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Oil Spill Remediation Techniques Can Have Different Impacts on Mixed Function Oxygenase Enzyme Activities in Fish

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Over the past decade, the use of biochemical indicators to measure xenobiotic exposure has been shown to be an effective tool, even when ambient concentrations can not be measured. The early detection of exposure to contaminants can lead to preventive management measures, as effects will be manifested at the subcellular level before effects are seen at higher levels of biological organisation (Stein et al. 1998).

The measurement of change in an organisms' biochemistry can assist in determining the types of impacts that may occur following exposure to anthropogenic inputs. Traditionally, the concentrations of chemicals present in the water column or in the tissue of the target species were determined and if these levels exceeded the guideline levels, measures were taken to improve conditions. However, this method of examining changes in the aquatic environment is non-specific and does not give an indication of the environmental impact of contaminants on biota (Australian and New Zealand Guidelines for Fresh and Marine Water Quality, 2000). This is especially the case when assessing the impacts of hydrocarbons in fish, as hydrocarbons are readily metabolised in fish and therefore, measuring residual hydrocarbon concentrations in tissue alone is of limited ecological and biological relevance (Collier et al. 1992).

A recognized method of studying site specific impacts of anthropogenic contaminants is to investigate biochemical responses in exposed organisms (Australian and New Zealand Guidelines for Fresh and Marine Water Quality, 2000). Biochemical markers (biomarkers) of exposure and effects are so reliable that they have been integrated into several large-scale monitoring programs such as NOAA's National Status and Trends Program, and the North Sea Task Force Monitoring Plan (Collier et al. 1995).

A common and reliable biomarker of exposure to petroleum hydrocarbons is the activity of hepatic microsomal ethoxyresorufin O-deethylase (EROD). EROD is an enzyme which utilises substrates representative of cytochrome P-450 isozymes. This enzyme is specifically induced following exposure to organic compounds and has been used as an effective biomarker of xenobiotic exposure in fish (Collier et al. 1992; Holdway et al. 1994a).

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The purpose of the present study was to investigate changes in EROD activity in fish livers following exposure to the water accommodated fraction (WAF) of Bass Strait crude oil, dispersed crude oil and burnt crude oil.

MATERIALS AND METHODS

This study monitored hepatic microsomal EROD activity after only a 4-day exposure to Bass Strait crude oil WAFs, followed by a 12-day depuration period in clean seawater to see if EROD activity was a useful biomarker of short term exposure.

Three month old Australian bass were obtained from New South Wales Fisheries and transferred to a flow-through seawater system at the Queenscliff Marine Station, Victoria, Australia for a two week acclimation period. The juvenile fish were fed starter pellets *ad libitum*. Fish were randomly distributed into 12 x 80 L tanks (n = 10 fish per tank) with a seawater flow-through rate of 150 ml/ minute, which ensured a 90 percent molecular replacement every seven hours (Sprague 1973). Fish weight at the beginning of the experiment was 1.08 ± 0.17 gram (mean ± S.E.M., n=10).

Crude oil WAF was prepared by pouring 13.5 litres of seawater into a 20 litre glass mixing chamber and creating a vortex using a magnetic stirrer. Bass Strait crude oil (1.5 litres) was then added (ratio crude oil:water, 1:9) and the solution was capped and mixed for 20 hours. After one hour settlement, the oil WAF was isolated via a tap at the bottom of the mixing chamber. The dispersed oil WAF was prepared using the same methodology, except that dispersant was added after the oil at the recommended ratio of 1:30 (dispersant: oil) (Gilbert 1996). Burnt crude oil WAF was prepared by mixing oil and seawater for 5 minutes, and after a one-hour settling time, the surface emulsion was ignited. After 20-minutes of burning, the burnt crude oil WAF was isolated via a tap at the bottom of the burn chamber. Stock solutions (WAFs) were prepared daily.

Initially, the various WAFs were added to the tanks (1.6 L WAF per tank) to commence the exposure to 2% WAFs. Immediately following the initial addition of WAF, the different WAFs were delivered at a rate of 3 ml/min by a peristaltic pump which dispensed the toxicant solutions into an overhead mixing chamber where seawater and stock solutions were mixed. This resulted in an exposure concentration in the aquariums equivalent to 2% WAF. The 2% concentrations were chosen, based on the 96-h LC_{50} values established by Cohen & Nugegoda (2000), as being 7.2%, 43.2%, and 49.8% for dispersed crude oil WAF, crude oil WAF and burnt crude oil WAF, respectively. In the present experiment, there were three treatments (crude oil WAF, dispersed crude oil WAF and burnt crude oil WAF), plus control groups. Control fish (n = 10 per tank, 4 tanks) were sampled at time zero, immediately prior to the addition of WAFs, to determine baseline EROD activity. Ten fish were sampled on Day 4 from each aquariums, after which the input of WAFs was stoped to commence the depuration period. On Day 16 (after 12 days of depuration in clean seawater), the remaining fish

were sampled for hepatic microsomal EROD activity. Physicochemical parameters were monitored every four days throughout the experiment in each aquarium. Seawater petroleum hydrocarbon concentrations were analysed by gas chromatography – flame ionization detection as described in Cohen et al. (2005). The detection limit of this method is 1 µg/L for individual PAHs. Stock solutions were analyzed instead of experimental concentrations in exposure chambers, because WAF stock solutions concentrations were believed to provide more accurate measurements than experimental concentrations, as the concentration in the exposure chambers would be in some instance, below laboratory detection limits.

All procedures in microsomal preparation were conducted at 4 °C according to the methods of Holdway et al. (1994b). Livers were weighed and added to homogenizer buffer (0.1M of K_2HPO_4 and KH_2PO_4 with 1mM dithiothreitol, 1mM EDTA, 0.1M KCL, 0.1M phenanthroline), pH 7.4. An Ultraturax homogeniser was used to produce a liver homogenate. The homogenate was then centrifuged using a Heraeus centrifuge (10,000g) at 4°C for 20 minutes. The supernatant (ie S9 fraction) was collected and used immediately. Resorufin was used to prepare the standard. For each assay four tubes were set up, with the test sample in triplicate and a blank. Tris buffer (0.45ml 0.1M), 0.25ml cofactor (10 mM magnesium chloride, 200 mM potassium chloride, 6 mM glucose-6-phosphate, 1.25 mM NADP and 100 U glucose-6-phosphate dehydrogenase), 0.1ml microsomes and 0.1ml BSA were added to each tube and preincubated at 30°C for two minutes. Ethoxyresorufin was dissolved in 1:1 methanol:DMSO and diluted to a working concentration in 0.1 M Tris buffer, pH 7.6. The reaction was started by adding 0.1 ml ethoxyresorufin to test vials only and tubes were incubated at 30°C for 10 minutes. Tubes were then removed from the water bath and placed on ice and the reaction was stopped with the addition of 2.5ml methanol. Tubes were then vortexed and 0.1ml of ethoxyresorufin was added to blanks and standards. Tubes were centrifuged at 2000 G for five minutes. The supernatant (1 ml) was removed from each tube and the fluorescence was recorded using a spectrofluorimeter at an excitation wavelength of 530 nm and an emission wavelength of 585nm. Protein analyses were performed on each sample using the method of Lowry et al. (1951). Results were reported in pmol resorufin min^{-1} mg $protein^{-1}$.

For EROD activity, analysis of variance (ANOVA) was applied to detect true differences amongst treatments. ANOVA was run on log_{10}-transformed data to achieve normal distribution. Several control fish had very low EROD activity; therefore to allow log transformations, a value of 1.0 was added to each measured EROD activity before transformation, and subtracted again after log means were decoded. Data normality was tested using a Shapiro-Wilk test. Homogeneity of variances was confirmed by examination of residual versus predicted values. A post-hoc Tukey multiple comparisons test was used to determine statistical differences amongst treatments at $p < 0.05$.

The appropriate graphical representation of log-transformed data uses geometric means, which has been computed as the antilog of the arithmetic mean of the

logarithm of the data (Zar 1984). Back-transformed standard deviations are uninterpretable and therefore, the variance of the geometric mean is represented as the back-transformed 95% confidence interval (C.I.) of the geometric mean (Zar 1984). When plotted on an arithmetic scale, the 95% C.I. result in asymmetrical confidence limits, reflecting the skewed distribution of the original data. Therefore, the Y-axis of any geometric mean and its associated C.I. has to be presented on a logarithm scale. This presents data in the original units, but plotted in a way that reflects the normal log distribution and therefore, 'log-symmetrical' C.I. (Zar 1984; Hodson et al. 1996).

RESULTS AND DISCUSSION

The physicochemical parameters (mean ± S.D., n = 48) remained stable over the experimental period. Salinity remained constant at 35 ± 0.2 ppt, pH at 8.1 ± 0.1, conductivity at 52.6 µs/cm, dissolved oxygen at 6.4 ± 0.2 mg/l and temperature at 17.7 ± 0.3°C.

The total petroleum hydrocarbon (TPH) concentrations in the stock solutions for the crude oil WAF, dispersed crude oil WAF and burnt crude oil WAF treatments were 6.3 mg/l, 15.8 mg/l and 4.9 mg/l respectively. The resulting nominal concentrations in the experimental tanks were 98 µg/l, 384 µg/l and 125 µg/l for the crude oil WAF, dispersed oil WAF and burnt crude oil WAF respectively. Petroleum hydrocarbon background levels in seawater were non-detectable (N.D.).

There was no significant difference in EROD activity in the control treatments between Days 0, 4 and 16 ($p > 0.05$, Figure 1). There was a significant increase in EROD activity in fish exposed to the WAF of Bass Strait crude oil, dispersed crude oil and burnt crude oil, compared to the control fish after 4 days of exposure ($p < 0.000$, Figure 1). The Post Hoc test indicated that on Day 4 of the experiment, the EROD activity in the dispersed oil group was significantly higher than in the other treatments, with hepatic microsomal EROD activity in fish of 42.3 pmol min^{-1} mg $protein^{-1}$. On day 4, fish exposed to crude oil WAF had significantly ($p < 0.05$) lower EROD activity (19.4 pmol min^{-1} mg $protein^{-1}$), compared to fish in the dispersed crude oil treatment. However, EROD activity levels in the crude oil WAF treatment were similar to those in the burnt crude oil treatment, the later resulting in EROD activity in fish of 16.2 pmol min^{-1} mg $protein^{-1}$ (Figure 1).

After the 12 day depuration period (day 16 of the experiment) there was no significant difference in EROD activity amongst treatments on that day, or relative to all other control groups ($p > 0.05$), indicating that the EROD activity in fish had returned to background levels following the 12 day depuration in clean seawater.

EROD-inducing petroleum hydrocarbons present in the dispersed crude oil treatment were bioavailable and were taken up by the fish, resulting in

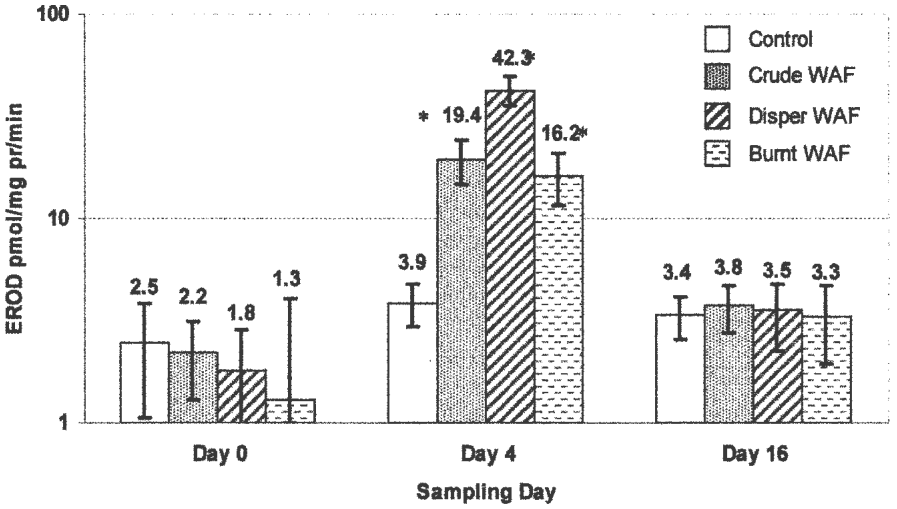

Figure 1. EROD activity (geometric mean ± 95% CI., n = 30 per treatment) in the liver of Australian bass exposed to WAF of Bass Strait crude oil, dispersed crude oil and burnt crude oil. *denotes differences between treatments at $p<0.05$.

significantly elevated EROD activity in the dispersed oil group following 4 days exposure. This was coincident with the high petroleum hydrocarbon concentration in the dispersed crude oil WAF treatment. Elevated liver EROD activities have been associated with high concentrations of petroleum hydrocarbons in fish flesh and in the ambient water (Arinc et al. 2000, Cohen et al. 2001). It has been established that the use of dispersant increases significantly not only total PAHs in the water column, but also increases the proportion of high molecular weight PAHs bioavailable to fish (Couillard et al. 2005). PAHs have been recognized as potent inducers of cytochrome P4501A enzymes (Arinc et al. 2000), with resulting PAH metabolites being potentially genotoxic (Collier et al. 1996). In this regard, results of the present study indicate that the use of dispersants to prevent short-term impacts on a given ecosystem (ie prevent the oil reaching a shoreline) has the potential to induce long-term damage to fish via molecular mechanism such as genotoxicity.

In the crude oil and burnt crude WAF-exposed groups, uptake of bioavailable petroleum compounds was occurring, but to a lesser extent than in the dispersed oil group. This resulted in lower EROD activity in these groups relative to the dispersed oil group, however the EROD activity was significantly higher in the crude oil WAF and in the burnt WAF groups than all control groups. Lower EROD activity might result in lower genotoxic potential as less PAH metabolites would be formed. In the present study, EROD activity correlates well to the concentrations of petroleum hydrocarbons in the water column. Under field situations where oil has been spilt however, petroleum hydrocarbon concentrations might be lower than the concentrations observed in the present study. One month after the Sea Empress spill off the coast of Wales in February

1996, petroleum hydrocarbons water column concentrations were less than 0.5 µg / L (Lunel and Lewis 1999). It is not expected that an induction of the EROD enzymatic activity would be observed under such conditions. It appears that the use of EROD activity induction as a biomarker of exposure to spilt crude oil would be limited to freshly spilt oil when highest concentrations of petroleum hydrocarbons occur in the water column, or in environments where the oil remains undegraded and undispersed for long periods eg when tar balls formation occur.

Despite the fact that petroleum hydrocarbons might return to undetectable levels within months following a large spill (Lunel and Lewis 1999), some persistent PAHs can still be bioavailable and taken up by aquatic biota. Monitoring long-term bioavailability of petroleum hydrocarbons to fish would be better performed by measuring PAH biliary metabolites. Following metabolisation of petroleum hydrocarbons by liver enzymes, PAH metabolites are directed into the bile where they accumulate awaiting elimination via the intestines. Benthic fish have been found to accumulate biliary PAH metabolites *in situ* for up to two years following an oil spill, confirming low level continuous exposure of fish to PAHs (Collier et al. 1996).

EROD activity as well as PAH biliary metabolites are biomarkers of exposure to crude oil, and no adverse biological effects have been directly associated to the measurement of these biomarkers. However, metabolisation of PAHs by the liver enzymes can lead to the formation of reactive metabolites which interact with bioproteins such as DNA. The formation of adducts between DNA and PAH metabolites has been related to increased cancer rates in field-caught fish populations (Collier et al. 1996). This relationship between biomarkers of exposure and effect emphasises the need to assess a suite of biomarkers when assessing the health of field-caught fish populations.

The present study has shown that between the techniques of 'natural weathering', chemical dispersion, or burning the spilt crude oil, chemically dispersing the crude oil resulted in the highest EROD induction in Australian bass over 4 days of exposure. A return to clean seawater for 12 days resulted in pre-exposure EROD levels. Ultimately, the decision of using dispersants on spilt crude oil will consider a variety of socio-economic and environmental factors with key elements such as local environmental conditions and receiving environment. These factors will influence the decision making, resulting in severe short-term effects, ie the use of chemical dispersants, or in chronic but subtle effects, ie natural weathering or burning of the oil. In all cases, a suite of biomarkers can inform on the health of fish populations inhabiting the receiving environment.

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