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ORIGINAL ARTICLE

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The use of innate immune responses as biomarkers in a programme of integrated biological effects monitoring on flounder (*Platichthys flesus*) from the southern North Sea

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Abstract Immunological biomarkers that reflect the effects of exposure to environmental contaminants in coastal marine habitats were sought in European flounder (*Platichthys flesus*) from five locations in the German Bight with different anthropogenic impacts. During a 2-year period of sampling, innate immune responses were monitored from a total of 331 individual flounder of a body length of 18 to 25 cm. From the fish, plasma lysozyme, phagocytosis and respiratory burst activity of head kidney leucocytes were analysed and implemented as part of an integrated biological effects monitoring programme. As the measurements of the parameters applied here varied within wide ranges at some locations, spatial differences could not always be established, but some general trends could be drawn: plasma lysozyme activity was decreased in flounder contaminated with DDT adducts and some PCBs, while cellular functions such as phagocytosis and respiratory burst were stimulated by some chlorinated hydrocarbons. Correlation analysis also revealed connections not only between the parameters applied here and some contaminants but also with some biochemical parameters used as biomarkers in pollution monitoring: in flounder with decreased integrity of hepatocyte lysosomal membranes, immune functions also were impaired, and plasma lysozyme as well as phagocytosis activity of head kidney cells were impaired when the activity of cytochrome P450 1A was induced. The data presented here indicate that innate immune responses may be useful parameters to monitor cellular functions in a battery of biomarkers of different levels of biological organisation.

Keywords Innate immune response · Biological effect monitoring · Immunological biomarkers · Fish · Pollution

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Introduction

In the last few decades, a possible influence of environmental pollution on the aquatic environment has gained considerable interest. Fish have become a favourable subject for research in this area, because temperature changes, habitat and water quality deterioration as well as aquatic pollution adversely affect fish health, which may result in mortalities and population decline. Among various biochemical, cellular and physiological systems, certain innate immune responses are considered as suitable biomarkers for monitoring biological effects of pollution [reviewed by Dunier and Siwicki (1993); Wester et al. (1994); Bols et al. (2001)]. Impairment of immune functions, which protect fish against invading pathogens, can lead to harmful consequences at the individual level, such as disease outbreak followed by death, and at the ecosystem level, as population reductions are followed by changes in the entire ecosystem. For fish populations, a link between environmental pollution and diseases has long been expected (Sniezko 1974; Sinderman 1979), and studies carried out under defined laboratory conditions concluded modulating influences of xenobiotics on fish immune responses [for review see Dunier and Siwicki (1993); Bols et al. (2001)]. Understanding the effect of toxicants on fish innate immunity supports the larger ecotoxicological goal of comprehending the actions of ecotoxicants on fish populations (Bols et al. 2001).

Given that the non-specific immune system of fishes constitutes their first line of defence against pathogens (Ingram 1980), cellular and humoral reactions of the innate immune system are of great importance. They are unspecific but effective against pathogens which try to invade the host. This leads, at the cellular level, to activation and infiltration of one specialized type of leucocytes into the tissue where invasion took place. This leucocyte subpopulation, which consists of granulocytes and macrophages, is able to ingest pathogens via endocytosis and kill them intra- or extracellularly by a process known as the respiratory burst (Halliwell and Gutteridge 1999). In fish it has been shown that these cell functions can be modulated by xenobiotics and it has been suggested that this may be a useful indicator of health (Bols et al. 2001). One important humoral component in the innate immune system is lysozyme (E.C.3.2.1.17), which attacks the peptidoglycan layer in the cell wall of predominantly Gram-positive bacteria and, to some extent, also Gram-negative bacteria. Lysozyme is localized in the lysosomes of neutrophils and macrophages and is released into the blood from these cells (Murray and Fletcher 1976). From previous studies, it is known that lysozyme activity in fish blood is sensitive to environmental contaminants (Bols et al. 2001).

Activity of phagocytic cells, such as endocytosis or respiratory burst, and plasma lysozyme levels are part of innate immune mechanisms, which respond immediately to pathogen challenge. Phagocytes migrate to areas of infection and their activity is influenced by pathogen factors or mediators secreted by other cells. Phagocyte redistribution and phagocyte activation as an immediate response to pathogen challenge [for review see Ellis (2001)] can be modulated by exposure to xenobiotics. These responses can be measured in simple and inexpensive spectrophotometer based assays, which make them suitable for field studies. As opposed to innate mechanisms, antigen-specific responses depend on a previous stimulation with a particular antigen, which obstruct their incorporation into a field monitoring programme.

In the present study, we implemented measurements of innate immune responses of fish in an integrated biological effect monitoring programme on European flounder (*Platichthys flesus*) in the North Sea. The flounder is widely distributed in different habitats of the North and Baltic Seas. Like other marine flatfishes, it lives in close contact with the sediment and feeds on various benthic organisms. Thus, marine flatfish species are frequently used as sentinel species in international monitoring programmes of biological effects of contaminants in coastal waters and estuaries (ICES 1996; 1999).

The data reported here are part of a monitoring programme which was conducted on flounder collected at several locations in the German Bight and which included the analysis of biochemical, pathological and parasitological parameters of the same individual as well as the measurement of some innate immune responses. The study was supplemented with chemical analysis of chlorinated hydrocarbon residues in the muscle of the same individual fish used for an assessment of biological effects. In addition, sediments and invertebrates (*Mytilis edulis*) were collected from the locations where the fish sampling took place. In this communication, findings of the immunological analysis are compared to chemical, biochemical and histochemical data for a more complete understanding of pollution impact on fish physiology.

Methods

Sampling

Four sampling campaigns in the North Sea were conducted in April and September 1999 and 2000. During these cruises with the research vessel '*Uthörn*' of the Alfred Wegener Institute, a total of 331 flounder (*Platichthys flesus* L.) were caught at five different locations (Elbe estuary, Inner Eider estuary, Outer Eider, Spiekeroog and Tiefe Rinne near Helgoland; Table 1). The stations were determined by their geographical positions. Fishing was conducted with a bottom trawl (opening 1.5 m, mesh width in the cod end 40 mm stretched mesh). Fishing period was limited to 30 min to keep fish stress as low as possible. Fish were sorted out immediately and kept in tanks with permanent seawater flow-through and aeration for up to 6 h until further processing took place. Only macroscopically healthy flounder in the size class 18–25 cm were used for this investigation. A maximum of 20 fish per site and campaign were collected and prepared for analysis.

Examination procedure

Onboard the research vessel, body length and weight of each fish were measured, and macroscopically visible ectoparasites were collected. Blood was drawn from the caudal vein into disposable syringes pre-filled with a lithium-heparin bead (Sarstedt, Germany). From the blood, the haematocrit was determined according to standard procedures (Houston 1990). The remaining blood was then transferred to centrifuge tubes, centrifuged at 2,000 *g* for 15 min at 4°C, and the supernatant plasma was collected and frozen at −80°C. Then the fish was killed and 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.) and stored at 4°C for up to 24 h for further processing. In addition, liver, kidney, intestine, gills and muscle samples were collected for parasitological, physiological and biochemical research as well as residual analysis. The results of these are reported in other contributions to this issue. From the morphological measurements, a whole body condition factor (CF) was determined for each fish according to the formula: CF = body weight [in g] × body length^3 [in cm] and used as an allometric index for overall health (Busacker et al. 1990).

Lysozyme assay

Lysozyme activity of flounder 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, Germany) in 0.05 M sodium phosphate buffer (pH 6.2) was mixed with 25 µl flounder 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 lysozyme activity. One unit of lysozyme activity is defined as the amount of sample causing a decrease in absorbance of 0.001 min^{-1}. Hen egg white lysozyme (Sigma-Aldrich, Germany) was used as an external standard, as described by Hutchinson and Manning (1996).

Table 1 Summary of the sampling programme on flounder (*Platichthys flesus*) for analysis of some innate immune parameter in 1999 to 2000. All parameters were measured from the same individual

Campaign/year	Site	Fish	Hem	Pin	ROS	ROS PMA	Lys
Spring 1999	Elbe	20	20	15	16	16	20
	Spiekeroog	9	9	8	9	9	9
	Helgoland	20	20	20	20	20	20
	Inner Eider	15	14	14	13	14	15
	Outer Eider	19	18	18	19	19	17
Autumn 1999	Elbe	20	20	14	20	20	20
	Spiekeroog	20	19	19	20	20	19
	Helgoland	20	19	20	20	20	18
	Outer Eider	20	19	19	20	20	19
Spring 2000	Elbe	20	20	17	19	19	20
	Spiekeroog	9	9	8	9	9	9
	Helgoland	20	20	16	18	18	20
	Inner Eider	18	18	11	15	15	18
	Outer Eider	20	20	9	17	17	20
Autumn 2000	Elbe	20	20	20	20	20	20
	Spiekeroog	20	20	7	7	7	20
	Helgoland	20	20	20	20	20	20
	Outer Eider	20	20	20	20	20	20

Fish: number of fish caught; *Hem:* haematocrit; *Lys*: plasma lysozyme activity, *Pin:* pinocytosis; *ROS*: basal reactive oxygen production; *ROS PMA*: production upon stimulation with phorbol-12-myristate-13-acetate (PMA) by head kidney leucocytes

Leucocyte isolation

Leucocyte isolation was done as described previously (Skouras and Steinhagen 2003). Briefly, 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 at 550 *g* for 10 min and resuspended in cell culture medium (RPMI-1640 supplemented with 100,000 IU l^{-1} penicillin, 100 mg l^{-1} streptomycin and 4 mM L-glutamine and 1% [v/v] carp serum; chemicals: Biochrom; carp serum: serum from 15 *Cyprinus carpio* L. individuals was pooled, heat inactivated for 30 min at 56°C, 0.2 µm 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 HKL

Generation of reactive oxygen species (ROS) by HKL was measured by means of the nitro blue tetrazolium salt (NBT) reduction assay after cell isolation, as described earlier (Skouras and Steinhagen 2003). Briefly, cells 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} phorbolmyristate acetate (PMA). The indicator NBT was added at 1 g l^{-1}. Wells without PMA served to determine the spontaneous ROS generation of 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 two times 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 of 2 M KOH and 150 µl DMSO per well (all chemicals: Sigma-Aldrich, Germany). The optical densities were recorded with a spectrophotometer at 650 nm. From the measurements, an ROS stimulation index was calculated as followed: SI = (PMA triggered ROS [OD]) / (unstimulated ROS [OD]).

Endocytosis activity of head kidney phagocytes

Endocytosis activity of head kidney phagocytes was measured by means of neutral red retention of isolated head kidney cells as described by (Mathews 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, Germany). All set-ups were made at least in triplicate. After incubation, each well was washed two times with 125 µl 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.

Analytical chemistry

Residue analysis was conducted by a commercial laboratory that had undergone an intercalibration exercise (Handels- und Umweltschutzlaboratorium Dr. Wiertz, Hamburg). Ten fish muscle tissue samples per location were analysed for the contents of standard chlorinated hydrocarbons and heavy metals (Hg, Pb, Cd, Cu). In addition, samples of the sediment from all locations and samples of *M. edulis* from the inner Eider Estuary, Elbe Estuary and Helgoland were taken and analysed for the same parameters. The results of this work are presented by Dizer et al. (2003).

Statistics

Normality of the data was tested using the Kolmogorov-Smirnow test. To determine the significance between groups, data were compared using Student's *t*-test, or when the data were not normally distributed, using Mann-Whitney's rank sum test, or using Kruskal-Wallis ANOVA and subsequent multiple comparison by means of the Student-Newman-Keuls method at a probability of error $P<0.05$. Correlations between data sets were tested using Pearson's product moment correlation test or using Spearman's rank correlation test. Correlations were considered as significant at a probability of error $P<0.05$. The following biochemical parameters assessed from the same flounder individuals were incorporated into the correlation analysis of this communication (detailed description of the parameters and the applied methods are given in the respective references):

Macrophage aggregate area (MAA), the mean size of macrophage aggregates in flounder liver (Broeg et al. 1999);

Macrophage aggregate activity (MAM), the activity of acid phosphatase in macrophage aggregates of flounder liver (Broeg et al. 1999);

Lysosomal stability (LY), the membrane stability of hepatocyte lysosomes (group of lysosomes, which display an early membrane breakdown; Broeg et al. 1999);

EROD activity (EROD), the activity of cytochrome P450-dependent monooxygenase ethoxyresorufin-O-deethylase (EROD; Broeg et al. 1999);

Choline esterase activity (ChE; Bressler et al. 1999; Dizer et al. 2003);

DNA-unwinding (DNA), DNA strand breaks in hepatocytes (Dizer et al. 2003);

Vitellogenin (Vit), concentration of vitellogenin in the plasma of male flounder (Dizer et al. 2003).

Results

Haematocrit

The haematocrit of flounder blood was measured as a simple, non-specific indicator for overall health (Blaxhall 1972; Anderson 1990). In the sample, measurements varied from 18 to 30 and had a mean of 23. Male flounder had a haematocrit of 24 (19–30) %, slightly higher than that of females which was 22.5 (18–26) %. In spring, a much wider variation of haematocrits was observed at all locations when compared to autumn samples. Differences between sample sites, however, could not be discerned. This indicates that major differences in health aberrations such as infections with micro-organisms or nutritional deficiencies did not occur between the locations of the study (Blaxhall 1972).

Plasma lysozyme activity

With plasma lysozyme activity, no dependence on the sex of analysed flounder could be observed. It increased, however, with increasing size and weight of the fish with R=0.15, P<0.006 in the size range examined here. Flounder collected in autumn had a lysozyme activity of 1,189 (743–1,882) units ml^{-1}, significantly (P<0.001) higher than flounder collected in April with 975 (484–1,618) units ml^{-1}. These seasonal differences were significant at the Elbe and Helgoland locations (Fig. 1) and were not seen at Spiekeroog and Outer Eider. Site-related differences were found in both spring and autumn samplings between flounder from the Elbe and Helgoland location (Fig. 1). In spring, flounder collected at Elbe and Inner Eider had significantly lower plasma lysozyme activity than flounder from Spiekeroog and Helgoland (Fig. 1).

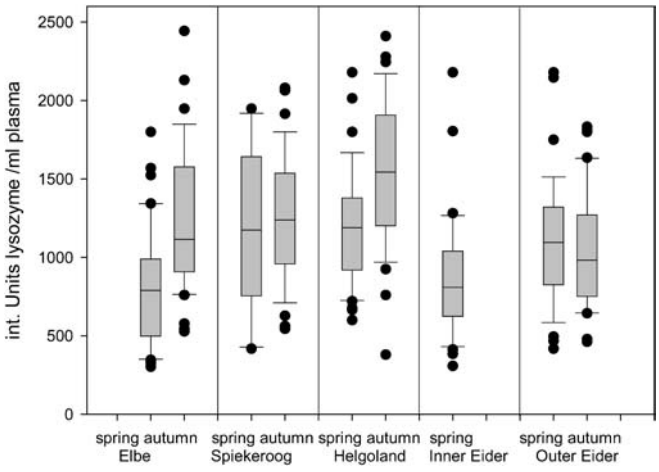

Fig. 1 Plasma lysozyme activity in flounder (*Platichthys flesus*) collected at five different sampling sites in spring and autumn sampling campaigns in 1999 and 2000. *n*: see Table 1. Significant differences in spring: Spiekeroog vs Elbe (P=0.003) and Inner Eider (P=0.023); Helgoland vs Elbe (P<0.001) and Inner Eider (P=0.003); Outer Eider vs Elbe (P=0.023). In autumn campaigns: Helgoland vs Outer Eider (P<0.001), Elbe (P=0.007) and Spiekeroog (P=0.009). Key for box plots: *boundary* of the boxes: 25th and 75th percentile; *line* in the box: median; *whiskers*: 10th and 90th percentiles; *dots*: outliers

When muscle residues were compared to plasma lysozyme activity of the same individual, a positive correlation was found to residues of γ-HCH. The plasma lysozyme activity was negatively correlated with β-HCH, o,p'-DDD, PCB 101 and PCB 118 residues in the muscle of the same individual (Table 2).

Table 2 Correlation between some heavy metal and chlorinated hydrocarbon residues in the muscle of flounder (*P. flesus*) and applied parameters from the same individual. The flounder were collected in 1999 and 2000 at five locations in the German Bight (*n*=153–182, see table 1): Spearman's Correlation on Ranks. The chemical data were provided by Broeg (personal communication)

Contaminant	Haematocrit	Lysozyme	Pinocytosis	Basal ROS	PMA activated ROS	Ratio activated/basal ROS
o,p–DDD	0.003	–0.18*	0.14	0.05	0.01	0.07
p,p–DDE	0.22**	–0.12	0.14	–0.07	–0.05	0.09
o,p–DDT	0.01	–0.09	0.16*	–0.06	0.13	0.07
p,p–DDT	0.006	–0.002	0.21**	–0.04	0.15*	0.17*
Dieldrin	0.002	–0.12	0.28**	0.15*	0.11	0.02
β–HCH	–0.04	–0.24**	–0.004	–0.06	–0.07	0.12
γ–HCH	0.08	0.17*	–0.12	0.08	–0.07	–0.06
PCB 101	0.15*	–0.16*	0.14	0.06	–0.02	–0,04
PCB 118	0.20**	–015*	0.21*	0.11	0.18*	–0.04
PCB 153	0.19*	0.11	0.24*	0.01	0.17*	0.17*
Sum PCB	0.22**	0.05	0.34**	0.01	0.20**	0.21**
Cu	0.09	0.06	–0.03	0.10	–0.20*	–0.19*

* P<0.05; ** P<0.001

Table 3 Cross-correlation between parameters measured in flounder (*P. flesus*) collected in 1999 and 2000 at five locations in the German Bight (*n*=270–320, see Table 1): Spearman's Correlation on Ranks

	Haematocrit	Lysozyme	Pinocytosis	Basal ROS	PMA activated ROS
Haematocrit	1	0.11*	0.10	0.04	0.05
Lysozyme	0.11*	1	–0.07	0.12*	0.23**
Pinocytosis	0.10	-0.07	1	–0.10	0.16**
Basal ROS	0.04	0.12*	-0.10	1	0.58**
PMA-activated ROS	0.05	0.23**	0.16**	0.58**	1

* $P<0.05$; ** $P<0.001$

Table 4 Correlation between some physiological and chlorinated biochemical parameters in flounder (*P. flesus)* and the parameters applied here, measured from the same individual. The flounder were collected in 1999 and 2000 at five locations in the German Bight (*n*= 242–322, see Table 1): Spearman's Correlation on Ranks. The physiological and biochemical data were provided by Broeg (Bremerhaven, personal communication). *MAA* Liver macrophages aggregate area; *MAM* acid phosphatase activity in liver macrophage aggregate; *Ly* stability of hepatocyte lysosomes; *EROD* 7 Ethoxyresorufine O-deethylase acticity in hepatocytes; *ChE* cholinesterase activity; *DNA* DNA strand breaks; *Vit*: Vitellogenin concentration in the plasma of male flounder

Biomarker	Haematocrit	Lysozyme	Pinocytosis	Basal ROS	PMA activated ROS
MAA	0.12*	0.04	–0.05	0.01	–0.05
MAM	–0.09	0.13*	0.17*	–0.06	0.23**
Ly	–0.04	0.15**	0.16**	–0.03	0.14*
EROD	0.16**	–0.21**	0.09	–0.16*	–0.14
ChE	–0.03	0.04	0.26**	–0.07	0.01
DNA	–0.22**	–0.07	–0.25**	0.17**	–0.05
Vit	–0.05	0.14*	–0.32**	0.24**	0.14*

* $P<0.05$; ** $P<0.001$

In addition, plasma lysozyme activity was correlated to PMA-stimulated ROS production by HKL (Table 3), lysosomal stability and activity of macrophage aggregations in the liver from the same individual. In individuals with increased induction of EROD activity in liver cells, the plasma lysozyme activity was decreased (Table 4).

While no correlation of plasma lysozyme activity was observed with the concentration of chlorinated hydrocarbons or heavy metals in the sediment, negative correlation of plasma lysozyme activity was seen with a standardized contamination load in *Mytilus edulis* collected from the sediments (Fig. 2, Spearman's Rank Correlation, $P<0.05$). It has to be noted that the *n* value for this correlation was very low ($n=10$), but nevertheless the contamination load of *Mytilus* might reflect the contamination of the food used by flounder at a particular location.

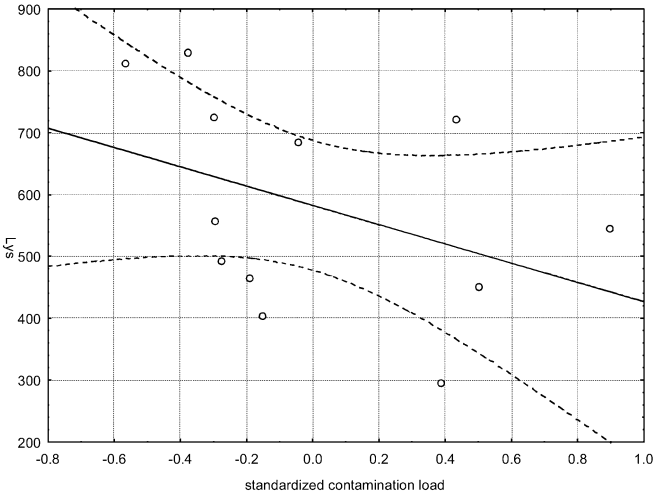

Fig. 2 Correlation between mean plasma lysozyme activity in flounder (*P. flesus*) and mean standardized contamination load in *Mytilus edulis* collected at the Helgoland, Spiekeroog and Eider sites. The contamination load of mussel tissue was calculated from standardized mussel residues. Spearman's Correlation Rank coefficient $R=-0.58$, $P<0.05$; *dotted lines*: 95% confidence interval; *Lys* lysozyme activity [international units ml^{-1} plasma]

Cellular responses:

Endocytosis by head kidney phagocytes

The baseline endocytosis activity of head kidney phagocytes was not different in cells from male or female fish but increased with size ($R=0.20$, $P<0.001$). Cells isolated from flounder in April showed a higher neutral red uptake than cells from fish collected in September ($P<0.001$). In addition, the variation in measurements was much higher in the spring samples. This was most obvious at Spiekeroog, but HKL phagocytes from some individual flounder were also highly active compared to the mean at the other locations. At the Elbe and Outer Eider locations, the wide variation in measurements was not as pronounced. Here, the majority of cells showed a very low endocytosis activity, significantly less than at the other locations ($P<0.05$, Fig. 3). In autumn, the variation in pinocytosis measurements was not as pronounced. Again, the activity of cells from Elbe flounder was decreased, but this was not statistically significant. At the other

Table 5 Summary of immune parameters of flounder (*P. flesus*) collected from selected locations in the German Bight during four sampling campaigns during spring and autumn of 1999 and 2000

Parameter	Measurements [mean (range)]				
	Summary	Male	Female	Spring	Autumn
Haematocrit [vol %]	23 (18–30)	24 (19–30)	22.5 (18–26)	23 (19–30)	23 (18–27
Plasma lysozyme [units ml^{-1}]	1,090 (606–1,816)	1,139 (600–1,833)	996 (628–1,767)	975 (484–1,618)	1,189 (743–1,882)
Endocytosis [OD]	0.046 (0.009–0.138)	0.042 (0.009–0.016)	0.047 (0.008–0.146)	0.057 (0.014–0.153)	0.023 (0.007–0.092)
Basal ROS [OD]	0.087 (0.025–0.499)	0.102 (0.029–0.603)	0.078 (0.025–0.489)	0.053 (0.024–0.367)	0.150 (0.036–0.618)
PMA activated ROS [OD]	0.322 (0.095–0.825)	0.331 (0.103–0.915)	0.317 (0.079–0.694)	0.335 (0.070–0.915)	0.319 (0.166–0.683)

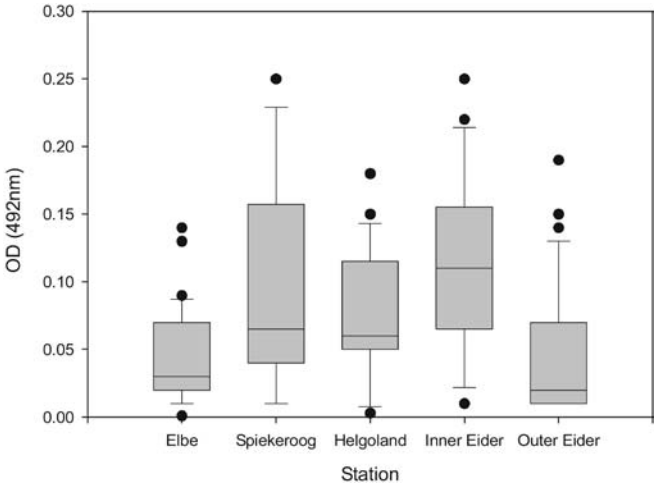

Fig. 3 Endocytosis activity of head kidney derived leucocytes from flounder (*P. flesus*) at five different sampling sites in spring and autumn sampling campaigns in 1999 and 2000. *n*: see Table 1. The activity of the cells was measured by means of neutral red uptake. Cells from flounder collected at the Elbe location showed significantly lower endocytosis activity than flounder from Spiekeroog, Inner Eider or Outer Eider ($P<0.05$). For key, see Fig. 1

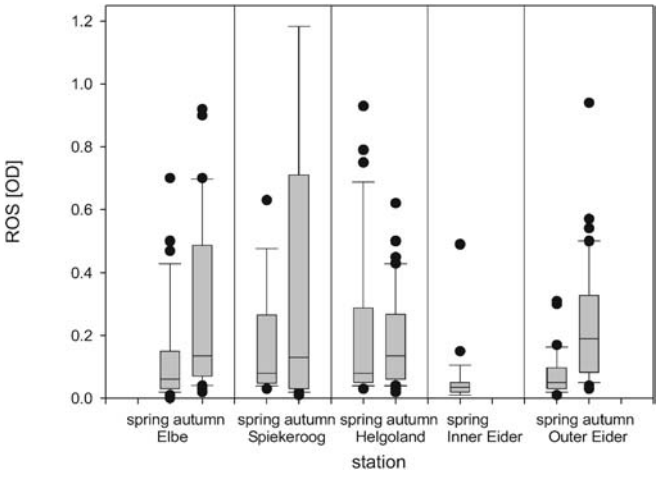

Fig. 4 Basal production of reactive oxygen species by head kidney-derived leucocytes collected from flounder (*P. flesus*) at five different sampling sites in spring and autumn sampling campaigns in 1999 and 2000. *n*: see Table 1. Inner Eider: no fish were caught during autumn campaigns. Differences in spontaneous ROS production between sites in spring and autumn (1999/2000). For key, see Fig. 1. There were no significant differences between the sites

locations, the activity varied within similar ranges and no site-specific differences could be detected (Fig. 3).

The pinocytosis activity of HKL was positively correlated to DDT metabolites, and the sum of PCBs and Dieldrin residues in the muscle of same individual (Table 5). The pinocytosis activity of head kidney cells was positively correlated to other cellular responses of the innate immune system (Table 3): HKL from flounder with increased pinocytosis activity correlated with increased ROS production to PMA stimulation, elevated activity of macrophage aggregations in the liver, higher lysosomal stability and increased cholin-esterase activity in neurons. In addition, individuals with decreased pinocytosis activity of HKL had increased ratios of DNA fragmentation significantly more often (Table 4).

Production of ROS

The production of ROS by HKL was not influenced by the sex of the specimen examined. It was, however, significantly influenced by the size of the fish: cells from larger fish had a higher base line as well as phorbol-ester-stimulated ROS production ($R=0.14$, $P<0.01$). The base-line respiratory burst was significantly ($P<0.001$) lower in spring compared to autumn samples (Fig. 4). This difference was not observed in cells stimulated with the phorbol ester PMA (Table 5). In spring, mean values of stimulated ROS production were similar at Spiekeroog, Helgoland and Outer Eider, and higher than the means at Elbe and Inner Eider. Head kidney phagocytes from some flounder could not be stimulated by PMA, while in other individuals the cells responded with a high NBT reduction upon stimulation (Fig. 5). At Helgoland and Outer Eider,

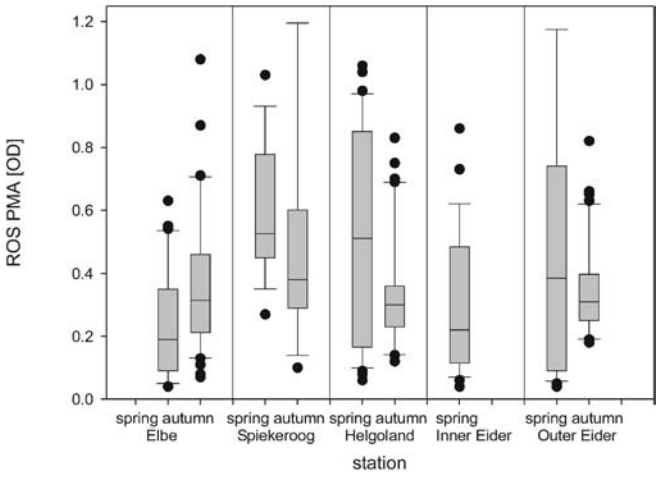

Fig. 5 Production of reactive oxygen species by head kidney-derived leucocytes upon stimulation with PMA collected from flounder (*P. flesus*) at five different sampling sites in spring and autumn sampling campaigns in 1999 and 2000. *n*: see Table 1. Inner Eider: no fish were caught during autumn campaigns. Autumn: significantly ($P<0.05$) lower values at Elbe compared with Helgoland and Spiekeroog; significantly ($P<0.05$) lower values at Inner Eider than Spiekeroog. Spring: no significant differences between sites. For key, see Fig. 1

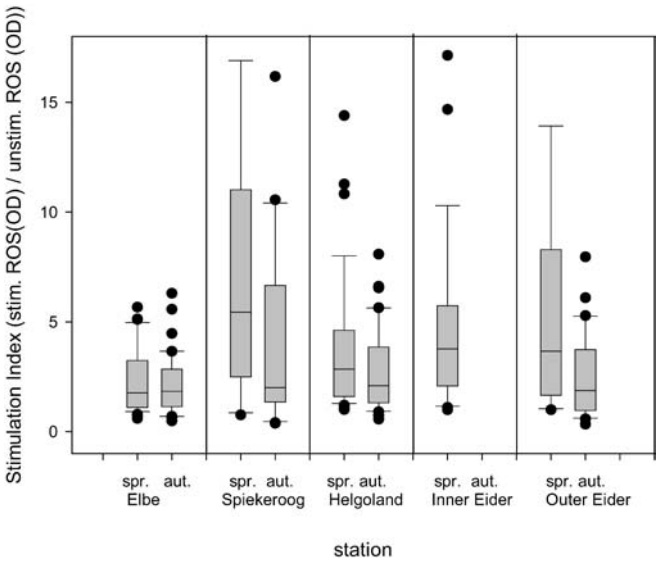

Fig. 6 Production of reactive oxygen species by head kidney-derived leucocytes from flounder (*P. flesus*) at five different sampling sites in spring and autumn sampling campaigns in 1999 and 2000. Ratio of ROS production upon PMA stimulation / basal production. *n*: see Table 1. Inner Eider: no fish were caught during autumn campaigns. Cells from flounder collected at Spiekeroog, Outer Eider and Inner Eider in spring had significantly higher values ($P<0.05$) than cells from Elbe flounder. In autumn no significant differences were observed. *spr.* Spring; *aut.* autumn. For key, see Fig. 1

this resulted in a high variation of measurements in the spring sampling campaigns. In autumn, this variation was much lower. Then, cells from flounder collected at the Elbe location produced significantly less ROS than cells from fish at the Helgoland and Spiekeroog sites ($P<0.05$).

When considering the ratio of ROS production upon stimulation versus baseline ROS production, cells from flounder collected at both of the Eider locations had significantly higher ratios than Elbe flounder (Fig. 6).

The respiratory burst activity of HKL was correlated to residues of several chlorinated hydrocarbons and to heavy metals in the muscle of the same individual. The baseline ROS production was positively correlated with dieldrin residues. The ROS response to phorbol ester stimulation was positively correlated to DDT and PCB residues in the muscle of the same individual, and there was a negative correlation with copper residues. The ratio of stimulated ROS versus baseline ROS production showed a positive correlation with DDT, PCB and Hg residues, while this ratio was reduced with increasing Cu residues. The PMA-activated ROS production of HKL was correlated to other indicators of innate immune response, such as activity of macrophage aggregates and lysosomal stability in the liver. Interestingly, baseline ROS production was lower in individuals with increased induction of the EROD system in liver cells, but this was not seen in PMA-activated cells (Table 4).

Discussion

From laboratory studies, it has become clear that environmental contaminants indeed modulate immune responses in fish [for reviews see Dunier and Siwicki (1993); Zelikoff (1993); Bols et al. (2001)]. However, with attempts to extrapolate experimental data to 'field' situations in monitoring studies, problems arise. In natural environments, fish are exposed to an undefined cocktail of various substances for an unknown period of time. To gain knowledge about the actual contamination level of the individuals studied, the present study was substantiated by extensive analytical chemistry of sediment as well as fish residues, which included chlorinated hydrocarbons and heavy metals [see Broeg et al. (1999); Dizer et al. (2003)]. In a previous study (Broeg et al. 1999) on flounder from the same locations, a contamination gradient with respect to residues of chlorinated hydrocarbons was observed with highest values in fish from the Elbe estuary and lowest values in fish from the Tiefe Rinne near Helgoland. This gradient could be established when the residue data were based on the fat content of the liver, which is considered to reflect the chemical burden of the habitat (Broeg et al. 1999). From sediment analysis, however, such a clear gradient could not be established, and when residue data from flounder based on wet weight were considered for analysis, individual flounder could not be related to different sample sites by means of residue analysis (Schmolke et al. 1999). This is most probably a consequence of a strong reduction of heavy metal as well as PCB influx into the southern North Sea during the last decade, which resulted in a decrease in heavy metal and PCB content in the sediment of between

45% and 85% (De Jong et al. 1999). In the German Bight of the southern North Sea, residues of these substances are now far below previous levels and far below contamination levels of other marine sites such as the Mediterranean coast near Haifa in Israel (Kress et al. 1999).

When considering heavy metal and chlorinated hydrocarbon contaminations in flounder muscle, very low residues were found in animals from all the sites, and there was no 'clean' site with all the residues 'below detection limit' (Schmolke et al. 1999; Dizer et al. 2003). In this situation, when analysing immunological data in their response to pollution, clear differences between sampling locations which could be confirmed in all the sampling campaigns could not be established in the present study. This was mainly an effect of the high variation in plasma lysozyme level, as well as phagocyte activity, at some of the locations. The variation in measurements was most probably a result of different contamination profiles of individual flounder: some correlations were found between chlorinated hydrocarbon levels of individual flounder and innate immune responses measured from the same individuals. It also has to be taken into account that contaminants may cause indirect effects such as elevated levels of cortisol, which have a marked modulatory potential on immune functions (Bennet and Wolke 1987a, 1987b). Stimulatory effects of contaminants, as observed in the present study, may also be a consequence of indirect effects of contaminants (Faisal et al. 1991). Some contaminants, however, such as copper in the present study, are clearly immunotoxic.

Cellular immune responses are considered to be sensitive indicators of biological effects of pollutants (Secombes et al. 1997). Broeg et al. (1999) showed that the stability of hepatocyte lysosomes was modulated in a delicate manner. Thus the integrity of hepatocyte lysosomes provided valuable information for the interpretation of the expression of cytochrome P450 1A in liver cells of the same individual. Likewise, we found a correlation between lysosomal stability of liver cells and the activity of plasma lysozyme as well as head kidney phagocytes in the same individuals, indicating an overall impairment of cell functions with decreased lysosome stability.

Experimental studies showed that flounder acquire contaminants with the food rather than by passive uptake via skin or gills (Mondon et al. 2001). Thus, contaminant residues in benthic invertebrates such as *Mytilus edulis*, which serve as prey for flounder, most probably have a higher biological relevance than residue measurements from the sediment. This is supported by the finding that flounder from a location with less contaminated *Mytilus* had a higher plasma lysozyme activity.

In conclusion, the results presented here underline that biological effects of environmental contaminants can be monitored by means of immunological assays in the 'field'. However, in a complex environment such as the German Bight of the North Sea, with a diffuse contamination of various compounds at a low level, it was difficult to separate polluted from less affected sites clearly, most likely because contaminated individuals were found at all locations. When the contamination load of individual flounder was considered, it was possible to spot pollution-mediated effects. The present study was part of an integrated monitoring programme on flounder, which has showed that in conjunction with other physiological data from the same individual, innate immune parameters also allowed the observation of pollution effects. Cellular function such as uptake of neutral red was impaired in individuals with increased proportions of DNA adducts or decreased stability of lysosomes. The activity of plasma lysozyme was also decreased in individuals with impaired lysosome stability, and showed some correlation to cytochrome P450 1A induction. This underlines that innate immune parameters such as plasma lysozyme activity or phagocyte functions form valuable parameters as parts of an integrated monitoring programme.

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References

Anderson DP (1990) Immunological indicators: effects of environmental stress on immune protection and disease outbreak. Am Fish Soc Symp 8:38–50

Bennet RO, Wolke RE (1987a) The effect of sublethal endrin exposure on rainbow trout, *Salmo gairdneri* Richardson. I. Evaluation of serum cortisol concentration and immune-responsiveness. J Fish Biol 31:375–385

Bennet RO, Wolke RE (1987b) The effect of sublethal endrin exposure on rainbow trout, *Salmo gairdneri* Richardson. II. The effect of altering serum cortisol concentrations on the immune response. J Fish Biol 31:387–394

Blaxhall PC (1972) The haematological assessment of the health of freshwater fish. A review of selected literature. J Fish Biol 4:593–604

Bols NC, Brubacher JL, Ganassin RC, Lee LEJ (2001) Ecotoxicology and innate immunity in fish. Dev Comp Immunol 25:853–873

Bressler V, Bissinger V, Abelson A, Dizer H, Sturm A, Kratke R, Fishelson L, Hansen PD (1999) Marine molluscs and fish as biomarkers of pollution stress in littoral regions of the Red Sea, Mediterranean Sea and North Sea. Helgol Mar Res 53: 219-243

Broeg K, Zander S, Diamant A, Körting W, Krüner G, Paperna I, von Westernhagen H (1999) The use of fish metabolic, pathological and parasitological indices in pollution monitoring. I. North Sea. Helgol Mar Res 53:171–194

Busacker GP, Adelman IR, Goolish EM (1990) Growth. In: Schreck CB, Moyle PB (eds) Methods for fish biology. American Fisheries Society, Bethesda, Md., USA, pp 363–387

De Jong F, Bakker JF, van Berkel CJM, Dankers NMJA, Dahl K, Gätje C, Marencic H, Potel P (1999) Wadden Sea quality status report. Wadden Sea Ecosystem No.9. Common Wadden Sea Secretariat, Trilateral Monitoring and Assessment Group, Quality Status Report Group. Wilhelmshaven, Germany

Dizer H, Fischer B, Bresler V, Unruh E, Krüner G, Broeg K, von Westernhagen H, Levikow S, Baumert H, Hansen PD (2003)

Neurotoxic, genotoxic, immunotoxic and encocrine effects of fish and molluscs in the North Sea, Mediterranean Sea and Red Sea. Helgol Mar Res (in press)
Dunier M, Siwicki AK (1993) Effects of pesticides and other organic pollutants in the aquatic environment on immunity of fish: a review. Fish Shellfish Immunol 3:423–438
Ellis AE (2001) The innate host defense mechanisms of fish against viruses and bacteria. Dev Comp Immunol 25:827–839
Faisal M, Marzouk MSM, Smith CL, Huggett RJ (1991) The effect of drilling cuttings on model marine sediment systems. Mar Environ Res 32:103–134
Halliwell B, Gutteridge JMC (1999) Free radicals in biology and medicine, 3rd edn. Oxford University Press, Oxford, UK
Houston AH (1990) Blood and circulation. In: Schreck CB, Moyle PB (eds) Methods for fish biology. America Fisheries Society, Bethesda, Md., pp 273–334
Hutchinson TH, Manning MJ (1996) Seasonal trends in serum lysozyme activity and total protein concentration in dab (*Limanda limanda* L.) sampled from Lyme Bay, UK. Fish Shellfish Immunol 6:473–482
ICES (1996) Report of the working group on biological effects of contaminants. ICES CM 1996/ENV: 5
ICES (1999) Report of the joint meeting of the working group on biological effects of contaminants and the working group on statistical aspects of environmental monitoring. ICES CM 1999/E: 9
Ingram GA (1980) Substances involved in the natural resistance of fish to disease: a review. J Fish Biol 16:23–60
Kress N, Herut B, Shefer E, Hornung H (1999) Trace element levels in fish from clean and polluted coastal marine sites in the Mediterranean Sea, Red Sea and North Sea. Helgol Mar Res 53:163–170
Mathews ES, Warinner JE, Weeks BA (1990) Assay of immune function in fish macrophages: Techniques used as indicators of environmental stress. In: Stolen JS, Fletcher DP, Anderson DP, Robertson BS, van Muiswinkel WB (eds) Techniques in fish immunology. SOS Publications, Fair Haven, N.J., USA pp 155–163
Mondon JA, Duda S, Nowak BF (2001) Histological, growth and 7-ethoxyresurufin *O*-deethylase (EROD) activity responses of greenback flounder *Rhombosolea tapirina* to contaminated marine sediments and diet. Aquat Toxicol 54:231–247
Murray CK, Fletcher TC (1976) The immunohistochemical localisation of lysozyme in plaice (*Pleuronectes platessa* L.) tissues. J Fish Biol 37:873–885
Parry RM, Chandau RC, Sahani RM (1965) A rapid and sensitive assay of muramidase. Proc Soc Exp Biol Med 119:384–386
Schmolke SR, Broeg K, Zander S, Bissinger V, Hansen PD, Kress N, Herut B, Jantzen E, Krüner G, Sturm A, Körting W, von Westernhagen H (1999) Multivariate statistical approach to the temporal and spatial patterns of selected bioindicators observed in the North Sea during the years 1995–1997. Helgol Mar Res 53:257–266
Secombes CJ, Tahir A, Stagg RM (1997) Immunocompetence in flatfish as a measure of the biological effect of exposure to sewage sludge or hydrocarbon contaminated sediments. In: Zelikoff JT (ed) Ecotoxicology: response, biomarkers and risk assessment, an OECD workshop. SOS Publications, Fair Haven, N.J., USA, pp 281–292
Sinderman CJ (1979) Pollution associated diseases and abnormalities of fish and shellfish: a review. Fish Bull 76:717–749
Skouras A, Steinhagen D (2003) Measuring some flounder (*Platichthys flesus*) innate immune responses to be incorporated in effect biomonitoring concepts. Helgol Mar Res (in press)
Snieszko SF (1974) The effects of environmental stress on outbreaks in infectious diseases of fishes. J Fish Biol 6:197–208
Wester PW, Vethaak AD, van Muiswinkel WB (1994) Fish as biomarkers in immunotoxicology. Toxicology 86:213–232
Zelikoff JT (1993) Metal pollution-induced immunomodulation in fish. Ann Rev Fish Dis 3:305–325