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Biomarkers and bioassays for detecting dioxin-like compounds in the marine environment ☆

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Abstract

The presence of toxic chemical contaminants in some marine organisms, including those consumed by humans, is well known. Monitoring the levels of such contaminants and their geographic and temporal variability is important for assessing and maintaining the safety of seafood and the health of the marine environment. Chemical analyses are sensitive and specific, but can be expensive and provide little information on the actual or potential biological activity of the contaminants. Biologically-based assays can be used to indicate the presence and potential effects of contaminants in marine animals, and therefore, have potential for routine monitoring of the marine environment. Halogenated aromatic hydrocarbons (HAHs) such as chlorinated dioxins, dibenzofurans, and biphenyls comprise a major group of marine contaminants. The most toxic HAHs (dioxin-like compounds) act through an intracellular receptor protein, the aryl hydrocarbon receptor, which is present in humans and many, but not all, marine animals. A toxic equivalency approach based on an understanding of this mechanism provides an integrated measure of the biological potency or activity of HAH mixtures. Biomarkers measured in marine animals indicate their exposure to these chemicals in vivo. Similarly, in vitro biomarker responses measured in cell culture bioassays can be used to assess the concentration of 'dioxin equivalents' in extracts of environmental matrices. Here, I have reviewed the types and relative sensitivities of mechanistically-based, in vitro bioassays for dioxin-like compounds, including assays of receptor-binding, DNA-binding and transcriptional activation of native (CYP1A) or reporter (luciferase) genes.

Abbreviations: AHH, aryl hydrocarbon hydroxylase; AHR, Ah (aryl hydrocarbon) receptor; CYP1A1, Cytochrome P450 1A1; DRE, dioxin response element; EC50, estimated concentration needed to produce 50% of the maximal response; ELISA, enzyme-linked immunosorbent assay; EROD, ethoxyresorufin *O*-deethylase; HACCP, Hazard Analysis and Critical Control Point; HAH, halogenated aromatic hydrocarbons; PAH, polynuclear aromatic hydrocarbon; P450, cytochrome P450; PCB, polychlorinated biphenyl; PCDDs, polychlorinated dibenzo-*p*-dioxins; PCDFs, polychlorinated dibenzofurans; SPMDs, semipermeable membrane devices; TCB, tetrachlorobiphenyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; TEF, toxic equivalency factor; TEQ, dioxin (TCDD) equivalents

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Examples of their use in environmental monitoring are provided. Cell culture bioassays are rapid and inexpensive, and thus have great potential for routine monitoring of marine resources, including seafood. Several such assays exist, or are being developed, for a variety of marine contaminants in addition to the dioxin-like chemicals. A battery of cell culture bioassays might be used to rapidly and sensitively screen seafood for the presence of contaminants of concern, including dioxin-like compounds as well as other contaminants such as natural toxins, hormonally active agents, and heavy metals. Such a battery of mechanism-based, in vitro bioassays could be incorporated into monitoring efforts under recently adopted hazard analysis and critical control point (HACCP) programs.

Keywords: Seafood; Cell culture bioassays; Biomarkers; Dioxins; Polychlorinated biphenyls; Cytochrome P450; CYP1A1; Hepatoma cells; Reporter genes

1. Introduction

It is well known that a wide variety of toxic chemicals are present in the world's oceans (Clark, 1986; Giam and Ray, 1987). Included among these are natural products as well as compounds of anthropogenic origin: marine toxins, inorganic and organic metals, petroleum and combustion-derived hydrocarbons, chlorinated pesticides, halogenated aromatic hydrocarbons, and many others. These contaminants can be found bound to sediments, dissolved in water (including pore water), in the sea-surface microlayer, and within various marine organisms, including marine animals used as food by humans and by other marine species. The highest concentrations of these chemicals are often found in urban harbors and other coastal areas (Farrington et al., 1983; Weaver, 1984; Dethlefson, 1988). However, there is also a more generalized, global contamination; persistent organic and inorganic pollutants have been documented even in remote locations such as the open ocean, polar regions, and in the deep sea (Stegeman et al., 1986; Muir et al., 1988; Mason and Fitzgerald, 1990; Ballschmiter et al., 1997; Stegeman et al., 2001).

Experimental or epidemiological studies have shown that marine pollutants are capable of producing a variety of toxic effects in exposed organisms; some of the most common include neurotoxicity, immune dysfunction, reproductive and developmental effects, and cancer. Some of the compounds, such as the algal toxins sometimes found in shellfish, are primarily acutely toxic, while others, such as dioxins and polychlorinated biphenyls (PCBs), are of concern primarily because of their potential for causing chronic effects following long-term, low-level exposure.

The presence of toxic chemicals in the marine environment presents two types of hazard: hazard to the health of humans exposed through consumption of contaminated seafood, and hazard to the health of marine organisms and ecosystems. The potential dangers of contaminated seafood are recognized by some consumers (Anonymous, 1992), though not by all (Tilden et al., 1997; Burger et al., 1998). For chemicals such as methyl mercury and PCBs, seafood represents the primary source of human exposure (excluding occupational and accidental exposures) (Friberg, 1988; Svensson et al., 1991; Egeland and Middaugh, 1997). For other chemicals, intake from seafood merely augments exposure from other sources. The evidence for acute and chronic health effects associated with consumption of chemicals from seafood has been reviewed (Swain, 1988; Boyer et al., 1991; Dawe and Stegeman, 1991; Kimbrough, 1991; Ahmed et al., 1993a; Grandjean et al., 1997; Longnecker et al., 1997).

In addition to their potential impact on human health, marine pollutants pose a well documented risk to the health of marine organisms and ecosystems. Some marine animals exhibit levels of certain contaminants, such as PCBs, that are among the highest ever reported (Tanabe and Tatsukawa, 1992; Elskus et al., 1994; Norstrom and Muir, 1994; Lake et al., 1995; Bello et al., 2001). In some cases, such as PAH-induced tumors in flatfish (Malins et al., 1985; Murchelano

and Wolke, 1985) or organotin effects in gastropod molluscs (Gibbs and Bryan, 1986; Alzieu, 1991), the data suggesting an adverse impact are dramatic and compelling. In other cases, such as the possible impact of PCBs and other organochlorines on marine mammal reproduction, unequivocal evidence of health effects has been difficult to obtain (Addison, 1989).

Because of the presence and potential impact of marine pollutants in humans and wildlife, the need for monitoring the fate and effects of these chemicals has been recognized for many years (Pearce and Despres-Patanjo, 1988; Ahmed, 1991; Pearce, 1997). The choice of environmental matrix to be monitored depends on the chemical of concern, potential targets, and specific questions being asked. For monitoring of actual human exposure, it is possible to analyze several human tissues such as blood, milk, urine, even hair or placenta. Marine organisms — including those used as seafood as well as other organisms not usually consumed by humans — can also be examined. Abiotic matrices such as sediments and water are often monitored as a source of potential exposure of humans and wildlife. For lipophilic organic contaminants, semipermeable membrane devices (SPMDs) are emerging as an efficient way to sample, in an integrative way, the bioavailable fraction of organic contaminants in aqueous environments (e.g. Huckins et al., 1996; Gale et al., 1997).

2. Approaches for monitoring the marine environment

There are several approaches that can be used to measure chemical contaminants in the marine environment (Table 1). For many chemicals, monitoring by analytical chemistry has provided an extensive database on levels of contamination in various sites and species. The utility of this approach is perhaps best illustrated by the US and International 'Mussel Watch' and 'Status and Trends' programs, in which concentrations of a variety of contaminants measured in bivalve molluscs have been used to document geographic and temporal differences in coastal pollution (Goldberg, 1975; Farrington et al., 1983, 1987).

The advantages of analytical chemical methods include their sensitivity and specificity. However, these methods are often quite costly, sometimes precluding their use in routine monitoring (Farrington et al., 1987). Alternatives to chemical analyses include indirect techniques such as immunoassays using chemical-specific antibodies (Szurdoki et al., 1996), or biosensors, which use antibodies or other recognition molecules coupled to electrochemical signal-transduction systems (Bender and Sadik, 1998).

Assays employing biomarkers offer another powerful alternative to chemical analyses. Methods based on biological effects and their underlying mechanisms can complement, and for some

Table 1
General approaches for environmental monitoring and specific application to HAHs (especially dioxin-like compounds)

General approaches	Examples for HAHs
• Chemical analysis	GC-ECD; GC-MS
• Immunoassay for chemicals	ELISA for PCBs or PCDDs
• Biosensors	Biosensor for PCBs
• In vivo biomarkers of exposure	CYP1A induction in vivo
• In vivo bioassays	Fish early life stage bioassay
• In vitro bioassays	
O Receptor-binding assays	Ah receptor binding assay
O Enzyme inhibition assays	NA
O DNA-binding assays	DRE-binding gel-shift assay
O Native responses in cell culture	CYP1A induction in cell culture
O Reporter gene assays	DRE-luciferase construct expressed in cell culture

See text and Tables 2 and 3 for references. *Abbreviations:* GC: gas chromatography; ECD: electron capture detection; MS: mass spectrometry; DRE: dioxin-responsive element; NA: not applicable.

applications could replace, the use of analytical chemistry in monitoring the marine environment. The major advantages of such biological, mechanism-based methods are their toxicological specificity, rapidity, and low cost. Here, 'toxicological specificity' refers to the relationship between the assay response and the toxic potential (rather than simply the contaminant concentrations) of the sample being analyzed. McLachlan (1993) called this 'functional toxicology'. Biological assays include in vivo biomarkers, in vivo bioassays, and in vitro bioassays.

2.1. In vivo biomarkers

Biomarkers are biochemical, physiological, or other types of biological changes that indicate the presence or effects of xenobiotic compounds (Committee on Biological Markers of the National Research Council, 1987; Henderson et al., 1989; Huggett, 1992; Decaprio, 1997). In addition to the commonly used biomarkers of *exposure* and *effect*, which are especially useful in biomonitoring, some biological characteristics can be used as biomarkers of *susceptibility* (Nebert, 1980; Nebert et al., 1996; Perera, 1997). The term 'in vivo biomarker' is used here in reference to changes occurring in organisms as a result of 'natural' exposure to contaminants in their environment. Numerous studies have shown strong relationships between in vivo biomarker responses and exposure to specific classes of marine contaminants. The various types of biomarkers that have been or might be used to monitor the marine environment, their advantages and disadvantages, chemical and biological specificity, and methods of analysis have been thoroughly reviewed (Huggett, 1992; Stegeman et al., 1992).

2.2. In vivo bioassays

In vivo bioassays involve the deliberate exposure of test animals to contaminants or contaminated materials. This might occur in the field (e.g. caging studies) or in the laboratory. In the context of seafood safety, the mouse bioassay for shellfish contaminated with algal toxins (Horwitz, 1990) is one example of an in vivo bioassay. Such assays have the advantage of measuring integrated responses at the whole-organism level. In addition, in vivo bioassays may be used to estimate the bioavailability of contaminants in environmental samples. Disadvantages include the costs and time required for studies using whole animals.

2.3. In vitro bioassays

Increasingly, bioassays employing cultured cells or cellular extracts are being developed and used to detect the presence of contaminants. Examples include assays that measure receptor-binding, enzyme inhibition, or changes in gene expression in cultured cells (Table 1). Such in vitro bioassays have numerous advantages over in vivo and chemical techniques, including speed, low cost, and biological (i.e. mechanistic) specificity. However, because the endpoints and exposure conditions may be quite different from those of concern in the target species, extrapolation of in vitro bioassay results to in vivo situations requires great caution. The features of the various monitoring approaches will be discussed below in more detail using dioxin-like compounds as an example.

3. Monitoring for the presence and effects of dioxin-like compounds

3.1. Halogenated aromatic hydrocarbons: multiplicity and mechanism of action

Halogenated aromatic hydrocarbons (HAHs) are among the most prominent marine contaminants due to their extensive production, their persistence, and the extreme toxic potency of some of the individual compounds (congeners). HAHs are also among the most controversial marine pollutants, because of the uncertainty surrounding estimates of the degree of hazard associated with present levels of exposure. Included among the HAHs are the polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polyhalogenated biphenyls (PCBs and PBBs), polyhalogenated diphenyl ethers (PCDEs and PBDEs), and several other classes of compounds (Poland and Knutson,

1982; Safe, 1990). Together, there are hundreds of HAH isomers and congeners, which vary in the number and position of their halogen substituents and thus in their environmental fates and toxic potencies. This multiplicity of compounds, fates and effects, along with known or potential species differences in sensitivity, contributes to the difficulty in evaluating their human and ecological risks.

There are several mechanisms by which various HAHs cause toxicity (Poland and Knutson, 1982; Fischer et al., 1998; Hansen, 1998). By far the most well known is that involving a high-affinity interaction with the aryl hydrocarbon receptor (AHR), a transcription factor that is activated by HAH binding. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic HAH, also has the greatest affinity for the AHR. Toxicity resulting from exposure to TCDD and other AHR ligands is thought to occur as a result of AHR-dependent changes in gene expression or interference with other signaling pathways, leading to the disruption of cell growth and differentiation (Poland and Knutson, 1982; Nebert, 1989; Whitlock, 1993). The biochemistry and molecular biology of the AHR and its role in the mechanism of HAH action have been reviewed (Swanson and Bradfield, 1993; Hankinson, 1995; Schmidt and Bradfield, 1996; Rowlands and Gustafsson, 1997; Hahn, 1998a).

3.2. The toxic equivalency (TEQ) approach

The subgroup of HAHs that act through the AHR are sometimes referred to as 'dioxin-like compounds'. These include the 2,3,7,8-substituted PCDDs and PCDFs, non-ortho-substituted (and some mono-ortho-substituted) PCBs and other HAH congeners that are able to achieve a planar configuration. There are large differences among these HAH in their affinities for the AHR, and consequently, in their biological potencies. In an attempt to deal with the multiplicity of HAH compounds and potencies and to express the potential biological activity of complex mixtures of HAH, a toxic equivalency concept has been developed (reviewed in Bellin and Barnes, 1985; Eadon et al., 1986; Safe, 1987, 1990; Ahlborg et al., 1992; van den Berg et al., 1998). In this approach, the biological or toxic potencies of individual HAH are expressed relative to a benchmark HAH, usually 2,3,7,8-TCDD. Using a variety of endpoints or responses, a relative biological potency or 'toxic equivalency factor' (TEF) can be determined for each HAH, and the TEF values can be used in conjunction with data on the concentrations of the individual PHAH to determine the 'calculated dioxin (TCDD) equivalents' (TEQ_{calc}) in a particular environmental sample. Similarly, the response to mixtures of HAHs in a bioassay can be expressed relative to that of TCDD, in the form of a 'bioassay-derived' TEQ value ($TEQ_{bioassay}$).

The toxic equivalency approach is an attempt to provide an integrated assessment of the toxic potential of environmental mixtures. In relies on a number of assumptions, including the absence of non-additive interactions (e.g. antagonism, synergism) among the components of the mixture (Safe, 1990; Ahlborg et al., 1992, 1994). Although not perfect, the TEQ concept is extremely useful in monitoring the presence of dioxin-like compounds in aquatic environments. In addition, TCDD equivalents are being used increasingly in risk assessments as a replacement for exposure measures based only on TCDD or total PCBs (Barron et al., 1994; van den Berg et al., 1998; Various authors, 1998).

3.3. Approaches for monitoring for dioxin-like compounds (Table 1)

3.3.1. Analytical chemistry, immunoassay, and biosensors

Over the past 20 years, congener-specific methods for detecting and quantitating HAHs in environmental matrices have been developed by several laboratories (Ballschmiter and Zell, 1980; Rappe et al., 1981; Safe et al., 1985; Norstrom et al., 1986; Tanabe et al., 1987; Duinker et al., 1988; Peterman et al., 1996). The methods currently in use employ gas chromatography with detection by electron capture or mass spectrometry and thus are exquisitely sensitive and specific. These methods have been used to detect dioxin-like compounds in a variety of marine environ-

ments, including remote regions such as the Arctic and Antarctic, the open ocean and the deep sea (Risebrough et al., 1976; Stegeman et al., 1986; Ono et al., 1987; Norstrom et al., 1988).

An alternative to analytical chemical detection is provided by immunoassays that utilize antibodies that recognize specific classes of HAHs. For example, antibodies against PCBs, PCDDs, and PCDFs have been developed and are being used in enzyme-linked immunosorbent assays (ELISA) to measure PCB contamination (e.g. Zajicek et al., 1996; Suguwara et al., 1998).

More recently, analytical methods based on biosensor technology have been developed. Many of these are based on the interaction of HAHs with specific antibodies immobilized on probes that transduce the physicochemical changes resulting from the antigen–antibody interaction into electrochemical signals that can be transmitted to a detector (e.g. Bender and Sadik, 1998). Though not yet widely used, these methods have the potential to provide continuous, real-time data on HAH concentrations in some environments.

3.3.2. In vivo biomarkers

The concept of biological changes or 'biomarkers' as useful indicators of exposure and effect has emerged over the past 15 years as our understanding of mechanisms of chemical toxicity has grown. The most commonly measured biomarker of exposure to dioxins and dioxin-like chemicals is the induction of cytochrome P450 1A (CYP1A). CYP1A is induced following the binding of these compounds to the AHR; it occurs in parallel with the AHR-dependent changes in gene expression that are responsible for dioxin toxicity. In experimental studies with individual compounds, dioxin-like toxicity and induction of CYP1A are highly correlated (Safe, 1987, 1990). In this way, the CYP1A induction response is a surrogate for AHR-dependent toxicity. In addition, induction of CYP1A can also be directly responsible for some forms of HAH toxicity. This may occur, for example, through the generation of reactive oxygen species (Toborek et al., 1995; Schlezinger et al., 1999, 2000). Such a mechanism could be important for some endpoints of concern, such as cardiovascular toxicity involved in early-life stage mortality in fish (Stegeman et al., 1989; Cantrell et al., 1996; Guiney et al., 1997). However, because the role of CYP1A in toxicity is not yet firmly established, this response is considered primarily a biomarker of exposure, and sometimes a biomarker of biochemical effect, but not a biomarker of toxic effect.

In the field, CYP1A induction has been shown to be highly correlated with the presence of AHR ligands in vertebrate animals and their environment (Stegeman and Hahn, 1994; Bucheli and Fent, 1995). Although most commonly assessed by measuring one of its catalytic activities (aryl hydrocarbon hydroxylase [AHH] or ethoxyresorufin *O*-deethylase [EROD]), CYP1A can also be determined by measuring immunodetectable CYP1A protein (Stegeman et al., 1986) or messenger RNA (Haasch et al., 1993). Induction of CYP1A has been used as a biomarker of exposure to dioxin-like compounds in fish (Payne, 1984; Goksøyr and Forlin, 1992; Stegeman and Hahn, 1994; Bucheli and Fent, 1995), birds (Rattner et al., 1989), marine mammals (White et al., 1994; Letcher et al., 1996), and humans (Wong et al., 1986; Lucier et al., 1987; McLemore et al., 1990; Vanden Heuvel et al., 1993).

As with any biomarker, the use of CYP1A induction to indicate HAH exposure is limited by the biological specificity of the response (Huggett, 1992; Stegeman et al., 1992). Measurement of CYP1A expression provides information relevant to exposure only in those organisms possessing the appropriate response mechanism (i.e. an intact AHR pathway, linked to regulation of CYP1A). In general, a functional AHR-CYP1A pathway exists in most vertebrates, including bony and cartilaginous fish, amphibians, birds, reptiles, and mammals (Hahn et al., 1992; Stegeman and Hahn, 1994; Hahn, 1998a). However, use of CYP1A induction as a biomarker is not appropriate for organisms whose ancestors diverged prior to the evolution of an HAH-responsive AHR/CYP1A system. For example, the presence of an HAH-responsive AHR pathway has not been confirmed in aquatic invertebrates (Denison et al., 1985; Hahn et al., 1994; Brown et al., 1995; Hahn et al., 1997; Livingstone et al., 1997; Hahn, 1998a) and many invertebrates or early verte-

brates appear to be non-responsive to dioxin-like compounds as assessed by CYP1A assay or toxicity testing (Goksøyr et al., 1991; West et al., 1997; Hahn et al., 1998). Moreover, invertebrate AHR homologs do not appear to bind TCDD or other typical AHR ligands (Powell-Coffman et al., 1998; Butler et al., 2001). Another situation in which CYP1A induction would not be an appropriate in vivo biomarker occurs when populations of normally responsive species develop HAH resistance or tolerance through physiological acclimation or genetic adaptation (reviewed in Hahn, 1998b). In such cases, the use of CYP1A expression as an index of exposure would be misleading, providing false negative data.

3.3.3. In vivo bioassays

Experimental exposure of animals to mixtures of contaminants in in vivo bioassays has been used to assess the potential health effects of consuming contaminated food and to determine the amount of biologically active components in environmental mixtures. For example, studies have determined the health effects of feeding contaminated fish, or feed made from such fish, to mice (Cleland et al., 1987), seals (Reijnders, 1986), mink (Heaton et al., 1995), birds (Summer et al., 1996), and fish (Leatherland and Sonstegard, 1982). Other investigators have examined the dioxin-like activity of various environmental extracts by exposing fish or bird eggs and monitoring early-life stage toxicity (Walker et al., 1996; Wilson and Tillitt, 1996; Powell et al., 1997) or development of tumors (Metcalfe and Sonstegard, 1985; Metcalfe et al., 1990). A major advantage of such in vivo bioassays is their direct relationship to endpoints of concern, such as reproductive and developmental effects or cancer, in exposed animals.

3.3.4. In vitro bioassays

Several types of in vitro bioassays are available for monitoring dioxin-like compounds (Table 2); these offer advantages in speed and cost as compared to many of the methods discussed above. As with in vivo biomarkers, the in vitro assays are mechanistically based, providing an integrated measure of the biologically active component of an environmental mixture. Although some of the in vitro assays are less sensitive than chemical analysis, others approach or equal the latter methods in this regard (Table 2).

One type of in vitro assay measures the ability of compounds or mixtures to compete with radiolabeled dioxin (TCDD or dioxin analog) for binding to the Ah receptor. Competitive receptor-binding assays using [^{125}I]2-iodo-7,8-dibromodibenzo-*p*-dioxin (Bradfield and Poland, 1988) and [^{3}H]TCDD (Hu et al., 1995; Schneider et al., 1995) have been described. A disadvantage of these assays is that they do not distinguish between receptor agonists (compounds that bind to the receptor and activate transcription) and receptor antagonists (compounds that bind but do not activate).

The ability of compounds or mixtures to bind to the AHR and activate or 'transform' it to its DNA-binding form can also be used as an in vitro bioassay for dioxin-like compounds. Receptor transformation and DNA binding are determined by an electrophoretic mobility shift or gel shift assay, in which specific protein (AHR)-DNA complexes are detected by their altered mobility during electrophoresis (Denison et al., 1988). Gel shift assays can be quite sensitive, and have been used to detect the presence of Ah receptor agonists in numerous types of samples (Denison et al., 1998; Seidel et al., 2000). However, like competitive binding methods, these assays do not necessarily distinguish between AHR agonists and antagonists, leading to high rates of false positive results (Seidel et al., 2000).

Bioassays using responses in cell culture (cell culture bioassays) are the most sensitive of the in vitro methods.[1] Cell culture assays to measure dioxin-induced changes in gene expression utilize either a native (i.e. intrinsic) response such as CYP1A induction, or increased expression from an artificial construct containing a reporter gene

[1] Cells in culture are referred to variously as in vitro, in vivo, or ex vivo, depending on the perspective and bias of the investigator. The term in vitro is used here in recognition of the artificial nature of these cell culture bioassays, as compared to responses measured in whole animals.

Table 2
Relative sensitivities of in vitro assays for dioxin-like compounds

Response or Endpoint	Cell/tissue type	EC_{50} (nM)	Minimum detection limit[a]			References
			(pM)	(pg/well)	(fmol/well)	
Ah receptor binding	mouse liver			100		(Schneider et al., 1995)
Ah receptor binding	mouse liver			3.2	10	(Bradfield and Poland, 1988)
DRE binding	guinea pig liver			0.04 −0.2		(Yao and Denison, 1992)
DRE binding	guinea pig liver	0.15	1–5			(Seidel et al., 2000)
EROD	H4IIE (rat)	0.028		10		(Bradlaw and Casterline, 1979 Trotter et al., 1982)
EROD	H4IIE (rat)	0.080				(Sawyer and Safe, 1982)
EROD	H4IIE (rat)	0.017		10	31	(Tillitt et al., 1991b)
EROD	H4IIE (rat)	0.020	2.4	0.19	0.6	(Sanderson et al., 1996)
EROD	H4IIE (rat)	0.006				(Wiebel et al., 1996)
EROD	HepG2 (human)	0.1	50	16[b]	50[b]	(Wiebel et al., 1996)
EROD	Chick embryo hepatocytes	0.015	1.0	0.16	0.5	(Kennedy et al., 1993b, 1995)
EROD	PLHC-1 (fish)	0.012	3	0.48	1.5	(Hahn et al., 1993, 1996; Hestermann et al., 2000)
EROD	PLHC-1 (fish)			0.25	0.76	(Villeneuve et al., 1997)
EROD	RTL (trout)	0.006	< 6	< 1	< 3	(Clemons et al., 1994, 1996; Bols et al., 1997)
EROD	RTH-149 (trout)		100			(Richter et al., 1997)
Uroporphyrin accumulation	Chick embryo hepatocytes	0.002	1	1.1	3.5	(Sinclair et al., 1997)
Alkaline phosphatase	T13 (mouse)	0.35	100			(El-Fouly et al., 1994)
Luciferase	101L (human)	0.35	1			(Postlind et al., 1993)
Luciferase	101L (human)	0.09	100	65	200	(Anderson et al., 1995; Jones and Anderson, 1999)
Luciferase	H1L1.1c2 (mouse) others	0.03	0.1–1.0			(Garrison et al., 1996)
Luciferase	H1L1.1c2 (mouse)	0.68	100	1.61		(Ziccardi et al., 2000)
Luciferase	H4IIE-Luc (rat)	0.0056	0.8	0.065	0.2	(Sanderson et al., 1996)
Luciferase	RTL 2.0 (trout)	0.064	4	0.32		(Richter et al., 1997)
Luciferase	H4IIE-Luc	0.010	1	0.16	0.5	(Murk et al., 1996)
Luciferase	H4IIE-Luc Hepa-1-Luc			0.032	0.1	(Murk et al., 1997)

[a] Minimum detection limit for TCDD or TEQ.
[b] Assuming 5 ml medium/plate.

(e.g. luciferase) under control of specific regulatory elements that are able to respond to dioxin-like compounds.

The use of CYP1A induction in cell culture bioassays as an integrated measure of dioxin-like compounds was first described by Bradlaw and colleagues more than 20 years ago (Bradlaw and Casterline, 1979; Bradlaw et al., 1980; Trotter et al., 1982) (see also Niwa et al., 1975). Since that time, other investigators have offered several improvements in speed and sensitivity, including the use of multi-well plates and fluorescent plate readers (Tillitt et al., 1991b; Donato et al., 1993; Kennedy et al., 1993b, 1995; Tysklind et al., 1994; Hahn et al., 1996). The methods have been further expanded to include measurements of immunodetectable CYP1A protein and mRNA (Herrero and Castell, 1994; Bruschweiler et al., 1996; Hahn et al., 1996; Zabel et al., 1996; Scholz et al., 1997). Published reports using such assays

are abundant; cell types employed have included primary cell cultures and continuous cell lines from numerous species (see below).

Although the value of cell culture bioassays has been demonstrated repeatedly, there has been increasing recognition of the potential pitfalls associated with the measurement of CYP1A catalytic activities in cultured cells. Biphasic concentration–response curves have been observed often in studies examining induction of CYP1A activities in cell culture (Sawyer and Safe, 1982; Hahn et al., 1993; Kennedy et al., 1993b) and recent studies have shown that some HAHs and PAHs, at high concentrations, can inhibit CYP1A activity (Gooch et al., 1989; Hahn et al., 1993; Besselink et al., 1998; Willett et al., 1998). The result of this inhibition is that the measured activity (e.g. EROD) does not reflect the amount of induced CYP1A protein (Hahn et al., 1996). One consequence of this is that concentration–response (activity) curves obtained in cell culture appear to be shifted to the left because of the inhibition of activity at high concentrations of inducer. This leads to a lower apparent EC_{50} for induction and thus to an overestimation of biological potency (Hahn, 1994, 1996; Hahn et al., 1996; Petrulis and Bunce, 1999). Measurement of CYP1A protein (Hahn et al., 1993, 1996; Bruschweiler et al., 1996) or mRNA (Zabel et al., 1996) provides more reliable estimates of in vitro CYP1A-inducing potency.

The possibility of artifacts associated with using the native CYP1A response, along with the potential for enhanced sensitivity, has stimulated the development of reporter gene systems for measuring dioxin-like compounds in cell culture bioassays (Postlind et al., 1993; El-Fouly et al., 1994; Murk et al., 1996). In these systems, reporter genes such as luciferase are inserted into a plasmid, under control of dioxin-responsive enhancer elements (DREs; also known as xenobiotic-responsive enhancers or XREs). When used together with a sensitive luminometer, cells expressing such reporter constructs offer approximately three- to 10-fold greater sensitivity than cells using the native CYP1A response (Sanderson et al., 1996; Richter et al., 1997); detection of as little as 0.1 fmol (32 fg) of TCDD is possible with this method (Table 2). Moreover, since luciferase activity appears not to be inhibited by HAHs, this artifact is avoided (Murk et al., 1996; Sanderson et al., 1996). Species-specific, recombinant cell lines have been engineered using cells from fish (Richter et al., 1997) and several species of mammals, including humans (Postlind et al., 1993; Garrison et al., 1996).

3.4. Applications of Cell culture bioassays for environmental monitoring of dioxin-like compounds

Cell culture bioassays have been used by many investigators to assess contamination of dioxin-like compounds in many different types of environmental samples (Table 3). In general, these assays have provided results that correlate closely with data from chemical analysis of dioxins and/or PCBs in the same samples or samples from the same sites (Jones et al., 1993; Kennedy et al., 1996; Giesy et al., 1997; Willett et al., 1997; Whyte et al., 1998). While most of these studies have not focused on seafood per sé, the methods developed could easily be applied to routine monitoring of dioxin-like contaminants in seafood or other marine samples. To avoid the uncertainty introduced by species-to-species extrapolation, it may be preferable to use human cells or cell lines (Postlind et al., 1993; Garrison et al., 1996; Wiebel et al., 1996) for screening of seafood.

As with any analytical technique, the use of cell culture bioassays must be accompanied not only by an understanding of the underlying mechanisms on which they are based, but also by an awareness of potential pitfalls. Problems associated with the inhibition of CYP1A activity were described above. In addition, it is important to keep in mind that a large number of structurally diverse compounds are capable of activating the AHR (Denison et al., 1998). Many PAHs, for example, are ligands for the AHR and induce AHR-dependent responses in bioassays (Willett et al., 1997; Bols et al., 1999; Jones and Anderson, 1999; Fent and Batscher, 2000; Seidel et al., 2000; Jung et al., 2001; Villeneuve et al., 2001). However, because of their lack of persistence, PAHs are not thought to produce dioxin-like toxicity unless exposure is sustained (Poland and

Table 3
Use of cell or tissue culture bioassays to monitor for environmental contamination by dioxin-like compounds: some examples from the literature

Matrix or sample type	Assay/endpoint:		
	DRE-luciferase	CYP1A[a] — H4IIE	CYP1A — other cell types; other endpoints
Blood plasma or serum	(Murk et al., 1997; Ziccardi et al., 2000)		
Fish or fish eggs		(Trotter et al., 1982; Zacharewski et al., 1989; Ankley et al., 1991; Hanberg et al., 1991; Smith et al., 1994; van den Heuvel et al., 1994; Giesy et al., 1997; Stegeman et al., 2001)	(Bols et al., 1997; Whyte et al., 1998)
Bird eggs or yolk sac		(Tillitt et al., 1991a, 1992; Jones et al., 1993, 1994; Rattner et al., 1994; Williams et al., 1995; Larson et al., 1996)	(Kennedy et al., 1993a, 1996; Hart et al., 1998)
Marine mammal tissues		(Hanberg et al., 1991)	
Other vertebrate animals		(Tillitt et al., 1996)	
Shellfish		(Willett et al., 1997)	(Engwall et al., 1997)
Sediments or soils	(Anderson et al., 1995; Murk et al., 1996)		(Engwall et al., 1996 Huuskonen et al., 1998b; Huuskonen et al., 1998c)
Water	(Murk et al., 1996)		(Villeneuve et al., 1997; Huuskonen et al., 1998b)
Effluent	(Zacharewski et al., 1995)		(Huuskonen et al., 1998a)
Air		(Chiarolini et al., 1997)	(Franzen et al., 1988)
Soot or fly ash		(Kopponen et al., 1994)	(Gierthy et al., 1984; Till et al., 1997)

[a]CYP1A includes EROD and AHH activities, or CYP1A protein or mRNA.

Glover, 1974; Francis and Smith, 1987; Fragoso et al., 1998; Billiard et al., 1999). Thus, a positive response in one of the assays listed in Table 2 could indicate contamination by PAHs or other AHR ligands, rather than — or in addition to — HAHs. In general, PAH- and HAH-dependent responses can be distinguished by the time course of the response: the former are transient, due to metabolic inactivation, whereas the latter are more persistent (Poland and Glover, 1974; Riddick et al., 1994; Wiebel et al., 1996; Celander et al., 1997).

It is important to keep in mind that most of the assays described above measure only the 'dioxin-like' (i.e. AHR-mediated) toxicity of complex mixtures. There are other mechanisms by which seafood contaminants, including PCBs that are not ligands for the AHR, could potentially be toxic to consumers of seafood (Fischer et al., 1998; Hansen, 1998). The development of assays to screen for the non-AHR-mediated component of PCB toxicity is an important future goal.

3.5. Bioassays for other marine contaminants

Although the focus of this presentation has been on methods for detecting dioxin-like compounds in the marine environment, many of the approaches described here for dioxins are being used, or could be used, to assess contamination of the marine environment — including seafood — by other types of contaminants (Pierce and Kirkpatrick, 2001). Cell culture bioassays, in particular, have great potential in this regard (Zacharewski, 1997; Fairey and Ramsdell, 1999; Rogers and Denison, 2000). Table 4 lists several cell culture bioassays that have been developed to measure marine toxins, metals, and other contaminants.

Table 4
In vitro bioassays with potential application for routine monitoring of contaminants in seafood

Toxin or toxicant	Mechanism of action	Bioassay	Reference
• **Natural toxins**			
PSP (saxitoxins)	Na^+ channel (blocker)	Competitive binding to sodium channel	(Doucette et al., 1997)
		Antagonism of ouabain and veratridine-induced cytotoxicity	(Manger et al., 1993, 1995 Fairey et al., 1997)
DSP (okadaic acid; dinophysiotoxins)	Inhibition of protein Phosphatase activity	Inhibition of protein phosphatase activity	(Simon and Vernoux, 1994; Vieytes et al., 1997)
ASP (domoic acid)	Glutamate analog	Competitive binding to kainic acid receptor	(Van Dolah et al., 1994)
NSP (brevetoxins) and ciguatera toxin (ciguatoxin)	Na^+ channel (enhancer)	Competitive binding to sodium channel	(Van Dolah et al., 1994)
		Potentiation of ouabain and veratridine-induced cytotoxicity	(Manger et al., 1993, 1995 Fairey et al., 1997 Dickey et al., 1999 Poli et al., 2000)
Maitotoxin	Activation of calcium channels	^{45}Ca flux	(Anonymous, 1998; Fairey et al., 1999)
Pfiesteria toxin	Unknown	Induction of c-fos/luciferase reporter gene expression	(Anonymous, 1998; Fairey et al., 1999)
• **Dioxin-like compounds** (dioxins, planar PCBs, etc)	AHR-dependent changes in gene expression	AHR competitive binding DNA-binding (gel shift) CYP1A1 (EROD) induction Induction of reporter genes under control of DRE	see Table 3
• **Environmental estrogens**[a]	ER agonist or antagonist activity	ER competitive binding DNA-binding (gel shift) Vitellogenin induction Induction of reporter genes under control of ERE	(McLachlan, 1993; Gray et al., 1997; Zacharewski, 1997; Rogers and Denison, 2000)
• **Retinoid mimics**[a]	Activation of gene expression controlled by retinoid receptor(s)	Induction of reporter genes under control of response elements for retinoic acid receptors (RAR) or retinoid X receptors (RXR)	(Todd et al., 1995; Blumberg et al., 1996)
• **Organophosphorous insecticides**	Inhibition of acetylcholinesterase	Inhibition of acetylcholinesterase	(Galgani and Bocquene, 1991)
• **Metals**	Various	Induction of reporter genes under control of metallothionein or heat shock response elements	(Todd et al., 1995; Klimowski et al., 1996)

[a]These two examples are intended to be representative of direct-acting agonists or antagonists of any member of the nuclear hormone receptor family, e.g. androgen receptor, thyroid hormone receptor, etc.

Like the dioxin-responsive assays, many of these are mechanism-based methods that integrate the biological activity of any compounds that share the same mode of action as the target chemical.

4. Conclusions

A stated goal of efforts to improve and ensure the safety of seafood is to develop an economical set of monitoring and inspection practices that will minimize the exposure of consumers to hazardous chemicals (Ahmed et al., 1993a,b). Thus, 'rapid and simple tests should be developed and used to screen potentially hazardous fish or shellfish at the point of harvest to reduce costs to the fishermen and to protect the consumer from toxins and dangerous contaminants' (Ahmed, 1991 p. 17). Chemical methods of analyses are sensitive and specific, but can be expensive and provide little information on the actual or potential biological activity of the contaminants. Biological indicators or biomarkers can be used to indicate the presence and (in some cases) biological effects of contaminants in marine animals. In vitro bioassays using mechanistically-based biomarker responses provide an integrated measure of the biologically active components of environmental mixtures. Such assays are rapid and inexpensive and thus offer great potential for routine monitoring of marine resources, including seafood. Cell culture assays such as those described in Table 4, in combination with other assays, might be incorporated into a battery of tests (e.g. MacGregor et al., 1995; Todd et al., 1995) to rapidly and sensitively screen seafood for the presence of contaminants of concern. The identity of contaminants in samples testing positive (i.e. above some action level) in screening tests could be confirmed if necessary using chemical analysis. In the United States and perhaps elsewhere, such a battery of mechanism-based, in vitro bioassays could be part of monitoring efforts under the recently adopted Hazard Analysis and Critical Control Point (HACCP) programs (Food and Drug Administration, 1994).

Improved monitoring of seafood for chemical contaminants is important for minimizing the potential for adverse human health effects due to these contaminants. However, it must also be recognized that in many ways our ability to measure these contaminants, whether by analytical chemistry or cell culture bioassay, has progressed beyond our ability to interpret the data in terms of the level of risk to human or environmental health. As we develop more efficient ways to detect ever lower concentrations of contaminants in the marine environment, we must also strive to improve our ability to accurately predict the risk of these low level exposures, and in the interim, to better communicate the uncertainty inherent in the current risk assessment process (Cordle et al., 1982; Maxim and Harrington, 1984; Kimbrough, 1991; Reinert et al., 1991; Barron et al., 1994). In addition, recommendations concerning consumption of seafood must consider not only the risks posed by contaminants, but also the benefits provided by this nutritious food source (Egeland and Middaugh, 1997).

Noted added in proof

Recently, a new cell culture bioassay for detecting dioxin-like compounds has been developed using green fluorescent protein (GFP) as reporter. This GFP-based assay has a number of advantages over earlier luciferase-based assays. See Nagy et al. (2001) for details. Additional information about HACCP programs can be found in National Advisory Committee on Microbiological Criteria for Foods (1998).

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