production of HKL also had lower AChE and GST activities ($P<0.01$, Table 2). Individuals with decreased plasma lysozyme levels had displayed induced EROD and GST activities in liver cells ($P<0.01$, Table 2). Correlations between the haematocrit values of individual dab and responses of physiological biomarkers were not found.

The presence or absence of obviously visible diseases or liver parasites had a marked impact on several of the innate immune parameters measured, but the pattern varied with the disease or parasitic infection (see Table 3). Individuals with lymphocystis had reduced plasma lysozyme levels with 892 (628–1,123) IU ml^{-1} compared with 1,007 (797–1,337) IU ml^{-1} in non-infected fish. In dab with pigment anomalies, haematocrits were decreased, at 22 (19–24)% compared with 23 (20–27)% in non-affected individuals, and basal as well as PMA-triggered ROS production of HKL from affected dab was increased, with 0.156 (0.0985–0.387) OD values for basal and 1.085 (0.546–1.772) OD values for PMA-stimulated ROS versus 0.105 (0.062–0.208) and 0.639 (0.375–1.169), respectively, in unaffected individuals. In individuals with skin ulcers or epidermal hyperplasia/papilloma, results of measurements of innate immune responses were not different from those of unaffected dab.

Infections with liver nematodes were accompanied by significantly reduced haematocrits (mean 22, range 19–24%, in infected and mean 23, range 21–27%, in uninfected dab), decreased plasma lysozyme levels (mean 966, range 694–1,189 IU ml^{-1} versus mean 991, range 797–1,369 IU ml^{-1}), reduced basal ROS production, but increased endocytosis activity of HKL. Infections of dab with liver acanthocephalans were accompanied by reduced endocytosis activity of HKL. Multiple regression analysis indicated that from the immune parameters considered here, haematocrits and endocytosis activity of HKL were mainly affected by nematode infections, plasma lysozyme levels by lymphocystis, and ROS production of HKL by pigment anomalies (Table 4).

Discussion

Numerous studies have demonstrated that water contamination has an impact on innate as well as on adaptive immune responses in fish (recently reviewed by Zelikoff et al. 2000; Bols et al. 2001). This became very clear

Table 1 Cross-correlation between immune parameters measured in dab (*Limanda limanda*) collected in the North Sea from five different locations (see Fig. 1)

	Lysozyme	Pinocytosis	Basal ROS	PMA activated ROS
Haematocrit	0.18**	0.12*	–0.06	–0.08
Lysozyme	1	0.24**	0.06	–0.13*
Pinocytosis		1	–0.06	–0.10
Basal ROS			1	0.50**

Spearman's correlation on ranks at *$P<0.05$, **$P<0.01$, N= 286–300

Table 2 Correlations between biochemical parameters of dab (*Limanda limanda*) and the immunological parameters applied. The measurements were done in the same individuals. The dab were collected in 1999–2001 at five different locations in the North Sea (for locations see Fig. 1, N=84–200)

Biomarker	Haematocrit	Lysozyme	Pinocytosis	Basal ROS	PMA activated ROS
EROD	0.00	–0.26**	–0.05	–0.44**	–0.29**
CYP	0.00	0.12	0.08	0.09	0.00
ACHE	–0.10	0.03	–0.06	0.35**	0.39**
GST	0.01	–0.37**	0.16	–0.23*	0.01

Spearman's correlation on ranks, marked are correlations at *$P<0.05$, **$P<0.01$
Abbreviations: *EROD* 7-ethoxyresorufin-O-deethylase assay, *CYP* cytochrome P450 1A protein concentration, *ACHE* cholinesterase activity, *GST* glutathion-4-S-transferase activity

Table 3 Comparison of the presence of grossly visible diseases and parasites in dab (*Limanda limanda*) and immunological parameters measured from the same individual. The dab were collected in 1999 – 2001 at five different locations in the North Sea (see Fig. 1). Compared were immune parameters of affected dab to measurements obtained from non-infected individuals by means of the Mann-Whitney rank sum test. Listed are *P* values obtained from the test. Statistically significant differences in the immune response between the groups at p<0.05 are marked in bold, *n*=286–300

Disease	Haematocrit	Lysozyme	Pinocytosis	Basal ROS	PMA activated ROS
Lymph	0.17	**0.01**(−)	0.18	0.47	0.32
Eppap	0.16	0.61	0.95	0.71	0.10
Ulc	0.17	0.73	0.47	0.19	0.36
Pigmel	**0.01**(−)	0.45	0.58	**0.01**(+)	**0.01**(+)
Nemato	**0.01**(−)	**0.04**(−)	**0.01**(+)	**0.02**(−)	0.69
Acanth	0.12	0.35	**0.02**(−)	0.17	0.64

(+) increased immune parameter in infected dab
(−) depressed immune parameter in infected individuals
Lymp lymphocystis, *Eppap* epidermal hyperplasia/papilloma, *Ulc* acute/healing skin ulceration, *Pigmel* anomaly in pigmentation, *Nemato* nematodes (liver), *Acanth* acanthocephalans (liver)

Table 4 Multiple linear regression between grossly visible diseases and parasites in dab (*Limanda limanda*) and the immune parameter measured in the same individual. The presence of grossly visible diseases or parasites is tested as explaining variable on the immune responses applied. Given are the *P* values obtained from the calculation

Disease	Haematocrit	Lysozyme	Pinocytosis	ROS	PMA activated ROS
Lymph	0.42	0.04*	0.40	0.20	0.20
Eppap	0.23	0.57	0.91	0.50	0.50
Ulc	0.14	0.72	0.72	0.74	0.74
Pigmel	0.09	0.55	0.18	<0.01**	<0.01*
Nemato	0.04*	0.20	0.02*	0.14	0.84
Acanth	0.86	0.60	0.12	0.72	0.88

An influence was considered to be significant at **P*<0.05, ***P*<0.001, *n*=286–300
Lymp lymphocystis, *Eppap* epidermal hyperplasia/papilloma, *Ulc* acute/healing skin ulceration, *Pigmel* anomaly in pigmentation, *Nemato* nematodes (liver), *Acanth* acanthocephalans (liver)

when fish were exposed to various substances such as metals, pesticides or insecticides under laboratory conditions, but could also be confirmed in studies on wild fish collected from contaminated sites. Thus several authors (Dunier et al. 1991; Dunier and Siwicki 1993; Wester et al. 1994; Zelikoff et al. 2000; Bols et al. 2001) recommended fish immune assays as useful techniques for predicting toxicological risk associated with contamination in aquatic environments. Innate immune responses that protect an organism against infections without depending on prior exposure to any particular pathogens are especially suitable biomarkers for assessing the adverse biological effects of contaminants (Wester et al. 1994).

In wild fish, biological parameters such as enzyme activities or immune responses underlie natural fluctuations and may be influenced by host-specific variation, and when considering these responses as biomarkers of environmental degradation, contaminant-mediated effects have to be distinguished from these factors. Therefore, a sufficiently large number of individuals of comparable size should be collected, which is most desirable in a long-term study (Anderson 1990). Thus in the present study, 336 individuals were analysed during three sampling campaigns in three consecutive years. When the data were analysed for the sampling campaigns separately, general results were achieved (data not shown). Measurements of haematocrit, endocytosis, basal and PMA-stimulated ROS, however, were significantly influenced by body weight and age of dab, even though the analysis was restricted to a defined size class of 20–24 cm total length. These findings are consistent with results from field studies on flounder (Skouras et al. 2003), Japanese medaka (*Oryzias latipes*, see Duffy et al. 2002) and from mammals, which reveal a decreasing sensitivity to toxic insult with increasing age (Parkinson and Safe 1987). In the present study, only female dab were collected during campaigns in August and September in order to reduce seasonal and sex-related variations, which were described in detail by Hutchinson and Manning (1996a). In addition, North Sea dab were collected at locations with similar characteristics in respect of salinity, in order to reduce variations caused by this factor.

When considering the North Sea sampling locations of the present study, a contamination gradient was discussed by Kammann et al. (2001) on the basis of PAH contamination of the sediments. The highest contamination, expressed as Σ of 16 PAHs, was measured at Ekofisk (P02) with 35.87 ng g^{-1} and the Firth of Forth (N06) with 27.47 ng g^{-1} dry matter, followed by Danfield (P01) with 13.78 ng g^{-1}. In the German Bight (JMP) (6.02 ng g^{-1}) and at the Dogger Bank (N04) (5.79 ng g^{-1}) lower levels of PAHs were measured. Along with this pollution gradient, the plasma lysozyme level and the respiratory burst activity of head kidney phagocytes was reduced in individuals from stations with higher contaminant levels (see Figs. 3, 4 and 5). In contrast, haematocrits and endocytosis activity of HKL were not altered in dab from regions with increased PAH contamination. These findings substantiate observations from other studies in dab.

In individuals caught after a major oil spill in the North Sea, serum lysozyme levels were negatively correlated with the PAH levels in the sediment (Secombes et al. 1997). Dab exposed to oil-contaminated sediment or sewage sludge had lower serum lysozyme levels (Tahir et al. 1993) and decreased ROS production by head kidney phagocytes relative to control groups (Secombes et al. 1991; Tahir et al. 1993). In vivo exposure of dab to different concentrations of cadmium was also related to a reduction in the ROS production by head kidney phagocytes when compared with unexposed individuals (Hutchinson and Manning 1996b).

The work reported here was part of an integrated field study, which included a simultaneous assessment of other biomarkers as recommended for monitoring programmes on biological effects (see Diamant and Westernhagen 1999), such as the induction of mono-oxygenase ethoxyresorufin O-deethylase (EROD) or glutathion-4-S-transferase (GST) in liver cells, the inhibition of acetylcholin esterase (AChE) in muscle, and grossly visible fish diseases and parasite infections. EROD is known to be a sensitive indicator of the exposure to lipophilic compounds such as PAHs, dioxins and coplanar PCB congeners (Goksoyr and Förlin 1992; Boer et al. 1993; Sleiderink et al. 1995). Cholinesterase (AChE) is widely used to estimate the neurotoxic impact of contaminants at the cellular level of marine organisms (Galgani et al. 1992; Bressler et al. 1999) and the induction of glutathion-4-S-transferase (GST) activity indicates an adaptation of the organism to enhanced contaminant stress (Bressler et al. 1999). In the present study, responses of the innate immune system and these biomarkers were recorded from the same individual dab, which allowed us to compare responses of the different parameters on the basis of individual fish. These comparisons showed that dab with induced EROD or GST activities also had lower lysozyme activity and decreased phagocyte responses, which indicates that in fish under contaminant stress, several functional systems were affected. The observations made in dab confirm findings in flounder from the German Bight (Skouras et al. 2003), where in individuals with decreased integrity of hepatocyte lysosomes, the EROD system was also induced and innate immune responses were impaired. In the present study on dab, correlation coefficients between biomarkers and immune parameters were much higher than those found for flounder (Skouras et al. 2003), most probably because a more pronounced contamination gradient existed between the sampling locations of the present study compared with the locations in the German Bight, where the xenobiotic load has decreased during the past decade (for details see De Jong 1999) and only a less pronounced contaminant gradient was found (Schmolke et al. 1999). In addition, the present study concentrated on female dab collected during the same period in every year, which probably reduced natural variation in the results.

A link between impaired immune functions of fish and disease susceptibility has been long suspected and forms the basis of programmes on the systematic monitoring of the occurrence and prevalence of grossly visible diseases in dab in the North Sea, which have been carried out by ICES member countries since the late 1970s (Dethlefsen et al. 2000; Lang 2002). In the present study, the prevalence of grossly visible diseases was recorded along with an analysis of some innate immune responses. Individuals affected by lymphocystis, liver nematodes or acanthocephalans displayed altered plasma lysozyme or head kidney phagocyte activities compared with unaffected dab. In parasite-infected individuals, lysozyme and respiratory burst activities were decreased, while in dab with lymphocystis, non-specific cellular responses appeared not to be affected. This is in contrast to observations in American plaice, *Hippoglossoides platessoides*, where head kidney cells displayed enhanced phagocytosis and respiratory burst activity in association with lymphocystis infection (Marcogliese et al. 2001). Other diseases, such as epidermal hyperplasia/papilloma or skin ulcers, were not observed to be associated with altered lysozyme or head kidney phagocyte activity.

In conclusion, the data presented here indicate that plasma lysozyme and head kidney phagocyte activities detected in North Sea dab display differences associated with a sediment contamination gradient. Innate immune responses were altered along with the physiological biomarkers GST or EROD and with the occurrence of fish diseases such as lymphocystis. The innate immune parameters applied in the present study can easily be integrated into biological effects monitoring programmes and will provide supplementary information about immunomodulatory effects associated with exposure to contaminants. In particular, plasma lysozyme, which can be analysed in an easy and inexpensive assay, is considered as a suitable parameter in a battery of other biomarkers.

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