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Sublethal Behavioral Effects of the Water Accommodated Fractions of Crude Oil to Gastropod Molluscs

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Crude oils, and their water-soluble fractions, are highly variable and often complex mixtures containing many potentially toxic compounds (Andersen et al. 1974). The organic constituents present, including petroleum hydrocarbons and poly-aromatic hydrocarbons (PAHs), have been shown to be the most harmful components to aquatic organisms (Di Giulio et al. 1989). The various toxicants may elicit a number of deleterious responses upon release into the natural environment, depending on the dosage and susceptibilty of the exposed species. Sub-lethal impacts on individuals exposed to low levels of oil contaminants may be manifested as biochemical, carcinogenic, physiological and behavioural effects (Suchanek 1994). These sub-lethal impacts may compromise an organism's survival and reproductive success (fitness) in the long-term, and ultimately may result in effects at the population and community levels *in situ*. Sub-lethal organism responses are thus important to establish. If sensitive dose-dependent responses are identified, they may be employed as early warning biomarkers of higher-level impacts (Depledge et al. 1995). The development and utilisation of these tools may allow early remedial action to be taken to alleviate future damage at the population or community level.

Aquatic oil contamination due to human activities including: intentional tank purging, sewage effluents, run-off from ports, and spillages, is most likely to impact rocky shorelines of coasts and estuaries (Suchanek 1994; Stephensen et al. 2000). Gastropod molluscs are ubiquitous within these habitats (Beesley et al. 1998). Employing behavioural alterations of marine gastropod molluscs as specific measures of oil-induced sub-lethal responses is thus ecologically relevant and may be extremely valuable for monitoring exposure to complex pollutant mixtures, such as those occurring in crude oils, especially as the specific contaminants responsible for toxicity are often difficult to ascertain (Depledge and Fossi 1994). This approach may also prove a more cost effective screening procedure than identifying all of the many possible individual toxic components that may occur in unknown organic mixtures.

Locomotion is a stereotypic behavioural element of motor activity common across gastropod taxa which meets established criteria for behavioural endpoints of contaminant exposure and effect (Rand 1985). Locomotion may be directly measured in a quantitative fashion and can be accurately defined (Bayley 2002).

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Locomotor behaviour is directly related to the fitness of an organism, not only an adaptive response in order to maintain an individuals metabolic, neurological and physiological homoeostasis, but negotiating the environment in an adaptive fashion. Perturbations to an individuals locomotor behaviour can potentially have major impacts for temporal and spatial distributions; foraging, resource selection and acquisition; intra and inter-specific interactions (including competition for resources, territoriality and predation), survival and fitness (Adkins-Regan 2002).

Gastropod locomotor responses have been employed with success as sensitive endpoints of sub-lethal toxicity to oil pollutants in a number of past studies. These have included the locomotor responses of *Theodoxus fluviatilis* to the water accommodated fraction (WAF) of crude oil (Lindén 1977), locomotor activity of *Ophicardelus quoyi* to naphthalene (Mackey and Hodgkinson 1996), crawling rates of *Littorina littorea* on exposure to oil and dispersants (Hargrave and Newcome 1973) and crawling rates of *Cerethidea fluviatilis* after exposure to the water soluble fractions of diesel (Nagarajah et al. 1985). All studies have shown significant alterations to locomotor behaviour on exposure to environmentally relevant sub-lethal concentrations of oil products.

In the current experiments, a range of crude oil WAF exposures were employed in the laboratory to examine sub-lethal effects on spontaneous locomotor responses of a number of endemic Australian gastropod molluscs.

MATERIALS AND METHODS

Animals were collected from Merewether Beach (32°57'123"S, 151°45'276"E) or Wangi Wangi Point (33°04'50"S, 151°36'40"E), NSW Australia (NSW Fisheries Permit, F97/180) on the first morning of experiments (ASTM, 1997). A number of species were chosen to represent abundant gastropod molluscs: from the low to high tidal range of marine and estuarine rocky shores in South-Eastern Australia (Underwood 1994); with differing trophic associations (Beesley et al. 1998); and which exhibited varying mortality responses to an environmental oil spill in inner Sydney Harbour in 1999 (MacFarlane and Burchett 2003). The species chosen for testing included the microalgal grazer *Austrocochlea porcata* (Trochidae), 15-20mm; the Littorinid macroalgal grazer *Bembicium nanum*, 11-19 mm; the microalgal grazer *Nerita atramentosa* (Neritidae)11-19 mm and the predatory whelk, *Morula marginalba* (Muricidae), 12-18mm. Individuals underwent an acclimation period of four hours in aerated seawater tanks in the laboratory prior to experimentation(Hargrave and Newcombe 1973).

A 1:10 WAF stock was prepared for behavioural bioassays. WAFs were prepared with crude oil (McKee crude-Maui condensate (53%-47%) blend from Shell Co. Australia) and seawater in the appropriate proportions of 1:10 (oil: total volume). The stock and seawater were used to make dilution concentrations from 100-0.5% and 0% (seawater control). WAF dilutions were added to 100mL glass measuring cylinders (n = 5 replicates per treatment, 1 individual per replicate). Locomotor activity was determined from the vertical distance moved over five-minute intervals, for 20 minutes (0 hours). Mean distance moved by the gastropods was plotted against time for each treatment. The slope of the line (locomotor activity, mm/min) was used as the index of activity. The observations were repeated after gastropods had been exposed to the toxicant treatments for 24 hours. Behavioural bioassays were conducted in a temperature-controlled room without feeding (ASTM 1996). Water quality parameter ranges for the duration of the trials were: salinity 35ppt; pH 7.5 ± 0.2; dissolved oxygen concentration 4.1-6.9mg/L; and, humidity 70-75%. A partitioned ANOVA (Statsoft 1998) was applied to data, as individuals in the higher WAF concentrations often displayed no locomotor activity after initial exposure.

For quantifiable comparison of contaminant concentrations among species, and to other studies, the total petroleum hydrocarbons or TPH (C6-C9, C10-C14, C15-C28, C29-C36; TPH average recovery = 89%) and the poly-aromatic hydrocarbon (PAH; average recovery = 93%) content of the 1:10 WAF of crude oil were measured by Gas Chromatography and Gas Chromatography /Mass Spectrometry, MS (Hewlett Packard 5890 Series II, Hewlett Packard MSD, 5971A; fused silica capillary column, 45-270^{o}C at 10^{o}C/min, H_2/He carrier, flame ionisation or MS detection) after hexane extraction.

RESULTS AND DISCUSSION

All species tested exhibited significant reductions in locomotor activity on initial exposure to low levels of 1:10 WAF of crude oil, at low environmentally relevant concentrations.

For *A. porcata*, significant decreases in locomotor activity were seen at concentrations of 50% WAF within the initial 20 minute exposure period. After 24 hours, overall activity had decreased and movement had ceased at concentrations of 10 % WAF and above. For those individuals for which movement was observed, there were no significant differences among treatments (Table 1a, Table 2a).

Significant decreases in locomotor activity for *M. marginalba* occurred at concentrations of 50% WAF within the initial exposure period. Activity had decreased substantially after 24 hours; individuals in 50% WAF had ceased to be active. There were no significant differences among treatments for which movement was recorded at 24 hours (Table 1b, Table 2b).

Within the initial exposure period locomotor activity was significantly decreased for *B. nanum*, with no movement observed at concentrations of 10 % WAF and above. Of those treatments where activity was recorded, activity was significantly lower than the control treatment at 5 % WAF. After 24 hours, activity across all treatments had decreased. No activity was recorded in treatments of 10% WAF and above. There was no significant difference among treatments for which movement was recorded at 24 hours (Table 1c, Table 2c).

Initial significant decreases in locomotor activity were observed at 5% WAF and above for *N. atramentosa,* with no movement observed at 50% WAF. After 24 hours, no decreases in activity were observed among control treatments. No movement was observed in treatments of 5% WAF and above at 24 hours. Of those treatments where activity was observed, activity had significantly decreased at concentrations of 1% WAF (Table 1d, Table 2d).

Table 1. Locomotor activity of four species of gastropod mollusc exposed to 1:10 crude oil WAF dilutions over 24 hours.

Species	% 1:10 WAF Treatment	locomotor activity (mm/min) 0hr	locomotor activity (mm/min) 24hr
a). *A. porcata*	Control	17.6 ± 13.0*a*	4.1 ± 4.6
	1	17.4 ± 12.5*a*	8.9 ± 9.6
	5	9.2 ± 14.7*a*	4.1 ± 4.8
	10	6.4 ± 5.3*ab*	0.0
	50	0.4 ± 0.8*b*	0.0
	100	0.3 ± 0.6*b*	0.0
b). *M. marginalba*	Control	14.1 ± 10.1*a*	1.3 ± 1.0
	0.5	12.0 ± 8.4*a*	1.5 ± 1.9
	1	12.4 ± 3.8*a*	1.4 ± 2.9
	5	6.2 ± 8.1*a*	1.7 ± 2.4
	10	6.8 ± 5.4*a*	1.8 ± 3.6
	50	0.1 ± 0.1	0.0
c). *B. nanum*	Control	19.0 ± 8.5 *a*	4.6 ± 6.9
	0.5	2.4 ± 4.1 *bc*	0.7 ± 1.6
	1	7.2 ± 6.4 *ab*	1.2 ± 2.7
	5	0.1 ± 0.1 *c*	1.7 ± 2.4
	10	0.0	0.0
	50	0.0	0.0
d). *N. atramentosa*	Control	28.7 ± 12.2*a*	22.7 ± 9.5*a*
	0.5	5.7 ± 1.4*b*	16.3 ± 6.3*ab*
	1	14.4 ± 7.4*ab*	11.4 ± 3.6*b*
	5	4.2 ± 4.3*b*	0.0
	10	0.6 ± 1.2	0.0
	50	0.0	0.0

Locomotor activity (mm/min) over a 20 minute observation period (mean ± SE, treatments within species and time period identified as similar are linked with identical letters).

Comparing among species, *A .porcata* and *M. marginalba* (LOEC = 50% WAF, equivalent to [TPH] = 16.4μg/mL) were less sensitive than *B. nanum* and *N. atramentosa* (LOEC = 5% WAF, 1.6 μg/mL [TPH]) for decreases in locomotor activity immediately after exposure to treatments. After 24 hours, *N. atramentosa* was found to be the most sensitive species with a LOEC for decreased locomotor activity of 1% WAF ([TPH] = 0.3 μg/mL), *A. porcata* and *B. nanum* of intermediate sensitivity with a LOEC of 10% WAF ([TPH] = 3.3 μg/mL) and *M. marginalba* the least sensitive with a LOEC of 50% WAF ([TPH] = 16.4 μg/mL).

Evidence from a number of invertebrate phyla suggests both seretonergic and octopaminergic neurons are involved in the regulation of stereotyped locomotor sequences (Adkins-Regan 2002). Gravitational orientation is co-ordinated by bilaterally paired statocysts within the foot (Deliagina et al. 1998). The gastropod foot generates continuous retrograde ditaxic pedal waves of muscular contraction from longitudinal muscles. Such wave patterns are initiated by command neurons in the cerebral ganglia, are controlled by a central pattern generator in the pedal ganglia and may be affected by hydrocarbon exposure (Morton 1979; Barron 2002). Alkanes, aromatics and polyaromatic compounds (especially naphthalene) are components of petroleum oil which are narcotic in nature, causing anesthetic effects, hypoactivity, and depression of sensory function (Mackey and Hodgkinson 1996; Barron 2002). The 1:10 WAF employed in experiments contained 32.8 ug/mL TPH composed of 69% C6-C9, 19% C10-C14, and 11% C15-C28 respectively. PAH's were present at 0.31 ug/mL, with naphthalene, fluorene and penanthrene the most significant constituent PAH's. Effects of these organics on locomotion may be due to sensory interference effects alone (Battrup and Bailey 1998), and/or accumulation and subsequent alterations to neural membrane structure, function and synaptic transmission (Barron 2002). Total cessation of locomotor activity can alternatively represent the observed response of gastropods retreating into their shell in an attempt to evade exposure to the toxicants present in WAF dilutions.

Table 2. F values for partitioned one-way analysis of variance for locomotor activity of four species of gastropod mollusc exposed to 1:10 crude oil WAF dilutions over 24 hours (* $p< 0.05$, ** $p <0.01$).

Planned comparisons	a). *A. porcata*		b).*M. marginalba*		c). *B. nanum*		d).*N. atramentosa*	
	0 hr	24hr	0 hr	24hr	0 hr	24hr	0 hr	24hr
Treatments	6.86 **	4.19 **	6.71 **	0.91	8.48 **	2.06	36.41 **	284.6 **
Act. Vs No Act.		20.56 **		3.61	21.09 **	5.57 *	56.77 **	1407 **
Activity		0.21		0.23	7.09 **	1.50	31.32 **	7.73 **
No activity		0.0		0.0	0.0	0.0	0.0	0.0

For most species, movement in control treatments had decreased substantially after 24 hours, with *N. atramentosa* being the notable exception. Decreased movement suggests habituation or conditioning of organisms to the test environment. The spontaneous locomotor activity measured is an admixture of a highly active motile response to the unidirectional pathway of the test chamber and a tendency to travel in a vertical fashion facilitated by statocysts. Continued exposure to the test conditions may result in adversive conditioning and thus may reduce response patterns over time (Bayley 2002). For most of the species tested, these locomotor reductions continued in controls up to 96 hours. Similar reductions in locomotor activity in control treatments have also been observed for other gastropods in behavioural oil toxicity experiments after much shorter exposure periods of one hour (Nagarajah et al. 1985). The current findings suggest future sub-lethal behavioural testing should be conducted after brief acclimatization periods, within short-term test exposure periods to most accurately quantify changes in spontaneous locomotor activity to toxicants, free from adversive conditioning effects.

In order for behavioural bioassays to be environmentally relevant and instructive, future field validation is required. Laboratory based studies obtaining gastropods from oil-impacted locales and control locations or alternatively along an oil pollution gradient and quantifying relative locomotor behaviour under lab conditions after acclimation is required. Field manipulative studies or mesocosms are also important in order to measure locomotor activity under environmentally relevant conditions (Chapman 2000). Establishing causal links between sensitive initial locomotor perturbations and later effects at higher organisational levels including survival, reproduction, population and community effects is the ultimate goal of behavioural biomarker development.

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