MFO INDUCTION IN ATLANTIC SALMON (SALMO SALAR) DURING AND AFTER EXPOSURE TO BASS STRAIT CRUDE OIL

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ABSTRACT

Biochemical markers of exposure (mixed function oxygenase: MFO as determined by ethoxyresorufin O-deethylase (EROD) activity) and of effect (sorbitol dehydrogenase: SDH) were investigated for use during and after exposure of Atlantic salmon (*Salmo salar*) to low levels of Bass Strait crude oil water-accommodated fraction (WAF) and dispersed crude oil. EROD activity was significantly induced after only two days of exposure to dispersed oil, while four days of exposure were necessary to significantly induce EROD in the WAF-exposed salmon. Following the termination of exposure, EROD induction remained elevated for eight days in both the WAF-exposed and the dispersed oil-exposed fish. Dispersing the oil using Corexit 9527 produced similar EROD activity levels in salmon relative to WAF only. Serum SDH activity confirmed that no hepatocellular injury was caused by exposure of salmon to these levels of WAF or to dispersed Bass Strait crude oil. It is concluded that MFO induction, as measured by changes in EROD activity, can be used for confirmation of low-level exposure of commercial salmon stocks to petroleum-contaminated waters for up to eight days after the event.

Key words: Atlantic salmon, oil spill, EROD, mixed function oxygenase

INTRODUCTION

Most biomarkers of exposure used in aquatic environmental monitoring have been developed and validated through chronic exposure of organisms to xenobiotics, while few biomarkers of short term sub-acute exposure have been identified. Although bioindicators of chronic exposure assist environmental managers in better understanding environmental health, indicators of short-term exposure could indicate if commercially important fish stocks have been pulse-exposed to contaminated waters for short periods of time.

In Australia, oil exploration, extraction and transport is a fast growing industry with many industrial activities centred in Bass Strait, in the South-East part of the continent. Oil slicks drifting from this area during a major oil spill could affect the commercial fisheries in this part of Australia by direct toxicity of the dissolved and dispersed oil in water, or by affecting the commercial quality of the edible fish. Atlantic salmon (*Salmo salar*) farming operations are a highly valuable resource in Tasmania where salmon are held in seacages from a juvenile stage to up to 20 months before they reach a marketable size.

Bass Strait crude oils are classified as light crude oils, with aromatic hydrocarbons accounting for up to 45% of the total hydrocarbons (Volkman *et al.* 1994). As aromatic hydrocarbons are relatively soluble in water, it is expected that the potential of this Australian crude oil to adversely affect aquatic organisms is higher than for heavier, less water-soluble crude oils.

It is known that fish can rapidly pick up and accumulate petroleum compounds from the water soluble fraction of crude oil, resulting in adverse physiological effects such as reduced androgen levels (Truscott *et al.* 1983), liver enlargement and depletion of reduced glutathione (GSH) (Steadman *et al.* 1991), DNA adduct formation (Collier *et al.* 1996) and the development of

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hepatocellular carcinomas (Van Veld *et al.* 1992). Commercial impacts such as tainting of the flesh can occur at very low levels of exposure (Heras *et al.* 1993).

Following exposure to petroleum hydrocarbons, the induction of fish hepatic mixed function oxygenases as measured by increased ethoxyresorufin O-deethylase (EROD) activity provides a sensitive method for monitoring the environmental exposure to petroleum-contaminated water mass (Collier *et al.* 1995). However, the potency of Australian crude oils to cause MFO induction in fish is unknown. It is useful to have information on the time necessary for significant EROD induction during exposure to petroleum compounds, along with information on the persistence of the induction following the termination of exposure in relation to hydrocarbon depuration, so that suspected exposure of commercial fish stocks to contaminated water masses may be evaluated within a determined time frame.

Exposure to petroleum compounds during an oil spill event may be increased by the use of dispersants (containing mainly surfactants, solvents and additives (Clayton *et al.* 1993). Dispersants are one of the first options considered in the event of an oil spill, and some companies have obtained pre-approval to use dispersants in offshore areas of Victoria (Volkman *et al.* 1994). Because of their amphipathic nature (ie. opposing solubility tendencies), surfactants reduce the oil-water interfacial surface tension and increase the amount of oil dispersed in the water column in the form of fine droplets. Therefore, the use of dispersants as an oil spill remediation technique may increase by several-fold the exposure level of aquatic organisms to petroleum compounds in the water column (Fucik *et al.* 1995).

In this study, juvenile Atlantic salmon were exposed to low levels of the water-accommodated fraction (WAF) of Bass Strait crude oil or dispersed Bass Strait crude oil for a period of four days. Fish were sampled during and after exposure to determine the possibility of using EROD induction as an indicator of exposure to Bass Strait crude oil or to dispersed crude oil. Determinations of sorbitol dehydrogenase (SDH) were also performed to investigate possible liver damage caused by exposure to petroleum hydrocarbons or to dispersant. Results presented in this publication are part of a larger study on the identification and validation of biomarkers of exposure suitable for the monitoring of short-term exposure to petroleum compounds as would occur during an oil spill event.

Relating biomarker responses to short term petroleum exposure offers considerable potential for a scientific assessment of commercial fish stock exposure to contaminated waters, and could also provide an estimate of the time necessary for depuration of fish prior to marketing. If the biomarker reveals petroleum exposure in a significant portion of the sample, it is likely to have direct relevance for the entire commercial fish stock. This study focuses on the use of EROD activity induction to detect exposure rather than adverse ecological effects. The experimental conditions applied are an attempt to bridge some of the gaps between the controlled laboratory assay and the more variable field conditions in an aquaculture set up, by combining characteristics of both.

METHODS

Fish stock

Juvenile Atlantic salmon (80-120g), all immature females, were obtained from Salmon Enterprises of Tasmania (Saltas) Ltd. Fish stocks had been previously subjected to the process of feminisation by the sex-inversed male parent method. This method uses female genitors functioning as males by the feeding of an androgenised diet (Bye and Lincoln 1986). Therefore, all eggs fertilised by a masculinised female result in female progeny. Fish were transported by ferry to Queenscliff Marine Station (Queenscliff, Victoria) in 1000 L of oxygenated brine water (10 ppt) and upon arrival were distributed into six 1000-L tanks containing water of similar salinity. Salt water pumped from Port Philip Bay was introduced at a rate of 10L/minute to transfer fish from brine to salt water. As variations in basal MFO and EROD levels have been observed for up to 40 days following transfer to saltwater (Vandermeulen *et al.* 1994), six weeks of acclimation have been allowed. During the six-week acclimation period, fish were fed *ad libidum* with salmon starter pellets, and tanks were cleaned daily to remove wastes. Mortality during the six weeks preceding the start of the experiment was <1%.

Crude oil and dispersant

Stabilised Bass Strait crude oil and the dispersant Corexit 9527 were provided by Esso Australia and Nalco Ltd respectively. The oil was kept in a sealed 200-L barrel with no head space until collection of a subsample for the preparation of the exposure solutions. Corexit 9527 is a *type III* concentrated dispersant, with surfactants as active ingredients; surfactants are specifically designed to have both hydrophilic and hydrophobic groups to help the formation of small oil droplets, facilitating breakdown and dispersion in the water column.

WAF and oil-dispersant mixtures

The water-accommodated fraction of Bass Strait crude oil was produced by mixing stabilised crude oil and seawater in a ratio of 1:100 (one part of oil in 99 parts seawater) for 23 hours. To obtain the oil-dispersant mixture, oil and seawater were mixed for one hour, and Corexit 9527 was added with a Pasteur pipette to the surface of the oil-seawater mixture at the ratio of 20:1 oil:dispersant (Gilbert 1996). Both mixtures were then allowed to settle for 1 hour prior to the isolation of the water-accommodated fraction from a tap located at the bottom of the 23 L mixing bottle (Girling 1989).

A toxicity range finder using WAF and dispersed oil established that exposure to a concentration of 500 ppm (0.05%) of oil-dispersant mixture for three hours was lethal to the fish. However, the animals apparently remained healthy following four days of exposure to 250 ppm (0.025%) of WAF or dispersed oil. Consequently, the exposure level for the experiment was set at 250 ppm WAF or oil-dispersant mixture, which was expected to cause physiological stress but no mortalities. The final concentration of chemical dispersant in the water column during the exposure (0.132 ppm) was not expected to cause any toxicity. Previous experimentation showed Corexit 9527 to be practically non-toxic (LC50: 100-1000 ppm) to *Oncorhynchus mykiss*, with similar results for fathead minnow *Pimephales promelas* and mummichog *Fundulus heteroclitus* (Gilbert 1996). Analysis of total petroleum hydrocarbons present in the seawater was not performed.

To realistically simulate time of exposure to dissolved petroleum compounds during an oil spill event, an initial two-day exposure to 250 ppm WAF or dispersed oil was followed by two days' exposure to 125 ppm and a depuration period of 57 days. Less than 1% mortality occurred during the 61 days of the experiment. Simultaneous exposures were run in duplicate, ie. two tanks exposed to WAF and two tanks exposed to dispersed oil were compared with two control tanks. Seawater was pumped from Port Phillip Bay and sand filtered before being distributed to the six tanks. Water quality parameters were measured daily at the outflow point of each tank and were very similar in all six tanks (Table 1).

Fish sampling

Eight Atlantic salmon per tank per sampling day were killed by a blow on the head and weighed. Blood was immediately collected by caudal puncture and allowed to clot on ice for 15 minutes before centrifugation at 5000 rpm for 5 minutes. Serum was isolated and kept frozen at -80°C until analysis for sorbitol dehydrogenase (SDH) levels by photometric determination (Sigma procedure No 50-UV).

The liver was removed, weighed, rinsed with an ice-cold solution of 0.15 M KCl, minced and frozen in liquid nitrogen until analysis for ethoxyresorufin-*O*-deethylase (EROD) activity by the fluorimetric method described in Hodson *et al.* (1991). Weights of carcasses (whole body minus viscera) were also recorded.

Table 1. Physicochemical characteristics of the seawater [mean (±SE) in the six tanks] during the experimental period.

Sampling Day/Date	DO (%)	Temperature (°C)	Salinity(ppt)	pH
Acclimation[a]	86.67 (0.93)	11.83 (0.10)	36.10 (0.04)	7.59 (0.01)
Day 0 : 29/07/96	87.95 (1.45)	12.36 (0.02)	36.42 (0.03)	7.70 (0.01)
Day 2 : 31/07/96	88.68 (1.05)	11.96 (0.01)	36.22 (0.04)	7.65 (0.01)
Day 4 : 02/08/96	88.45 (0.75)	12.21 (0.01)	36.55 (0.02)	7.72 (0.01)
Day 6 : 04/08/96	90.65 (0.69)	11.93 (0.004)	36.45 (0.03)	7.73 (0.01)
Day 8 : 06/08/96	92.48 (0.46)	11.08 (0.003)	35.65 (0.08)	7.70 (0.004)
Day 10 : 08/08/96	95.83 (0.67)	9.68 (0.01)	35.80 (0.05)	7.68 (0.01)
Day 12 : 10/08/96	98.78 (0.34)	11.17 (0.01)	35.52 (0.04)	7.76 (0.01)
Day 19 : 17/08/96	NA[b]	11.50 (0.01)	NA	NA
Day 33 : 31/08/96	88.57 (0.71)	12.41 (0.01)	36.08 (0.02)	7.90 (0.01)
Day 61 : 28/09/96	89.70 (1.19)	12.38 (0.12)	35.33 (0.08)	7.86 (0.01)

[a] Averaged over 6 weeks (June 12 to July 29, 1996)
[b] NA: not available

Statistical analysis

In this hierarchical study design, mixed ANOVAs (two-way analysis of variance with treatment and time as orthogonal factors and tanks nested in the treatment) were applied to detect true differences among sampling days or treatment (control, crude oil or dispersed oil-exposed fish) (Zar 1984). Mixed ANOVAs were run on original SDH data, as for liver somatic index (LSI = 100x liver weight/body weight) and condition factor (CF = 100{W/L^3}). EROD activities were log-transformed to achieve normal distribution, which was tested with a Shapiro-Wilk test. Homogeneity of variance was confirmed by examination of residual versus predicted values (Zar 1984). Post-hoc Tukey Compromise tests were used to determine statistical differences between sampling times and groups at $p \leq 0.05$, when applicable.

As no differences were identified between replicate tanks in SDH levels and log-transformed EROD activities, replicate tanks were pooled for graphical presentation.

RESULTS

A significant ($p < 0.05$) increase in liver EROD activity occurred on the second day of exposure in fish exposed to dispersed Bass Strait crude oil, while salmon exposed to WAF of crude oil did not show a significant ($p < 0.05$) increase in EROD activity until after four days of exposure (Figure 1).

Although exposure terminated on day four of the experiment, EROD induction reached a maximum on day six for salmon exposed to dispersed oil, and two days later (on day eight) for fish exposed to the WAF. In both cases, maximum induced EROD activities were five times higher than in control fish (Figure 1). EROD activity remained significantly ($p < 0.05$) elevated up to eight days after the termination of exposure in both dispersed oil and WAF exposed salmon.

Circulating serum SDH remained at low levels in all groups, varying between 2.65 and 4.87 mU in control fish, between 3.53 to 5.26 mU for crude oil WAF-exposed fish, and from 2.81 to 5.17 mU for dispersed oil-exposed salmon. No significant differences ($p = 0.76$) were found between treatments or sampling days, indicating that no significant hepatic injuries were caused by chemical exposure during the experiment (Ozretic and Krajnovic-Ozretic 1993).

No statistical differences ($p > 0.05$) among treatment groups or sampling days were identified in liver somatic index or in condition factor, indicating that fish were feeding well and were in good physical condition throughout the experiment.

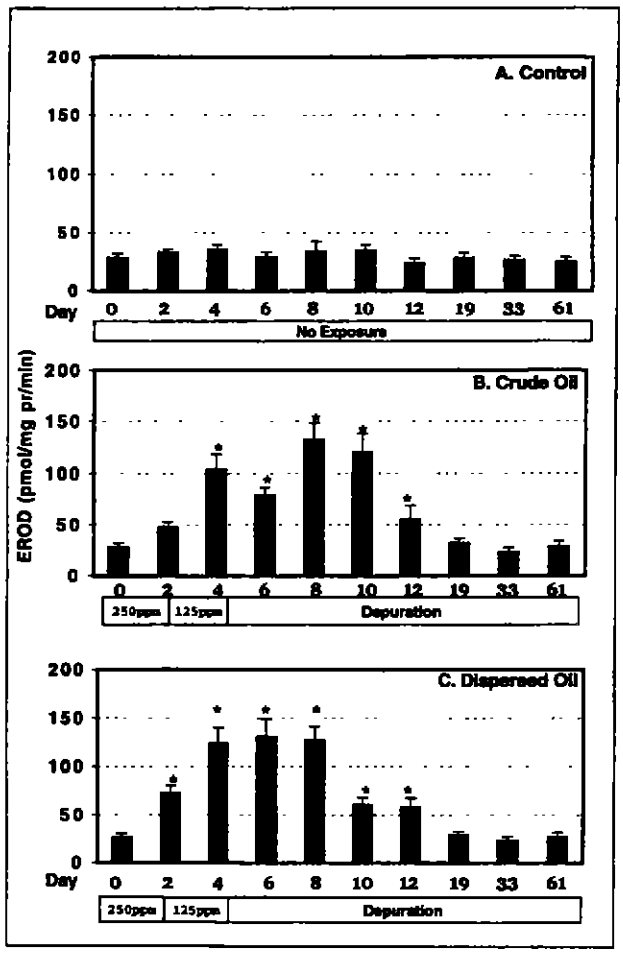

*Figure 1. EROD induction in Atlantic salmon in a non-exposed group (graph A), and following exposure to the water-accommodated fraction of Bass Strait crude oil (graph B) or to dispersed oil (graph C). * = significantly different from control fish sampled on that day.*

DISCUSSION

This research shows that it is possible to use MFO induction, as measured by EROD activity, to monitor low level exposure of Atlantic salmon to Bass Strait crude oil. Induction of MFO in WAF and dispersed oil-exposed Atlantic salmon indicated that a significant amount of petroleum hydrocarbon was transferred from Bass Strait crude oil to the seawater. In an oil spill situation, fish would be exposed for a number of days to the water-soluble fraction of crude oil, and to the chemically or naturally dispersed oil droplets in the water column. Induction of the detoxication system can confirm significant exposure of commercial Atlantic salmon stocks only two days after the beginning of a spill event if oil dispersants are used, and after four days if stocks are exposed to the water-accommodated fraction. In both cases, MFO induction persisted for

up to eight days after the end of the exposure, during which sampling for confirmation of MFO induction can be performed.

The faster MFO induction that occurred when crude oil was chemically dispersed can be explained by increased exposure levels to petroleum products. By their amphipathic nature, dispersants reduce the surface tension of crude oil, which results in oil droplets being dispersed into the water column (Volkman *et al.* 1994). It is also possible that the presence of surfactants in the dispersed oil facilitates uptake of the contaminants through the gills. Hence, fish are exposed to higher concentrations of petroleum products in the water column, which may explain the faster MFO induction when chemical dispersants were applied. The use of chemical dispersants, however, did not increase the level of EROD activity despite the apparently higher exposure levels.

The results obtained in the present study are comparable to other studies. EROD activity in Atlantic salmon parrs and smolts increased to a maximum following seven days of exposure to the WAF of Hibernia crude oil (Vignier *et al.* 1996). Collodi *et al.* (1984) determined that aryl hydrocarbon hydroxylase (AHH) activity of coho salmon (*Onchorhynchus kisutch*), which often co-varies with EROD, showed significant induction between two and five days of exposure to the water soluble fraction of Cook Inlet crude oil, and that AHH levels in exposed fish recovered to control levels by the seventh day of depuration. Measured MFO induction in these studies reflected the short-term exposure and, therefore, indicates a definite time during which fish stocks can be sampled for confirmation of exposure to petroleum hydrocarbons.

The relative sensitivity of MFO induction is an important consideration in selecting a biomarker of exposure to petroleum compounds. The EROD assay is thought to be one of the most sensitive physiological effects that can be measured following exposure to a wide range of environmental contaminants (Collier *et al.* 1995). We have shown in this study that MFO induction can provide an important tool for estimating the geographical magnitude of any potential impact caused by oil industry development or by an oil spill event. Past spill events have shown that broad areas may be impacted through contaminated water bodies, even if the oil slick did not reach the precise geographical area. For example, walleye pollock (*Theragra chalcogramma*) sampled at more than 640 km from where an oil spill event originated showed evidence of exposure to petroleum compounds (Collier *et al.* 1996).

The long-term biological significance of such induction as it may relate to more serious physiological changes in the flesh of salmon has not been adequately addressed. The induction at the biochemical level by the water-soluble petroleum compounds indicates that chemicals reach critical cellular sites, and suggests potential for other biological effects. From a commercial point of view, it is of primary importance to determine whether oil-exposed fish are safe for human consumption. Findings of Hom *et al.* (1996) suggested that there is little risk involved in the consumption of flesh from oil-exposed fish because of the extensive metabolism and excretion of petroleum compounds by fish. However, certain reactive compounds such as PAH metabolites have the potential to create DNA adducts in fish, a step that is widely held as the initiating point in the multistep process of chemical carcinogenesis (Stein *et al.* 1995).

Heras *et al.* (1993) showed that while it took 17 days in clean seawater for an almost complete depuration of the muscle tissues of Atlantic salmon following exposure to the water-soluble fraction of Flotta North Sea crude oil, the white muscle of the fish which comprise most of the edible portion depurated 80% of the hydrocarbons within less than a day. George *et al.* (1995) showed that there were no significant petroleum compounds in muscle tissues from the fish sampled three months after the oil spill incident, but that oil components had in fact been assimilated by the fish and had induced increases in the measured mixed function oxygenase levels. It could be speculated that EROD activity would increase more readily and persist longer in younger, leaner fish relative to fattier adults who could store petroleum hydrocarbons in lipid tissues. To date, however, varying EROD induction capabilities with body size and fat content have not been documented in *Salmo salar*. Although EROD induction can be significantly inhibited by the presence of oestrogenic compounds (Gagnon *et al.* 1995; Goksoyr and Larsen 1991), farmed salmon are grown and harvested prior the onset of reproductive activity; inhibition of EROD activity by oestrogenic compounds is expected to be minimal in this situation. The temperature at which fish were held during this experiment (9.7°C-12.4°C) are typical water temperatures for Tasmanian waters where Atlantic salmon are held (HR King, Saltas Ltd, Tasmania, pers. comm.); slightly higher temperatures may be encountered during the summer months (occasionally up to 23°C), increasing the fish's metabolism which may result in increased uptake of petroleum hydrocarbons; again, the influence of water temperature on the uptake of hydrocarbons and temporal course of EROD induction has not to date been documented. The results of the present study

confirmed the sensitivity and adequacy of MFO induction as measured by increased EROD activity in sexually immature fish for assessing the bioavailability of petroleum hydrocarbons after an environmental pollution incident, and for assessment of their spatial and temporal effects in Tasmanian waters.

There are at least three advantages of using MFO induction over chemical residue analysis to assess recent exposure of fish stocks to petroleum hydrocarbons: 1) MFO analysis is 5-10 times less expensive (George *et al.* 1995), 2) the induction of hepatic enzymes is more persistent than retention of petroleum compounds in the flesh (Heras *et al.* 1993), and 3) MFO induction confirms an actual uptake and depuration of petroleum hydrocarbons, while chemical analysis may confirm a long term storage of petroleum compounds in adipocytes (Zhou *et al.* 1997). In addition, MFO activity as measured by EROD in the liver of rainbow trout (*Oncorhynchus mykiss*) have been shown to appear before the formation of bile metabolites (Upshall *et al.* 1993). Measurement of EROD activity can therefore provide a faster assessment of fish exposure to petroleum compounds than measurement of bile metabolites.

CONCLUSIONS

In the event of an oil spill, measurement of MFO as monitored by increased EROD activity would provide evidence of, or refute significant exposure of commercial fish stocks to petroleum hydrocarbons. This would assist environmental managers in providing scientific evidence to confirm or refute public debate about the exposure and viability of fish stocks. Such debate could result in the requirement of costly stock surveys to be undertaken over wide-ranging areas associated with petroleum hydrocarbon development or an oil spill event (Payne *et al.* 1987). The present study shows that immature Atlantic salmon can be sampled as soon as two days following initial exposure to petroleum hydrocarbons, and up to eight days following the termination of such an exposure.

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