WATER QUALITY AND STRESS INDICATORS IN MARINE AND FRESHWATER ECOSYSTEMS: LINKING LEVELS OF ORGANISATION (INDIVIDUALS, POPULATIONS, COMMUNITIES)

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Molecular biomarkers and toxic consequences of impact by organic pollution in aquatic organisms

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Organic contaminants are readily bioaccumulated by aquatic organisms. Exposure to and toxic effects of contaminants can be measured in terms of the biochemical responses of the organisms (i.e. molecular biomarkers). The hepatic biotransformation enzyme cytochrome P4501A (CYP1A) in vertebrates is specifically induced by organic contaminants such as aromatic hydrocarbons, PCBs and dioxins, and is involved in chemical carcinogenesis via catalysis of the covalent binding of organic contaminants to DNA (DNA-adducts). Hepatic CYP1A induction has been used extensively and successfully as a biomarker of organic contaminant exposure in fish. Fewer but equally encouraging studies in fish have used hepatic bulky, hydrophobic DNA-adducts as biomarkers of organic contaminant damage. Much less is known of the situation in marine invertebrates, but a CYP1A-like enzyme with limited inducibility and some potential for biomarker application is indicated. Stimulation of reactive oxygen species (ROS) production is another potential mechanism of organic contaminant-mediated DNA and other damage in aquatic organisms. A combination of antioxidant (enzymes, scavengers) and pro-oxidant (oxidised DNA bases, lipid peroxidation) measurements may have potential as a biomarker of organic contaminant exposure (particularly those chemicals which do not induce CYP1A) and/or oxidative stress, but more studies are required. Both CYP1A- and ROS-mediated toxicity are indicated to result in higher order deleterious effects, including cancer and other aspects of animal fitness.

Introduction

A wide variety of potentially toxic organic contaminants enters marine and other aquatic environments, and is readily taken up into the tissues of resident organisms (Walker & Livingstone 1992). Such chemicals include aliphatic hydrocarbons, polynuclear aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and dibenzofurans, tributyl tins, nitroaromatics, phthalate esters and organochlorines. Uptake of organic contaminants can take place from the sediment, water-column and food, and increases with increasing bioavailability, lipophilicity/hydrophobicity and external concentration of the chemical. Bioaccumulation of contaminants depends on the balance between their rate of uptake and rate of metabolism and/or elimination from the organism. Thus, readily metabolizable contaminants, such as PAHs, tend to bioaccumulate to highest tissue concentrations at the bottom of food chains in invertebrates, where rates of uptake exceed metabolism, rather than at the top of the chains in vertebrates, where rates of metabolism are comparable to uptake (Table 1). In contrast, relatively poorly metabolized compounds, such as many PCBs, bioaccumulate along food chains, reaching highest concentrations in the tissues of top predators (Table 1).

Table 1. *Different patterns of bioaccumulation of readily metabolizable (polynuclear aromatic hydrocarbons) and poorly metabolizable (polychlorobiphenyls) organic contaminants along food chains.*

Amounts of contaminants (range of values) are given in micrograms per gram wet weight for various whole tissues and individual tissues, e.g. liver, eggs (birds) and blubber (whales).
Dry weight concentration was converted to wet weight by a factor of x 0.2.
PAHs = polynuclear aromatic hydrocarbons;
PCBs = polychlorobiphenyls.

Data compiled from Neff 1979, Walker & Livingstone 1992, Widdows & Donkin 1992 and Livingstone *et al.* 1994.
–, indicates no information.

Contaminant	Mollusc	Crustacean	Fish	Bird	Sea mammal
Total PAHs	0.1–75	0.1–60	0.01–0.1	–	–
Total PCBs	0.002–13.9	0.03–7.7	0.44–7.3	0.4–340	1–800

The toxicity of foreign compounds (xenobiotics), such as organic contaminants, can be effected by the parent compound, by metabolism (biotransformation) to free radicals or electrophilic metabolites, and by stimulation of reactive oxygen species (ROS) production (Livingstone 1991a; Sahu 1991; Kehrer 1993). Induction of biotransformation enzymes, alterations in endogenous metabolism (e.g. steroids, redox balance), genotoxicity and damage to other key molecules can all play a part in toxicity, with potential consequences for reproduction, diseases such as carcinogenesis, and other aspects of animal fitness (Walker & Livingstone 1992).

The need to detect and assess the impact of pollution, particularly low concentrations of increasingly complex mixtures of contaminants, on environmental quality has led to the development of molecular indicators (biomarkers) of exposure to, and effects of, contaminants on organisms (Haux & Förlin 1988; McCarthy & Shugart 1990; Livingstone 1993). Exact definitions of biomarkers vary but include "measurements which indicate in molecular terms the presence of contaminants, and/or their deleterious effects, and/or the magnitude of the host response" (modified from McCarthy & Shugart 1990). Such diagnostic and prognostic early-warning tests offer the potential of specificity (e.g. induction of cytochrome P4501A1 and metallothioneins to detect impact by respectively organic and metal contaminants), sensitivity and application to a wide range of organisms. The advantages, limitations and applications of molecular biomarkers in pollution monitoring have been discussed extensively elswhere (McCarthy & Shugart 1990; Stegeman *et al.* 1992; Livingstone 1993). Whereas earlier definitions of biomarkers (variously called "stress indices" or something similar) linked the contaminant-caused biological response of the organism with a necessary decrease in animal health as a result of that response (Bayne *et al.* 1985), these two aspects have now tended to become separated, leading to the identification of biomarkers of organic contaminant exposure (e.g. induction of cytochrome P4501A) and organic contaminant damage (e.g. bulky, hydrophobic DNA-adducts).

The aims of this paper are to (1) briefly describe certain established and potential biomarkers of organic contaminant exposure and damage, and (2) explore the links between the molecular mechanisms of toxicity which the biomarkers reflect and the higher order deleterious effects in organisms. Thus the paper focusses on two major mechanistic areas of molecular toxicity, *viz.* (i) cytochrome P450-mediated mutagen production, and (ii) stimulation of ROS production leading to oxidative damage.

Biomarkers of organic contaminant exposure

Cytochrome P4501A

Cytochrome P4501A1 (CYP1A1) is the terminal component of the mixed-function oxygenase (MFO) system and an oxidative enzyme of central importance in the metabolism of many PAHs and certain PCB congeners. [CYP1A1 is the enzyme product of the *CYP1A1* gene of the P450 multi-gene family – see Nebert *et al.* 1991 for nomenclature convention which is based on sequence homology of the different genes/isoenzymes]. Induction of the enzyme by organic contaminants, such as PAHs, certain PCBs, dioxins and many other chemicals, forms the basis of its use as a biomarker of organic pollution.

Induction of hepatic CYP1A has been used extensively and successfully worldwide in many field studies with over 25 species of fish (N.B. the use of the more general term CYP1A is recommended for the fish enzyme unless the "CYP1A1 sequence" has been established – Stegeman 1992). Thus, subject to characterization of aspects of variability in new species, application of CYP1A as a biomarker in teleost fish can be considered routine (Goksøyr & Förlin 1992; Livingstone 1993).

Biochemical, toxicological and other functional and regulatory aspects of the MFO system in fish have been the subject of a number of recent reviews (Andersson & Förlin 1992; Goksøyr & Förlin 1992; Stegeman 1993; Stegeman & Hahn 1994). In addition, attention has focussed on the practical aspects of using CYP1A induction in fish as a biomarker in pollution monitoring (Goksøyr & Förlin 1992; Förlin *et al.* 1994a). A single gene/protein with properties related to the CYP1A subfamily in mammals has been identified (Heilmann *et al.* 1988; Leaver *et al.* 1993). Characteristically, the CYP1A enzyme is inducible, via an *Ah* (aromatic hydrocarbon) regulatory element in the 5'-upstream regulatory region of the *CYP1A* gene (see later), by a variety of aromatic compounds, including PAHs, PCBs, dioxins and benzofurans. This induction can be analysed with suitable probes at the levels of mRNA, protein or enzyme activity (see Stegeman *et al.* 1992), although the response kinetics can differ for each method

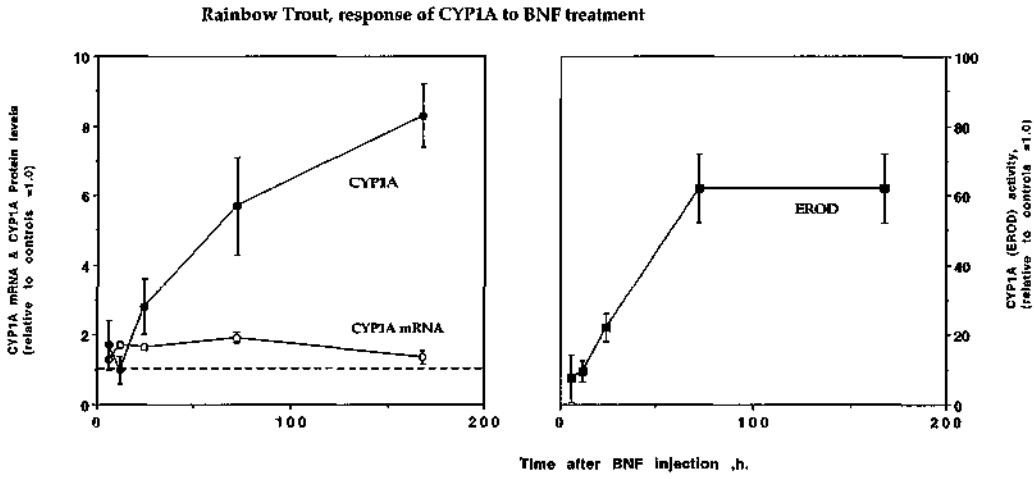

Figure 1. Kinetics of the response of the rainbow trout (*Oncorhynchus mykiss*) liver CYP1A1 system to a single injection of the classical inducer ß-naphthoflavone (BNF). Fish were injected intraperitoneally with 50 mg kg^{-1} BNF, sacrificed at intervals, and livers were assayed for *CYP1A* mRNA with a trout *CYP1A* cDNA probe, CYP1A protein with anti-perch (*Perca fluviatilis*) antiserum, and 7-ethoxyresorufin (EROD) activity. (Redrawn from Celander *et al.* 1993).

of analysis. This is illustrated in Figure 1 for liver of rainbow trout (*Oncorhynchus mykiss*) exposed to the classical CYP1A-inducer, ß-naphthoflavone. Aryl hydrocarbon hydroxylase (e.g. benzo[a]pyrene hydroxylase – BPH) and 7-ethoxyresorufin *O*-deethylase (EROD) activities are the most commonly used measurements of CYP1A induction in fish because they are rapid and no specific probes (antibodies or gene probes) are required for the assays. However, the measurement of CYP1A protein or mRNA is also recommended for routine field monitoring because CYP1A catalytic activity can be inhibited in certain situations, e.g. very high levels of pollutants, or the presence of particular contaminants such as certain PCB congeners, metals and hepatotoxins (see Livingstone 1993). For example, in a recent study in which flounders *Platichthys flesus*, held in mesocosms, were exposed to dredged sediments from Rotterdam harbour, elevations of hepatic *CYP1A* mRNA and CYP1A protein, but not EROD activity, were detected, indicating induction of the enzyme but inhibition of its catalytic properties (Eggens *et al.* 1994). Co-ordinated use of the different methods can also provide more detailed information on the sequelae of toxic events occurring in a tissue. For example, induction of CYP1A in an organ can be measured using enzyme activity assays, and the response within particular cell types studied with immunocytochemistry (Smolowitz *et al.* 1991; Husøy *et al.* 1993).

Table 2. *Microsomal EROD activity and CYP1A protein cytochrome concentrations in liver, and TCDD equivalents in muscle of female pike* (Esox lucius) *from three sites in Lake Vänern, Sweden.*

CYP1A = cytochrome P4501A, in A_{410} absorbance units.
EROD = 7-ethoxyresorufin *O*-deethylase, in nmol min^{-1} mg^{-1} protein.
TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, in pg g^{-1} wet weight,
calculated according to the Nordic model (Ahlborg 1989).
All values are means and standard errors ($n = 5$ (TCDD) or 10).
*$P < 0.05$ compared to reference site (site 3). Data from Förlin *et al.* 1992.

Site	TCDD	EROD	CYP1A
1 (north)	1.04 ± 0.05*	0.20 ± 0.07*	0.35 ± 0.04*
2 (middle)	0.38 ± 0.38	0.09 ± 0.06	0.33 ± 0.06
3 (south)	0.15 ± 0.05	0.05 ± 0.03	0.28 ± 0.05

Nearly twenty years ago, it was proposed that the induction response of MFO (CYP1A) activity could be used as a biomarker for monitoring environmental organic pollution (Payne & Penrose 1975). Subsequently, the kinetics and dose-responses for induction of CYP1A in (principally) liver, but also in other tissues (e.g. kidney, intestines) of fish, have been characterised in numerous laboratory studies (e.g. George & Young 1986; Leaver *et al.* 1988, 1994; Celander *et al.* 1993), and the response has been validated by many field studies showing elevated CYP1A-dependent (EROD and AHH) activities in fish from waters contaminated with petroleum products, industrial and municipal effluents (e.g. Stegeman *et al.* 1988; Goksøyr *et al.* 1991). Detailed examples of the field application of hepatic CYP1A induction in fish include the correlation of EROD activity and CYP1A protein level with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents in pike *Esox lucius* from Lake Vänern, Sweden (Table 2), and two studies of hydrocarbon pollution in Scottish waters, namely the operation of the Grangemouth petrochemical complex in the Forth estuary, and the recent MV Braer oil-tanker incident in the Shetlands. A measurable impact of the refinery discharges from Grangemouth on *P. flesus*, consistent with the known hydrography of the region, was demonstrated by maximal hepatic microsomal EROD activities in fish occurring in the area of

turbidity maximum of the Forth estuary, between Kincardine and Grangemouth (Fig. 2). In the MV Braer incident, the tanker ran aground at Garth's Ness, southern Shetland, releasing 85,000 tonnes of Gulfaks crude oil, and contaminated a large area of the coastline (Fig. 3a). Although, due to severe weather and other factors, the coastline appeared to be clean again 10 days later, hepatic EROD activity in rockling (*Ciliata* sp.), a small subtidal gadoid fish, was still up to x18-fold higher at St Ninian (10 km from the wreck) and Burra Isle (northern limit of reported slicks) than at two reference sites (Lunna and Voe) 3 months after the incident (Fig. 3b), demonstrating that biological impact had occurred and persisted. EROD activities returned to reference levels 8 months after the incident. Levels of CYP1A protein also showed direct proportionality with EROD activities, indicating that the high levels of hydrocarbons were not inhibitory to CYP1A.

In contrast to fish, much less is known of the existence, fundamental properties and gene regulation of CYP1A in marine invertebrates, and few studies have been carried out (Livingstone 1991b). The evidence of a CYP1A-like enzyme, or isoform, which is readily inducible by organic pollutants such as particular PCBs and PAHs, is as yet unclear (Livingstone 1991a). EROD activity is catalysed solely by CYP1A in vertebrates, but is either not detectable or only present in low activity in invertebrates (Livingstone 1991a), although microsomal EROD activities of 24 to 60 pmol min^{-1} mg^{-1} were recently reported in digestive gland of the bivalves *Donax trunculus* and *Brachidontes variabilis*, and the gastropods *Patella caerulea* and *Avicularia gibbosula* (Yawetz *et al.* 1992), using the spectrophotometric assay of Klotz *et al.* (1984), rather than the more commonly used fluorometric assay (see references to work on fish, above). BPH activity is mainly catalysed by CYP1A, plus some other CYP isoenzymes, in vertebrates (Åstrom & DePierre 1986; Goksøyr & Förlin 1992) and in contrast to EROD activity is widely detectable in marine invertebrates (Livingstone 1991a). Apparent induction of the MFO system with exposure to PAHs or PCBs has been indicated for some marine invertebrate species, e.g. spiny crab *Maja crispata* (Batel *et al.* 1988), but not for

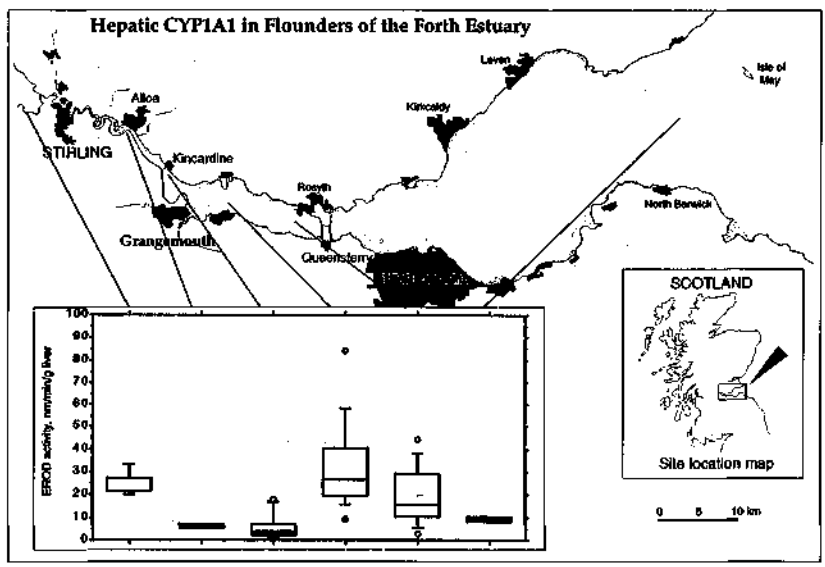

Figure 2. Monitoring the impact of the Grangemouth petrochemical complex on flounder (*Platichthys flesus*) in the Forth estuary using hepatic CYP1A measurements. Flounder were caught from the stations shown and analysed for 7-ethoxyresorufin *O*-deethylase (EROD) activity; values shown are means and percentiles. (Data from Sulaiman *et al.* 1991).

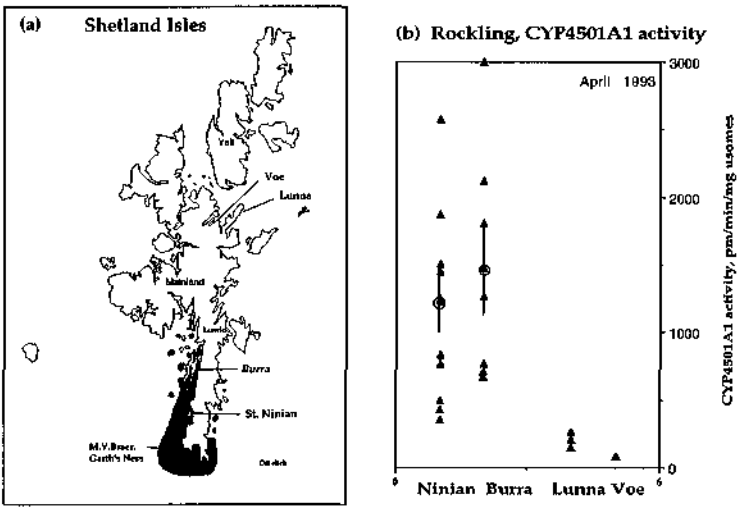

Figure 3. Wreck of the oil-tanker MV Braer in Shetland, in January 1993, and impact on hepatic CYP1A of a representative inshore fish species (rockling, *Ciliata* sp.). Fish were caught by trapping at the sites shown and analysed for 7-ethoxyresorufin *O*-deethylase (EROD) activity by standard procedures. (a) Sampling sites and extent of surface oil contamination; (b) EROD activities in rockling from the different sites in April 1993. (Unpublished data from S. G. George and co-workers).

others, e.g. spiny lobster *Panuliris argus* (James & Little 1984). Overall, responses are variable and to an extent absent (Livingstone 1991b), and certainly much lower than for fish, e.g. 3-fold increases in BPH activity in pyloric caeca of the starfish *Asterias rubens* (Den Besten *et al.* 1993) compared with up to several hundred-fold increases for hepatic EROD activity in fish. This limited response could reflect a less sophisticated mechanism of induction than in vertebrates, which can be dampened down by interactions with other environmental variables, such as season and reproductive state. In field studies with mussels (*Mytilus* sp.) and other molluscs, correlation of putative induction of the digestive gland MFO system with exposure to organic contaminants has been seen for BPH activity (Narbonne *et al.* 1991), total cytochrome P450 (Yawetz *et al.* 1992) and the "418-peak" (putative denatured cytochrome P450) (Livingstone 1988), but as yet no single parameter has emerged as a widely used biomarker for exposure to organic pollution in molluscs, and a multi-parameter approach has been suggested (Livingstone 1991b). Thus, for example, in the case of *D. trunculus* exposed to an oil-spill, an increase in total cytochrome P450 content was accompanied by a drastic decrease in EROD activity (Yawetz *et al.* 1992). Deleterious interactions by other contaminants have also been indicated; e.g. levels of cytochrome P450, cytochrome b_5 and NADPH-cytochrome c (P450) reductase activity in digestive gland microsomes of the marine gastropod *Monodonta turbinata* were reduced with exposure to cadmium, mercury or chromium (Manelis *et al.* 1993).

More recently, additional evidence has been obtained for the existence of a CYP1A-like enzyme in digestive gland of molluscs. Immunoquantitation (Western blotting) with polyclonal antibodies to CYP1A of *O. gairdneri* indicated the presence of a hydrocarbon-inducible CYP1A-like enzyme in the digestive gland of the chiton *Cryptochiton stelleri* (Schlenk & Buhler 1989). Similarly, Western blotting of partially purified cytochrome P450 from digestive gland of mussel (*Mytilus edulis*) with polyclonal antibody to perch (*Perca fluviatilis*) CYP1A, gave a single band of 54 kD (Porte *et al.* 1994); Northern blotting with *O. gairdneri CYP1A1* cDNA probe gave a single mRNA band (Wootton *et al.* 1994). These measurements of a

CYP1A-like protein and *CYP1A*-like mRNA, plus *in vitro* metabolism of benzo[a]pyrene to free metabolites, have been applied to a field study of mussels (*Mytilus galloprovincialis*) in the Venice area (Table 3). All three parameters were higher, or indicated to be higher, at an industrial site (Canale Vitorio Emanuele) in the Venice Lagoon, compared to a cleaner site in the Adriatic Sea, correlating with x 2.4 to x 7.9 higher levels of hydrocarbons and PCBs in mussels at the former site. Thus, the results argue for further study to investigate the biomarker potential of this enzyme in marine invertebrates.

Table 3. *Amounts of some chemical contaminants and biomarkers in digestive gland of mussels* (Mytilus galloprovincialis) *from the Venice Lagoon and the Adriatic Sea.*

[a]Unresolved complex mixture (UCM) of aliphatic hydrocarbons, in μg g^{-1} dry weight.
[b]Total polynuclear aromatic hydrocarbons (PAHs) and polychlorobiphenyls (PCBs), in ng g^{-1} dry weight.
[c]Arbitrary units.
[d]Free polar metabolites (sum of dihydrodiols, diones and phenols), in pmol min^{-1} mg^{-1} protein.
*$P < 0.05$ comparing sites.
All values are means and standard errors ($n = 3$–6). Data from Livingstone *et al.* 1994.

Measurement	Platform Site (Adriatic Sea)	Canale Vitorio Emanuele (Venice Lagon)
UCM[a]	339	814
PAHs[b]	149	443
PCBs[b]	185	1461
CYP1A-like mRNA[c]	10.5 ± 5.6*	39.5 ± 0.9
CYP1A-like protein[c]	6.0 ± 1.3	10.9 ± 3.8
BaP metabolism[d]	1.25 ± 0.61	3.02 ± 0.93

Antioxidant enzymes

Reactive oxygen species (ROS) such as the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) are continually produced in biological systems as toxic by-products of normal oxidative metabolism. ROS production can be increased by interactions with organic xenobiotics by various mechanisms (see later). Detoxication and removal of ROS and other oxidants is effected by antioxidant defence systems, including specific antioxidant enzymes such as superoxide dismutase (SOD; converts O_2^- to H_2O_2; EC 1.15.1.1), catalase (converts H_2O_2 to H_2O; EC 1.1.1.6), glutathione peroxidase (GPX; converts H_2O_2 to H_2O utilizing reduced glutathione; EC 1.11.1.9), aldehyde dehydrogenase (ALDH; converts aldehydes to acids; EC 1.2.1.3) and DT-diaphorase (DTD; prevents quinone-mediated O_2^- production; EC 1.6.99.2). Elevation of antioxidant enzymes in response to increased ROS production offers the potential of a biomarker of contaminant-mediated oxidative stress (Di Giulio 1991; Stegeman *et al.* 1992), although such changes are indicated to be much less marked (i.e. basal activities are already high to cope with endogenous ROS production), more variable and less specific (ROS production can be increased by metals and oxygen tension) than for CYP1A (Livingstone *et al.* 1990; Winston & Di Giulio 1991). However, much yet remains to be elucidated of these processes in marine organisms and it could be that biomarker specificity will be increased by a combination of antioxidant (enzymes, scavengers, stress proteins) and pro-oxidant (ROS production, oxidative damage) measurements (Di Giulio 1991; Stegeman *et al.* 1992; Sanders 1993; Lemaire & Livingstone 1994a). Also, both antioxidant enzyme activities and oxidative damage have the advantage that they may be increased by

organic contaminants which do act via induction of CYP1A and/or metabolism to bulky DNA-adducts (see later).

Pro-oxidant and antioxidant processes in fish and marine invertebrates have been the subject of several reviews (Di Giulio *et al.* 1989; Livingstone *et al.* 1990; Di Giulio 1991; Winston 1991; Winston & Di Giulio 1991; Lemaire & Livingstone 1994a). Antioxidant enzyme activities are widely distributed in the tissues of marine organisms, and are generally highest in liver of fish or digestive gland or equivalent in marine invertebrates. In addition to SOD, catalase and GPX, recent studies have also indicated the widespread presence of hepatic ALDH and DTD in fish (Förlin *et al.* 1994b). These two enzymes are of particular interest because in mammals they are part of the same gene battery as CYP1A (so-called [*Ah*] gene battery) and may be coinduced with exposure to organic xenobiotics (Nebert *et al.* 1990). Increases in hepatic antioxidant enzyme activities have been seen or indicated with experimental exposure to contaminants, but the changes can be transient. Thus, with exposure to sediments contaminated with PAHs, PCBs and other chemicals, increases in hepatic SOD and catalase activities were seen in channel catfish *Ictalurus punctatus* after 2 to 28 days (Di Giulio *et al.* 1993), and in dab *Limanda limanda* after 80 but not 140 days (Livingstone *et al.* 1993), but not in *P. flesus* after 6 months (Bergman *et al.* 1994). In contrast, increased DTD and ALDH (benzaldehyde dehydrogenase) activities were seen in liver of *O. mykiss* after 12 months exposure to the carcinogen aflatoxin B_1 (Parker *et al.* 1993). Higher hepatic antioxidant enzyme activities in fish from polluted field sites were seen for SOD and catalase in *L. limanda* from the North Sea (Livingstone *et al.* 1992), and on occasions for catalase and putative DT-diaphorase in male goby *Zosterisessor ophiocephalus* from the Venice Lagoon (Livingstone *et al.* 1994), but seasonal or other environmental interactions were also indicated. In *Mytilus* sp., slight increases have been variously seen in digestive gland SOD, catalase, GPX and putative DT-diaphorase activities with experimental (Livingstone *et al.* 1990) and field (Porte *et al.* 1991) exposure to PAHs and PCBs.

Biomarkers of organic contaminant damage

DNA-adducts

Many organic xenobiotics such as PAHs and PCBs are metabolically activated to electrophilic metabolites which bind to nucleic acids and other macromolecules, forming covalent adducts. DNA-adduct formation integrates contaminant uptake, metabolism and macromolecular repair, and is the initial event in chemical carcinogenesis. Thus, it is currently being used in humans as a biomarker for exposure to environmental and occupational carcinogens (Santella 1991), and a similar role as a biomarker of contaminant exposure and damage is proposed for aquatic organisms (McCarthy & Shugart 1990; Dunn 1991; Jones & Parry 1992). Experimental studies in fish have shown that hepatic DNA-adducts are persistent, sometimes lasting for months, and in the case of PAHs are retained longer than parent compounds or unbound metabolites (Varanasi *et al.* 1992). The ^{32}P-postlabelling method (which can detect 1 adduct in 10^9 normal bases – see Jones & Parry 1992) has been used for the few field studies carried out so far on marine organisms, and the majority of these have shown increased formation of bulky, hydrophobic aromatic adducts with higher levels of contaminant exposure (Livingstone 1993). The studies include fish species from contaminated waters of New York and Michigan (Maccubbin *et al.* 1990), Boston and Long Island Sound (Varanasi *et al.* 1992) and Puget Sound (Stein *et al.* 1991) (all USA), and the Damsui river, Taiwan (Liu *et al.* 1991). DNA-adducts were also observed in juvenile mussels (*M. galloprovincialis*) from near an oil refinery, but not from a clean site (Kurelec *et al.* 1990). An aspect of some concern, however, has been the observation of DNA-adducts of apparently natural (non-pollutant) or endogenous

origin, present in both fish (Kurelec *et al.* 1989) and marine invertebrates (Garg *et al.* 1992). Such adducts are indicated to be species-specific, endogenously regulated (i.e. change with season/reproduction) and independent of contaminant exposure. However, overall, measurement of DNA-adducts is considered to have great potential for application in pollution monitoring (Livingstone 1993).

Oxidative damage

Somewhat fewer studies, particularly in the field, have been carried out on oxidative damage, compared to genotoxicity, in marine organisms. Oxidative damage has the disadvantage that it may be caused by certain non-organic as well as organic contaminants, but has the advantage that the wide range of toxic pro-oxidant contaminants includes those which may not be detected by CYP1A induction or bulky DNA-adduct formation. The contaminants include oxidizing air pollutants (such as ozone, sulphur dioxide and various nitrogen oxides) and other direct acting oxidants (such as H_2O_2, organic peroxides, and waterborne nitrite and chlorine), plus stimulators of ROS production (including quinones, nitroaromatics, azo dyes, hydrazines, bipyridyl herbicides and transition metals) (Di Giulio 1991).

Studies on marine organisms have focussed principally on oxidative damage to lipid (i.e. lipid peroxidation producing malonaldehyde-like breakdown products) and DNA (Di Giulio 1991; Lemaire & Livingstone 1994a). For example, increases in lipid peroxidation were seen in digestive gland of mussel (*Mytilus* sp.) exposed to benzo[a]pyrene (Livingstone *et al.* 1990) or copper sulphate (Viarengo *et al.* 1990), and in the liver of catfish *Heteropneustes fossilis* exposed to mercuric chloride (Bano & Hasan 1989), and of *L. limanda* (Livingstone *et al.* 1993) and *I. punctatus* (Di Giulio *et al.* 1993) exposed to sediments containing, principally, PAHs and PCBs. The oxidised base 8-hydroxy-deoxyguanosine was present in DNA of digestive gland of *M. edulis* and liver of *L. limanda*, but was not increased respectively with exposure to pro-oxidant chemicals (menadione, nitrofurantoin) (Marsh *et al.* 1993), or along a pollution gradient in the North Sea (Chipman *et al.* 1992). In contrast, levels of hepatic 8-hydroxy-deoxyguanosine increased in English sole *Parophrys vetulus* exposed to nitrofurantoin (Nishimoto *et al.* 1991), and in *O. mykiss* exposed to hydrogen peroxide with the hepatocarcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Kelly *et al.* 1992). The oxidised base 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) was present in neoplastic livers of *P. vetulus* from a polluted environment (Malins *et al.* 1990).

Molecular mechanisms of toxicity and links with higher order effects

CYP1A-catalysed metabolism, DNA-adduct formation, chemically-caused cancer and other higher order effects

Hepatic neopasms and other pathologies in fish have been correlated with both experimental (Fabacher *et al.* 1991; Schiewe *et al.* 1991) and field (Myers *et al.* 1991) exposure to organic contaminants such as PAHs, PCBs and dioxins. Induction of CYP1A by such compounds occurs via their binding to a soluble protein known as the *Ah* receptor, the resulting complex of which interacts with the regulatory region of the *CYP1A* gene (see before). This protein has been found in the liver of seven species of teleost and elasmobranch fish (Hahn *et al.* 1991). Although many organic xenobiotics are detoxified by CYP1A, other are activated to more toxic and possibly mutagenic species. Induction of CYP1A by PAHs, PCBs and dioxins, metabolism of PAHs to mutagenic species, and formation of DNA-adducts have been implicated in the etiology of chemically caused carcinogenesis in fish (Stein *et al.* 1990; Stegeman & Lech

1991). Considerable mechanistic evidence therefore exists linking CYP1A with higher order (carcinogenic) effects.

Links between CYP1A (and other P450s) and other higher order effects in fish, such as reporoduction and other aspects of animal fitness, are far more tenuous and generally correlative rather than mechanistic (McCarthy & Shugart 1990). A battery of physiological and biochemical responses, including CYP1A induction, were investigated in studies on the environmental impact of bleached kraft mill effluents (BKME) (Andersson *et al.* 1988; Södergren 1989; Förlin *et al.* 1991). These co-ordinated field and laboratory exposure studies in various fish species showed that the animals were markedly affected by BKME, producing altered plasma ion levels and blood cell counts, reduced gonad size, and induced hepatic EROD activity as the strongest signal. In these and other field studies in Scandinavia and North America, it is apparent that CYP1A induction was indicative of areas where the health of fish was adversely affected by BKME (Lindström-Seppä & Oikari 1990; Hodson *et al.* 1991; Munkittrick *et al.* 1991). This conclusion is supported by the simultaneous occurrence of elevated EROD activity and adverse biological effects, including effects at the population level, *viz.* reduced survival rates of both larvae and adults, and altered patterns of growth (see Södergren 1989). Thus, although no mechanistic links are established between CYP1A induction and higher order effects, such as reproduction and growth, the extensive correlative data-set produced from the fish BKME studies indicate that CYP1A induction can be used as an early warning biomarker of pollutant impact at higher levels of biological organisation.

Much less is known of the situation in marine invertebrates. Many organic contaminants, including chlorinated paraffins, PAHs, PCBs, aromatic amines, nitroaromatics, chlorophenols and phthalate esters, are metabolized to macromolecular adducts by the MFO system and other biotransformation enzymes in a wide range of marine invertebrates (Livingstone 1991a; Marsh *et al.* 1992, 1993; Walker & Livingstone 1992). A CYP1A-like enzyme is indicated, with limited inducibility (see before) and the ability to metabolize PAHs such as BaP to bacterial mutagens (Michel *et al.* 1993) and DNA-adducts (Livingstone 1991a; Marsh *et al.* 1992). Regulation of *CYP1A*-like mRNA levels is indicated in *Mytilus* sp. (Wootton *et al.* 1994), but the *Ah*-receptor protein was not detected in a wide range of marine invertebrates (Hahn *et al.* 1992), indicating either that levels of the protein were below the level of detection of the assay procedure, or there is possibly an alternative (maybe more primitive) mechanism of induction as, for example, is indicated for induction of EROD activity by *N*-benzylimidazole in *Ah*-nonresponsive strains of mice (Manning & Franklin 1992).

Our understanding of the relationship between contaminant exposure and cancer in marine invertebrates is similarly limited. Previous surveys of the literature found that there was little evidence to associate neoplastic diseases in bivalve molluscs with environmental pollution (Mix 1986). However, experimental studies have demonstrated chemical induction of tumours in the gastropod *Ampullarius australis* by 3-methylcholanthrene (Krieg 1972), in the oyster *Crassostrea virginica* by a mixture of PAHs, PCBs, amines and metals (Gardner *et al.* 1992), and in planarians by a range of mammalian carcinogens, including BaP (Schaeffer 1993). Thus, combined with the molecular studies described above, an involvement of enzyme-mediated bioactivation of organic contaminants to carcinogens in chemically-caused cancer is indicated. The bioactivation of organic contaminants and/or the occurrence of cancer is also indicated to have an effect on animal fitness. Thus, exposure of the snail *Lymnaea stagnalis* to 2,2'– or 4,4'–PCBs affected the latency of oviposition and the number of egg masses and eggs (Wilbrink *et al.* 1987), and field experiments on the clam *Mya arenaria* demonstrated that under natural conditions mortality was higher in animals with hematopoietic neoplasia than those without neoplasia (Brousseau & Baglivo 1991).

ROS generation, oxidative damage, cancer and other diseases

Much evidence exists for mechanisms of xenobiotic-stimulated ROS production and ROS-mediated oncogene activation and anti-oncogene inactivation in mammals. However, what is uncertain is the extent to which such processes contribute to chemically-caused carcinogenesis and other diseases *in vivo*, although the evidence for a significant role is compelling (Sahu 1991; Kehrer 1993). Mechanisms of xenobiotic-stimulated ROS production include disruption of electron-transfer systems, enzyme induction, redox cycling and organic radical production, plus secondary processes such as lipid peroxidation producing more oxidants (Borg & Schaich 1984; Livingstone 1991a). ROS can activate oncogenes by point mutation (via direct oxidation, gene amplification, and chromosomal translocation), DNA cross-link formation and DNA strand breakage; and influence oncogene activation by altering membrane function (via lipid peroxidation and protein degradation) which in turn can change signal transduction, protein kinase activation and growth factors and their receptors (Sahu 1991; Kehrer 1993).

Little is known of the role of disruption of electron-transfer systems by xenobiotics in marine organisms, although many highly lipophilic contaminants such as PAHs and PCBs will readily penetrate membrane systems (Livingstone 1991a; Walker & Livingstone 1992). Enzyme loci for ROS generation include proteins involved in electron-transfer (e.g. flavoprotein reductases) and oxygen metabolism (e.g. cytochrome P450s, amino oxidases). Induction of total cytochrome P450 content (rather than specific forms, i.e. CYP1A), or cytochrome P450 reductase and cytochrome b_5 reductase activities, with contaminant exposure, rarely occurs in fish (Goksøyr & Förlin 1992) but has been seen in bivalve and gastropod molluscs (Livingstone 1991a; Yawetz *et al.* 1992). Redox cycling of xenobiotics such as quinones, nitroaromatics and aromatic amines involves their univalent reduction to anion radicals, followed by autoxidation to produce $O_2^{\cdot-}$, which in turn can give rise to H_2O_2 and the highly reactive •OH (Borg & Schaich 1984). Redox cycling of model and pollutant xenobiotics, including quinones derived from PAHs (BaP) and present in pulp mill effluents, has been demonstrated variously for liver or digestive gland microsomes of several fish species (Winston & Di Giulio 1991; Lemaire *et al.* 1994) and *Mytilus edulis* (Livingstone *et al.* 1990; Garcia Martinez *et al.* 1992; Garcia Martinez & Livingstone 1994). Studies on hepatic microsomes of *P. flesus* have shown the involvement of cytochrome P450 reductase in redox cycling by the nitroaromatic nitrofurantoin (Lemaire & Livingstone 1994b), and minimal stimulation of ROS production by the non-redox-cycling pesticide, lindane, presumably by free radical interactions (Lemaire *et al.* 1994).

Redox cycling and other contaminants capable of stimulating ROS-production may be taken up from the environment, or produced by biotransformation. Given the observations with lindane, the range of these contaminants, and therefore their cumulative effects on ROS production, may be considerable. Normal metabolism of PAHs, such as BaP, to redox-cycling quinones is indicated to be low in fish, crustaceans and echinoderms (major metabolites are phenols and dihydrodiols), but higher in molluscs (Stegeman 1989; Livingstone 1991a; Den Besten *et al.* 1992). Much higher levels of quinones are produced in microsomes of fish (*P. flesus*), crustaceans (crab *Carcinus maenas*) and echinoderms (*A. rubens*) by hydroperoxide-dependent metabolism of BaP (i.e. peroxidase activity of cytochrome P450) (Den Besten *et al.* 1992; Lemaire & Livingstone 1994c; Lemaire *et al.* 1993). Combined with the observations of contaminant-stimulated lipid peroxidation (see before), this indicates the possibility of a toxicity cycle of lipid peroxidation leading to enhanced quinone formation and ROS production, leading to more oxidative damage, including lipid peroxidation. The oxidative damage may also include oxidation of DNA bases, as has been described earlier for the effects of the pro-oxidants H_2O_2 and nitrofurantoin on hepatic 8-hydroxy-deoxyguanosine levels.

Very few experimental studies have been carried out in marine organisms on the link between ROS generation and chemical-caused cancer; nevertheless a relationship is indicated. Thus, in liver of *O. mykiss*, activities of the antioxidant enzymes DTD and ALDH were elevated in aflatoxin B_1-induced tumors (Parker *et al.* 1993), and levels of 8-hydroxy-deoxyguanosine were correlated with the tumour-enhancing effect of H_2O_2 and MNNG-initiated carcinogenesis (Kelly *et al.* 1992). Increased ROS generation was indicated in livers of *L. limanda* along a pollution gradient in the North Sea (Moore, 1992) and unique oxidised DNA lesions (FapyGua) were present in hepatic tumors of *P. vetulus* from polluted sites (see before). The extent of any oxidative damage will depend upon the degree to which antioxidant defences are overwhelmed by ROS production (Halliwell & Aruoma 1991; Kehrer 1993). The former may be particularly high in organisms which regularly experience other forms of oxidative stress, such as changing oxygen availability, e.g. many molluscs (Livingstone *et al.* 1990).

Conclusions

Although induction of CYP1A need not necessarily lead to toxic consequences because, for example, reactive metabolites can be detoxified by phase II conjugases (see Livingstone 1991a; George 1994), considerable evidence exists in fish to link CYP1A function and DNA-adduct formation with higher order deleterious effects, principally contaminant-caused cancer. Alterations in these biomarkers therefore signals a cause for concern for the individual organisms and the population. Much less is known, in fish, about contaminant-stimulated ROS production and the contribution of oxidative stress to higher order deleterious effects, but the evidence for a significant role is growing. Similarly, less is known about a CYP1A-like enzyme and ROS production in marine invertebrates, but roles for both in contaminant-mediated toxicity seem likely.

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