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Assessment of Hepatic Mixed Function Oxidase Induction in Winter Flounder (*Pseudopleuronectes americanus*) as a Marine Petroleum Pollution Monitoring Technique, with an Appendix Describing Practical Field Measurements of MFO Activity

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Assessment of Hepatic Mixed Function Oxidase Induction in Winter Flounder (<u>Pseudopleuronectes</u> <u>americanus</u>) as a marine petroleum pollution monitoring technique, with an Appendix describing practical field measurements of MFO activity.

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#### Abstract

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The rationale and potential for using measurements of hepatic mixed function oxidase (MFO) enzyme activity in winter flounder (<u>Pseudopleuronectes americanus</u>) as an index of exposure to petroleum hydrocarbons are discussed. In flounder from an uncontaminated environment, several indices of hepatic MFO activity (ethoxyresorufin O-de-ethylase (EROD) activity, benzo(a)pyrene (B(a)P) hydroxylase activity, and concentrations of cytochrome P-450) showed some seasonal variation which was related to changes in the reproductive cycle. This variation was small, however, in comparison with the changes in EROD and B(a)P hydroxylase activity observed during dose-response experiments involving even low levels of certain hydrocarbons. Polychlorinated biphenyls, which also induce MFO activity, did so only at environmentally unrealistic dose levels. Thus, provided seasonal and sex differences are controlled by careful sampling, measurement of hepatic MFO induction in flounder is a potentially specific and sensitive monitoring technique to show the presence of petroleum hydrocarbons.

An Appendix is provided in which practical methods, suitable for field measurement of fish hepatic MFO activity, are described. sur la terrain de l'activité des MFO hépatiques chez les poissons.

#### Résumé

Addison, R.F. and Payne, J.F. 1986. Assessment of hepatic mixed function oxidase induction in winter flounder (<u>Pseudopleuronectes americanus</u>) as a marine petroleum pollution monitoring technique, with an Appendix describing practical field measurements of MFO activity. Can. Tech. Rept. Fish. Aquat. Sci. 1505.

On discute de la possibilité d'utiliser les mesures de l'activité enzymatique des mono-oxygénases à fonction mixte (MFO) du foie chez la plie rouge (Pseudopleuronectes <u>americanus</u>) comme indice de l'exposition à des hydrocarbures dérivés du pétrole. On expose également la justification scientifique de cette application des MFO. Chez la plie provenant d'un environnement non contaminé, plusieurs indices de l'activité des MFO hépatiques, comme l'activité éthoxyrésorufine O-déséthylase (EROD), l'activité benzo(a)pyrène (B(a)P) hydroxylase et les concentrations de cytochrome P-450, montrent une certaine variation saisonnière qui a été liée à des changements touchant le cycle reproducteur. Toutefois, cette variation est petite en comparaison des changements touchant l'activité EROD et B(a)P hydroxylase observés au cours d'experiénces visant à établir la relation dose-effet dans le cas de certains hydrocarbures et ce, même pour des doses faibles. Les PCB, qui induisent également l'activité MFO, ne le font qu'à des concentrations très elevées par rapport aux concentrations observées dans l'environnement. Ainsi, une fois que l'on aura déterminé les différences saisonnières et les différences liées au sexe à l'aide d'un échantillonnage rigoureux, la mesure de l'induction de l'activité des MFO hépatiques chez la plie pourrait se révéler une méthode de surveillance à la fois sensible et spécifique pour déceler la présence des hydrocarbures dérivés du pétrole.

On décrit en annexe des méthodes pratiques pour la mesure

#### Introduction

Interest in the use of mixed function oxidases (MFOs) as an environmental monitoring tool derives from basic research on enzymatic detoxification mechanisms carried out since the early 1960's (see Addison, 1984, for a review). This work showed that there existed in various animal tissues an enzyme system with the following characteristics:

(1) it was usually, but not exclusively, reported in mammalian liver, associated with the smooth endoplasmic reticulum (SER), and it catalysed the degradation of both endogenous and exogenous lipophilic substrates to polar watersoluble products which were more easily excreted than the substrate;

(2) it required molecular O2, NADPH and involved a CObinding cytochrome, "P-450", so-called from the absorption maximum of its CO complex;

(3) it was present at relatively low activity in normal animals, where its function appeared to be to degrade endogenous lipophilic substrates such as steroids, but if the animal were exposed to certain foreign compounds --- some of which were persistent environmental contaminants such as the insecticide DDT, polycyclic aromatic hydrocarbons (PAHs) and the polychlorinated biphenyls (PCBs) --- its activity would increase dramatically, apparently to enhance the degradation and clearance of the offending compounds.

This final point attracted the interest of environmental toxicologists, for it implied that the activity of the MFO system in "naturally" contaminated organisms might be a measure of the chemical stress to which they had been exposed; MFO activity would therefore be an indirect and general measurement of environmental quality --- a sub-lethal bioassay. Since the mid-1970's, various groups have explored this possibility, focussing specifically on fish.

Subsequent research has shown that the MFO system in fish is similar in its general features to that of mammals; the system is found mainly, though not exclusively, in liver; it is associated with the SER, and has the same biochemical characteristics. The principal differences are that uninduced activity in fish is considerably lower than that in mammals, and that the MFO system in fish appears to be inducible only by those chemicals which induce a "modified" P-450-based system in mammals --- principally PAHs and some PCB congeners. There are two practical results from these differences. The lower overall MFO activity in fish requires that the assays to measure MFO activity be more sensitive than those used in mammals; hence there is a trend towards using enzymatic reactions whose products can be determined fluorimetrically, gas chromatographically or radiometrically. The absence of induction in fish by "classical" mammalian inducers such as phenobarbitone or DDT means that fish MFOs respond quite selectively to induction by petroleum hydrocarbons, including PAHs, or by PCBs.

# Field Trials Associated with Hydrocarbon Pollution.

Field studies in which elevated MFO activities in fish have been shown to be associated with hydrocarbon pollution are summarised in Fig. 1. The first such studies were carried out in the early 1970's in Newfoundland, where it was shown that brown trout (<u>Salmo trutta</u>) from a small urban lake in St. John's, which had a history of hydrocarbon contamination, had elevated hepatic benzo(a)pyrene (B(a)P) hydroxylase activities (Payne and Penrose, 1975). Other field studies were carried out near a large oil refinery at Placentia Bay, Nfld., where

both liver and gill tissue from cunner (Tautogolabrus adspersus) were found to contain elevated MFO activity (Payne, 1976). Similar observations were made near a small boat marina putatively contaminated with hydrocarbons. On the basis of these field observations with both freshwater and marine species, MFO induction in fish was suggested to be a sensitive indicator for use in biological monitoring of the presence of Further field studies carried out hydrocarbons. in the northern Adriatic Sea supported these initial results (Kurelec et al., 1977): blennies (Blennius parvo) collected near a spill of No. 2 fuel oil showed marked MFO induction, with activities remaining elevated for a two-to-three week period. In other studies, blennies taken in the area of a refinery outfall in the Adriatic had highly elevated hepatic MFO activity (Britvic et al., 1983). An interesting series of observations has been made on fish from the site of a natural oil seep in the Santa Barbara Channel, off California. Two species of sand-dabs (Citharichthys sardidus and C. stigmeus) and white perch (Phanerodon furcatus) collected in the vicinity of oil seeps contained elevated B(a)P hydroxylase levels (Spies et al., 1980; 1982). Both Burns (1976) and Stegeman (1978) have reported slightly elevated MFO levels (as indicated by aldrin epoxidase and B(a)P hydroxylase, respectively) in mummichog (Fundulus heteroclitus) captured in the vicinity of oil spill sites in Massachusetts. Given the low level of hydrocarbons expected around oil rigs in the open sea, it is particularly interesting that Davies et al. (1984) have reported elevated MFO levels in cod (Gadus morhua), haddock (Melanogrammus aeglefinnus) and whiting (Merlangus merlangus) around oil rigs in the U.K. sector of the North Sea. Although most field studies to date have focussed on liver tissues as the source of MFO enzymes, Payne et al. (1984) have also reported elevated kidney MFOs in winter flounder (Pseudopleuronectes americanus) collected at the site of a spill of No. 2 fuel oil at Baie Verte, Nfld. Finally, the most recent study of "field" hepatic

MFO induction comes from Finland, where Lindström-Seppä et al. (1985) found that perch (<u>Perca fluviatilis</u>) collected at the site of an oil spill in the Vaasa Archipelago had elevated MFO levels, in comparison with fish collected at control sites. In this study, fish still had elevated levels four months after the spill, but activities had returned to control values after nine months. Taken together, these field studies of the impact of hydrocarbon exposure on various species of fish from widely differing environments provide convincing evidence that MFO induction provides a potentially useful tool to monitor point sources of hydrocarbons, and to demonstrate "recovery" from their effects.

#### <u>Objectives</u>

The objectives of the work described in this report were:

(1) to select a suitable bottom-dwelling fish which would be available in sufficient numbers to study in detail, and which could be placed on a "short list" of potential monitoring species;

(2) to establish the natural variability in MFO activity in an uncontaminated population of the species;

(3) to define the dose-response relationship between hydrocarbon exposure and MFO activity;

(4) to assess the potential for pollutants such as PCBs to interfere with the use of MFOs as a petroleum monitoring system.

#### Experimental

#### "Calibration" of MFOs.

(a) Species selection.

The requirements for a species to be considered as a suitable candidate organism for monitoring were as follows:

(1) the species should be readily available and widely distributed around the Atlantic provinces and its life history should be reasonably well described;

(2) it should be robust and amenable to laboratory culture and experiment, yet its MFO system should be sensitive to petroleum hydrocarbon components;

(3) it should be large enough that samples of specific tissues or organs could be obtained from individuals for analysis;

(4) ideally, it should be a bottom dweller, since this is one of the interfaces at which petroleum introduced to the sea will accumulate.

On the basis of these criteria, winter flounder (<u>Pseudopleuronectes \_ americanus</u>) Its basic was selected. biology is well known (e.g., Leim and Scott, 1966), and several studies of its distribution and population dynamics have been carried out on specific populations (e.g., Levings, 1972). It has been used experimentally for MFO induction (Payne and Fancey, 1982) and related studies (e.g., Fletcher et al., 1982) and could be held successfully under laboratory conditions. In addition, it occupies a wide variety of habitats, ranging from

shallow inshore systems of varying salinity to relatively deep and stable offshore environments, and its habit of burying itself in soft sediments seemed likely to enhance its exposure to sediment hydrocarbons. Finally and most importantly, it was easily obtained either by trawling or by divers.

In addition, since MFO activity and inducibility was expected to vary with gonad maturation, it was decided to examine these variables in cunner throughout the spawning period. Cunners were selected because they have a contracted gonadal maturation and spawning period, and could be obtained in suitable numbers at well-defined stages of pre-spawning, spawning and post-spawning. They have also been used successfully in field validation of hydrocarbon pollution at the site of the Come-by-Chance Refinery in Placentia Bay, Nfld. (Payne, 1976).

(b) Analytical procedures

Measurement of MFO activity involves the following steps:

(1) dissection of appropriate tissue and preparation of a homogenate in an appropriate buffer;

(2) centrifugation of homogenate to yield a postmitochondrial supernatant (PMS, 10,000 X g supernatant) which could be further centrifuged at 100,000 X g to yield a microsomal pellet;

(3) incubation of the MFO enzyme source (10,000 X g supernatant or re-suspended microsomal pellet) with appropriate substrates;

(4) measurement of Cytochromes P-450 and bs levels (both of which are electron transport components of the MFO system) in microsomal preparations;

(5) determination of protein content in the enzyme preparation.

<u>Preparation of homogenates</u>: Tissue was homogenised (1:4, w:v) in 1.15% KCl using 6 strokes at 1750 rpm with a teflonglass Potter-Elvehjem homogeniser. The crude homogenate was centrifuged at 10,000 X g for 20 min and an aliquot of the supernatant (PMS) was centrifuged for 1h at 100,000 X g; the resulting microsomal pellet was re-suspended in 0.1 M phosphate buffer, pH 7.8.

Ethoxyresorufin O-de-ethylase (EROD): EROD was determined by the procedure of Burke and Mayer (1974). The incubation mixture consisted of 2 ml 0.1M phosphate buffer, pH 7.6, for routine assays; NADPH (Sigma) at a final concentration of 200  $\mu$ M; and enzyme preparation at a final concentration of approx. 1 mg protein • ml<sup>-1</sup>. Ethoxyresorufin, prepared as described by Burke and Mayer (1974) was added at a final concentration of 2.5  $\mu$ M. Fluorescence was read at 585 nm (excitation at 510 nm) and compared to a standard curve prepared with resorufin (Pierce) in the 10-50 nM range.

Benzo(a)pyrene (B(a)P) hydroxylase: This enzyme was determined by a modification of the method of Nebert and Gelboin (1968). The incubation mixture contained 0.5 ml microsomal preparation μM; substrate was and NADPH at a final concentration of 20 added in 10  $\mu$ l acetone at a final concentration of 0.3  $\mu$ M. After incubating at 25°C the reaction was stopped by the addition of 0.5 ml ice-cold acetone, and the mixture was 2 ml cold extracted with petroleum ether, which was centrifuged and back-extracted with 2 ml NaOH. Fluorescence of the NaOH extract (excitation 400 nm, emission 522 nm) was read routinely against a secondary standard of quinine

dihydrochloride; a calibration curve was prepared using 3hydroxybenzo(a)pyrene as a primary standard.

<u>Cytochromes P-450 and b5</u>: Cytochromes P-450 and b5 were determined by a modification of the method of Omura and Sato (1964). Cytochrome b5 was determined by adding sodium dithionite to a microsomal preparation in the sample cuvette of a double beam spectrophotometer and scanning from 400 to 500 nm against an unreduced sample in the reference beam; cytochrome b5 was determined from the difference spectrum, assuming its absorbance to be 171 • cm<sup>-1</sup> • mM<sup>-1</sup>. Cytochrome P-450 was determined after addition of sodium dithionite to the reference beam and bubbling CO through the sample cuvette and scanning as above; absorbance at 450 nm was assumed to be 91 • cm<sup>-1</sup> • mM<sup>-1</sup>.

<u>Protein determinations</u>: Protein was determined on PMS or microsomal suspensions by the method of Lowry et al., (1951), using bovine serum albumin as standard.

<u>Statistical Analyses</u>: Data were analysed by regression and analysis of variance, using the BMDP package (Dixon et al., 1983).

# Results

#### (a) <u>Dose-response relationships</u>

An integral component of this study was to determine the threshold levels of sediment hydrocarbons required for MFO induction and to compare the sensitivity of this response with various other indices. The general concentration range required to effect MFO induction in flounder was established in exposures carried out over a three-month period. This was followed, a year later, with an expanded experiment in which fish were exposed to sediments contaminated with total PAH

concentrations (nominal values) as low as  $0.5 \ \mu g$  •  $g^{-1}$ . In terms of lipid soluble material determined by micro-gravimetry, the total concentration of aliphatic and aromatic hydrocarbons in the lowest dose levels was only twice the concentration found in control sediments. In addition to MFO induction measurements, other indices were investigated: these included (a) liver, kidney, gonad and spleen condition indices, (b) gill and liver histopathology and (c) muscle and liver concentrations of protein, glycogen and lipid. MFO induction was by far the most sensitive response, with differences from control fish being detectable at the lowest level of sediment hydrocarbon contamination (Fig. 2). In contrast, most of the other indices studied displayed little or no change, even at the higher exposure levels. This is illustrated in Figs. 3 and 4, which compare the percentage change in MFO enzyme induction with the other indices.

This study with flounder has established the sensitivity of enzyme response over various other indices the MFO for assessing the effects of biologically available PAH in contaminated sediments. Similar conclusions about the sensitivity of the MFO response emerged from field studies at a petrochemical complex in Finland. In this case, twenty-five different indices were investigated in rainbow trout (Salmo gairdneri) held in cages near the waste-water discharge, and the only clear change attributable to waste-water among these was an increase in the detoxification enzymes of the MFO system and glucuronyl transferase (Nikunen, 1985).

# (b) Natural Variability of MFOs in Uncontaminated Flounder

The basic features of MFO activity in winter flounder were studied in an apparently uncontaminated population sampled from Head Harbour, St. Margaret's Bay, N.S. (This area is free from any obvious industrial or domestic source of pollution.)

Pooled liver homogenates from several individual fish were examined to determine optimum pH, temperature, and substrate and enzyme concentrations in B(a)P hydroxylase and EROD assays; the results of these studies are shown in Figs. 5-10. (These data have been summarised elsewhere: Edwards et al., 1987.)

The variation of MFO activity was studied in this population by sampling at approximately monthly intervals over a two year period, between November 1983 and October, 1985. Samples were taken with a small otter trawl, towed in shallow water (< 3m) for 20 mins, to minimise stress on the captured fish; samples were transported in aerated sea water to BIO, and held in flowing sea-water, and were usually analysed for MFO activity the following day. A total of 336 fish were examined; usually, not fewer than eight fish of each sex were analysed at each sampling point. However, during late winter, when there was extensive ice cover in Head harbour, fish could only be sampled with difficulty, although a few individuals could usually be trawled along the ice edge.

Table 1 summarises the mean values for hepatic MFO activity, separated by sex, over the entire sampling period. There were no significant differences between sexes when data were grouped on this scale, because factors such as seasonality introduced considerable variance to the data. The main indices of MFO activity varied as follows (<u>cf</u>. Table 2-5):

(1) Both EROD and B(a)P hydroxylase varied seasonally (Figs. 11-14), usually reaching a maximum in late spring and falling to a minimum in winter. Similar variation occurred in both sexes, but was more pronounced in females. Variation within sex and between season was approx. 6-fold for EROD and 4-fold for B(a)P hydroxylase; between sex and within season, it did not exceed 2-fold for either enzyme.

(2) EROD and B(a)P hydroxylase activity were consistently slightly higher in males than in females at any sampling point.

(3) EROD and B(a)P hydroxylase were themselves highly correlated ( $\underline{r} = 0.476$ , P < 0.01 in males (n = 164);  $\underline{r} = 0.661$ , P < 0.01 (n = 172) in females).

(4) In females only, both EROD and B(a)P hydroxylase were significantly correlated with Cytochrome P-450 content, and inversely correlated with body weight and liver weight; in males, only B(a)P hydroxylase was correlated with Cytochrome P-450, and also (inversely) with liver protein content.

(5) Cytochrome P-450 content was inversely related to both liver weight and liver protein content in females, and with liver protein content only in males.

As steroid hormone status influences MFO activity in mammals, and since the period of gonadal maturation and spawning is accompanied by variation in fish enzyme activity (Payne, 1984; Payne et al., 1986) it was important to determine if MFO induction occurred during this period of physiological change. As indicated above, cunners were chosen for this study. Male and female fish were collected from Conception Bay, Nfld., at intervals during summer 1985 and were exposed to a pulse of diesel oil in water. The exposure regime was such that the oil concentration increased rapidly to a maximum of 40  $\mu g \cdot ml^{-1}$ after six h, and decreased slowly to background levels  $(1 \ \mu g \cdot ml^{-1})$  within 18 h. Although basal MFO levels varied, induction was readily detectable in male and female fish in both hepatic and extra-hepatic tissues in all of the trials during the pre-spawning period (June), spawning (July-August) and post-spawning period (September). The high levels of induction observed in liver, heart and kidney are summarised in Fig. 15.

Only recently have field studies of MFO activity in experimental and reference sites been carried out on a seasonal

basis. Studies at a petrochemical complex on the Adriatic coast have documented elevated MFO activity in blennies throughout various seasons (Britvic et al. 1983). Swedish workers have also noted induction at various seasons in fish taken from coastal regions receiving effluents from a pulp and paper industry (Förlin et al., 1985). These field studies corroborate the conclusions from the cunner studies, and show that MFO induction can be used as a sensitive biological indicator of chemical "stress" year round. However, to obtain maximum sensitivity, both sample and reference populations should be examined at the same season.

# (c) <u>Induction by non-petroleum compounds.</u>

As noted above, hepatic MFO enzymes in fish are induced only by those chemicals which in mammals induce MFO systems involving Cytochrome P-448 or other modified P-450s. In practice, the only environmental chemicals likely to bring about such induction. other than polycyclic aromatic hydrocarbons, are the polychlorinated biphenyls (PCBs). Accordingly, an experiment was carried out in which PCBs (as Aroclor 1254) were injected into flounder and hepatic MFO response was measured, as indicated by benzpyrene hydroxylase activity. Fig 16 summarises the results: benzo(a)pyrene hydroxylase activity was not induced by an injection of Aroclor 1254 at 110  $\mu$ g • g<sup>-1</sup>, on a whole body wt. basis, but it was induced at dose levels of 215  $\mu$ g • g<sup>-1</sup> PCB. However, PCB concentrations expected in fish tissues from uncontaminated environments like St. Margaret's Bay are in the range of 1 - 5  $\mu g \cdot g^{-1}$  at most, so induction by environmental PCBs seems likely to occur only at massive levels which would occur only at the site of a major PCB spill. In other words, hepatic MFO induction seems relatively specific for petroleum hydrocarbons, to the extent that background levels of PCBs would usually not be expected to be confounding inducers around offshore or

coastal petroleum development sites.

#### (d) <u>Time course of MFO induction</u>.

hepatic MFO induction was assessed The time course of PCBs (Aroclor 1254) at a dose of following injection of approx. 200  $\mu g \cdot g^{-1}$  and analysing MFO activity at appropriate intervals afterwards. Results are summarised in Fig. 17. Induction of B(a)P hydroxylase activity was not detectable 3d after injection, though there was a slight (non-significant) increase in EROD activity, but by 6d after injection, both B(a)P hydroxylase and EROD were dramatically induced. Induction continued until at least 10d after injection. Itthus appears that induction is reasonably rapid and persistent.

# Assessment of flounder hepatic MFO induction as an environmental monitoring technique.

The data summarised above show that induction of hepatic MFO in winter flounder is highly sensitive to the presence enzymes of petroleum hydrocarbons at environmentally realistic levels. Thus, the concentrations of sediment PAH at which induction was observed (at least over relatively long-term exposures) was well within the range of PAH concentrations reported in sediments contaminated by oil spills, which are often in the range of several hundred µg • g<sup>-1</sup> (e.g., Teal et al., 1978). Petroleum is, of course, a complex mixture, and since only some its components are hepatic MFO inducers, the absolute of sensitivity of the MFO response will vary with the composition of the oil. However, if appreciable amounts of PAH are present, an effect on MFO activity is virtually assured, even if PAH content is low and evanescent, as in natural gas condensate (Mahon et al., 1987). It is worth noting, however, that if PAH are essentially absent --- as in the highly refined base oils used for some oil-based drilling muds --- MFO induction may be low or undetectable (Addison et al., 1984;

Payne et al., 1985).

In addition to being highly sensitive to petroleum hydrocarbon exposure, the flounder hepatic MFO induction response is also quite selective. The only pollutants other than PAH which are likely to be encountered in practice, and which are capable of inducing fish hepatic MFOs are the PCBs (and even then, only selected congeners of the series). However, the dose of PCBs required to cause detectable induction of hepatic MFO enzymes is so large as to be likely to arise only from major PCB contamination, and such events are likely to be recorded by means other than subtle biological effects monitoring techniques. (However, there is evidence that environmental PCBs may cause changes in the hepatic MFO system by inducing specific P-450 isozymes, as opposed to indicator MFO enzymes: Stegeman et al., 1986.) For practical purposes, then, induction of hepatic B(a)P hydroxylase or EROD seems to be a specific indicator of the presence of PAH, whose most likely source would be petroleum pollution.

The time course of hepatic MFO induction seems slower in flounder than in other species; in trout, for example, induction comes about within 3d or so of initial exposure to PCBs, in contrast to the 6d apparently required in flounder. The slower process in flounder may simply reflect the lower environmental temperatures and generally lower metabolic rates at which these fish function. In practice, this may not be a disadvantage: MFO activity remains elevated for at least 10d after initial exposure, so even if a pollution event was relatively transient, lasting only a few days, it seems that the MFO response would still be recorded, several days after the event. However, in practice, few petroleum spills last for only a few days: most continue for weeks or months. This is certainly true of the major spills or blow outs of oil (such as the Arrow spill in Chedabucto Bay, N.S., in 1969, traces of which are still visible) or the Venture gas and condensate blow out of February 1984, which continued for about a month.

However, we envisage the use of the flounder hepatic MFO induction "bioassay" not as confirming the obvious visible evidence of a major spill or blow out, but as indicating the general quality of an environment potentially threatened by relatively low level "chronic" releases of petroleum. Such releases are, by their nature, more likely to be continuous, or at least frequent, though on a small scale, rather than being rare and spectacular. It seems that the flounder hepatic MFO response would be relatively sensitive to these.

This final point leads us logically into a discussion of the rationale for regarding MFO induction measurements as a practical tool for "environmental managers". The argument is frequently made that although MFO induction may be a sensitive response at the biochemical level, it cannot be translated into a whole organism, population or community response. This disregards the possibility that induction must have some adaptive significance --- even if we cannot yet define it--but, more importantly, it assumes that only responses at the population or community level are significant enough to be considered by environmental managers. But, to draw an analogy with the impact of human disease, would we wait for an effect at the population or community level before trying to control the outbreak of cholera, or would we prefer to have an early warning of the possibility of such an outbreak? The answer is obvious.

From a regulatory point of view, one of the most valuable features of sensitive responses such as MFO induction is that they may define spatial and temporal boundaries of the effects of pollution. In considering this, we may take as an example the concerns which continue to be expressed about the environmental impact of offshore development in the North Sea, or (closer to home) in the Grand Banks of Newfoundland, which is one of the world's richest fishing grounds. Various laboratory studies have shown that, depending on the dose and duration of exposure, petroleum fractions may have both acute

and sub-lethal effects on fish. If these data are taken out of context, they can justify concerns expressed about the potential for offshore development to harm commercial fish stocks. Although these concerns are entirely justified, scientific evidence is available --- from MFO induction monitoring in the North Sea --- to show that at least in that environment, "chronic" effects are very limited. Thus, in three species of fish, MFO activities are only marginally induced in the vicinity of drilling rigs, and induction is observed within only a few km of the rig (Davies et al., 1984). If such a sensitive biochemical "early warning" of the possible impact of petroleum is manifested at only a low level, and in a limited area, it seems highly unlikely that impacts would occur at the higher levels of biological organisation, the In such cases, it would seem population or community level. unnecessary --- from a scientific perspective, at least --- for environmental managers to mount extensive and costly stock surveys over wide areas (even assuming that such surveys would identify changes attributable to petroleum effects: cf. Longhurst, 1982). On the other hand, evidence of wide distribution of MFO induction, far from drilling sites, would provide better justification for large scale and continuing the potential impact of offshore petroleum surveys ofdevelopment.

# Conclusions

Indices of hepatic MFO activity in winter flounder vary with the sex of the fish, and seasonally, probably in response to changes in the reproductive cycle. Of various indices of MFO activity examined, the enzymes ethoxyresorufin O-de-ethylase and benzo(a)pyrene hydroxylase showed the most consistent variability with natural factors; these indices were themselves highly correlated. In addition, these enzyme activities responded with most sensitivity to exposure of the fish to petroleum. MFO inducers other than petroleum, exemplified by the polychlorinated biphenyls (PCBs) did not induce hepatic MFO activity at environmentally realistic concentrations.

It therefore appears that in the laboratory, at least, winter flounder hepatic MFO activity responds in a predictable and sensitive way to the presence of petroleum. If flounder are to be used as a laboratory bioassay test organism to assess water quality, obvious sources of natural variability in the MFO response (sex, seasonal differences, size etc.) should be eliminated --- even though the variation caused by these natural factors is usually smaller than that caused by the effects of petroleum.

In using MFO enzyme systems as an environmental monitoring technique, it is important to sample comparable groups of organisms from "experimental" and "reference" sites at the same time. Since it is possible that widely differing diets may affect MFO activity, we favour the practice of carrying out investigations over an expected pollution "gradient" in which natural environmental factors should not change too rapidly. Finally, it should be stressed that from a regulatory perspective, the main value of such sensitive assays as MFO induction is as an early warning of possible impact, and as an approach to identifying boundaries of affected areas.

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Teal, J.M., Burns, K. and Farrington, J. (1978). Analyses of aromatic hydrocarbons in intertidal sediments resulting from two spills of No. 2 fuel oil in Buzzards Bay, Massachusetts. <u>J.</u> <u>Fish Res. Bd. Can.</u> **35** 510-520. Table 1. Summary of biological variables and indices of hepatic mixed function oxidase activity in winter flounder (<u>Pseudopleuronectes americanus</u>) from St. Margaret's Bay, N.S., from Nov. 1983 to Oct. 1985. Data as mean ± S.D. (no. of samples).

Variable	Females	Males
Body weight (g)	361 ± 83.9 (172)	317 ± 55.2 (164)
Liver weight (g)	$4.74 \pm 1.98$ (172)	$3.23 \pm 1.02$ (164)
Liver % Body weight (Liver weight X 100 ÷ Body weight)	1.23 ± 0.41 (135)	$1.01 \pm 0.24$ (141)
Microsomal protein (mg•g liver <sup>-1</sup> )	$13.9 \pm 4.73$ (172)	$14.3 \pm 5.13$ (164)
EROD (nmoles•mg protein <sup>-1</sup> •min <sup>-1</sup> )	$0.380 \pm 0.359$ (172)	$0.577 \pm 0.470$ (164)
EROD turnover (EROD•P-450-1)	$2.57 \pm 4.11$ (96)	$3.67 \pm 6.69$ (102)
B(a)P hydroxylase (nmoles•mg protein <sup>-1</sup> • hr <sup>-1</sup> )	9.22 ± 9.18 (172)	$13.1 \pm 11.0$ (164)
B(a)P hydroxylase turnover (B(a)P hydroxylase•P-450 <sup>-1</sup> )	46.8 ± 46.6 (108)	71.6 ± 120 (117)
Cytochrome P-450 (nmoles• mg protein <sup>-1</sup> )	$0.21 \pm 0.16$ (172)	$0.28 \pm 0.18$ (164)
Cytochrome b5 (nmoles•mg protein <sup>-1</sup> )	$0.11 \pm 0.07$ (172)	$0.12 \pm 0.09$ (164)

Table 2. Variation of EROD in winter flounder with biological factors. Regression is of the form:

# y = ax + b

where  $\underline{y} = \text{EROD}$  (nmoles•mg protein<sup>-1</sup>•min<sup>-1</sup>) and  $\underline{x}$  is the independent variable;  $\underline{a}$  and  $\underline{b}$  are fitted coefficients.

Independent variable	<u>a (± S.E.)</u>	<u>b</u>	<u>r</u>	P		
	Males					
Liver weight (g)	0.016 ± 0.036	0.524	0.036	N.S.		
Body weight (g)	0.0004± 0.001	0.437	0.052	N.S.		
P-450(nmoles•mg protein <sup>-1</sup> )	$0.145 \pm 0.021$	0.537	0.054	N.S.		
Females						
Liver weight (g)	-0.068 ± 0.013	0.704	0.377	<0.01		
Body weight (g)	$-0.001 \pm 0.0003$	0.877	0.321	<0.01		
P-450(nmoles•mg protein <sup>-1</sup> )	$0.655 \pm 0.164$	0.242	0.294	<0.01		

Table 3. Variation of B(a)P hydroxylase in winter flounder with biological factors. Regression is of the form:

y = ax + b

where  $\underline{y} = B(\underline{a})P$  hydroxylase (nmoles•mg protein<sup>-1</sup>•hr<sup>-1</sup>) and  $\underline{x}$  is the independent variable;  $\underline{a}$  and  $\underline{b}$  are fitted coefficients.

<u>Independent variable</u>	<u>a (±S.E.)</u>	<u>b</u>	<u>r</u>	<u>P</u>

# <u>Males</u>

Liver protein (mg•g liver <sup>-1</sup> )	-0.434 ± 0.165	19.3	0.203	<0.01
P-450 (nmoles•mg protein <sup>-1</sup> )	$12.4 \pm 4.83$	9.65	0.197	<0.01

# Females

Liver weight (g)	-1.75	±	0.330	17.5	0.377	<0.01
Body weight (g)	-0.024	±	0.008	17.9	0.218	<0.01
P-450(nmoles•mg protein <sup>-1</sup> )	29.0	Ŧ	3.77	3.10	0.508	<0.01

Table 4. Variation of cytochrome P-450 in winter flounder with biological factors. Regression is of the form:

# y = ax + b

where y = P-450 (nmole•mg<sup>-1</sup>) and <u>x</u> is the independent variable; <u>a</u> and <u>b</u> are fitted coefficients.

Independent variable	<u>a (±S.E.)</u>	<u>b</u>	<u>r</u>	<u>P</u>
	Males			
Liver protein (mg•g liver <sup>-1</sup> )	-0.005 ± 0.003	0.350	0.153	<0.05
	<u>Females</u>			
Liver weight (g)	.021 ± 0.006	0.312	0.261	<0.01
Liver protein (mg•g liver-1)	-0.006 ± 0.003	0.289	0.165	<0.05

Table 5. Variation of liver protein content in winter flounder with biological factors. Regression is of the form

y = ax + b

where  $\underline{y}$  = protein (mg•g-1) and  $\underline{x}$  is the independent variable;  $\underline{a}$  and  $\underline{b}$  are fitted coefficients.

Independent variable	<u>a (±S.E.)</u>	b	r	<u>P</u>
	Males			
Liver weight (g)	$-1.24 \pm 0.383$	18.3	0.248	<0.01
	Females			
Body weight (g)	$-0.008 \pm 0.004$	17.0	0.150	<0.05
Liver weight (g)	$-0.504 \pm 0.179$	16.3	0.211	<0.01

# Appendix

# Practical field measurement of MFO activity

#### Introduction

The methods summarised here are based on conventional laboratory procedures for estimation of MFO activity which have been modified, usually by choosing equipment robust enough to be suitable for field use. The basic facilities required include some bench space and a source of 110V 60 Hz single phase power, maximum 10A. The methods and equipment described have been used in such diverse environments as aboard ship (CSS Hudson Arctic cruises), in a tent at Resolute Bay, NWT (using power supplied from a gasoline generator) and on Sable Is., N.S.

Our strategy has been to prepare in advance as many reagents and solutions as possible. Most, such as those required for protein determinations, are stable and will withstand freezing and thawing if contained in plastic bottles. It is usually not possible to prepare nucleotide co-enzyme solutions in advance, however, and since (usually) small amounts of these are needed and as they are relatively expensive, it is desirable to preweigh appropriate amounts of these, and keep them (cooled and desiccated) in small vials. Finally, we have selected instruments which are simple and robust examples of their type and which have simple maintenance or support requirements, but which are adequately sensitive or precise.

Weight determinations present special problems. They are particularly difficult to perform accurately on ship because of its motion, unless a weighing table on gimbals is available. A partial solution to the problem is to use an "averaging" balance of the sort that integrates over a period of several seconds; even this, however, is not satisfactory when dealing with weights below 1g, in our experience. Fortunately, most

variables of interest in MFO assays can be expressed in terms of protein contents, which in turn can be measured aboard ship using only volumetric manipulations.

Safety deserves special attention, especially if these techniques are to be used in the field where medical help is available only with difficulty. The majority of chemicals and equipment items recommended below are relatively harmless, provided they are not abused or mistreated. It is simply good laboratory practice to treat any chemical or equipment item with respect, even if it is known to be harmless; and furthermore, the toxicology of some chemicals (such as ethoxyresorufin) has not been investigated. We therefore recommend the use of disposable items wherever possible and the use of safety items such as "propipettes", gloves, bench-top soakers etc. The benzo(a)pyrene hydroxylase assay presents special problems, since the substrate is carcinogenic: the assay is best carried out in an area dedicated to the work (which is desirable in any case, as the assay should be done under safe lights since the products are photo-sensitive); protective clothing MUST be worn, pipetting by mouth MUST be avoided, and the working area MUST be protected with soakers which MUST be combined with carcinogenic waste for special disposal.

#### (1) Dissection and isolation of tissue

Equipment: top-pan balance weighing to 0.1g, conventional dissection instruments, bench space with adequate lighting, ice bucket plus ice and range of small beakers.

Kill fish, usually by a blow to the head and/or severing spinal cord; weigh fish with appropriate accuracy. Dissect out issue (usually liver) avoiding rupturing the gall bladder, since bile contains MFO inhibitors. Place weighed tissue in beaker on ice, pending homogenisation.

(2) Homogenisation of tissue.

Equipment: electric drill capable of 1750 rpm; Potter-Elvehjem teflon-glass homogeniser (5 or 15 ml); homogenisation solution (usually 1.15% KCl prepared in advance and stored frozen or cooled in plastic bottles); measuring cylinder (10 or 25 ml capacity).

Mince weighed tissue with scissors and place in homogeniser tube on ice; add KCl solution in ratio 4:1, v:w. Homogenise with 6 vertical strokes at 1750 rpm, keeping tube cooled in ice. This yields the "crude homogenate".

(3) Preparation of 10,000 X g homogenate.

Equipment: Beckman "Microfuge" or equivalent. This instrument is not self-cooled, but it may be run in a refrigerator or outside in the Arctic.

Place 1.5 ml aliquots of crude homogenate in centrifuge tube and spin for 15 min. Supernatant is nominally 10,000 X g supernatant ("post-mitochondrial supernatant", PMS) or 12,500 X g supernatant, depending on instrument. Protein concentrations in supernatant prepared in this way do not differ significantly from those prepared by conventional centrifuging (5 min at 500 X g followed by 15 min at 10,000 X g) in refrigerated centrifuges. Retain supernatants for analyses.

A single centrifuge tube usually provides sufficient sample for benzo(a)pyrene hydroxylase, ethoxyresorufin O-de-ethylase and protein determinations. Obviously, if activity or protein concentrations are low, or if subsequent samples are required for, say, electrophoretic studies of P-450 isozymes, further aliquots of the crude homogenates can be centrifuged.

(4) Protein determinations (Lowry et al., 1951).

Equipment: 15 ml test-tubes and rack, micropipettes with disposable tips to deliver 10, 25 or 500  $\mu$ l, graduated glass pipettes to deliver 2 ml and 10 ml, 250 ml glass beakers. Distilled water, 2% aq. copper sulphate solution, 2% aq. sodium potassium tartrate solution and 2% aq. sodium carbonate in 0.1

N sodium hydroxide, prepared in advance as described by Lowry et al. (1951). Protein standard, e.g., bovine serum albumin, diluted (accurately) to approx.  $100 - 200 \ \mu\text{g} \cdot \text{ml}^{-1}$  (so that appropriate volumes can be pipetted conveniently to prepare standard curves). Folin-Ciocalteu phenol reagent, diluted 1:1 v/v immediately before use. Vortex mixer, Spectronic 20 or equivalent simple robust single beam spectrophotometer with wavelength range to 660 nm and cuvettes.

Prepare a standard curve for each set of determinations. Use four points (plus a blank) in the range 20 - 200  $\mu$ g protein. Make up to final volume 1 ml with distilled water.

Pipette duplicate 10  $\mu$ l or 25  $\mu$ l samples of PMS preparations and add 1 ml distilled water. Prepare reagent "A" as described by Lowry et al. (1951) and add 5 ml reagent "A" to each standard and sample tube. Mix on vortex mixer and allow to stand about 20 mins. With each tube being mixed on the vortex mixer, pipette 0.5 ml diluted Folin-Ciocalteu phenol reagent, mix and allow to stand a further 20 min. Read at 660 nm v. distilled water.

(5) Ethoxyresorufin O-de-ethylase (EROD) determination.

This is based on the method of Burke and Mayer (1974) in which the substrate (ethoxyresorufin) is incubated in a fluorimeter cuvette with enzyme preparation and cofactor (NADPH) in appropriate buffer, and the fluorescence increase due to resorufin production is recorded.

Equipment: 0.1M phosphate buffer in range 7.5 - 8.5; ethoxyresorufin (substrate) 100  $\mu$ M in water plus 1% Tween 80; resorufin (product) in range 10  $\mu$ M in ethanol (both substrate and product should be protected from light); NADPH, 5 mg in pre-weighed vial; micropipettes with disposable tips to deliver 10, 25, 50, 100, 200  $\mu$ l and glass pipette to deliver 2 ml; fluorimeter functioning in range excitation 510 nm and emission 585 nm with 15 nm bandwidth in each beam (Turner 430 or similar is suitable), and connected to strip chart recorder or other

recording device.

Place 2 ml buffer in fluorimeter cuvette and add NADPH to a final concentration of 200  $\mu$ M; add appropriate amount of enzyme (10,000 X g supernatant) and mix. Record fluorescence with excitation at 510 nm and emission at 585 nm. (There should be no increase over time.) After 1 min add ethoxyresorufin to as final concentration of 1.25 mM and record increase in fluorescence for 2 min. Finally add a known concentration of resorufin and note increase in fluorescence; express gradient of enzymatic fluorescence increase in terms of product concentration. (A typical run is shown in Fig. 6.)

#### (6) Benzo(a)pyrene hydroxylase determination

This is based on the procedure of Nebert and Gelboin (1968) in which the substrate (benzo(a)pyrene) is incubated with an enzyme preparation and base-extractable phenolic products are isolated and determined fluorimetrically.

THE READ PARAGRAPH ON SAFETY. BENZO(A) PYRENE IS CARCINOGENIC. IF SUITABLE FACILITIES FOR HANDLING CARCINOGENS ARE NOT AVAILABLE, USE AN ALTERNATIVE SUBSTRATE SUCH AS DIPHENYLOXAZOLE (PPO) TO ASSESS AROMATIC HYDROCARBON HYDROXYLASE (AHH) ACTIVITY.

Equipment: disposable polypropylene test-tubes (16 ml) (Falcon #2006 is suitable); micropipettes with disposable tips, 10 µl to 100 µl; waste pail dedicated to carcinogenic waste and lined with plastic bag; clinical centrifuge, preferably with disposable liners in buckets; fluorimeter capable of functioning at 395 nm (excitation) and 520 nm (emission) (corresponding wavelengths for assay based on PPO are 345 and 520 nm); water bath set at 27°C; safe light (25W red lamp); test-tube racks, Pasteur pipettes, disposable tissue, gloves and bench-top soaker, timer; buffer solution 0.05M Tris-0.25M sucrose-1% KCl; acetone; hexane, spectro-grade (redistilled from KMnO4 if necessary); benzo(a)pyrene, 2mM in methanol or propanol; NADPH, 1.25 mg·ml-1 in Tris-sucrose-KCl

buffer (may be kept frozen 60d).

To a 16 ml polypropylene tube add 500 µl Tris-sucrose-KCl buffer, 100 µl NADPH solution, 20 µl 2mM benzo(a)pyrene, and Add 10-100 µl 10,000 X g homogenate or bring to 27°C. microsomal preparation and adjust volume to 1.02 ml with Trissucrose-KCl buffer. Incubate with gentle shaking for 15 min. under safelights. Stop reaction by addition of 1 ml acetone and add 5 ml hexane to each tube. Mix 30 sec on Vortex mixer and centrifuge 3 min. (Wrap the tubes and use liners to prevent leakage at these steps.) Transfer hexane phase (Pasteur pipette) as completely as possible to second tube and back extract with 5 ml 1N NaOH. Mix 30 sec and centrifuge 3 min. Remove hexane layer to incubation tube to minimise waste. Measure fluorescence of the NaOH phase against a standard curve (secondary standard) of quinine sulphate, which in turn, is standardised against 3-hydroxybenzo(a)pyrene.

Seal all disposable tubes and add to waste. Monitor area with long wave UV lamp and clean with solvent and/or NaOHsoaked tissues. Incinerate all waste.



Fig. 1 (a). Summary of field observations of hepatic mixed function oxidase induction in fish resulting from (inferred) environmental pollution. Data shown as histograms with are "control" values on left values and induced on right (comparative data only).



Summary of field observations of hepatic mixed Fig. 1 (b). resulting from (inferred) fish function oxidase induction in shown as histograms with environmental pollution. Data are and values on right "control" values on left induced (comparative data only).



Fig. 2. Assessment of the sensitivity of MFO induction (ethoxyresorufin O-de-ethylase) in flounder as an indicator of hydrocarbon bioavailability in petroleum-contaminated sediments. The total PAH level in treatment E5 was approx. 0.5  $\mu$ g • g<sup>-1</sup> measured by HPLC. Units of activity are nmoles product • mg protein <sup>-1</sup> • min<sup>-1</sup>. Statistical analysis was by ANOVA.



Fig. 3. Comparison of the sensitivity of MFO induction (ethoxyresorufin O-de-ethylase) with other biological indices in flounder exposed to hydrocarbon-contaminated sediments.



Fig. 4. Comparison of the sensitivity of MFO induction (ethoxyresorufin O-de-ethylase) with other biological indices in flounder exposed to hydrocarbon-contaminated sediments.



Fig. 5. Variation of benzo(a)pyrene hydroxylase activity in a pooled sample of winter flounder hepatic microsomes with incubation time.



Resorufin production by pooled winter flounder Fig. 6. hepatic microsomal ethoxyresorufin O-de-ethylase (EROD) as a function of incubation time.



Fig. 7. Temperature dependence of the rate of benzo(a)pyrene hydroxylase and ethoxyresorufin O-de-ethylase (EROD) activity.



Fig. 8. Dependence of the rates of benzo(a)pyrene hydroxylase and ethoxyresorufin O-de-ethylase activities on pH.



Fig. 9. Variation of product formation by winter flounder hepatic microsomal benzpyrene hydroxylase and ethoxyresorufin O-de-ethylase with protein concentration.



Fig. Lineweaver-Burk plots of winter flounder hepatic 10. microsomal benzpyrene hydroxylase and ethoxyresorufin O-deethylase activities.



Fig. 11. Seasonal variation of hepatic microsomal ethoxyresorufin O-de-ethylase activity in female winter flounder from St Margaret's Bay, N.S., sampled from November 1983 to October 1985.



Fig. 12. Seasonal variation of hepatic microsomal ethoxyresorufin O-de-ethylase activity in male winter flounder from St Margaret's Bay, N.S., sampled from November 1983 to October 1985.



 $3^-$  Hydroxy-benzo(a) pyrene production (nmoles • mg protein <sup>-1</sup>• hr<sup>-1</sup>)

Fig. 13. Seasonal variation of hepatic microsomal benzo(a)pyrene hydroxylase activity in female winter flounder from St Margaret's Bay, N.S., sampled from November 1983 to October 1985.



Fig. 14. Seasonal variation of hepatic microsomal benzo(a)pyrene hydroxylase activity in male winter flounder from St Margaret's Bay, N.S., sampled from November 1983 to October 1985.



Fig. 15. Induction of ethoxyresorufin O-de-ethylase in liver, heart, gill and kidney tissue of cunner exposed to a pulse of diesel oil at various periods of gonadal maturation or spawning. Units are as nmoles product • mg protein<sup>-1</sup> • min<sup>-1</sup>.



\* SIGNIFICANTLY DIFFERENT FROM CONTROLS (P<0.05)</pre>

Fig. 16. Benzo(a)pyrene hydroxylase (BPOH) activity in winter flounder hepatic microsomes in response to intraperitoneal injection of PCBs (Aroclor 1254).







**\*** SIGNIFICANTLY DIFFERENT FROM CONTROLS (P<0.05)

DAYS AFTER PCB INJECTION

Fig. 17. Time course of induction of benzo(a)pyrene hydroxylase (BPOH) and ethoxyresorufin O-de-ethylase (EROD) activities in winter flounder injected with the PCB mixture Aroclor 1254 at 200  $\mu$ g • g<sup>-1</sup>. Data as means ± s.d.