Monitoring of pollutant biochemical effects on marine organisms of the French coasts

Biological monitoring
Enzymes
Mytilus edulis
Limanda limanda
Callionymus lyra

Surveillance biologique
Enzymes
Mytilus edulis
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ABSTRACT

The establishment of a network to assess pollutant effects is a current concern of international bodies such as the North Sea Task Force, the International Council for the Exploration of the Sea and the Intergovernmental Oceanographic Commission. With respect to the French coasts, three major considerations have been defined : the choice of monitoring areas ; the determination of a target species according to different criteria such as wide distribution, limited migration and contact with pollutant-rich sediments; and the selection of suitable parameters.

This paper concerns the strategy adopted for the French coast. Ethoxyresorufin-O-deethylase, a specific cytochrome P450-dependent monooxygenase, measured by a simple microplate assay, was selected as an indicator of polyaromatic hydrocarbon and polychlorobiphenyl effects in *Callionymus lyra* and *Limanda limanda* in measurements conducted in the Seine Bay. Assessment of metallothionein in oysters in the Gironde Estuary indicated the absence of induction along a cadmium contamination gradient.

Acetylcholinesterase was selected to measure the effects of organophosphorus and carbamates in *Mytilus edulis* along the French coasts and in *Limanda limanda* and *Pleuronectes platessa* from the Seine Bay.

The results clearly demonstrate the feasibility of studying such parameters in the field and provide the scientific and technical basis for a network for the monitoring of pollutant effects along the French coasts.

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RÉSUMÉ

Surveillance des effets biochimiques des polluants sur les organismes marins des côtes françaises

La mise en place d'un réseau de surveillance des effets des polluants est un problème d'actualité pris en charge par les organisations internationales comme la North Sea Task Force (NSTF), le Conseil International pour l'Exploration de la Mer (CIEM) et la Commission Intergouvernementale de l'Océanographie (CIO). Dans le contexte des côtes françaises, les contraintes majeures ont été identifiées. La première concerne le choix de la région à surveiller. La deuxième est le choix d'une espèce cible qui réponde aux critères de la surveillance comme une large répartition, de faibles migrations et un contact avec les sédiments riches en polluants. La troisième contrainte est le choix de paramètres adéquats.

La stratégie retenue pour les côtes françaises est décrite. L'ethoxyresorufin-O-deethylase (EROD), une enzyme cytochrome P450 dépendante, mesurée à l'aide d'une technique simple utilisant les lecteurs de microplaques, a été sélectionnée comme indicateur de l'effet des hydrocarbures polyaromatiques (HAP) et les polychlorobiphényls (PCB) chez les espèces *Callionymus lyra* et *Limanda limanda*. Les mesures ont été réalisées en baie de Seine.

Les métallothionéines ont été mesurées sur des huîtres autour de la Gironde. Les résultats démontrent l'absence d'induction de ce paramètre le long d'un gradient de contamination par le cadmium.

L'acétylcholinestérase a été sélectionnée pour mesurer les effets des organophosphorés et des carbamates à la fois chez *Mytilus edulis* le long des côtes françaises et chez *Limanda limanda* ainsi que *Pleuronectes platessa* de la baie de Seine. Les résultats démontrent clairement l'adéquation de tels paramètres pour la surveillance de terrain et donnent les bases scientifiques et techniques de la mise en place de réseaux de surveillance des effets de pollution le long des côtes françaises.

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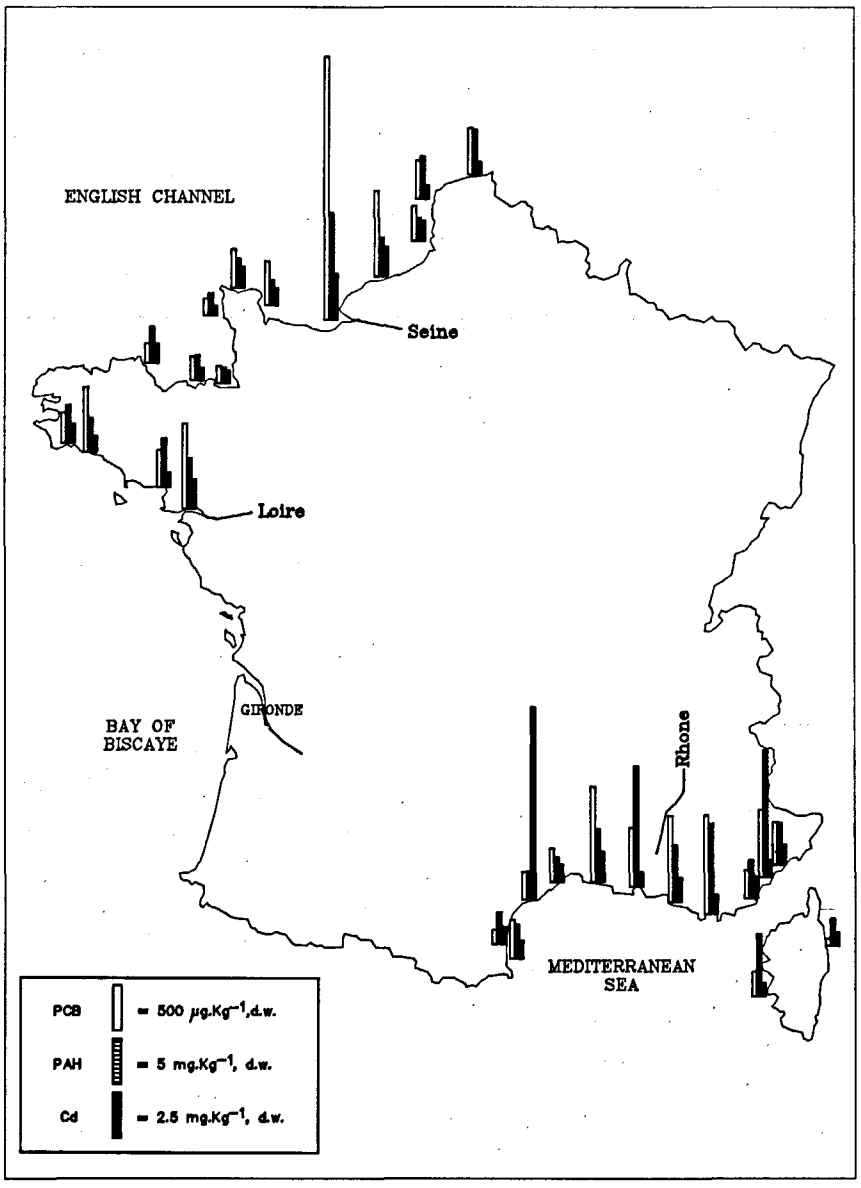
INTRODUCTION

Monitoring of pollutants in living matter is carried out in many countries to obtain concentration measurements and data on their movement and distribution. However, the effects of pollutants, as well as the changes in these effects with time, remain largely unknown. Only very meticulous studies can indicate how pollutants alter the metabolism of organisms. The evaluation of their effects over time will make it possible to determine their targets, the areas affected and the temporal variations in their action. The problem is especially crucial for marine organisms, since recent studies have shown alterations in their metabolism in highly polluted areas. The setting up of monitoring networks is thus a matter of current concern, and the problem has been taken into account by international organizations. The ICES (International Council for the Exploration of the Sea) and the IOC (Intergovernmental Oceanographic Commission), in particular, have recently organized experimental oceanographic cruises to validate the methods of measuring pollutant effects in marine organisms (ICES/IOC, 1992). Moreover, the North Sea Task Force has included the start of North Sea biological monitoring in its programmes for 1991. The example of the French coasts is typical. The National Monitoring Network (RNO) has been evaluating contamination levels in marine organisms since 1979, and the monitoring of pollutant effects is now being undertaken in conjunction with the international programs.



A

Three major factors are involved in the setting up networks to monitor pollutant effects on marine organisms: the sites to be monitored, the studied species and the parameters indicative of pollutant effects.

France has 3 200 km of coastline and a population of around 55 million (Fig. 1), but the areas of greatest population concentration and agricultural, urban and industrial waste are not uniformly distributed with respect to the coastline. In these circumstances, there is considerable advantage in establishing networks for monitoring pollutants in water, living matter and sediments. The RNO (Fig. 1) has located the areas of highest pollution where the risks of environmental contamination are greatest. The high concentrations of polychlorinated biphenyl (PCB) and hydrocarbons found in living marine organisms in the Seine Bay (ranging from 3 to 105 µg/g dryweight for PCB and from 10 to 30.5 µg/g dryweight for hydrocarbons in *Mytilus edulis*) indicate that this is a priority area for monitoring biological effects. Likewise, the cadmium levels detected in the Gironde estuary (from 10 to 129 µg/g dryweight for the species *Crassostrea gigas*) point to the high risk for organisms in that area.

Although existing networks have determined the priority of certain sites for monitoring, available data cannot always be processed effectively. There are recognized polluted areas for which no techniques for the measurement of impact exist. Moreover, for some contaminants, difficulties of analysis prevent any determination of the levels in organisms. In this context, the monitoring of effects is essential, to the extent that the results indicate the presence of contaminants even when analysis is difficult or impossible. This is the case for organophosphate and carbamate pesticides, which cannot at present be accurately and confidently detected in marine organisms. High concentrations of these products are sometimes found in river outflows (Capel *et al.*, 1988), but their persistence has not been demonstrated. Under these circumstances, the choice of monitoring sites is based on knowledge of the areas of use and disposal of this type of product. Most of the insecticides used in agri-

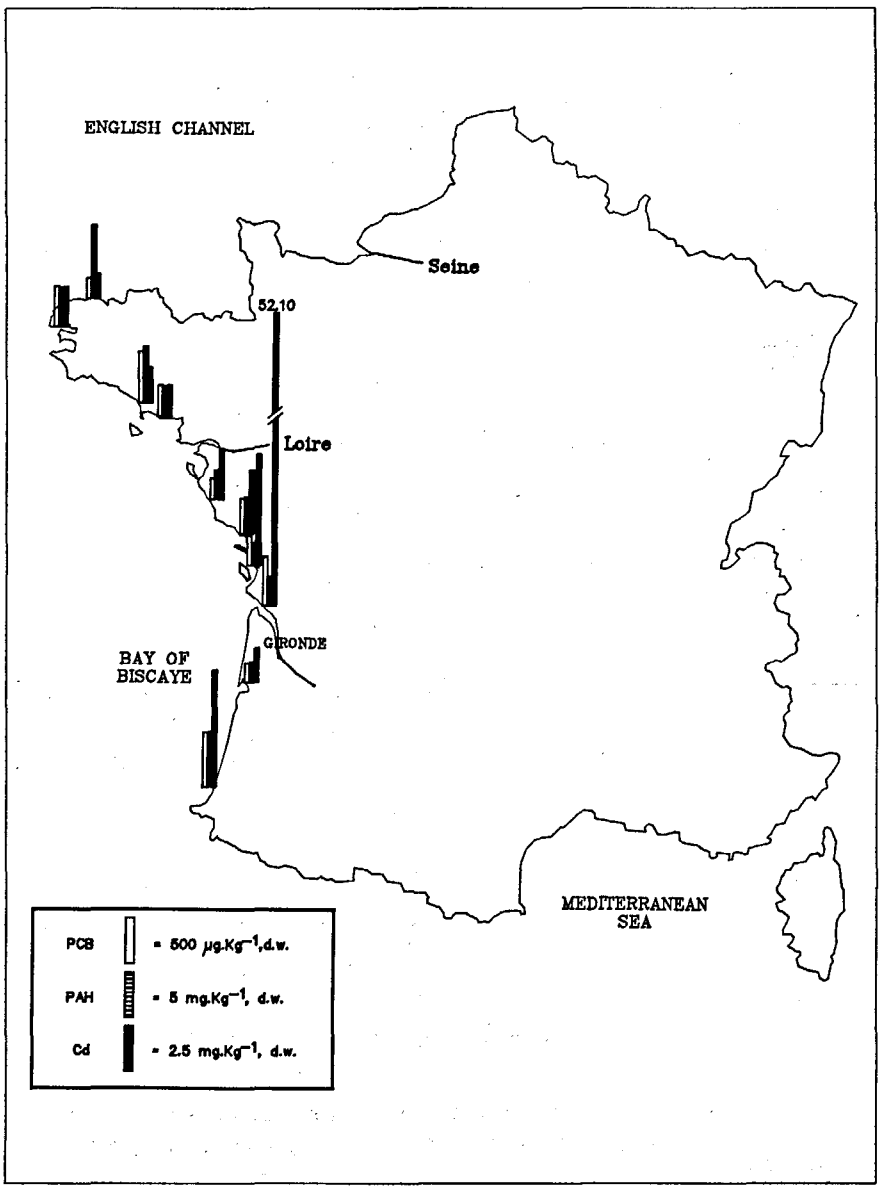


Figure 1

Mean concentrations in polychlorinated biphenyls (PCB), polyaromatic hydrocarbons, (PAH) and cadmium (Cd) in mussels (A) and oysters (B) of French coasts. Data were provided by the French National Monitoring Network from results described by Claisse (1989). Results are mean values measured four times per year between 1978 and 1988.

Concentrations moyennes en polychlorobiphényles (PCB), hydrocarbures polyaromatiques (HPA) et en cadmium (Cd) dans les moules (A) et les huîtres (B) des côtes françaises. Les données proviennent du Réseau National d'Observation, d'après Claisse (1989). Les résultats sont des valeurs moyennes mesurées quatre fois par an entre 1978 et 1988.

B

culture are molecules of organophosphate and carbamate types (Tronczynski, 1990) known to inhibit transmission of the nerve impulse. Monitoring of the effects of this type of molecule should thus be carried out in areas of major agricultural activity.

Relative to existing parameters, we chose to perform studies on metallothioneins mixed oxygenase function and acetylcholinesterase. Metallothioneins are low molecular weight proteins (Petering and Fowler, 1986) which can complex certain trace metals because of their high content in thiol groups. They can be activated by intoxication due to the presence of cadmium in food or in the environment (Viarengo, 1985; Olsson and Hogstrand, 1987). Studies based on the use of metallothioneins to evaluate trace metal effects have been performed for several years, but few field studies have been carried out on marine organisms to confirm the potential value of this parameter for monitoring biological effects.

Interest in mixed function oxidase (MFO) as a monitoring tool derives from basic research carried out over the last twenty years (review *in* Payne *et al.*, 1987). The MFO system catalyses the degradation of both endogenous and exogenous lipophilic substrates to polar water-soluble products which are more easily excreted. It is present at relatively low rates of activity in animals, and a dramatic increase in activity, apparently to speed up degradation and clearance of organisms, could be an indicator for measuring the degree of chemical stress. A number of field studies have been conducted in which elevated MFO activity in fish was found to be associated with hydrocarbon pollution (Payne *et al.*, 1987).

The MFO system requires molecular O_2 and NaDPH and involves cytochrome P-450, a CO-binding protein. In marine fish, two model reactions of the MFO system - aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin-O-deethylase (EROD) - have been studied most thoroughly (Klotz *et al.*, 1983; Klotz *et al.*, 1986; Goksoyr, 1987; ICES/IOC, 1992). Both AHH and EROD (the latter being more specific) are catalysed by the cytochrome P-450_{IA1} subfamily (Stegeman and Kloepper-Sams, 1987). In mammals, the cytochrome P-450_{IA1} subfamily contains two isozymes, cytochrome P-450_{IA1} and cytochrome P-450_{IA2}, but in fish only the former appears to be inducible by contaminants (Stegeman and Kloepper-Sams, 1987). The cytochrome P-450_{IA1} isozyme in fish can be induced by polycyclic aromatic hydrocarbons (PAH), polychlorinated dibenzodioxins (TCDD) and certain PCB that prefer a planar configuration. This has been reported many times in laboratory studies (Gooch *et al.*, 1989) as well as in mesocosms (Melancon and Lech, 1983; Goksoyr, 1985; Spies *et al.*, 1989) and field studies (Payne *et al.*, 1987; Goksoyr, 1987; Stegeman *et al.*, 1987; Vignier, 1985). It has recently been shown that specific isoforms of this protein are involved in the metabolism of xenobiotics. Thus, an increase in this specific isoform is a good indicator of the induction of the MFO system by pollutants. Determination of EROD activity constitutes a specific assay for the xenobiotic-inducible form of cytochrome P-450, thus making measurement of this activity in fish liver a good tool for evaluating fish response to PAH contamination.

The direct target of organophosphate and carbamate insecticides is a well-known enzyme, acetylcholinesterase (AChE). The role of this enzyme is essential in the correct transmission of the nerve impulse since AChE is responsible for hydrolysis of the chemical mediator acetylcholine which propagates the impulse (Massoulie and Bon, 1982). Acetylcholine released at synapses is quickly inactivated by AChE, but inhibition of this enzyme leads to an accumulation of the mediator which thus maintains the excitation. This situation generally causes tetany, paralysis and finally death. The importance of this enzyme for monitoring purposes is thus considerable. Health controls for workers in the agrochemical industry who handle these pesticides require regular monitoring of serum AChE concentrations. The assay of these levels is generally used to determine the degree of human and animal exposure to pesticide compounds (Trundle and Marcial, 1988). In biochemical terms, AChE is a true cholinesterase possessing a high degree of molecular polymorphism and essentially localized in nerve tissue, muscle and brain. Most marine organisms have AChE activity (Bocquéné *et al.*, 1990; Habig *et al.*, 1988), and its natural variations (Hogan, 1970) and *in vitro* sensitivity (Klaverkamp and Hobden, 1980; Kobayashi *et al.*, 1986; Galgani and Bocquéné, 1990) are beginning to be known. However, data on induction thresholds and detection of effects are scarce. Recent experiments have shown extremely low thresholds for induction of inhibitory effects (Habig *et al.*, 1986; Bocquéné and Galgani, 1991). These results suggest that detection of effects is possible after two or three weeks of exposure of marine organisms such as *Palaemon serratus* and *Ictalurus punctatus* to pesticide concentrations of around 0.5-5 µg/l. The most commonly used AChE activity assay is the colorimetric method of Ellman (Ellman *et al.*, 1961). Its use as a monitoring tool in fish for certain freshwater inhibitory contaminants was suggested in the 1960s and 1970s (Weiss and Gakstatter, 1964; Holland *et al.*, 1967; Coppage and Matthews, 1974). Data for the marine environment were practically inexistent up to a few years ago, but recent work has demonstrated that this parameter is also a valid monitoring tool for use there (Galgani and Bocquéné, 1992). The present paper presents the results of our studies of pollutant effects carried out during the past four years and the strategy for setting up a network to monitor these effects along the French coasts.

MATERIALS AND METHODS

Strategy

The biochemical parameters and key factors relative to sites and species allowed us to limit the field of study for monitoring of the French coasts. Metallothioneins were assessed in the Gironde estuary. The metabolization system for EROD is well known in fish and is under study in molluscs. Given the specificity of the EROD response (Gooch *et al.*, 1989; Payne *et al.*, 1987), we plan to carry out monitoring in fish in the Seine Bay, an area highly contaminated

by MFO inducers (Fig. 1). Acetylcholinesterase was assessed in *Mytilus edulis* along the French coast and in fish in the Seine Bay.

There are three current methods of assaying EROD activity : measurement of enzymatic activity; quantification of the isoform by ELISA techniques (Goksoyr, 1991); and quantification of RNA (Haasch *et al.*, 1989) specific for the isoform IA1 using a nucleic acid probe. In determinations in which enzymatic activity is directly related to the quantity of isoform (Stegeman *et al.*, 1987), an alternative to these techniques is to measure enzymatic activity directly on the microplate readers traditionally used in immunochemistry (Grzebyk and Galgani, 1991; Galgani and Bocquéné, 1989; Galgani et Bocquéné, 1991). This technique, validated during international meetings, allows measurements to be carried out in large series without the inconveniences of immunochemical assays (preparation and specificity of antibodies, standardization of measurements and operational difficulties). Moreover, this alternative method can be performed on fresh samples during oceanographic cruises. Monitoring networks concerned with the effects of organic pollutants on marine organisms, such as the RNO in France and the North Sea Task Force, have adopted this technique (Galgani and Payne, 1991).

AChE is an appropriate parameter for monitoring biological effects. However, a lack of information on inducer pollutant sites required us to determine first the critical areas for monitoring. As there is no species limitation for this parameter, it seemed suitable and more economical to use existing networks such as the RNO. This organization regularly conducts mollusc sampling along French coasts, and its network provided us with an adequate infrastructure for monitoring the effects of products harmful to phytoplankton. The data will enable us to demarcate the danger areas and subsequently limit the number of surveillance sites.

The choice of technique depends on economic criteria, which means that the role of biotechnology is important. As for EROD measurements, the development of simple, fast and inexpensive techniques (Grzebyk and Galgani, 1991) is essential for biological monitoring.

Biochemical measurements

Samples were obtained during three scientific cruises (Subio 1, 2 and 3) and from RNO stations. Sampling locations and the species investigated are indicated in the Results section. Ten samples were sampled for each site.

For EROD measurements, liver was washed in buffer (Tris 50 mM, pH 7.4; KCl 150mM; EDTA 1 mM; glycerol 20 % vol) and then minced (5 ml/g of tissue) for 5 to 10 s in an Ultra Turrax. Centrifugation was performed at 9 000 g for 15 min at 4° C. Supernatant was used as enzyme solution. The protein assay was performed using the method of Bradford (1976) with bovine serum albumin as standard. Measurements were done on a spectrophotometer plate reader at 595 nm. Assays were performed in buffer (Tris 0.1 M, pH 8; NaCl 0.1 M) containing 25 µm of 7-ethoxyresorufin and 0.25 mM of NADPH. Activity was determined by kinetic measurements at 20° C on supernatants diluted according to Addison and Payne (1986), and the quantity of resorufin (the specific product of EROD activity) was measured. Fluorometry was performed according to a modification of the method of Burke and Mayer (1974). Excitation occurred at 544 nm, and resorufin fluorescence emission was measured at 584 nm. Kinetics was determined using supernatant extracts diluted to 1 % final. Classical readings were obtained on a Turner 430 apparatus. Plate measurements were done on a Fluoroskan II (Grzebyk and Galgani, 1991): illumination by excitation light, as well as reception of emission light due to resorufin fluorescence, was done vertically for each well of the plate. Determinations for an entire plate require about 1 min. Resorufin was used for calibration of the apparatus. Reaction kinetics was linear for more than 10 minutes. Results are expressed as moles of resorufin released/min /mg of protein.

For cholinesterase measurements, fresh tissues were suspended in buffer (2/1 v/w) and homogenized for one minute using an Ultra Turrax. Extracts were then centrifuged at 16,000 g for 30 min (Sigma MK3 centrifuge). The method of Bradford (1976) was used for quantitative protein determination, with bovine serum albumin as standard. All assays of enzymatic activity were done in quadruplicate and performed directly on board ship as previously described (Galgani and Bocquéné, 1991). AChE was determined spectrophotometrically using acetylthiocholine as substrate. The method of Ellman *et al.* (1961) was used, as modified for microtitration plate reading (Galgani and Bocquéné, 1991). For each microplate well, 300 µl of Tris buffer 0.1 M (pH 8), 20 µl of dithiobis nitrobenzoic acid (DTNB) 0.01 M and 10 µl of enzyme solution were successively added. Substrate (10 µl 0.1 M) was added before enzymatic reaction was started, and optical density (OD) was monitored on a microplate reader (Titerteck MCC 340) at 405 nm. One unit of AChE activity is the variation of 0.001/0D. Results are given as units min^{-1} or units min^{-1} mg $protein^{-1}$ for specific activities. All assays were done in quadruplicate.

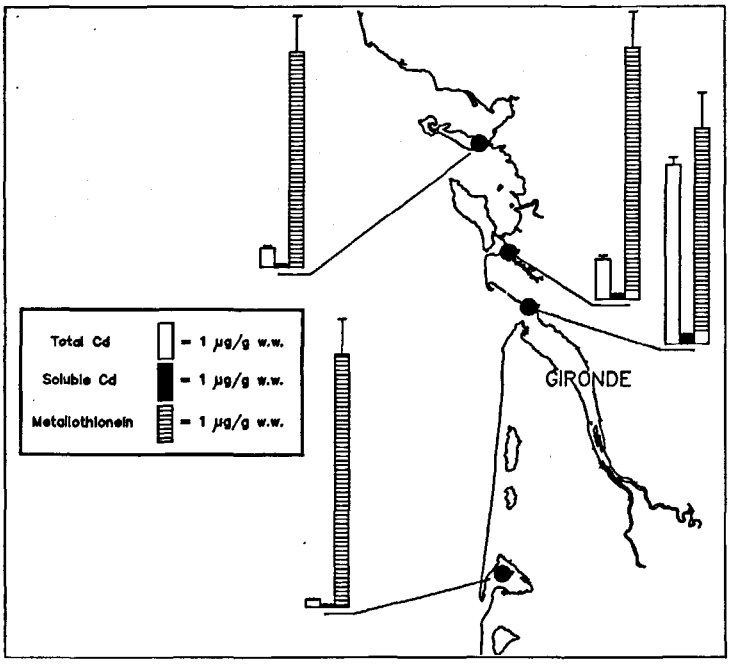
For metallothioneins, soft tissues from oysters (3 g) were partially thawed in 4 ml of buffer ($NaHPO_4$ 0.1 M, NaCl 0.5 M, PMSF 1 mM, pH 8.0), homogenized and centrifuged for one hour at 10,000 g at 0-4° C. Four ml of supernatant were then heated at 80° C for 3 min and centrifuged again for one hour at 0-4° C. Assays were performed in microplates using successively 300 µl supernatant, 50 µl dithiobis nitrobenzoate in buffer and 10 µl 1 % SDS. Optical density was reached after 30 min at 405 nm in a Multiskan MCC 340 apparatus. The principle of the reaction has been described by Dieter *et al.* (1987) and compared with other assays such as chromatography and immunoassays. For the standard curve, purified metallothionein (MT1) from rabbit liver (Sigma Chemical Co) was used instead of glutathione. The results are expressed in terms of metallothionein-like contents.

Total cadmium and soluble cadmium were assessed by AAS according to the method of Auger (1989).

Figure 2

Concentrations in cadmium (total and soluble) and metallothioneins in Gironde estuary oysters. Cadmium was assayed by atomic absorption spectrophotometry according to the method of Auger (1989). The thiol groups and metallothioneins involved in the complexing of metals were assayed by the DTNB reaction method (Dieter et al., *1987) as adapted to microplate readers according to Chiffoleau (1990; see Materials and Methods). Each value represents the mean of three measurements for five samples from one site.*

Concentrations en cadmium (total et soluble) et en métallothionéines dans les huîtres de l'estuaire de la Gironde. Le cadmium a été mesuré par spectrophotomètrie d'absorption atomique selon la méthode d'Auger (1989). Les groupements thiols et les métallothionéines impliquées dans la complexation du métal ont été dosés par le DTNB (Dieter *et al.*, 1987), selon une méthode en microplaque (Chiffoleau, 1990). Chaque valeur représente la moyenne de trois mesures pour cinq échantillons par station.



RESULTS AND DISCUSSION

Figure 2 gives the results of metallothionein measurements around the Gironde estuary. Four stations were sampled and for each station, cadmium concentration was measured in tissues. Values were found to range from 0.05 to 0.26 µg/g dryweight (soluble Cd) and from 0.21 to 5.0 µg/g dryweight (total Cd) in soft tissues. For the four stations, the mean metallothionein concentration was between 6 and 7 µg/g dryweight as determined using rabbit metallothionein as standard. No relation was found with cadmium content. Cadmium concentration in oysters from the Gironde estuary is one of the highest worldwide (Boutier and Chiffoleau, 1986), and our results indicate the absence of induction of thiols containing proteins such as metallothioneins in the presence of a high cadmium concentration. Consequently, this parameter was not retained for monitoring purposes in the context of our study.

Figure 3 shows the results for EROD measurements performed in March 1990 on *Callionymus lyra* (Fig. 3 A), which clearly demonstrating induction of EROD activity in Seine Bay fish. Induction was marked at the mouth of the Seine river where values were two to eight times higher than those obtained in organisms sampled out at sea. The results

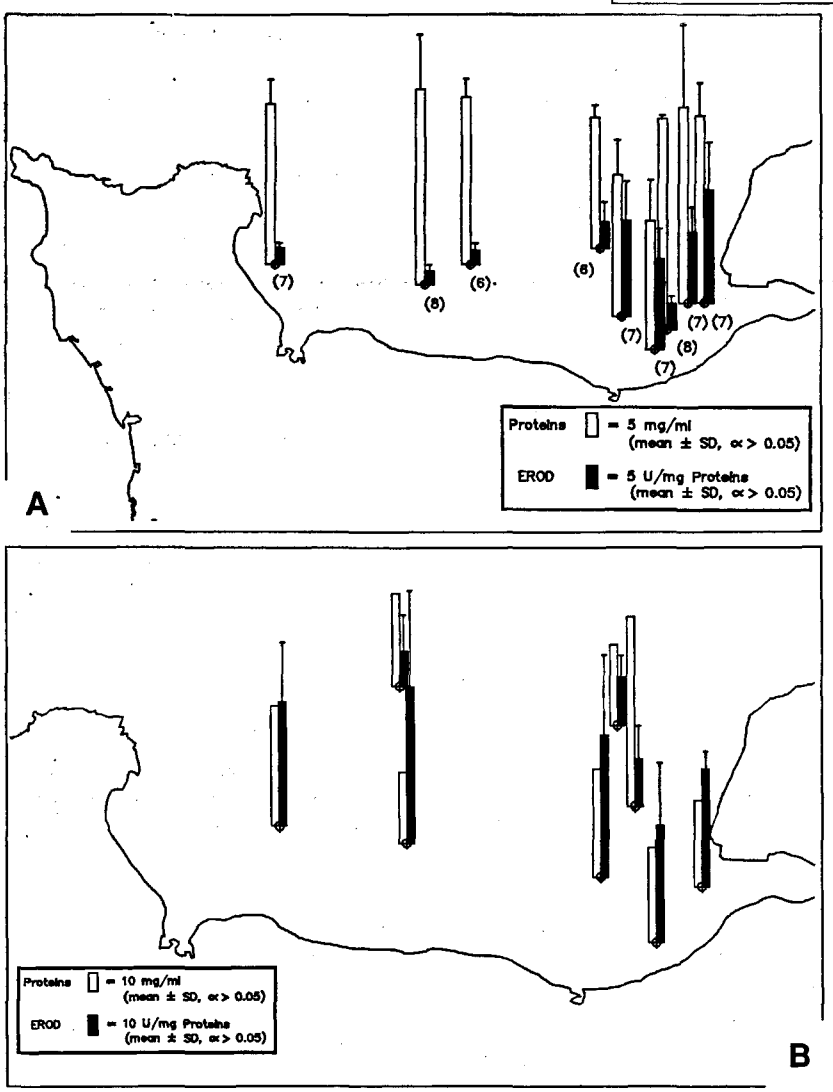


Figure 3

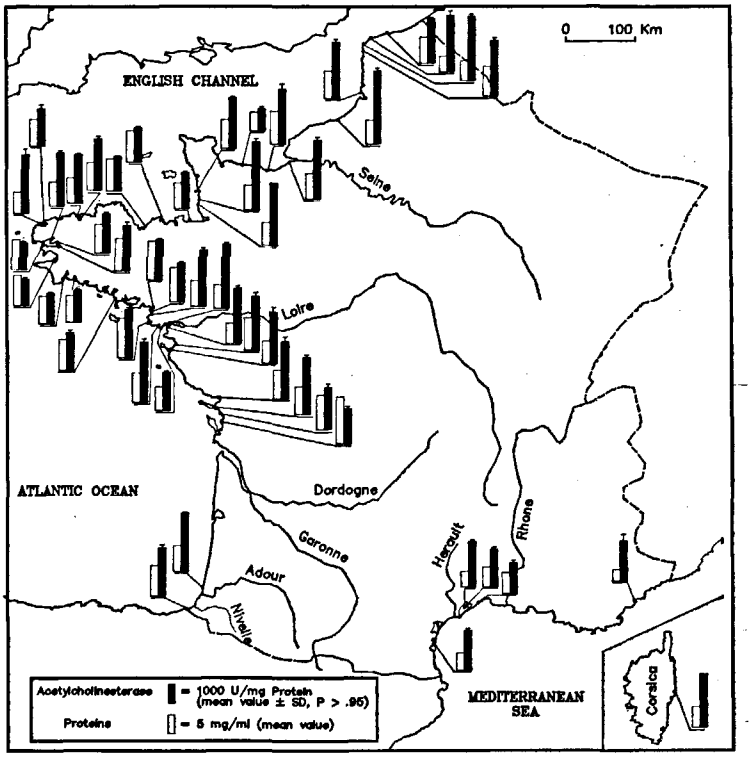
Soluble proteins and enzymatic activity of ethoxyresorufin deethylase (EROD) (mean values 1 S m) in livers of Callionymus lyra *(A) and* Limanda limanda *(B) sampled in the Seine Bay, (n = ?) number of samples. For* Limanda limanda *(B), ten samples were measured per station. For each sample, four assays were performed.*

Protéines solubles et activité enzymatique de l'éthoxyrésofurine dééthylase (EROD ; valeur moyenne ± t S m) dans le foie de *Callionymus lyra* (A) et *Limanda limanda* (B), échantillonnées dans la baie de Seine (n : nombre d'échantillons). Pour la limande (B), dix échantillons ont été mesurés par station. Pour chaque échantillon, quatre dosages ont été réalisés.

Figure 4

Soluble proteins and AChE activity of the adductor muscle of Mytilus edulis *sampled along French coasts. For each station, four assays were performed on an enzyme extract from one pool of ten adductor muscles.*

Protéines solubles et activité de l'acétylcholinestérase du muscle adducteur de *Mytilus edulis* des côtes françaises. Pour chaque station, quatre dosages ont été réalisés sur un extrait d'enzyme de dix muscles adducteurs.



for *Limanda limanda* (Fig. 3 B) obtained during a series of samples performed in March 1991 demonstrate the existence of variable EROD concentrations, with elevated values throughout Seine Bay and much lower concentrations out at sea.

From a regulatory viewpoint, one of the most valuable features of sensitive responses, *e. g.*, to EROD induction, is that they serve to define the spatial and temporal boundaries of pollution effects. Data from field experiments show that induction of EROD activity in flat fish is highly sensitive to the presence of petroleum hydrocarbons at environmentally realistic levels. However, as petroleum is a complex mixture, only some of its components are hepatic MFO inducers, so that the absolute sensitivity of the EROD response will vary according to the composition of the oil. Moreover, if PAH are essentially absent, MFO induction may be low or undetectable. The only pollutants other than PAH likely to be encountered in practice, or capable of inducing hepatic MFO in fish, are PCB. However, the PCB dose required to cause direct induction of hepatic MFO enzymes is so great that the level is unlikely to be reached except in major contamination events. The time-course of hepatic MFO induction, though apparently species-dependent, occurs within a few days. The slower course in some species simply reflects environmental temperatures and the generally lower metabolic state at which these fish function. Most petroleum spills are unusual events, even though there is quite visible evidence of contamination for weeks or months. The EROD induction technique in fish is not intended to confirm the obvious pollution from major oil spills or blowouts but to indicate the general quality of an environment potentially threatened by relatively low-level "chronic" petroleum release. Such events are likely to be continuous or at least frequent on a small scale, and it seems likely that fish hepatic MFO response would be relatively sensitive to them. Within the scope of this study, the first results in a series of monitoring experiments point to the existence of a gradient of biological effects along the dilution axis of the Seine river.

Figure 4 shows muscle cholinesterase levels in mussels sampled at French RNO stations in June 1990, a period of high pesticide diffusion. The lowest concentrations were found in organisms from Breton river estuaries, and the Thau lagoon on the Mediterranean coast. The low activities, representing 40 % of the highest levels found in the northern part of the English Channel, correspond to important inhibitory effects. Figure 5 indicates the cholinesterase concentrations in *Limanda limanda* sampled in March 1991 outside the pesticide diffusion period. The constant levels without inhibition serve to define a time-zone free of pesti-

Table

Soluble proteins and AChE activity in Limanda limanda *and* Solea solea *muscles from two Seine Bay stations in September 1990 values (x ± t S m). Four measurements were performed for each individual.*

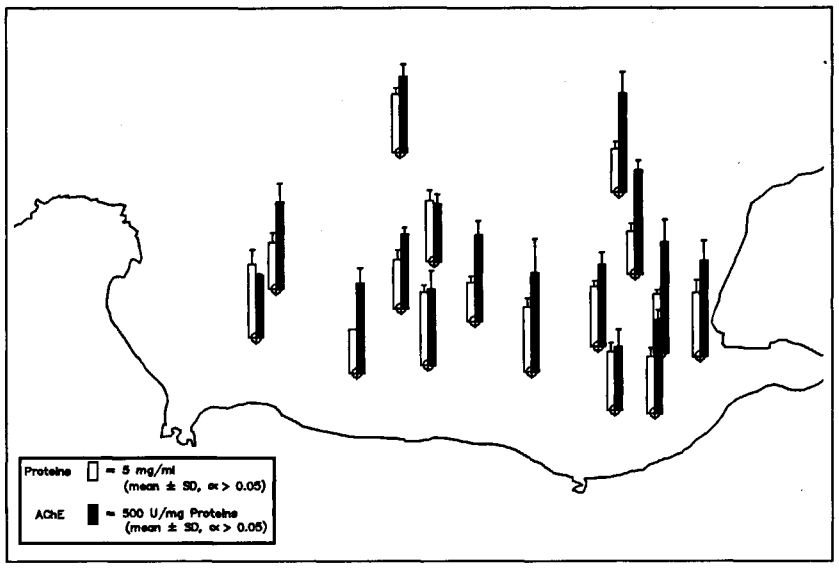
Protéines solubles et activité de l'AChE du muscle de *Limanda limanda* et de *Solea solea* d'échantillons de la baie de Seine prélevés en mars 1991. Les valeurs correspondent à des moyennes (x ± t S m). Quatre mesures ont été réalisées pour chaque station.

Position		Species	Number of samples	Proteins (mg/ml muscle extract)	AChE U/mgP^{-1}
LAT.	LONG.				
49°28'63	1°11'18	*Limanda limanda*	8	12.48 ± 0.93	2 776 ± 612
		Solea solea	10	7.88 ± 0.89	1 546 ± 301
49°25'94	2°58	*Limanda limanda*	8	15.7 ± 2.50	2 046 ± 352
		Solea solea	8	12.97 ± 1.91	1 031 ± 119

Figure 5

Soluble proteins and AChE activity in Limanda limanda *muscle from samples taken in the Seine Bay in March 1991. Values are mean values (x ± t S m) obtained in ten different organisms per station. Four measurements were done for each sample.*

Protéines solubles et activité de l'AChE du muscle de *Limanda limanda* d'échantillons de la baie de Seine prélevés en mars 1991. Les valeurs correspondent à des moyennes (x ± t S m), obtenues sur dix organismes différents par station. Quatre mesures ont été réalisées pour chaque station.



cide outflows. The Table shows variations in AChE activity in organisms sampled in September 1990, *i. e.*, at the end of the period of pesticide use. The rate of inhibition (26 % for *Limanda limanda* and 33 % for *Solea solea*) corresponds to unnatural variations. All these results raise the problem of interpreting variable levels of specific cholinesterase activity. The detection and monitoring of organochlorine pesticides, which is facilitated by their persistence in the environment, has already been performed in France by the RNO. Concentrations of DDT, its metabolites and those of lindane (HCH) are checked regularly. However, the great variety and relative fragility of organophosphate and carbamate molecules complicates analysis by chemical methods (Tronczynski, 1990), creating a considerable monitoring problem which can only be solved (and merely for a few molecules) by sophisticated techniques. The importance of controlling such nuisances and the convenient existence of monitoring networks have led scientists concerned with these problems to develop biological detection methods. The idea behind this approach is to determine the identity of a disturbing element by considering the way the disturbance affects an internal mechanism. This idea is not new, but its application to the control of contaminants and their effects on the environment requires thorough knowledge of all the intervening mechanisms involved.

The use of such a tool in an ocean environment must be based on an important preliminary study (Galgani and Bocquéné, 1990). Organophosphates and carbamates are not the only molecules capable of inhibiting cholinesterases. Several studies (Olson and Christensen, 1980) relating to the inhibitory effects of various chemical families on AChE activity have demonstrated the strong inhibitory capacity of certain metallic ions such as arsenic or copper, or the inhibitory effects of some compounds or neurochemical agents (atropine, nicotine, muscarine, ergotamine). These organic products, which are unlikely to occur in the marine environment at inhibitory concentrations comparable to those cited in these studies, cannot be considered responsible for the effects observed in that environment (on the order of 10^{-3} M).

The choice of such biological markers helps in determining the impact of chemical pollutants on the marine environment and thus in satisfying the national and international need for monitoring of biological effects in marine organisms. However, further studies must be carried out to specify the nature of the molecules involved. In this context, the monitoring of biological effects will be of true value in identifying the problems caused by pollutant effects.

CONCLUSION

The setting up of networks to monitor biological effects should be an international activity. North Sea countries are establishing such a monitoring network under the aegis of the North Sea Task Force. In fact, the ICES and the IOC have organized oceanographic cruises to validate the methods used and to determine the scientific difficulties involved in such an undertaking.

France has defined a strategy which includes measurements of biochemical parameters. The role of marine biotechnology is to develop sophisticated analytic tools which will permit measurements on a molecular scale to provide assistance in understanding problems on an oceanographic scale. As a result of technical achievements and the use of existing data on the presence of pollutants and the biology of species, the setting up of monitoring networks will be relatively simple and inexpensive. Moreover, the development of this type of monitoring will provide new data which will gradually allow us to replace the fragmentary and isolated results of existing research work and to evaluate the long-term tendencies of alteration of organisms. This will in turn facilitate our understanding of marine ecosystem adaptation mechanisms to chemical pollutants as well as our interpretation of the results in terms of the effects on human consumption.

In the context of the French coast, EROD measurement has been retained as the first molecular marker for monitoring the effects of contaminants in both the Seine Bay and the gulf of Fos-sur-Mer.

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REFERENCES

Addison F. and J.F. Payne (1986). 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. *Can. tech. Rept Fish. aquat. Sci.*, **1505**.

Auger D. (1989). Méthode de dosage du cadmium, du cuivre, du plomb et du zinc dans la chair de poissons. Rapport IFREMER, DERO-89-07-MR.

Bocquéné G. and F. Galgani (1991). Acetylcholinesterase activity in the common prawn (*Palaemon serratus*) contaminated by Carbaryl and Phosalone: choice of a method for detection of effects. *Ecotoxicol. Environ. Saf.*, **22**, 3, 337-345.

Bocquéné G., F. Galgani and P. Truquet (1990). Characterisation and assay conditions for use of AChE activity from several marine species in pollution monitoring. *Mar. environ. Res.*, **30**, 75-89.

Boutier B. and J.-F. Chiffoleau (1986). La contamination par le cadmium en Gironde et son extension sur le plateau continental. Rapport IFREMER, DERO-86-12-MR.

Bradford M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Analyt. Biochem.*, **72**, 248-254.

Burke M. and R. Mayer (1974). Ethoxy resorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug. Metab. Disp.*, **2**, 538-588.

Capel P.D., W. Gigen, P. Reichert and O. Waren (1988). Accidental input of pesticides into the Rhine river. *Environ. Sci Technol.*, **22**, 9, 992-997.

Chiffoleau J.-F. (1990). Les métallothionéines: méthodes d'analyse et utilisation comme bio-indicateurs, Rapport IFREMER DRO-90-11-MR. 54 pp.

Claisse D. (1989). Chemical contamination of French coasts: the results of ten years mussel watch. *Mar. Pollut. Bull.*, **20**, 523-528.

Coppage D.L. and E. Matthews (1974). Short-term effects of organophosphate insecticides on cholinesterases of estuarine fishes and pink shrimp. *Bull. environ. Contamin. Toxicol.*, **2**, 5, 438-488.

Dieter H.A., L. Muller, J. Abel and K.H. Summer (1987). Metallothionein determination in biological materials : interlaboratory comparison of 5 current methods. *Experientia*, **52**, 351-358.

Ellman G.L., K.O. Courtney, V. Andres and R.M. Featherstone (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.*, **7**, 88-95.

Galgani F. and G. Bocquéné (1989). A method for routine detection of organophosphates and carbamates in sea water. *Environ. technol. Letts*, **10**, 311-322.

Galgani F. and G. Bocquéné (1990). *In vitro* inhibition of acetylcholinesterase from four marine species by organophosphates and carbamates. *Bull. environ. Contamin. Toxicol.*, **45**, 243-249.

Galgani F. and G. Bocquéné (1991). Semi-automated colorimetric and enzymatic measurements in aquatic organisms using a plate reader. *Wat. Res.*, **25**, 2, 147-150.

Galgani F. and J. Payne (1991). Biological effects of contaminants: microplate method for the measurement of ethoxyresorufin-O-deethylase (EROD) in Fish. ICES Times series No. 13, 11 pp.

Galgani F., G. Bocquéné and Y. Cadiou (1992). Evidence of variation of cholinesterase activity in fishes along a pollution gradient in the North Sea. *Mar. Ecol.-Prog. Ser.*, in press.

Goksoyr A. (1985). Purification of hepatic microsomal cytochrome P-450 from β-naphtoflavone treated Atlantic Cod (*Gadus morrhua*), a marine teleost fish. *Biochem. Biophys. Acta*, **840**, 409-417.

Goksoyr A. (1987). Characterization of the cytochrome P450 Monooxygenase system in fish liver metabolism and effects of organic xenobiotics. *Ph.D. thesis, University of Bergen, Norway.*

Goksoyr A. (1991). A semi-quantitative cytochrome P-4501A1 Elisa: a simple method for studying the monooxygenase induction response in environmental monitoring and ecotoxicological testing of fish. *Sci. total Environment*, **101**, 255-262.

Gooch J.W., A.A. Elkuus, P.J. Kloepper-Sams, M.E. Hahn and J.J. Stegeman (1989). Effects of ortho and non-ortho substituted polychlorinated biphenyl congeners on the hepatic monooxygenase system in scup (*Stenotonus chrysops*). *Toxicol. Appl. Pharmacol.*, **98**, 422-423.

Grzebyk D. and F. Galgani (1991). Measurement of organic pollution on marine organisms. Rapid determination of EROD induction using plate readers. *Aquat. Liv. Resour.*, **4**, 53-59.

Haasch M.L., P.J. Wejksnora, J.J. Stegeman and J.J. Lech (1989). Cloned rainbow trout liver P1 450. Complementary DNA as a potential environmental monitor. *Toxicol. Appl. Pharmacol.*, **98**, 362-368.

Habig C., R.T. Digiulio, A.A. Nomeir et M. Abou-Donia (1986). Comparative toxicity, cholinergic effects, and tissue levels of S, S, S,-TRI-n-Butyl Phosphorotrithioate (DEF) to Channel Catfish (Ictalurus punctatus) and Blue Crabs (Callinectes sapidus). *Aquat. Toxicol.*, **9**, 193-206.

Habig C., R.T. Digiulio and M.B. Abou-Donia (1988). Comparative properties of Channel catfish (*Ictalurus punctatus*) and Blue Crab (*Callinectes sapidus*) acetylcholinesterases. *Comp. Biochem. Physiol.*, **91 C**, 2, 293-300.

Hogan J.W. (1970). Water temperature as a source of variation in the specific activity of brain cholinesterase of Bluegills. *Bull. environ. Contam. Toxicol.*, **5**, 347-354.

Holland H.T., D.L. Coppage and P.A. Butler (1967). Use of fish brain acetylcholinesterase to monitor pollution by organophosphorous pesticides. *Bull. environ. Contam. Toxicol.*, **2**, 3, 156-162.

ICES/IOC (1992). Report of the ICES/IOC workshop on the biological effects of contaminants in the North sea. *Mar. Ecol.-Prog. Ser.*, in press.

Klaverkamp J.F. and B.R. Hobden (1980). Brain acetylcholinesterase inhibition and hepatic activation of acephate and fenitrothion in Rainbow Trout (*Salmo gairdneri*). *Can. J. Fish. aquat. Sci.*, **37**, 1450-1453.

Klotz A.V., J.J. Stegeman and C. Walsh (1983). An aryl hydrocarbon hydroxylating hepatic cytochrome P-450 from the marine fish (*Stenotomus chrysops*). *Arch. Biochem. Biophys.*, **22**, 578-592.

Klotz A., J. Stegeman, B. Woodin, E. Snowberger, P. Thomas and C. Walsh (1986). Cytochrome P-450 isozymes from the marine teleost *Stenotomus chrysops*: their role in steroid hydroxylation and the influence of cytochrome P-450. *Arch. Biochem. Biophys.*, **249**, 326-338.

Kobayashi K., Y. Nakamura, R.M. Rompas and N. Imada (1986). Difference in lethal concentration *in vivo* between Fenitrothion and its oxo-form in Tiger Shrimp *Penaeus japonicus*. *Bull. japan. Soc. scient. Fish.*, **52**, 2, 287-292.

Massoulie J. and S. Bon (1982). The molecular forms of cholinesterase and acetylcholinesterase in vertebrates. *Annu. Rev. Neurosci.*, **5**, 57-106.

Melancon M.J. and J.J. Lech (1983). Dose-effect relationship for induction of hepatic monooxygenase activity in rainbow trout and carp by Aroclor 1254. *Aquat. Toxicol.*, **4**, 51-61.

Olson D.L. and G.M. Christensen (1980). Effects of water pollutants and other chemicals on fish acetylcholinesterase inhibition. *Bull. environ. Contamin. Toxicol.*, **21**, 502-506.

Olsson P.E. and C. Hogstrand (1987). Subcellular distribution and binding of cadmium to metallothionein in tissues of rainbow trout. *Environ. Toxicol. Chem.*, **6**, 867-874.

Payne J.F., L.L. Fancey, D.A. Rahimtula and E. Porter (1987). Review and perspective on the use of mixed function oxygenase enzymes in biological monitoring. *Comp. Pharmacol. Physiol.*, **86 C**, 233-245.

Petering D.H. and B.A. Fowler (1986). Role of metallothionein and related proteins in metal metabolism and toxicity: problems and perspectives. *Environ. Hlth Perspect.*, **65**, 217-224.

Spies R.B., D.W. Rice and J.F. Felton (1989). Effects of organic contaminants on reproduction of the starry flounder *Platichths stellatus* in San Francisco Bay. I: Hepatic contamination and mixed function oxidase (MFO) activity during the reproductive season. *Mar. Biol.*, **98**, 181-189.

Stegeman J.J. and P.J. Kloepper-Sams (1987). Cytochrome P-450 isozymes and monooxygenase activity in aquatic animals. *Environ. Hlth Perspect.*, **71**, 87-95.

Stegeman J.J., F Teng. and E. Snowberger (1987). Induced cytochrome P450 in winter flounder (*Pseudopleuronectes americanus*) from coastal Massachussetts evaluated by catalytic assay and monoclonal antibody probes I. *Can. J. Fish. aquat. Sci.*, **44**, 1270-1277.

Tronczynski J. (1990). Les produits phytosanitaires en zone littorale et estuarienne. Rapport IFREMER/DRO n° 90-05, 39 pp.

Trundle D. and G. Marcial (1988). Detection of cholinesterase inhibition: the significance of cholinesterase measurements. *Annls Clinic. Lab. Sci.*, **18**, 5, 345-352.

Viarengo A. (1985). Biochemical effects of trace metals. *Mar. Pollut. Bull.*, **16**, 4, 153-158.

Vignier V. (1985). Études des systèmes monooxygénasiques à cytochrome P450 dépendants chez la plie (*Pleuronectes platessa*). *Ph. D. thesis, Université de Bretagne Occidentale, Brest, France.*