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Immunotoxicity and oxidative stress in the Arctic scallop *Chlamys islandica*: Effects of acute oil exposure

Marie L. Hannam [a,*], Shaw D. Bamber [b], A. John Moody [a], Tamara S. Galloway [c], Malcolm B. Jones [d]

[a] Ecotoxicology Research and Innovation Centre, School of Biomedical and Biological Sciences, University of Plymouth, Drake Circus, Plymouth, PL4 8AA, UK
[b] IRIS – Biomiljø, Mekjarvik 12, 4070 Randaberg, Norway
[c] School of Biosciences, University of Exeter, Hatherly Laboratories, Prince of Wales Road, Exeter, EX4 4PS, UK
[d] Marine Institute, University of Plymouth, Drake Circus, Plymouth, PL4 8AA, UK

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ABSTRACT

With increasing oil exploration in Arctic regions, the risk of an accidental oil spill into the environment is inevitably elevated. As a result, concerns have been raised over the potential impact of oil exposure on Arctic organisms. This study assessed the effects of an acute oil exposure (mimicking an accidental spill) on the immune function and oxidative stress status of the Arctic scallop *Chlamys islandica*. Scallops were exposed to the water accommodated fraction of crude oil over 21 d (maximum ΣPAH 163 $\mu g\,l^{-1}$) and immune endpoints and oxidative stress parameters were measured. Mortalities were recorded during the exposure and reductions in immunocompetence were observed, with significant impairment of phagocytosis and cell membrane stability. Scallops were also subjected to oxidative stress, with a significant reduction in glutathione levels and induction of lipid peroxidation. After the acute oil exposure had subsided, no recovery of immune function was observed indicating potential for prolonged sublethal effects.

1. Introduction

Currently, around one tenth of the world's oil supply is produced in Arctic regions, and cumulative production is estimated at 13 400 million m^3 (AMAP, 2007). With the current expansion in oil production in the Arctic, there is growing concern over the effects of oil exposure on Arctic organisms. There is a zero discharge policy in place in some areas of the Arctic such as the Barents Sea, and adherence to strict regulations and guidelines can help to reduce oil emissions. However, pollution cannot be reduced to zero; the risks of tanker spills, blow outs and pipeline leaks cannot be eliminated, and transport of the extracted oil extends the risk of accidental release beyond the production area (AMAP, 2007). For marine ecosystems, oil spills pose the largest environmental threat and, when difficult to contain, can cause impacts over large areas. Even small-scale oil spills can have substantial effects on localised marine biota. After the acute effects of an initial spill have subsided, chronic seepage from residual oil in sediments can mean PAH levels remain elevated in benthic invertebrates (AMAP, 1998) such as bivalves, which have a tendency to accumulate these compounds within their tissues through passive diffusion (Meador, 2003).

The ecological impacts of oil spills in Arctic environments are unclear. What is known is that the chemical behaviour of oil is altered at low temperatures, affecting its distribution, composition and physical state, which in turn influence the bioavailability of its components (Payne et al., 1991). In addition, cold-water marine invertebrates possess biological adaptations such as altered cell membrane composition (Gillis and Ballantyne, 1999) enabling them to survive at low temperatures. However, these adaptations may influence their susceptibility to oil-induced toxicity; polar organisms often exhibit a higher degree of unsaturated fatty acids in cell membranes in order to maintain fluidity and functioning at cold temperatures (Viarengo et al., 1994), which may make them more susceptible to oxidative damage (Camus et al., 2002). Biological recovery from oil-induced damage is also predicted to be much slower in Arctic than in temperate systems because of the low growth rates, higher generation turnover times and increased age at maturation that are characteristic of many Arctic organisms (AMAP, 1998).

Due to its complexity, an organism's immune system is extremely vulnerable to xenobiotic stress (Galloway and Depledge, 2001), and there is evidence that oil can impact immune function in bivalve molluscs (Bado-Nilles et al., 2008; Dyrynda et al., 1997; Hamoutene et al., 2004; McCormick-Ray, 1987). Growth, disease and survival of an organism are partly determined by the capability of the immune system (Blaise et al., 2002), therefore, immune function is important in assessing sublethal effects of contaminant exposure (Luengen et al., 2004).

* Corresponding author. Fax: +44 1752 584605.
E-mail address: marie.hannam@plymouth.ac.uk (M.L. Hannam).

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Polycyclic aromatic hydrocarbons (PAHs) are natural constituents of crude oil. Once taken up by an organism, these PAHs undergo biotransformation reactions which can stimulate the production of reactive oxygen species (ROS) (Livingstone, 1991). In addition, increased levels of dissolved oxygen in polar regions may provide an increased source of ROS (Viarengo et al., 1995). ROS are produced continually in living cells, however, an imbalance between formation and neutralisation of these reactive species can induce oxidative damage (Valavanidis et al., 2006). The proliferation of ROS, and subsequent oxidative stress, has been suggested as a mechanism of contaminant toxicity in exposed organisms, with previous studies suggesting a link between oxidative stress and altered immune function in both vertebrates (Koner et al., 1997, 1998) and invertebrates (Hannam et al., 2010).

Bivalves, particularly the mussel *Mytilus edulis*, have been used widely for pollution effect studies and the Arctic scallop *Chlamys islandica* has potential as a sentinel species in the Arctic and Sub-Arctic regions, where *M. edulis* is absent (Baussant et al., 2009). Previous results for *C. islandica* indicate that immunotoxicity from low levels of mechanically dispersed oil is reversible (Hannam et al., 2009a); however, with the elevated risk of accidental spills due to increased oil exploration in the Arctic, the consequences of an acute oil exposure are yet to be established. Immunocompetence and oxidative stress measurements are promising tools for biological-effects monitoring (ICES, 2007) and here we investigate the effect of an acute oil exposure, simulating an accidental spill, on immune function and oxidative stress in *C. islandica.*

2. Materials and methods

2.1. Experimental design

Arctic scallops, *C. islandica* (70–100 mm shell length), were collected by divers from Porsangerfjord, Norway (70°1′ N, 25°1′ E) in March 2008, and transported by air to the exposure facility at Stavanger, Norway (58°57′ N, 5°43′ E). Upon arrival, organisms were transferred to 600 l fibreglass tanks with a continuous flow of filtered seawater (FSW) at 6 ± 1 °C. Scallops (180 total) were maintained in these holding tanks and fed daily (Instant Algae® Shellfish Diet) for 6 weeks prior to their transfer to the exposure system.

Scallops were exposed to the water-accommodated fraction (WAF) of North Sea crude oil via a bead column to simulate a weathered oil spill (Carls et al., 1999). This column exposure system delivers an initial high concentration of PAHs to the exposure tanks followed by a progressive decline in PAH exposure (Camus and Olsen, 2008; Kennedy and Farrell, 2005; Olsen et al., 2008). The polyvinyl chloride column was filled with a layer of large glass beads (15 mmϕ), before adding 20 kg of soda glass beads (3 mmϕ) coated in crude oil (35 ml oil kg^{-1} beads). FSW was passed through the column for 24 h to remove the most volatile and soluble fractions that would usually evaporate during the initial hours after a spill. The column was then connected into a continuous flow system (CFS) of filtered seawater, with a flow rate of 0.5 l min^{-1} entering the exposure tanks. Scallops ($n=80$ per treatment) were subjected to either the WAF or the control (FSW) treatment for 21 days (8 ± 1 °C) and fed daily on the microalgae concentrate Shellfish Diet 1800, Instant Algae® (approx. 6×10^9 cells per tank d^{-1}). Due to space constraints and the inherent variability in PAH concentration of replicate WAFs, replicate exposure tanks were not used in the current study. All chemicals used during this study were obtained from Sigma-Aldrich, Norway, unless otherwise stated.

2.2. Chemical analyses

Seawater samples were collected from the exposure tank at time zero and after 1, 2, 4, 7, 14, and 21 d exposure (one sample per time point), and analysed for PAHs. The 16 priority PAH pollutants (U.S. EPA, 2009), along with the alkyl homologues of naphthalene, chrysene, dibenzophiothene and phenanthrene/anthracene, were measured based on the method described by Jonsson et al. (2004). Eight deuterated PAHs were added as quantitative internal standards (QIS) and mixed on a magnetic stirrer for 15 min prior to liquid–liquid extraction with 50 ml cyclohexane. The extraction was repeated a further two times to ensure PAHs were extracted into the solvent phase. Combined extracts were dried with anhydrous $NaSO_4$, concentrated to 0.5 ml (TurboVap 500, Zymark Corporation USA). and analysed using gas chromatography (HP5890, Hewlett Packard, USA) connected to a mass spectrometer (Finnigan SSQ7000, USA).

2.3. Biological analyses

Scallops were checked daily for mortalities, and any moribund animals were removed from the exposure system. Eight animals from the exposed and control (FSW) treatments were sampled at time zero and after 1, 2, 4, 7, 14 and 21 d exposure. At each sampling time, visual observations were carried out and notes were made on the valve gape, tissue condition and mantle retraction of the scallops. Haemolymph (0.9 ml) was withdrawn from the striated region of the adductor muscle using a 21 gauge needle (outer ∅ 0.8 mm) and transferred to a siliconised Eppendorf® tube. Due to the large amount of haemolymph extracted from the scallops, haemolymph samples were not pooled for analysis. Samples for determination of total haemocyte count, cell membrane stability, phagocytosis, cytotoxic capacity, and total protein content were stored on ice to minimise cell aggregation. Samples for glutathione analysis were prepared by centrifuging 100 µl haemolymph at $200 \times g$ for 5 min (4 °C), the supernatant was then removed and cells re-suspended in physiological saline (0.02 M HEPES, 0.4 M NaCl, 0.1 M $MgSO_4$, 0.01 M KCl, 0.01 M $CaCl_2$; pH 7.4). Haemocytes were lysed through sonification (30% duty cycle, 3×15 s; Ultrasonic Processor W-385, Heat Systems Ultrasonics, USA) in an ice bath and the resulting haemocyte lysate stored at −80 °C until analysis. Unprocessed haemolymph, for lipid peroxidation determination, was also stored at −80 °C until required. All measurements conducted on haemolymph samples were carried out in triplicate and absorbances determined using a Labsystems Multiskan RC microplate reader (Labsystems, USA).

After haemolymph collection, scallop dimensions were recorded and the soft tissue removed. The tissue wet weight was recorded, before and after drying at 60 °C for 18 h, and the condition index (CI) calculated:

$$CI = \frac{\textit{tissue dry weight}\,(g)}{\textit{shell length}\,(mm)} \times 100$$

For the determination of total cell counts, 20 µl haemolymph samples were diluted 1 in 4 with Baker's formol calcium (BFC: 2% sodium chloride, 1% calcium acetate, 4% formaldehyde) immediately after extraction, in order to fix cells and prevent cell aggregation. Total haemocyte counts were carried out using an Improved Neubauer haemocytometer under $\times 40$ magnification.

Cell membrane stability was assessed by measuring retention of neutral red (NR) dye (Babich and Borenfreund, 1992) described in detail by Hannam et al. (2009b). Haemolymph samples (50 µl) were pipetted onto a microplate in triplicate. After 45 min incubation at 4 °C, non-adhered cells were removed by rinsing with physiological saline and 200 µl aliquots of 0.004% NR solution were added to each well. After 3 h incubation at 20 °C, excess NR solution was removed by rinsing with physiological saline, and 200 µl acidified ethanol was added to breakdown cellular membranes and resolubilise the dye. The optical density (OD) of the retained neutral red was measured spectrophotometrically at 550 nm and expressed as a function of protein content.

Phagocytic activity of haemocytes was assessed by measuring the uptake of neutral red stained zymosan particles (from *Saccharomyces cerevisiae*) based on the method of Pipe et al. (1995) as previously described by Hannam et al. (2009a). Briefly, haemolymph samples were incubated at 4 °C for 1 h after which non-adhered cells were removed by rinsing with physiological saline (100 µl × 2) and 50 µl of dyed zymosan suspension (50×10^7 particles ml^{-1}) was added. The microplate was incubated for 30 min (20 °C), after which the reaction was halted through the addition of 100 µl BFC. Excess zymosan suspension was removed by rinsing with physiological saline and 100 µl acidified ethanol was added to solubilise the dye before recording the absorbance at 550 nm. Phagocytic uptake of zymosan particles by haemocytes was determined against a standard curve and expressed as a function of protein content in the 50 µl sample.

The ability of haemocytes to lyse target mammalian erythrocytes was used as a measurement of cytotoxicity (Raftos and Hutchinson, 1995). Haemolymph samples were extracted and diluted to a concentration of 2×10^6 cells ml^{-1} in physiological saline. One ml of sheep erythrocytes (TCS Biosciences Ltd.) was centrifuged at $200 \times g$ for 5 min before being re-suspended in phosphate buffered saline (PBS: 150 mM NaCl, 10 mM $NaPO_4$; pH 7.4). The suspension was centrifuged again and 125 µl of the red blood cell pellet was re-suspended in 1 ml of PBS; this was further diluted with 15 ml of Tris-buffered saline (10 mM Tris–HCl, 150 mM NaCl, 10 mM $CaCl_2$, pH 7.4). Triplicate 100 µl samples of diluted haemolymph were pipetted into round-bottomed microplates, along with duplicate controls of 100 µl PBS (for spontaneous lysis) and 100 µl PBS with 2 µl of 2% Triton-X100 (for maximum lysis). One hundred microlitres of the erythrocyte suspension was added to each well and the plate was incubated at 25 °C for 1 h. After centrifuging the plate at $100 \times g$ for 10 min, the supernatant was transferred to a flat-bottomed microplate and the percentage lysis was determined by measuring the haemoglobin released into the supernatant by its absorbance at 405 nm. The cytotoxicity was expressed as a percent lysis of all available target erythrocytes (the maximum lysis observed using PBS with 2% Triton-X100).

Determination of total glutathione (GSH+GSSG) was based on the cyclic reduction assay of Owens and Belcher (1965). Haemolymph lysate samples

(80 μl) were thawed in ice before adding 80 μl DTNB solution (10 mM DTNB, 100 mM KH_2PO_4, 5 mM EDTA). Aliquots of 40 μl DTNB-treated samples were transferred to a microplate and 210 μl of glutathione reductase solution (2.06 U ml^{-1} glutathione reductase, 100 mM KH_2PO_4, 5 mM EDTA; pH 7.5) was added. After allowing samples to equilibrate for 1 min, 60 μl of 1 mM NADPH was added to start the reaction and the change in absorbance measured kinetically at 405 nm for 10 min. Concentrations of total glutathione were determined against a 40 μM GSH standard, and expressed per mg protein.

Oxidative damage, in the form of haemocyte lipid peroxidation (LPO), was assessed using a modified method of thiobarbituric acid reacting substances (TBARS) (Camejo et al., 1999). Haemolymph samples were thawed on ice and transferred in 40 μl aliquots onto a microplate containing 10 μl BHT (1 mM 2,6-di-*O*-tert-butyl-4-metylphenol in absolute ethanol) to prevent further LPO. One hundred microlitres of extraction buffer (20 mM Tris–Cl, 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA; pH 7.6) was added to each well, followed by 50 μl TCA solution (50% w/v trichloroacetic acid) and 75 μl TBA solution (1% w/v thiobarbituric acid in 50 mM NaOH). After 60 min incubation (60 °C), the plate was cooled on ice and the absorbance at 530 nm recorded. Results were measured as malondialdehyde equivalents (MDA_e) determined against a standard curve using 1,1,3,3-tetraethoxypropane (0–24 μM), and expressed per mg protein.

Total protein concentrations were determined using the modified microplate method of Bradford (1976). Briefly, diluted haemolymph samples (1:3 physiological saline; 0.02 M HEPES, 0.4 M NaCl, 0.1 M $MgSO_4$, 0.01 M KCl, 0.01 M $CaCl_2$, pH 7.4) were transferred in 5 μl aliquots to a microplate. Five microlitre aliquots of a blank (physiological saline) and 5 μl protein standards (0.2–1.0 mg ml^{-1} bovine serum albumin (BSA)) were also added in triplicate. Two hundred microlitres of diluted BioRad reagent (1:5 distilled water) was added to each well and the absorbance (595 nm) recorded after 20 min incubation at 20 °C. The protein concentration was determined against the BSA standard curve and used to express cell membrane stability, phagocytosis, total glutathione and lipid peroxidation as a function of protein content.

2.4. Statistical analyses

Biological measurements were taken from 8 individuals from each treatment at each time point with mean values reported. Results were analysed using Statgraphics 5.1 (StatPoint Technologies Inc, USA). Data sets were checked for homogeneity of variance and univariate analysis was performed using two-way ANOVA or Kruskal–Wallis where appropriate. Post-hoc pairwise comparisons were conducted (Fisher's LSD) to identify where significant differences occurred at or above the 95% confidence level (associated probability < 0.05).

3. Results

3.1. Chemical analyses

Chemical analysis indicated that after an initial increase in the total PAH (sum of 27 PAHs) exposure concentration in the first 48 h, levels then decreased during the remaining exposure period (Table 1). The maximum total PAH concentration (163.4 μg l^{-1}) was measured 48 h after the start of the exposure, this then decreased to just 8% of this maxima after 21 d, with a recorded total PAH concentration of 13.3 μg l^{-1}. Naphthalene, and its alkyl homologues, dominated the PAHs throughout the exposure period accounting for ~96% of the total PAH concentration (Table 1); homologues with 1-methyl substitution (C1) had the highest concentration up to day 7, after which C3-naphthalene was the most abundant PAH. Only 5 other priority PAHs were recorded in the exposure system over the 21 d (acenaphthene, fluorene, phenanthrene, pyrene and chrysene), of which, only fluorene and phenanthrene were detected at concentrations in excess of 1 μg l^{-1}. The highest concentration of these two PAHs was measured after 7 d (Table 1), reflecting the shift from smaller more volatile PAHs (e.g. naphthalenes) to larger less soluble PAHs (e.g. phenanthrene).

3.2. Biological analyses

Mortalities were recorded during the acute oil exposure with only 62.5% of individuals surviving after 21 d (Fig. 1a); however

Table 1
PAH water concentrations in the exposure tank at different intervals after the start of the exposure ($n=1$). US EPA priority pollutants are indicated by an asterisk (*) and their method detection limits (MDL) are also detailed.

PAH	MDL (μg l^{-1})	Concentration (μg l^{-1})						
		1 h	24 h	48 h	96 h	7 d	14 d	21 d
*Naphthalene	0.005	17.82	53.87	49.70	31.84	17.30	3.28	0.56
C1-Naphthalene		20.10	61.44	70.59	66.20	51.92	20.39	3.17
C2-Naphthalene		11.01	28.49	34.88	35.74	35.01	22.12	5.92
C3-Naphthalene		2.22	4.58	5.77	6.68	7.39	6.69	2.36
*Acenaphthylene	0.005	–	–	–	–	–	–	–
*Acenaphthene	0.005	–	–	–	–	0.14	0.07	0.03
*Fluorene	0.005	0.30	0.90	1.02	1.11	1.18	0.57	0.30
*Phenanthrene	0.005	0.31	0.75	0.89	1.03	1.14	0.75	0.23
*Anthracene	0.005	–	–	–	–	–	–	–
C1-Phen/Anthr		0.36	0.45	0.53	0.61	0.75	0.68	0.33
C2-Phen/Anthr		–	–	–	–	–	0.31	0.23
Dibenzothiophene		–	–	–	–	0.16	0.10	0.04
C1-Dibenzothiophene		–	–	–	–	–	0.13	0.10
C2-Dibenzothiophene		–	–	–	–	–	–	–
*Fluoranthene	0.005	–	–	–	–	–	–	–
*Pyrene	0.005	–	–	–	–	–	0.01	–
*Benzo(a)anthracene	0.005	–	–	–	–	–	–	–
*Chrysene	0.005	–	–	–	–	–	0.01	–
C1-Chrysene		–	–	–	–	–	–	–
C2-Chrysene		–	–	–	–	–	–	–
*Benzo(b,j)fluoranthene	0.005	–	–	–	–	–	–	–
*Benzo(k)fluoranthene	0.005	–	–	–	–	–	–	–
*Benzo(b,j,k)fluoranthene		–	–	–	–	–	–	–
*Benzo(a)pyrene	0.005	–	–	–	–	–	–	–
*Indeno(1,2,3-cd)pyrene	0.01	–	–	–	–	–	–	–
*Benzo(g,h,i)perylene	0.01	–	–	–	–	–	–	–
*Dibenzo(a,h)anthracene	0.01	–	–	–	–	–	–	–
ΣPAH		52.11	150.48	163.38	143.20	114.98	55.11	13.29

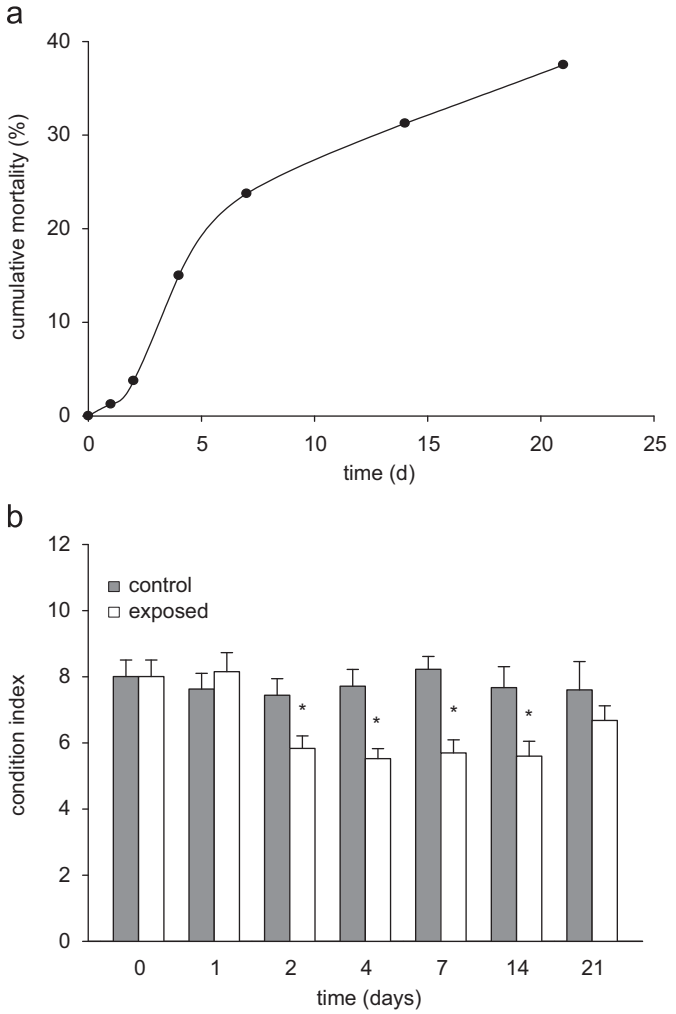

Fig. 1. (a) Cumulative mortality recorded and (b) condition index of surviving Arctic scallops (*Chlamys islandica*) after an acute exposure to the water accommodated fraction of crude oil for up to 21 d. CI data is expressed as mean values ($n=8$) ± 1 standard error and significant differences from the control ($P<0.05$) are indicated by an asterisk (*).

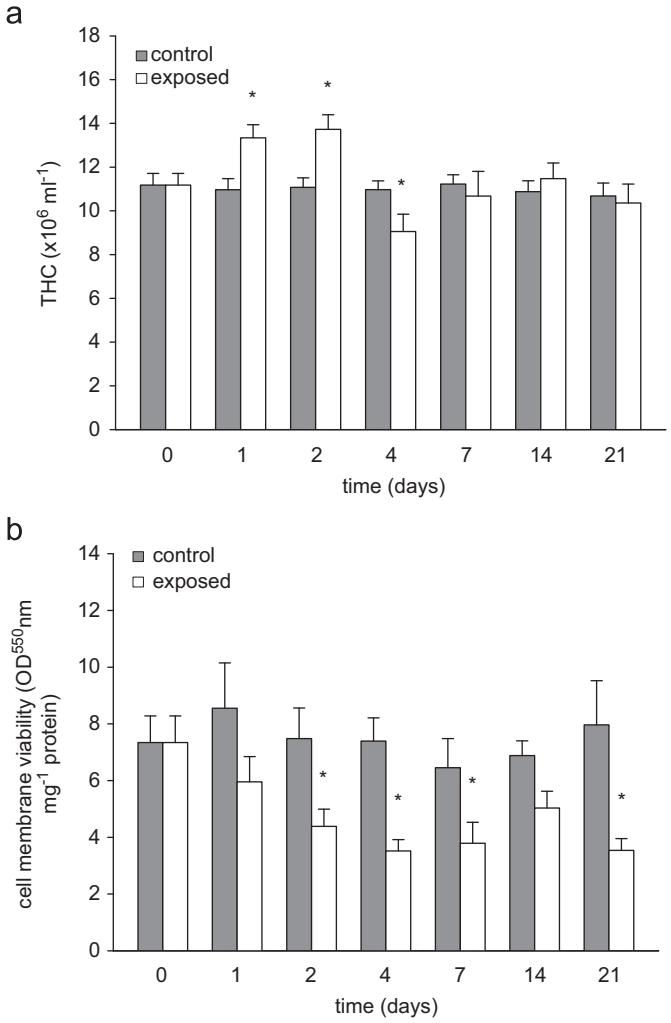

Fig. 2. Effects of acute oil exposure on the immune cells of *Chlamys. islandica*: (a) total haemocyte count (THC) and (b) cell membrane stability. Data is expressed as mean values ($n=8$) ± 1 standard error and significant differences from the control ($P<0.05$) are indicated by an asterisk (*).

no mortalities were recorded in the control treatment. The majority of the mortalities observed in the exposed scallops occurred in the first 7 days of the exposure, with 19 moribund animals observed, compared to only 5 mortalities recorded during the last 7 days of the exposure period (Fig. 1a). The condition index (CI) of *C. islandica* showed a significant interaction between treatment group and exposure time ($F_{6,97}=2.62$, $P<0.05$) with a reduction in the CI of scallops observed after 2 days. This reduction in CI corresponded to individuals exhibiting narcosis-like effects including mantle retraction and a slowed valve response in response to stimuli (visual observations only). The lowest CI was recorded after 4 d exposure; 28% less than the value recorded for the control scallops. The significant reduction in CI persisted up to day 14, after which the CI returned to similar values to those observed in the control organisms (Fig. 1b).

The number of circulating haemocytes in *C. islandica* was affected by both treatment group and exposure time with a significant interaction between these two factors ($F_{6,97}=3.14$, $P<0.01$). After 24 h the haemocyte count increased in contaminated animals compared to controls, with the highest THC (13.78×10^6 ml^{-1}) occurring in exposed scallops after day 2 (Fig. 2a). However, on day 4, the cell count declined to a level (9.06×10^6 ml^{-1}) below that of the control scallops, with 35% fewer cells observed compared to that recorded 2 days previous. As the exposure time progressed to 7 days, the THC returned to levels similar to scallops from the control treatment, and no significant difference in THC between exposed and control organisms was observed for the remainder of the 21 day experimental period (Fig. 2a). Cell membrane stability was significantly affected by acute oil exposure (Kruskal–Wallis=38.84, $P<0.001$). After 24 h, the cell membrane stability in exposed animals was impaired relative to the control, with a significant reduction observed after 2, 4, 7 and 21 days (Fig. 2b). The greatest decrease in cell membrane stability with an OD of 3.52 mg^{-1} protein was observed after 4 days exposure; 52% lower than the control scallops sampled for that respective time point.

Phagocytic activity in *C. islandica* was significantly altered by acute oil exposure (Kruskal–Wallis=66.05, $P<0.001$), with the number of particles phagocytosed increasing from 26.0×10^8 at the start of the exposure to 47.5×10^8 in oil-treated scallops after 24 h (Fig. 3a). This stimulation of phagocytosis changed to inhibition after 2 days exposure, with phagocytic ingestion reduced to 10.1×10^8 particles mg^{-1} protein. Impairment of the haemocyte phagocytic capability persisted throughout the remaining exposure period, with oil-exposed scallops exhibiting phagocytosis at 41–57% of the levels observed in the control

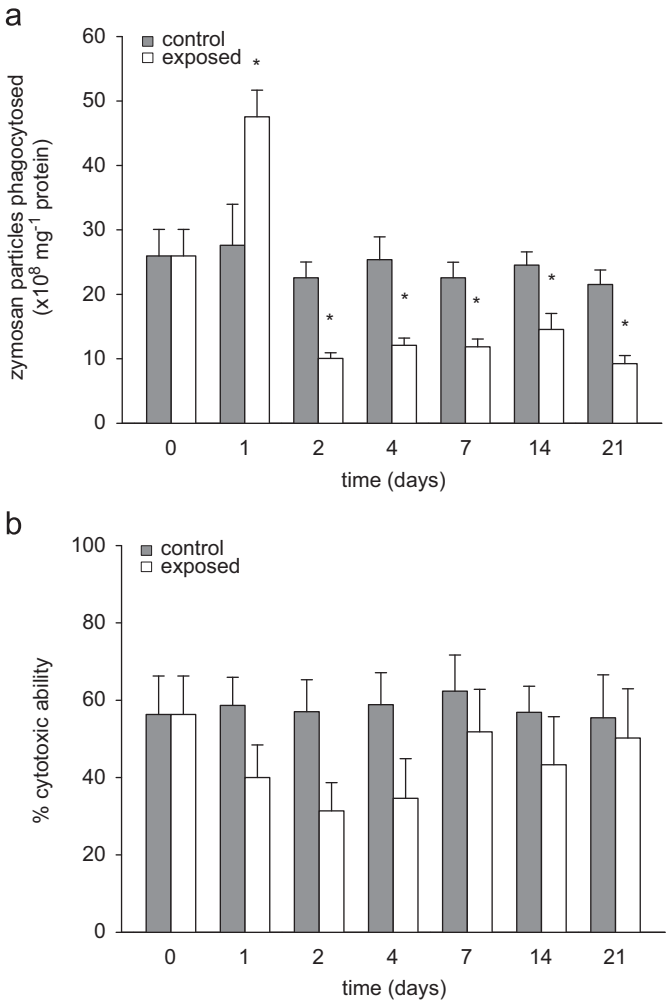

Fig. 3. Effects of acute oil exposure on the immune function of *Chlamys islandica*: (a) phagocytic activity and (b) cytotoxic ability. Data is expressed as mean values ($n=8$) ± 1 standard error and significant differences from the control ($P<0.05$) are indicated by an asterisk (*).

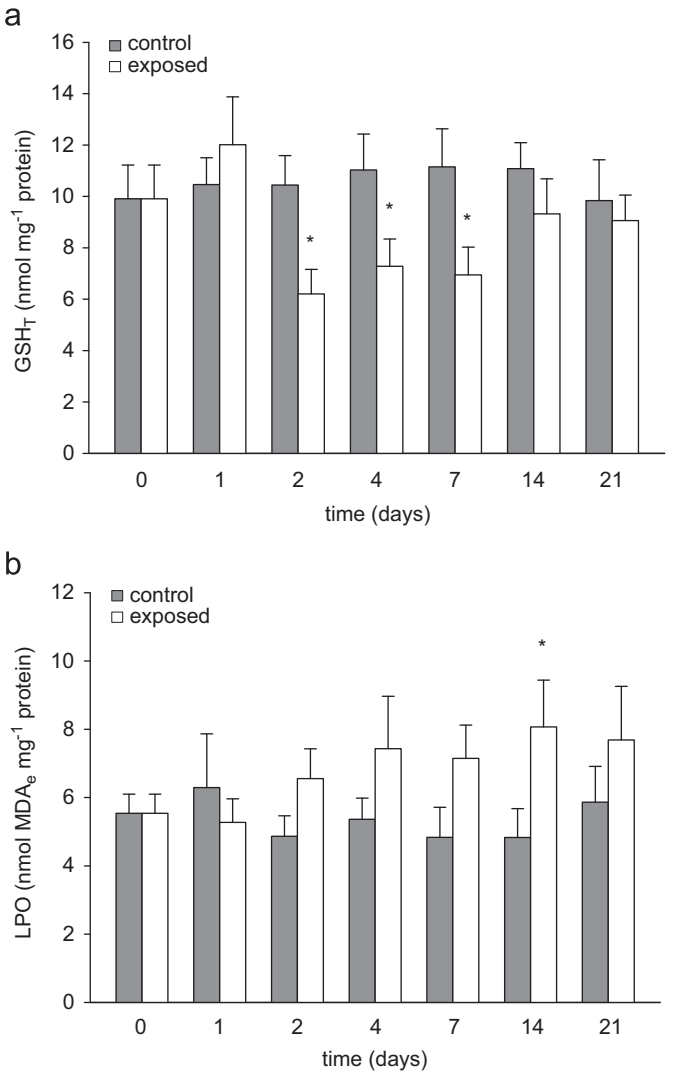

Fig. 4. Oxidative stress in *Chlamys islandica* following acute oil exposure: (a) total glutathione concentration (GSH_T) and (b) lipid peroxidation (LPO). Data is expressed as mean values ($n=8$) ± 1 standard error and significant differences from the control ($P<0.05$) are indicated by an asterisk (*).

groups for each time point (Fig. 3a). The ability of haemocytes to elicit a cytotoxic response to foreign cells was reduced in oil-exposed animals after just 24 h. Cytotoxic capability demonstrated the greatest inhibition after 2 days exposure, with 31.4% cytotoxicity compared to 57.0% in the control group (Fig. 3b). However, oil-exposed scallops demonstrated increased variation in the cytotoxic response and neither treatment group ($F_{1,97}=7.38$, $P=0.078$) nor exposure time ($F_{6,97}=0.50$, $P=0.810$) had a statistically significant effect on the cytotoxic capacity of *C. islandica*.

A significant depletion in glutathione (GSH+GSSG) was observed in *C. islandica* as a result of acute oil exposure ($F_{1,97}=7.46$, $P<0.01$). Total glutathione levels were lowest (6.2 nmol mg^{-1} protein) after day 2, 40% less than the concentration recorded in scallops from the control group. This reduction in total glutathione was also observed up to day 7, after which levels returned to control levels for the remainder of the exposure period (Fig. 4a). Lipid peroxidation (LPO) was significantly affected by exposure ($F_{1,97}=8.48$, $P<0.005$). LPO increased after 2 days in scallops from the acute oil treatment group and remained elevated up to day 21. Exposed organisms showed elevated LPO concentrations between 23% and 67% above the respective control groups (Fig. 4b). However, only the highest level of LPO, recorded after 14 days at 8.1 nmol mg^{-1} protein in exposed animals was significantly higher than the control value of 4.8 nmol mg^{-1} protein.

4. Discussion

PAHs are known to be a toxic component of the WAF of crude oil and were present at a concentration of 150 $\mu g\,l^{-1}$ at the beginning of exposure (24 h) followed by a decline in concentration over the 21 d. After an oil spill, PAH concentrations in the water column will vary according to environmental conditions and will also be dependent upon the quantity of oil released (Lee and Anderson, 2005). Total PAH concentrations in the water column have been reported to be in excess of 500 $\mu g\,l^{-1}$ after the Ekofisk blow out (Law, 1978), and Kingston (1999) reported high seawater levels of $\sim$1600 $\mu g\,l^{-1}$ following the Braer oil spill. Other studies have reported much lower concentrations after accidental oil spills, with levels of 6 $\mu g\,l^{-1}$ detected after the Exxon Valdez spill; however, this may reflect the rapid reduction in seawater PAHs post-spill, since these measurements were made 14 d after the initial spill (Short and Harris, 1996). After the

North Cape spill, Reddy and Quinn (1999) reported PAH levels of 115 $\mu g\,l^{-1}$, but predicted the initial concentrations following the spill to be in $>200\,\mu g\,l^{-1}$, indicating the PAH concentrations in the current study to be environmentally realistic.

Whilst PAHs concentrations were measured in the current study, many other compounds are also present in crude oil (Neff et al., 2000); PAHs only account for 1.6% of the composition of North Sea oil (Baussant et al., 2009). N-alkanes are a constituent of crude oil, although work by Skadsheim et al. (2000) only detected their presence in crude oil droplets, not in the WAF, so they are unlikely to be a source of toxicity in the current study. Alkylphenols (APs) are often present in the WAF of crude oil (Tollefsen et al., 2008). Whilst alkylated phenols may have contributed towards the immunotoxic effects here, the sublethal toxicity normally associated with APs concerns oestrogenic (Aarab et al., 2004) and genotoxic effects (Baršiene and Andreikenaite, 2007). Often, conventional GC analysis of crude oil indicates the presence of unresolved complex mixtures (UCM), which have also been reported in the WAF (Melbye et al., 2009). Branched alkylaromatic hydrocarbons, such as branched alkylbenzene, are likely to be present in this UCM and can be accumulated by marine bivalves (Booth et al., 2007); however, these branched alkylbenzenes were not found to have any effect on the cell membrane stability of *M. edulis* (Scarlett et al., 2008), so it is concluded that they are unlikely to be a contributing factor towards the immunotoxic effects observed here. Polar compounds are also a major component of oil and are reported to dominate the WAF of Norwegian crude, accounting for 70% of the organic compounds present (Melbye et al., 2009). These polar fractions are largely made up of cyclic and aromatic sulphoxide compounds, and have been shown to be genotoxic to fish (Tollefsen et al., 2008). Since polar compounds dominate WAF of crude oil, it should be noted that in addition to the measured PAHs, polar compounds may also account for a proportion of the toxicity to *C. islandica* observed in the current study.

For *C. islandica*, a simulated oil spill resulted in up to ~40% mortality after 21 d; similar oil-spill induced mortalities were reported in the field for the bivalves *Protothaca staminea* (Fukuyama et al., 2000) and *Mya arenaria* (Gilfillan and Vandermuelen, 1978). The scallops that survived the initial high dose of PAHs had a reduced condition index, and many individuals exhibited retraction of the mantle and a valve gape. Such a reduction in tissue condition has been reported previously for bivalves transplanted to an oiled environment (Culbertson et al., 2008) and exposed to oil-based drilling mud (Cranford et al., 1999). In scallops, glycogen is an essential metabolic energy reserve important in maintaining tissue condition (Barber and Blake, 2006). The metabolism of PAHs in the oil-exposed scallops may deplete these glycogen stores, limiting the energy available for tissue growth. A contaminant-induced increase in glycogen utilisation may therefore be a contributing factor in the observed reduction in condition index. Previous studies have also demonstrated a significant correlation between tissue glycogen content and condition index in mussels along a pollution gradient (Smolders et al., 2004), with mussels subjected to heavily contaminated sites having lower glycogen levels and an impaired condition index (Pridmore et al., 1990; Smolders et al., 2004).

The immune system of bivalve molluscs is dependent largely on the circulating haemocytes that carry out immune surveillance (Auffret, 2005). The number of circulating haemocytes can fluctuate under stressed conditions (Livingstone et al., 2000). For example haemocyte numbers increased in bivalves following oil spills in the field (Auffret et al., 2004; Dyrynda et al., 1997) and following exposure to fluoranthene (Coles et al., 1994); elevated haemocyte counts were observed in the current study during the initial stages of the exposure. Such a rapid increase in the number of circulating cells may reflect the stimulation of cell migration from the tissues (Auffret, 2005; Pipe et al., 1999) and, in this instance, is likely to be a compensatory mechanism for the compromised cell function demonstrated by the reduced cell membrane stability observed at the beginning of the exposure period. This is supported by the effects of chronic dispersed oil exposure on *C. islandica* where a significant increase in THC corresponded with a reduction in immune function (Hannam et al., 2009a). However, in addition to the increase in cell count, acute oil exposure in the current study also resulted in a decrease in the number of circulating haemocytes as the exposure period progressed. With PAHs reported to cause cytolysis in lysosome-rich cells, such as haemocytes (McCormick-Ray, 1987), the significant decrease in THC on day 4 is the likely result of PAH-induced cell death. Other studies have reported decreased haemocyte counts persisting for up to day 20 in bivalves following PAH exposure (Jeong and Cho, 2005; Pichaud et al., 2008). The recovery in cell numbers observed here as early as day 7 is likely to reflect the reduction in PAH concentration throughout the exposure and the proliferation of new haemocytes (Pipe et al., 1999). The current results suggest that the toxicity of oil to haemocytes and the subsequent effect on the number of circulating immuno-surveillance cells is dependent upon exposure period and concentration and can result in either an increase or decrease in THC.

With PAHs able to penetrate model membrane systems (Nelson et al., 1990), the reduced cell membrane stability, observed here, may be a result of lipophilic PAHs binding to membrane lipids which compromise basic cellular function by altering fluidity and ionic pumps (Camus, 2002). Such a decrease in cell membrane stability has also been reported in bivalves as a result of PAH (Camus et al., 2002), produced water (Hannam et al., 2009b) and dispersed oil exposure (Baussant et al., 2009).

In some instances, low exposure concentrations or short exposure times can result in an immuno-stimulatory effect; in the present study, acute oil exposure initially induced stimulation of the phagocytic activity in *C. islandica*. A short-term, low-dose stimulation of phagocytosis has also been reported in bivalves in response to contaminants including produced water (Hannam et al., 2009b), pesticides (Rickwood and Galloway, 2004), WAF of diesel (Hamoutene et al., 2004) and metals (Pipe et al., 1999; Sauvé et al., 2002). Whilst the increase in phagocytosis observed here in *C. islandica* was not associated with a low-dose exposure, it occurred after just a relatively short exposure on day 1, corresponding with an increase in the number of circulating haemocytes and prior to the compromised cell membrane stability observed from day 2 onwards. Due to the high energetic costs associated with phagocytic activity (Cheng, 1981), an initial immune-stimulation cannot be maintained and often changes towards suppression after longer exposure periods (Cheng and Sullivan, 1984; Pipe et al., 1999) as demonstrated by the current results. In addition, phagocytic processes are dependent upon the membrane properties of haemocytes; PAHs can interfere with the fluidity of cell membranes (Camus, 2002), restricting the deformation of the membrane essential to the phagocytic endocytosis process (Grundy et al., 1996). The reduced phagocytosis observed here on day 2, corresponded with significantly impaired cell membrane stability, suggesting that reduced cell membranes may have contributed towards the lower phagocytic activity.

In a range of molluscs, exposure to oil (Bado-Nilles et al., 2008; Dyrynda et al., 1997; Sami et al., 1992) and PAHs (Frouin et al., 2007; Gopalakrishnan et al., 2009; Matozzo et al., 2009; Pichaud et al., 2008; Wootton et al., 2003) results in a reduction in phagocytic activity; present results showed an inhibition of phagocytosis after 2 d exposure to the WAF of oil. Since the first

stage of phagocytosis relies upon recognition of the foreign body (Pipe and Coles, 1995), changes in the membrane receptors, such as those reported for oyster haemocytes exposed to PAHs (Sami et al., 1993), may affect the phagocytic capability of the cells.

Whilst the large variation in cytotoxic activity may have masked any significant effects, there was a general decrease in the cytotoxic reaction following acute oil exposure. The latter corresponded with the compromised cell membrane integrity, reflecting the fact that the ability of the haemocytes to lyse the target red blood cells is dependent upon cell to cell contact (Raftos and Hutchinson, 1995) and is therefore also dependent upon the membrane properties of the circulating haemocytes (Galloway et al., 2002).

The glutathione molecule (GSH) is a key component in an organism's antioxidant defence system with increased total glutathione concentrations reflecting an up regulation of antioxidant defences (Cheung et al., 2001). However, overwhelming of the antioxidant capacity can result in mass oxidation of GSH leading to excretion of the oxidised molecule (GSSG) from the cell resulting in a reduced intracellular concentration of total glutathione (Regoli et al., 1998). This excretion of GSSG makes intracellular total glutathione a more sensitive measure of oxidative stress than the intracellular ratio of GSH:GSSG. Results from the acute oil exposure in the present study suggest stimulated ROS production through PAH metabolism exceeded the neutralising capabilities of the antioxidant system after day 2 and reduced the total glutathione concentration. A similar reduction in total glutathione has been reported in bivalves exposed to organophosphorus compounds (Peña-Llopis et al., 2002), metals (Canesi et al., 1999) and transplanted to contaminated sites (Regoli and Principato, 1995), and also in crustaceans exposed to PAHs (Vijayavel et al., 2004). In bivalves, phagocytosis is accompanied by the production of oxyradicals to aid the destruction of invading foreign material (Pipe and Coles, 1995). However, the results from the current study indicate that this source of ROS is unlikely to be a contributing factor to the observed oxidative stress since reduced glutathione corresponded to a lower level of phagocytic activity.

Whilst the total glutathione concentration recovered to control levels by day 14, a significant increase in lipid peroxidation (LPO) was observed at this time point. This delay in the occurrence of LPO, following the overwhelmed antioxidant capacity observed on days 2–7, suggests that a reduced total glutathione concentration is a precursor to the oxidative damage of lipid membranes as proposed by Ringwood et al. (1999). The occurrence of LPO in bivalves following PAH exposure has been widely reported (Cheung et al., 2004; Kaloyianni et al., 2009; Pan et al., 2005) and can perturb membrane structure and function, which may contribute toward the reduced cell membrane stability observed during the later stages of the exposure period despite the reduction in PAH concentration.

5. Conclusion

Increased oil production in Arctic regions inevitably increases the risk of an accidental oil spill into the environment. Present results indicate that a simulated oil spill causes mortalities in the Arctic Scallop *C. islandica*. In addition, the individuals that survived the initial high dose of oil were subjected to immunotoxic effects and oxidative damage. The inhibition in immune function observed in *C. islandica* following an acute oil exposure is similar to that reported for exposure to chronic levels of dispersed oil (Hannam et al., 2009a). However, the reduction in these parameters as a result of chronic dispersed oil appeared reversible, with responses returning to control levels after a 7 d recovery period (Hannam et al., 2009a). In contrast, in the current study, cell membrane stability and phagocytic activity remained suppressed even at the end of the exposure period despite the reduction in ΣPAH exposure concentration, indicating the potential for prolonged sublethal effects following an oil spill. Whilst previous studies have indicated a higher oxyradical scavenging capacity in *C. islandica*, compared to temperate species (Regoli et al., 2000), present results suggest that this species is susceptible to oxidative stress. However, the depressed levels of total glutathione and the increase in lipid peroxidation in *C. islandica* had returned to control levels 21 d after the initial exposure suggesting that oxidative stress in exposed organisms may only be a short term effect of an accidental oil spill.

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