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Effect of Water Soluble Fractions of Diesel and an Oil Spill Dispersant (Corexit 9527) on Immune Responses in Mussels

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Hemocytes are considered to be the first line of defence against pathogens in bivalves. In the marine mussel, *Mytilus edulis*, an increase or a decrease of circulating hemocytes has been observed after exposure to pollutants (Drynda et al. 1997; Pipe et al. 1999; St-Jean et al. 2002a, 2002b). In animals exposed to waterborne butyltins phagocytosis was stimulated by low doses and decreased with higher concentrations (St-Jean et al. 2002a; 2002b). Although not as well documented as in vertebrate cells, cytoskeletal elements also contribute to the motility and phagocytic capability of invertebrate hemocytes (Alvarez et al. 1989). Any damage to cytoskeleton proteins could lead to a loss in hemocyte immune function because cell adhesion, motility, endocytic ability and the capacity to phagocytose foreign particles depend on the cytoskeleton (Gomez-Mendikute et al. 2002).

In this study, we investigated the short-term effect of water soluble fractions (WSF) of diesel oil and of emulsions of an oil spill dispersant (Corexit 9527) on cellular immune responses in mussels (*Mytilus sp.*). Different immune responses were investigated in hemocytes including: number, capacity to phagocytose zymosan particles, ability to adhere to surfaces and maintenance of cytoskeleton integrity. Studies were carried out *in vitro* by preincubating hemocytes with diesel WSF or Corexit 9527 emulsions prior to measuring phagocytosis and cytoskeleton integrity. *In vivo* immune responses were also investigated by 1) exposing test animals to WSF or Corexit emulsions to establish dose-response relationships and 2) by injecting mussels with foreign particles (zymosan) and measuring phagocytosis and cell numbers in the same individuals before and after exposure to WSF or Corexit emulsion.

MATERIALS AND METHODS

Mussels 5-10 cm in length were collected from an unpolluted site in Trinity Bay, Newfoundland. Diesel or corn oil WSF (1/50 and 1/100) were prepared by gentle stirring for 24h and allowing mixtures to settle for 1h in a separatory funnel. The aqueous fractions were collected for stock solutions and were used directly within 2 days. Diesel concentrations measured by fluorescence (total oil) (Hellou and Upshall 1993) ranged from 0.5 to 11 ppm (1-22 ppb chrysene equivalents). Corexit 9527

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emulsions (1 to 500 ppm) were prepared by dissolving dispersant in seawater and shaking thoroughly before use. For the preparation of the mixture diesel WSF + dispersant, 11 ppm of diesel was used with a dispersant concentration 20 times lower (0.55 ppm) in accordance with the effective dispersant-to-oil ratio generally established by scientists and manufacturers (Wells 1984).

Hemolymph was collected from posterior adductor muscles and counts were made using a Neubauer chamber. Cell adherence percentages were evaluated by counting cell numbers on randomly selected 0.1 mm^2 squares on a slide. Cells were counted on 20 squares and mean counts calculated. Percentage adherence was evaluated in relation to the total cell count (cells/mL), the hemolymph volume deposited on the slide, and the total surface covered by cells.

The phagocytosis assay was conducted following La Peyre et al. (1995) using zymosan (yeast extracts) as foreign particles. Counts of at least 100 cells were made for each individual. The results were expressed as the percentage of phagocytic hemocytes (number of hemocytes with at least one zymosan particle/total number of hemocytes).

The cytoskeleton labelling test was performed on microplates as described by Misamore and Lynn (2000) and Olabarietta et al. (2001) with some modifications. Hemolymph was fixed with 18.5% formaldehyde in seawater for 1h to 1h30'. Pooled samples of hemolymph were used with cell numbers varying from 5 $x10^4$ to 16 $x10^4$ cells per microplate well. Following a washing step with mussel buffer (Misamore and Lynn 2000), cells were permeabilized with 0.1% Triton-X 100 for 15 min. Triton-X was removed by washing twice with mussel buffer. To label microfilaments, 50 µl of TRITC-conjugated phalloidin (10 µg/mL in mussel buffer) was added to the cells and incubations were conducted for 30 min. Incubation with phalloidin was followed by 2 washing steps with mussel buffer. Fluorescence was measured in a fluorescence microplate reader (Excitation: 540 nm, Emission: 570 nm). As a positive control of cytoskeleton damage, testing of the technique was performed by preincubating cells with concentrations of hydrogen peroxide (H_2O_2) ranging from 20 to 1200 µM. Cells were also observed using a fluorescence microscope (Zeiss, Axioplan). Cell viability was measured using the Trypan Blue exclusion test (Conn 1953) and showed no significant increase of cell mortality with the concentrations used. In parallel with diesel WSF *in vitro* incubations, we conducted hemocyte incubations with corn oil WSF. Fluorescence measurements were also performed with different concentrations of diesel or corn oil WSF without cell incubation to monitor any changes of fluorescence (increase or quenching) due to oil components in seawater. Results were expressed as a percentage of fluorimetric values observed in controls after blank subtraction (Leira et al. 2001).

In vitro exposures were conducted by preincubating hemocytes with diesel WSF (0.5, 1.1, 2.2, 8.22 and 11 ppm), corn oil WSF or dispersant emulsion (100 , 200 and 500 ppm) 1h prior to testing. Control hemocytes were incubated in seawater for the same

length of time. All incubations were conducted on ice. Corn oil incubations were performed to check the possible existence of any "physical" effect of oil in water fractions when in direct contact with hemocytes. *In vivo* exposures were performed by exposing animals for 4 days to diesel WSF or dispersant emulsions (n=10 mussels per group). One control group was established for every 4 concentrations tested (diesel and/or dispersant) to avoid handling more than 50 hemolymph samples per experiment. Another set of experiments was conducted to evaluate any change of immune response in animals by injecting mussels with zymosan particles in seawater (0.5 ml at 1mg/ml) and assessing phagocytosis and cell numbers at 0, 30 min, 1h and 2h (t0, t30', t1h, t2h) after injection. The same mussels (n=5 per group) were then exposed for 2 days to diesel WSF, dispersant emulsions or seawater (controls) and their immune response was reevaluated at t0, t30', t1h and t2h after the second injection. Prior testing was performed to determine the zymosan concentration to be injected in mussels. A volume of 0.5 mL of zymosan at 10 mg/mL proved to be too high for hemocyte capacity response as most of the cells burst 30 min after injection. The dose selected to be injected was 0.5 ml at 1 mg/mL of zymosan.

Aromatic hydrocarbons contained in diesel WSF were analyzed by gas chromatography/mass spectrometry (GC/MS) (Hellou and Upshall 1993) by a certified analytical laboratory with a detection limit of 0.01 ppb (PSC Analytical Services, Newfoundland, Canada).

Arcsine transformations of the data were used in all analyses of phagocytosis and cell adherence percentages (Sokal and Rohlf 1981). Unpaired t-tests were performed to compare immune responses of treated cells or animals at every toxicant concentration with control cells or mussels. A probability level of $p \leq 0.05$ was considered significant.

RESULTS AND DISCUSSION

A study on the potential impact of petroleum hydrocarbons and dispersant emulsions was carried out on cellular immune responses of mussels. Diesel (No. 2 fuel oil) water soluble fractions can be used as surrogate sources of soluble hydrocarbons from crude petroleum and production waters (National Research Council U.S. 1989). Cytoskeleton integrity was investigated by labelling actin filaments with a fluorescent probe: phalloidin. Such measurements serve as a guide to the degree of preservation of the actin cytoskeleton under different conditions. Gomez-Mendikute et al. (2002) have observed an obvious change of actin cytoskeleton distribution after incubation of hemocytes with benzo(a)pyrene. Preliminary results obtained with H_2O_2 showed a decrease of fluorescence after 1h *in vitro* incubation of hemocytes (Figure 1B). Our results also showed a significant decrease of fluorescence starting at 0.5 ppm with diesel WSF with no important changes with increase of diesel concentrations. The decrease of fluorescence observed after cell incubation with diesel WSF is more important than the overall effect of H_2O_2.

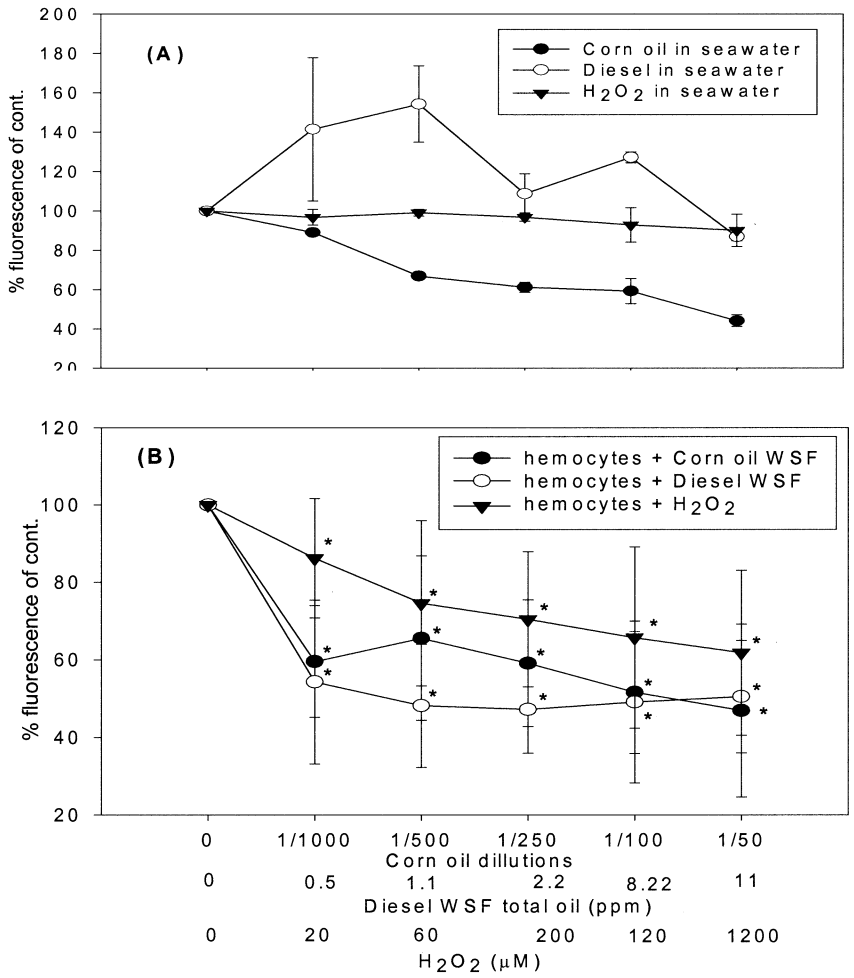

Figure 1. Fluorescence measurement (% fluorescence of controls)(A) H_2O_2, corn oil or diesel solutions (seawater) containing phalloidin, (B) hemocyte cytoskeleton labelled with phalloidin after incubation with H_2O_2, corn oil or diesel WSF (n=7, hemolymph of 4 mussels combined per sample).* Unpaired t-test, $p<0.05$.

Incubations conducted in parallel with corn oil WSF showed a similar pattern (Figure 1B). Nonetheless, the quenching observed with corn oil WSF incubated with phalloidin makes any conclusion on the effect of corn oil on cells difficult (Figure 1A). These results could suggest either a great sensitivity of hemocytes to diesel WSF or a "physical" effect of oily components of both corn oil and diesel WSF on cells. Further testing is still necessary to understand the diesel WSF effect on the actin cytoskeleton.

In vitro testing of phagocytosis showed no effect of diesel WSF at concentrations of 0.5 and 1.1 ppm but a non-significant decrease was observed after exposure to 2.2 ppm, 8.22 and 11 ppm of diesel oil . Similarly, a non-significant decrease of phagocytosis was recorded at 100, 200 and 500 ppm of Corexit. On the other hand, *in vivo* testing showed that Corexit and diesel concentrations responsible for the decrease in phagocytosis *in vitro* caused an increased response when mussels were exposed for 4 days (Table 1). The stimulation of immune responses has been considered by several authors to be an indication of lesser toxicity than an inhibition of these same responses (Pipe et al. 1999; St-Jean et al. 2002a; 2002b). As expected, *in vitro* testing provoked inhibitions while *in vivo* exposure often resulted in an increase in immune response. Similarly, in a study on metal immunotoxicity, Sauvé et al. (2002) have shown that concentrations required to obtain 50% suppression of phagocytic activity *in vivo* were lower by several orders of magnitude compared to *in vitro* results.

Table 1. Hemocyte cellular response after 4 days exposure of mussels to Corexit 9527 emulsions, diesel WSF or diesel + Corexit (n=10 mussels per concentration).

Group	Cell numbers (10^6 cells/ml)	% cell adherence	% phagocytosis
Controls	1.82±0.94	17.00±8.21	92.10±6.92
200 ppm Corexit	2.75±2.28	4.36±3.14 *	98.20±1.28 *
500 ppm Corexit	4.94±4.87	12.20±25.80	89.40±11.70
8.22 ppm Diesel	2.90±0.94 *	13.80±6.71	95.90±4.45
11 ppm Diesel	2.87±1.03 *	12.40±8.31	97.80±2.99 *
Controls	1.48±1.69	Not performed	80.90±23.00
11 ppm Diesel +0.55 ppm Corexit	1.36±1.09	Not performed	96.80±3.55 *

* Unpaired t-test, $p<0.05$.

Preliminary results (not presented) obtained after the first set of *in vivo* experiments (4 days exposure) showed no effect on phagocytosis and cell numbers with diesel or Corexit concentrations less than 8 ppm and 200 ppm respectively. Short-term exposure to diesel WSF (≈ 10 ppm total oil) resulted in a significant increase in cell numbers and phagocytosis (Table 1). Similarly, Drynda et al. (1997) found that total hemocyte counts in mussels sampled after an oil spill increased and subsequently returned to background levels as hydrocarbon concentrations in mussel tissues declined. On the other hand, Sami et al. (1992) found no consistent pattern for phagocytosis responses after exposing oysters to PAH contaminated sediments (12 ppm total PAH) for up to 11 weeks. Pipe et al. (1999) concluded that the energetic costs of immune function stimulation due to low level toxicant presence may be high with possible detrimental implications for long-term chronic exposure.

The principal hydrocarbons in the diesel WSF (11 ppm) were mono and diaromatics (Benzene, 18 ppb; Toluene, 248 ppb; Xylene, 473 ppb; Naphthalene, 65 ppb; 1-Methylnaphthalene, 9.1 ppb; 2-Methylnaphthalene, 9.1 ppb). Concentrations of 3 ring

compounds were very low (Fluorene, 0.22 ppb; Phenanthrene, 0.08 ppb), while 4-5 ring compounds were absent at a detection limit of 0.01 ppb. The lack of detectable large polycyclic aromatics in the diesel WSF suggests that the observed effect on hemocytes could be linked to low molecular compounds such as mono and diaromatics. A similar hypothesis was concluded by Hamoutene et al. (2002) while measuring DNA damage in mussel hemocytes exposed to crude oil WSF.

The Corexit 9527 concentrations responsible for alteration of mussel immune functions were higher than 100 ppm. These results are confirmed by most dispersant toxicity studies where dispersant sublethal thresholds ranged between 100 and 1000 ppm (Wells 1984), we can conclude that Corexit 9527 is not likely to affect cellular mussel functions if used in "normal" oil spill conditions. The cell number increase found after exposure to diesel WSF was not observed when dispersant was added to the diesel WSF (Table 1). Pipe et al. (1999) observed a similar effect on mussel cell numbers with increasing copper concentrations. This result could be due to enhanced toxicity of copper causing cell death or movement of hemocytes out of the circulation to sites of tissue pathology resulting from copper exposure (Pipe et al. 1999).

The second set of *in vivo* experiments consisted of testing the concentration displaying the most significant effect observed in the first short-term exposure. An inhibition of cell numbers ($p<0.05$) and phagocytosis ($p=0.083$) was found 30 minutes after injection (Figures 2A and 2B). This could indicate loss of ability to stimulate immune functions after the stress represented by zymosan injection.The production of free radicals known to occur in *M. edulis* blood cells when killing invasive pathogens (Pipe 1992) and in *Perna perna* hemocytes during zymosan phagocytosis (Barracco et al. 1999) could constitute a negative factor compromising any immune system stimulation as a defence mechanism. This increased "sensitivity" is confirmed by the fact that exposure to 2 days of Corexit emulsions (200 and 500 ppm) resulted in the death of mussels injected with zymosan.

We have investigated the potential for WSF of petroleum and a common oil spill dispersant to affect cellular immune responses in mussels. Relatively high concentrations of hydrocarbons (10 ppm range), which can be expected to occur under oil slicks (e.g. GESAMP 1993), were shown to have potential to affect some endpoints. However, immune responses were not enhanced by concentrations of dispersant at operational doses. Furthermore, analysis of the WSF suggest that toxicity could be mainly associated with mono and diaromatics which would be expected to evaporate from oil slicks relatively quickly (GESAMP 1993). This study also demonstrated the use of a new fluorimetric microplate assay for investigating the effects of pollutants on cytoskeleton integrity in mussels.

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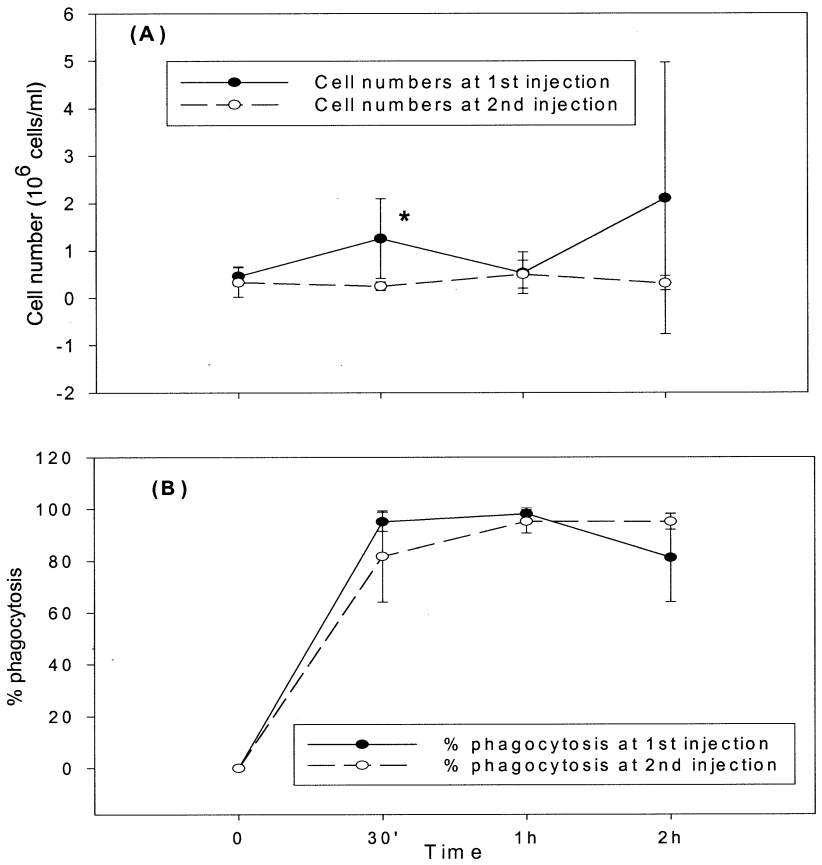

Figure 2. (A) Cell numbers measured after 2 injections of zymosan (at 0, 30 min., 1 h and 2 h after injection) separated by 2 days exposure to diesel WSF 11 ppm, n=5. (B) Percentages of phagocytosis measured at 0, 30 min., 1 h and 2 h after injection.*Unpaired t-test, $p<0.05$.

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