Effect of short-term exposure to oil-contaminated sediments on the immune response of dab, 
*Limanda limanda* (L.)

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Dab *Limanda limanda* (L.), were exposed to sediment containing 4, 8, 12 and 16% (w/w) diesel oil-based drilling mud. After 2 and 4 weeks of exposure a number of immune parameters were monitored. There was a tendency to increased haematocrits and lymphocyte numbers at low diesel oil doses, whereas high doses (particularly with longer exposure) gave significantly decreased values. Serum lysozyme levels were decreased, but serum bactericidal and anti-protease activities tended to increase following exposure. Kidney phagocyte respiratory burst activity and cell migration also showed a trend to lower levels relative to control fish, whereas the number of antibody-secreting cells from the kidney were increased with high drilling mud concentration. Lastly, there was a dose-dependent hepatomegaly.

Key words: Drilling mud; Immune response; Dab

INTRODUCTION

Several North Sea surveys have shown that sediments around oil and gas drilling platforms have relatively high hydrocarbon levels (Davies et al., 1984; Massie et al., 1985). These levels are primarily due to the discharge of oiled drill cuttings from such platforms (Leaver, 1987). Effects upon the benthic fauna occur mostly within 500 m of the platforms, and can be correlated with the sediment oil levels (Davies et al., 1984). For example, the induction of phase I and phase II enzyme systems in fish, such as cytochrome P450 monooxygenase, is a known biological consequence of 'natural' exposure to hydrocarbons in the North Sea (Davies and Bell, 1984). Exposure to high environmental hydrocarbon levels is also known to affect the immune system of fish (Faisal et al., 1991a,b; Seeley and Weeks-Perkins, 1991). However, immunological studies have been particularly focussed on fish from the highly pollut-
ed lower Chesapeake Bay region, with virtually nothing known about the effects of hydrocarbon exposure on the immunological status of fish in the North Sea.

Experiments on wild-caught fish suffer from a number of inherent difficulties (Secombes et al., 1991a). Standardisation of the length of exposure to potential toxicants being one of the main problems for pollution studies, particularly with respect to marine studies where highly mobile species may have more chance to move away from chemicals than in highly polluted river systems. Experimental exposure to contaminated sediments overcomes such problems and has been used in a number of studies looking at the immunological consequences of exposure of fish to pollutants (Bucke et al., 1989; Payne and Fancey, 1989; Secombes et al., 1991a, 1992). In addition, an experimental exposure system mimicking hydrocarbon levels seen in oil-contaminated cuttings about 500 m from drilling platforms in the North Sea has already been established (Leaver, 1987). This latter study was used as a guide for the present study, where some immunological effects of short term (2 and 4 weeks) exposure of dab *Limanda limanda* to diesel-based drilling mud were examined.

**MATERIALS AND METHODS**

*Animals*

Dab (*Limanda limanda*, L.) were collected off the coast at Aberdeen and Stonehaven, and acclimatized for at least 7 days in 450-l tanks in which recirculated sea water was passed through at approx. 5-1 min⁻¹, before being placed in the experimental tanks. The mean temperature was 10.9 ± 0.62°C (SE), varying from 9.0–13.0°C during the experiment (08/12/91 to 12/06/92). The mean weight was 233.1 ± 0.15 g (SE) and the mean length was 28.73 ± 0.76 cm (SE), for 72 fish.

*Drilling mud exposure*

Diesel-based drilling mud (SOAFD reference standard), containing 20–40% diesel oil, of which approx. 6% was naphthalenes, was added to 500 g coastal sand at a concentration of 4, 8, 12, and 16% (w/w) (based on Leaver, 1987). The oiled sediments were then added to 20-l of sea water in a 40-l tank and left for 24 h. Three tanks were employed for use with each concentration of drilling mud, and three further tanks were used with clean sand as a control for each concentration. The fish exposure was carried out in a static renewal sea water (34 ppt) system, with constant aeration, at three fish per tank. Two-thirds of the water in the tanks was replaced every 2 days by gently siphoning the water out from the top of the tank. Since the oil-exposed fish would not feed, both groups of fish were starved to prevent discrepancies between these groups. The experiment was terminated after exposure to drilling mud for 2 and 4 weeks, and the immunocompetence of the fish assessed as described below. Whilst no analysis of in vivo hydrocarbon levels was carried out a clear induction (3-fold
increase) of phase I enzyme systems (cytochrome P450 1A measured by ethoxyresorufin-O-deethylatation activity) was found in the livers of the exposed dab (Burke, personal communication) under these tank conditions. The fish were weighed, measured (total length) and the condition factor \( K = \frac{\text{weight}}{\text{length}^3} \times 10^5 \) calculated. Livers were also weighed and the hepatosomatic indices \( HI = \% \text{ liver weight of total body weight} \) calculated.

**Blood analysis**

Blood was collected from the caudal vein and smears made for differential leucocyte counts, after staining with 10% Giemsa (Gurr, BDH). Lymphocytes, granulocytes and monocytes were counted, but thrombocytes were excluded because of inconsistencies found between individuals. Blood cells were identified according to Lehmann and Sturenberg (1986). Haematocrits were determined and the remaining blood allowed to clot overnight at 4°C, and the serum removed after centrifugation and stored at −70°C. Serum was analysed for protein, lysozyme, anti-protease and bactericidal activity.

Protein concentrations were measured by the Bradford (1976) method. Lysozyme was measured using a suspension of *Micrococcus lysodeikticus* (0.075%) in 0.1 M phosphate-citrate buffer (pH 5.8) containing 0.09% NaCl. To a 96-well flat-bottomed microtitre plate containing 175 µl *M. lysodeikticus* suspension per well, 25 µl of various standard concentrations of hen egg white lysozyme (HEWL, Sigma) or serum were added. The plate was immediately shaken and then read at 450 nm at 15-s intervals over 5 min on a multiscan spectrophotometer (MDC) and the serum concentrations calculated in equivalents of HEWL using a Softmax programme.

Serum anti-protease activity, regarded as a putative defence against bacterial proteases, was determined by the inhibition of a known amount of trypsin, based on a test-combination kit for trypsin (BCL catalog No. 125024). Four doubling dilutions of the serum samples were made in 0.15 M PBS (pH 7.2) in a round-bottomed 96-well plate. Five µl of each diluted sample or PBS as a blank was placed into wells of a flat-bottomed 96-well plate. To this, 15 µl trypsin (100 µg ml⁻¹ PBS) was added and incubated for 5 min. Then, 200 µl N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA, 0.5 mg ml⁻¹ M Tris/CaCl₂ buffer, pH 7.8) was added and the plate read immediately at 450 nm every 30 s for 15 min. The difference in absorbance was converted to units of trypsin per litre, and the concentration of serum required to inhibit 85% of trypsin activity calculated by regression analysis.

The bactericidal activity of dab serum was assessed by using a 24 h (log phase) suspension of *Aeromonas salmonicida* (strain MT004) adjusted to a concentration of 5 × 10⁸ cells ml⁻¹ tryptic soy broth (TSB, Gibco). To a bacterial suspension (75 µl) in a 96-well plate, was added 25 µl of serum samples or broth as a blank. The plate was then incubated for 2 h at 19°C with gentle shaking every 30 min. Finally, 10 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, 5 mg ml⁻¹ wa-
ter, Sigma) was added to every well, the plate gently shaken and left in the dark for 15 min before being read at 600 nm on a multiscan spectrophotometer (MDC).

**Head kidney leucocyte analysis**

The head kidney of dab was removed, and disrupted through a 100-μm nylon mesh with L-15 medium (Gibco) containing 10 units ml⁻¹ heparin (Sigma) and penicillin (100 μg ml⁻¹)/streptomycin (100 units ml⁻¹) (P/S, Gibco) as described by Secombes et al. (1991b). Isolated kidney cells were washed twice in phenol red-free Hanks' balanced salt solution (HBSS, Gibco) containing 10 units ml⁻¹ heparin, by centrifugation at 400 × g for 15 min. A viable cell count was made by Trypan blue (0.5%) dye exclusion and the cells used immediately to determine respiratory burst activity (production of reactive oxygen species), the number of antibody-secreting cells, and the ability of cells to migrate.

The reduction of ferricytochrome c was employed to detect the production of superoxide anion (O₂⁻, a reactive oxygen species) as described by Secombes et al. (1988). Kidney cells were adjusted to a concentration of 10⁶ cells ml⁻¹ (except for the 2-week exposure group using 8% and 12% drilling mud, and their respective controls, in which 10⁵ cells ml⁻¹ was used), centrifuged and resuspended in phenol red-free HBSS containing 2 mg ml⁻¹ ferricytochrome c. These suspensions were then divided into two tubes, to which either phorbol myristate acetate (PMA, Sigma) or PMA plus superoxide dismutase (SOD, Sigma) was added, to give overall concentrations of 1 μg ml⁻¹ and 300 units ml⁻¹, respectively. The PMA served as a stimulant to trigger the respiratory burst and the SOD confirmed the specificity of the reaction. All treatments were performed in triplicate and incubated for 15 min at room temperature before removing the cells by centrifugation, and reading the supernatants (100 μl) at 550 nm on a multiscan spectrophotometer (MDC). Wells containing PMA/SOD from each individual served as blanks.

The number of antibody-secreting cells were assessed using an ELISPOUT assay as described by Secombes et al. (1991b). Kidney cell suspensions were resuspended in L-15 medium containing 5% FCS, and diluted to 5 × 10⁴ and 10⁴ cells ml⁻¹. These were added to wells of a millilitre-HA 96-well filtration plate (Millipore) coated overnight at 4°C with protein A purified anti-dab Ig (50 μg ml⁻¹ PBS). After 6 h at 18°C the cells were washed off with PBS and PBS containing 0.05% Tween (TPBS) and the plate immersed in TPBS for 5 min. One hundred μl of anti-dab Ig-HRP diluted 1:4000 was then added per well overnight at 4°C. Finally, the wells were washed and 100 μl HRP-chromogenic substrate consisting of 258 μg 3-amino-9-ethyl carbazol ml⁻¹ in 0.05 M acetate buffer (pH 5) containing 0.04% (30%) H₂O₂ was added to each well for 10 min. After stopping the reaction by washing in tap water, the plate was allowed to dry, the red spots counted under a binocular microscope (×25) and results expressed per 10⁶ kidney cells.

Cell migration activity was performed in a microchemotaxis apparatus. The kidney
cells were resuspended in L-15 containing heparin (but without serum) to $10^7$ cells ml$^{-1}$. To the lower chamber of the apparatus 27 µl of L-15 or 1:50 diluted dab serum (chemoattractant) were added. A 3-µm pore filter separated the lower and upper chambers. The cell suspension (43 µl) was added in triplicate per treatment per fish to the upper wells, and the apparatus incubated for 90 min at 19°C in a moist environment. At the end of this period the chamber was dismantled and the top surface of the filter immersed in PBS three times and wiped on a rubber blade to remove non-migrated cells. The filter was then dried, stained with Giemsa, rinsed in Gurr buffer and dried again. The migrated cells were counted under a binocular microscope (three HP fields per well) and the data expressed as a migration index which was obtained by dividing the mean number of cells migrating toward the chemoattractant by the mean number migrating to the L-15.

Values from control and oil-exposed fish were compared by Student's $t$-test analysis. Since all the exposure doses could not be carried out simultaneously, the data were also expressed relative to the control values within each experiment, to enable comparisons of dose effects between experiments. The control values were not pooled to obtain these relative values since they differed significantly between experiments. Dose effects were compared at each timing by one-way ANOVA.

RESULTS

The mean control values for all parameters investigated are given in Table 1.

Exposure of dab to various concentrations of drilling mud for 2 and 4 weeks was found to affect several of the parameters studied. Whilst there was virtually no effect upon the condition factor ($K$) at either timing (only exposure to 4% drilling mud for 2 weeks had a significant impact; Table 2), significant differences were seen between the hepatosomatic indices ($HI$) after 4 weeks exposure (Table 3). Here, the $HI$ was

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$n$</th>
<th>Mean ± SE</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition factor ($K$)</td>
<td>29</td>
<td>0.96 ± 0.09</td>
<td>0.69-1.12</td>
</tr>
<tr>
<td>Hepatosomatic index ($HI$)</td>
<td>29</td>
<td>1.69 ± 0.44</td>
<td>0.78-4.00</td>
</tr>
<tr>
<td>Haematocrit (%PCV)</td>
<td>29</td>
<td>21.41 ± 2.64</td>
<td>7.0-28.00</td>
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<tr>
<td>Lymphocytes (%)</td>
<td>23</td>
<td>51.63 ± 29.99</td>
<td>64.2-97.00</td>
</tr>
<tr>
<td>Lysozyme ($\mu g/ml$)</td>
<td>29</td>
<td>10.88 ± 3.40</td>
<td>3.45-19.7</td>
</tr>
<tr>
<td>Protein (mg/ml)</td>
<td>24</td>
<td>32.59 ± 7.97</td>
<td>15.72-59.99</td>
</tr>
<tr>
<td>Bactericidal (% bacteria killed)</td>
<td>27</td>
<td>49.03 ± 16.23</td>
<td>10.00-86.00</td>
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<tr>
<td>Antiprotease (units trypsin inhibited/µl serum)</td>
<td>24</td>
<td>20.21 ± 8.07</td>
<td>8.07-50.73</td>
</tr>
<tr>
<td>Respiratory burst (nmol/10^6 cells)</td>
<td>25</td>
<td>9.65 ± 2.70</td>
<td>0.82-14.31</td>
</tr>
<tr>
<td>Antibody secreting cells (No./10^6 cells)</td>
<td>20</td>
<td>11092 ± 7216</td>
<td>2800-50800</td>
</tr>
<tr>
<td>Cell migration index</td>
<td>13</td>
<td>5.23 ± 2.42</td>
<td>0.65-11.16</td>
</tr>
</tbody>
</table>
TABLE 2
Haematocrits, proportion of lymphocytes, condition factors (K), and hepatosomatic indices (HI) of 2 weeks drilling mud-exposed dab relative to their controls (%)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>4%</th>
<th>8%</th>
<th>12%</th>
<th>16%</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>87 ± 8*</td>
<td>99 ± 6</td>
<td>97 ± 4</td>
<td>99 ± 5</td>
</tr>
<tr>
<td>HI</td>
<td>88 ± 19</td>
<td>111 ± 19</td>
<td>129 ± 28</td>
<td>98 ± 17</td>
</tr>
<tr>
<td>Haematocrits (%)</td>
<td>126 ± 14</td>
<td>105 ± 11</td>
<td>92 ± 8</td>
<td>95 ± 7</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>N.D.</td>
<td>100 ± 11</td>
<td>99 ± 8</td>
<td>89 ± 7</td>
</tr>
</tbody>
</table>

*Proportion of lymphocytes from 200 leucocytes counted.

*P < 0.05 compared to control.

n = 5–9.

N.D., not determined

significantly higher compared with the controls using 8% (P < 0.05), 12% (P < 0.01) and 16% (P < 0.05) drilling mud. In addition, the size of the livers increased significantly as the concentration of the drilling mud increased (P < 0.05). Blood cell parameters also showed significant differences, again particularly at week 4. Exposure of dab to drilling muds for 2 or 4 weeks gave an increased haematocrit at the lowest dose used (4%), and this increase was significant in the latter case (P < 0.05), whereas exposure to the highest dose (16%) for 4 weeks resulted in a significantly decreased haematocrit (P < 0.05) (Table 3). The differential leucocyte counts showed similar effects after 4 weeks exposure, with the 8% drilling mud group having a significantly higher (P < 0.01) number of lymphocytes and the 16% group significantly lower numbers (P < 0.05) (Table 3).

TABLE 3
Haematocrits, proportion of lymphocytes, condition factors (K), and hepatosomatic indices (HI) of 4 weeks drilling mud-exposed dab relative to their controls (%)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>4%</th>
<th>8%</th>
<th>12%</th>
<th>16%</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>95 ± 22</td>
<td>91 ± 5</td>
<td>90 ± 1</td>
<td>96 ± 6</td>
</tr>
<tr>
<td>HI</td>
<td>92 ± 7</td>
<td>131 ± 10*</td>
<td>184 ± 28**</td>
<td>205 ± 42*</td>
</tr>
<tr>
<td>Haematocrits (%)</td>
<td>159 ± 18*</td>
<td>100 ± 13</td>
<td>93 ± 6</td>
<td>75 ± 12*</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>107 ± 2</td>
<td>124 ± 3**</td>
<td>104 ± 12</td>
<td>94 ± 3*</td>
</tr>
</tbody>
</table>

*Proportion of lymphocytes from 200 leucocytes counted.

*P < 0.05 compared to control.

**P < 0.01 compared to control.

n = 4–6.
Fig. 1. Effect of 2 weeks exposure to various concentrations of oil-contaminated drilling mud on dab serum lysozyme, bactericidal and anti-protease activity and protein level. Data are means plus standard errors of values expressed relative to control values from nonexposed dab. N.D. = not determined. *P < 0.05; **P < 0.01 compared to respective control, n = 5-9.

Serum samples from the drilling mud-exposed dab showed a tendency to have lower lysozyme levels relative to control dab (Figs. 1 and 2). The mean lysozyme levels were lower in all the dose and time combinations and were significantly lower in four of the eight groups, 4% (P < 0.05), 8% (P < 0.01) and 12% (P < 0.01) after 2

Fig. 2. Effect of 4 weeks exposure to various concentrations of oil-contaminated drilling mud on dab serum lysozyme, bactericidal and anti-protease activity and protein level. Data are means plus standard errors of values expressed relative to control values from nonexposed dab. *P < 0.05; **P < 0.01 compared to respective control, n = 5-6.
Fig. 3. Effect of 2 weeks exposure to various concentrations of oil-contaminated drilling mud on respiratory burst activity, number of antibody secreting cells and chemotaxis of dab kidney leukocytes. Data are means plus standard errors of values expressed relative to control values from nonexposed dab. N.D. = not determined. n = 4–6.

weeks exposure and 12% \( P < 0.05 \) after 4 weeks exposure. This was not a reflection of lower serum protein levels, which showed no significant differences in any of the groups, and there was no dose effect. In contrast, serum bactericidal activity tended to increase following exposure to drilling mud and was significantly higher than the controls using 12% drilling mud for 2 \( P < 0.05 \) and 4 weeks \( P < 0.01 \) (Figs. 1 and 2). One-way ANOVA on the 2-week exposure data revealed that there was also a significant \( P < 0.05 \) effect of drilling mud dose on bactericidal activity at this time. After 2 weeks exposure, serum anti-protease activity also tended to be high relative to controls and this effect was significant \( P < 0.05 \) using 16% drilling mud (Fig. 1). No significant differences were seen at week 4 and there was no dose effect at either timing.

Of the cellular assays carried out (Figs. 3 and 4), both respiratory burst activity and cell migration of phagocytes were significantly lower than their respective controls after 4 weeks exposure to 4% drilling mud. No other doses at either time had a significant effect on these activities, despite the very low mean value for the migration index seen after 2 weeks exposure to 16% drilling mud \( P = 0.066 \) (Fig. 3). One-way ANOVA of the respiratory burst data revealed there was a significant dose effect after 2 \( P = 0.01 \) and 4 \( P < 0.01 \) weeks exposure, but no consistent trend was seen. Finally, the number of kidney antibody-secreting cells was more clearly affected by exposure to drilling mud, with a significant increase seen after exposure to 16% drilling mud for 4 weeks \( P < 0.05 \). A trend to increasing numbers at higher doses was also seen after exposure to drilling mud for 2 weeks, but no significant effects were seen at this timing (relative to controls or to dose) due to the large standard deviations present.
The cellular assays examined also showed opposite effects, dependent on the cell type examined. Phagocyte respiratory burst activity and cell migration tended to be lower than control levels, whereas the number of lymphocytes secreting antibody was increased with high drilling mud concentration. Cell migration has been shown previously to be sensitive to the effects of polycyclic aromatic hydrocarbon (PAH) pollution (Weeks et al., 1986). Similarly, respiratory burst activity in dab was shown to be inhibited by exposure to sewage sludge (Secombes et al., 1991a). Precedents for an increased B cell responsiveness following exposure to pollutants also exist. For example, Robohm (1986) has shown that cadmium exposure can increase anti-Bacillus cereus antibody titres in striped bass, *Morone saxatilis*, Carballo et al. (1992) have shown that exposure to sublethal concentrations of copper increases total antibody levels in rainbow trout, *Oncorhynchus mykiss*, and Faisal et al. (1991a) have shown that leucocyte LPS responsiveness increases in PAH-exposed *Leiostomus xanthurus*. Stimulatory effects of some toxic pollutants on nonspecific immunological parameters have also been demonstrated in fish (Cossarini-Dunier, 1987; Elsasser et al., 1987; Rice and Weeks, 1989).

A suppressive impact of a pollutant is easy to explain, since many potent immunotoxic chemicals are known, including heavy metals, aromatic hydrocarbons and halogenated hydrocarbons (Wong et al., 1992). How a pollutant can have a stimulatory effect is less clear and may involve several mechanisms. With respect to antibody production, possibilities include direct stimulation of B cells by the chemical(s), an increase in T helper cell activity, a decrease in T suppressor cell activity, or an impairment of antigen or antibody degradation. Since enhancement of B cell responses can be concomitant with inhibition of T cell responses (Faisal et al., 1991a), there is the possibility that compensatory mechanisms exist within the immune system, making the overall impact of in vivo exposure to pollutants complex.

Finally, the dose-dependent hepatomegaly observed in this study, presumably reflects the liver’s importance as a detoxifying site. In a similar exposure study Leaver (1987) has shown that plaice, *Pleuronectes platessa* hepatic microsomal monooxygenase activity (both benzo[a]pyrene hydroxylase and ethoxyresorufin-O-deethylation activity) was increased following exposure to diesel contaminated drill cuttings at 50 ppm for 20 days (equivalent to 8% in the current study). However, the presence of generalised toxic effects such as hepatomegaly does not imply immunomodulation has also occurred, as seen using Aroclor 1254 (a halogenated aromatic hydrocarbon) in rainbow trout, where despite weight loss and hepatomegaly natural cytotoxic cell activity and antibody activity were unaffected (Cleland and Sonstegard, 1987; Cleland et al., 1988).

In conclusion, dab immune responses were affected by exposure to diesel-based drilling mud and both inhibitory and stimulatory effects were apparent. Whilst it is difficult to predict the in vivo consequences of this immune modulation, the effects of longer term exposure and the potential for return to normal immune responsiveness following transfer to clean water are worthy of further investigation.
ACKNOWLEDGEMENT

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REFERENCES


