Measurement of the effect of organic pollution on marine organisms: rapid determination of EROD induction using plate readers

Daniel Grzebyk and François Galgani

IFREMER, Laboratoire Effets Biologiques des Nuisances,
rue de l'île d'Yeu, B. P. n° 1049, 44037 Nantes Cedex 01, France.

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Abstract

The purpose of this study was to adapt the ethoxyresorufin O-deethylase (EROD) assay to the microplate technique. In addition to showing the practical advantages related to the intrinsic properties of such systems, we wished to demonstrate their potential value for biological monitoring of contamination in natural environments. A simplified procedure was developed for the preparation and fluorimetric assay of EROD activity using only basic laboratory equipment. The use of plate readers allows measurements to be done rapidly and provides numerous advantages in comparison with traditional methods. Within the context of our study, this test could be substituted to advantage for the enzyme-linked immunosorbent assay (ELISA).

Keywords : Mixed function oxidase, ethoxyresorufin O-deethylase, cytochrome, enzyme, plate reader, organic pollution.

Mesure de l'effet de la pollution organique sur les organismes marins : détermination rapide de l'induction de l'EROD à l'aide des microplaques.

Résumé

L'objet de cette étude est d'adapter le dosage de l'éthoxyrésorufin O-deethylase (EROD) à la méthode des microplaques. Au-delà des avantages liés aux propriétés du système, nous démontrons le potentiel de cette technique pour la surveillance de l'effet de la contamination de l'environnement naturel par les polychlorobiphényls (PCB) et les hydrocarbures polyaromatiques (HAP). Une procédure simplifiée a été développée pour la préparation et la réalisation du dosage de l'activité enzymatique n'utilisant que des équipements de base d'un laboratoire. L'utilisation du lecteur de plaque permet la mesure rapide de nombreuses activités et présente de nombreux avantages par rapport aux méthodes traditionnelles. En particulier, ce test peut remplacer avantageusement les tests immunoenzymatiques (ELISA).

Mots-clés : Éthoxyrésorufin O-deethylase, lecteur de plaques, enzyme, surveillance biologique, environnement.

INTRODUCTION

Evaluation of the response of aquatic organisms contaminated by pollutants present in natural environments has been the subject of numerous studies in recent years. At the same time, an attempt has been made to relate the level of contamination of the natural environment to organism response in order to evaluate the contamination itself. Implementation

of biological monitoring to survey changes in this response depends on the availability of a battery of biochemical tests capable of indicating rapidly and with good sensitivity the response of organisms subjected to a pollutant effect.

In this respect, the cytochrome P-450 dependent mixed function oxidase (MFO) enzymatic system is of considerable interest. Thanks to several of its isozymes, cytochrome P-450 is implicated in the degradation of numerous endogenous (vitamins, steroids) and xenobiotic [e.g. petroleum products and polychlorinated biphenyl (PCB) derivatives] organic molecules. Some of these isoforms are specifically induced by pollutants used as substrates and enter into the processes of decontamination and detoxification of organisms. These isozymes, which have broad substrate specificities, can be detected as a result of the particular catalytic activities exercised on standard substrates. EROD, one of these MFO activities, is supported by induced specific forms of cytochrome P-450 which has been purified in several species of teleosts, including scup (*Stenotomus chrysops*) (Klotz *et al.*, 1983), rainbow trout (*Oncorhynchus mykis*) (Williams and Buhler, 1983) and cod (*Gadus morrhua*) (Goksoyr, 1985). These isoforms are induced in natural environments by polycyclic aromatic hydrocarbons (PAH) present in petroleum and by PCB. EROD activity is characterized by the degradation (dealkylation) to 7-ethoxyresorufin in resorufin.

At the present time, there are two established methods for assessing the level of EROD induction. The first is by direct assay of enzymatic activity using either a fluorimetric (Burke and Mayer, 1974) or spectrophotometric (Klotz *et al.*, 1984) technique to measure the appearance of resorufin. The second is to assay the specific isoform of EROD by immunochemical tests (Stegeman *et al.*, 1987, 1988). A third method, currently being developed, is the mRNA assay (Lloepper-Sams and Stegeman, 1989). The microplate technique has been shown to be well adapted to routine use for colorimetric and enzymatic assays (Galgani et Bocquené, 1989). The conditions for optimal measurements of this enzymatic activity were studied in several species (from the N. E. Atlantic) useful for a monitoring system.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma Chemical Co. (St-Louis, MO): 7-ethoxyresorufin, NADPH, metyrapone and polyethylene glycol 8000 (PEG 8000). Goat-LM4 rabbit polyclonal antibodies were provided by C. Dalet (INSERM, Montpellier, France).

Animals

Plaice (*Pleuronectes platessa*) were obtained from St-Brieuc Bay, and flounder (*Platichthys flesus*) and eels (*Anguilla anguilla*) from the Loire estuary. Several species of invertebrates from a Nantes fish market were also tested: mussels (*Mytilus edulis*), oysters (*Crassostrea gigas*), crayfish (*Nephrops norvegicus*), shrimp (*Crangon crangon*) and crabs (*Cancer pagurus*). All these animals were collected in March and April.

Preparation of extracts

Organs were removed from living animals, washed in buffer A (Tris 50 mmol, pH 7.4; KCl 150 mmol; EDTA 1 mmol and glycerol 20% vol) and then minced (5 ml/g of tissue) for 5 to 10 secondes in an Ultraturrax. Centrifugation was performed at 9000 g for 15 minutes at 4°C. Supernatant was removed and stored at −85°C. The protein assay was performed using the method of Bradford (1976), with spectrophotometer plate reader at 595 nm.

EROD activity assay

This assay was performed in buffer (Tris-HCl 0.1 M, pH 8; NaCl 0.1 M) containing 2 µmol of 7-ethoxyresorufin and 0.25 mmol of NADPH (Nicotiamide ademine dinucleotide phosphate). Activity was determined by kinetic measurements at room temperature on supernatants diluted according to Addison and Payne (1986), and the quantity of resorufin, a specific product of EROD activity, was measured. The method of Klotz *et al.*, was used for spectrophotometric determinations. In this case, supernatant
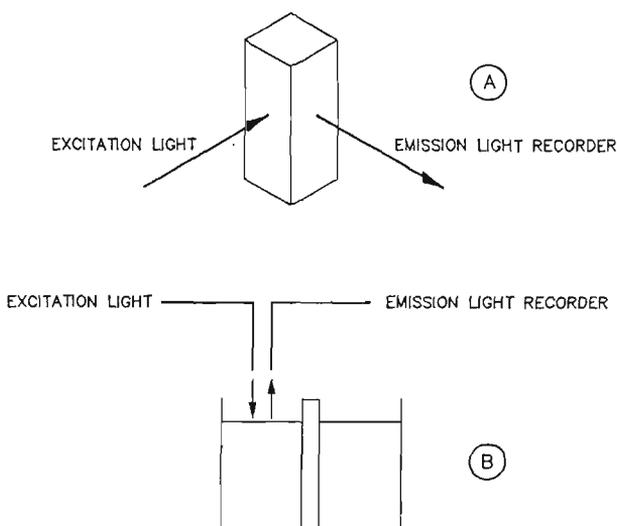

Figure 1. − Two ways of measuring fluorescence: (A) with a cuvette fluorimeter, (B) with a microplate fluorimeter.

dilution levels ranged from 2 to 25. Classical spectrophotometry was done at 572 nm using a Hitachi 2 000 apparatus. Plate measurements were performed at 578 nm using a MCC/340 P Multiskan which can provide a complete reading in 7 seconds. Each plate contained 96 wells which were filled with 380 μl to obtain an optical course of 10 mm.

Fluorimetry was performed according to the method of Burke and Mayer (1974). Excitation occurred at 544 nm, and resorufin fluorescence emission was maximum at 584 nm. Enzyme kinetics were determined using supernatant extracts diluted to 1%. Classical readings were obtained on a Turner 430 apparatus. Plate measurements were done on a Fluoroskan II: illumination by excitation light, as well as reception of emission light due to resorufin fluorescence, was done vertically for each well (*fig.* 1). Determination for an entire plate required about 1 minute.

Microsome preparation

Microsomes were prepared according to the technique of Van Der Hoeven (1981) using PEG 8 000 at a final solution (w/v) of 5%. After 10 minutes of incubation, the supernatant was centrifuged at 8 000 g for 10 minutes, and the pellet representing the microsomal fraction was resuspended in 1 vol of buffer.

RESULTS

In spectrophotometry , sensitivity was comparable for readings of microplates and classical cuvettes in the resorufin dilution assay (*fig.* 2). However, kinetic

(A)

(B)

Figure 3. — Relationship between fluorimetric microplate and classical cuvette measurements: (A) Assays for a resorufin dilution scale from 0.140 to 1.050 μmol. (B) EROD activity assays for various samples values are expressed in fluorimetric units.
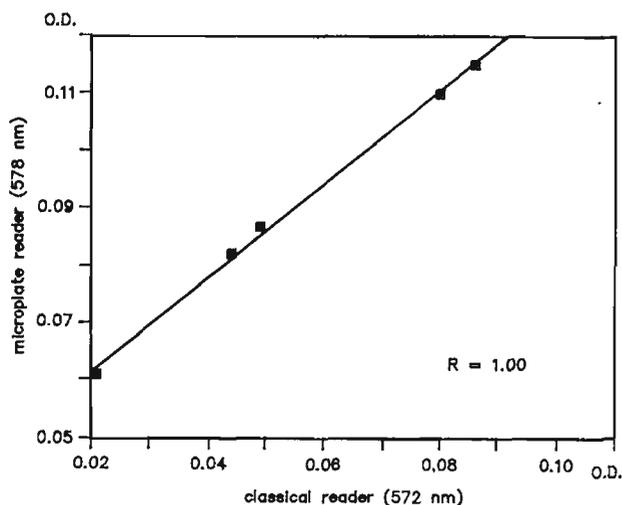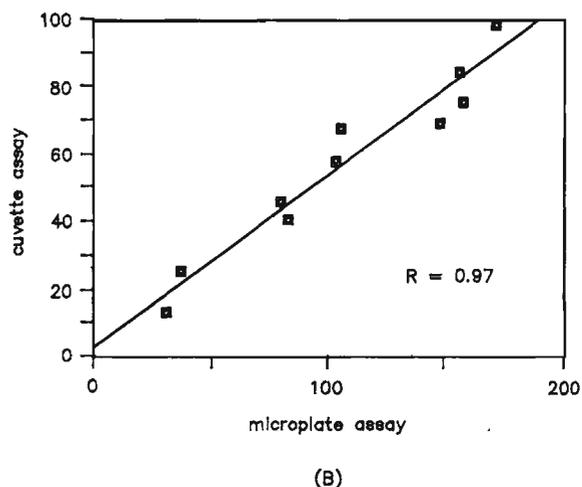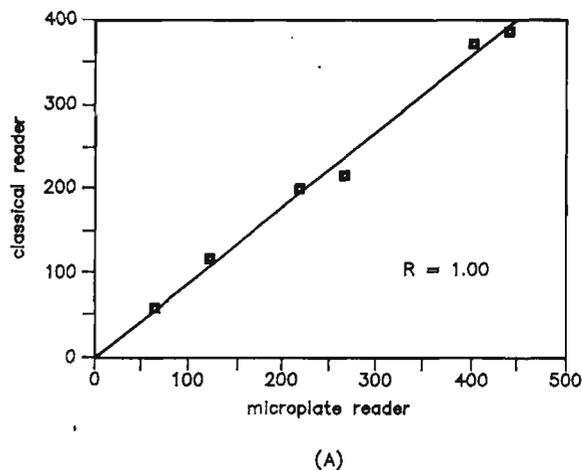

Figure 2. — Relationship between a classical cuvette spectrophotometer at 572 nm and a microplate spectrophotometer at 578 nm for a resorufin dilution scale from 0.140 to 1.050 μmol.

studies performed in plates with supernatants of uninduced plaice liver extracts showed only slight variations in optical density. This low sensitivity was undoubtedly due to the excessive turbidity of the reaction solution which contained a high proportion of supernatant.

In fluorimetry, microplate and cuvette readers gave closely correlated results (*fig.* 3). However, the plate reader was twice as sensitive as the classical cuvette reader for the EROD activity assay. This may have been due to the way in which excitation lighting and fluorescence reception were performed on each apparatus. For the cuvette reader, excitation light passed through the reaction solution and may have been reduced in intensity by a slight turbidity and a greater dispersion of fluorescence. This effect, as
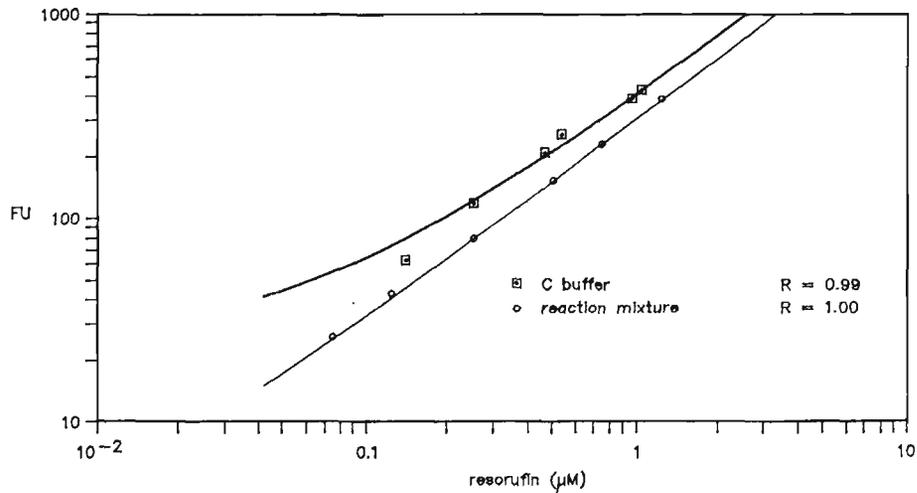
Figure 4. − Dilution scales of resorufin in (C) buffer and in reaction mixture [buffer, diluted supernatant and NADPH] obtained with a microplate fluorimeter.

determined by comparison of curves for resorufin dilution in buffer C and the reaction solution, is apparently not as great for plates (*fig.* 4).

With the plate reader, the coefficient of variation was less than 7%. The fluorimetric method thus proved to be markedly more sensitive, whereas the supernatant concentration in the reaction solution was much more reduced than with the spectrophotometric method.

Our essential results concern three species of fish (plaice, flounder and eel), and our study was limited to determining EROD activity in the livers of these fish since it seemed apparent that this organ had the highest EROD levels (*fig.* 5).

Variations in EROD activity as influenced by pH showed a peak at pH 8 with our standard assay buffer. However, a slightly improved activity can be noted at pH 7.0 to 7.2 for plaice and eels (*fig.* 6). The kinetic parameters (K_m, V_m) for the three species are shown in *table* 1. It should be noted that V_m values relate to total supernatant proteins ($nmol.min^{-1}$), among which microsomal cytochrome P-450 proteins represented only a part and those proteins responsible for EROD activity much less [2 to 50% of cytochrome P-450 values according to the level of EROD activity (Stegeman *et al.*, 1987)].

As the MFO system was NADPH-dependent, its affinity for this cofactor was determined. The values (*table* 1) indicate that NADPH saturation of the system was attained in our reaction solution.

The effect of temperature on the maintenance of EROD activity was also of importance in determining the best experimental conditions. The extract was exposed to different temperatures for 15 minutes, and
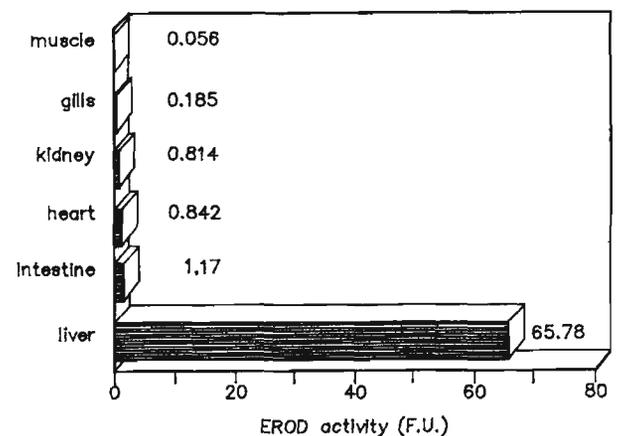

Figure 5. − EROD activity in various organs from flounder. Activities were obtained with the same dilution level of 9 000 g supernatant.

Table 1. − Kinetic parameters for EROD activity of liver from plaice flounder and eel. K_m: Michaelis constant (nmol per mg of protein); V_m: maximum velocity.

Species	K_m(nmol)	V_m (nmol.min^{-1} mg protein)	NADPH affinity (µmol^{-1})
Plaice	140	0.057	1.368
Flounder	237	0.087	—
Eel	415	0.009	3.05

residual EROD activity was then measured and compared to the room temperature (22°C) activity of an extract not subjected to this latency period. The results indicate that the activity was well maintained
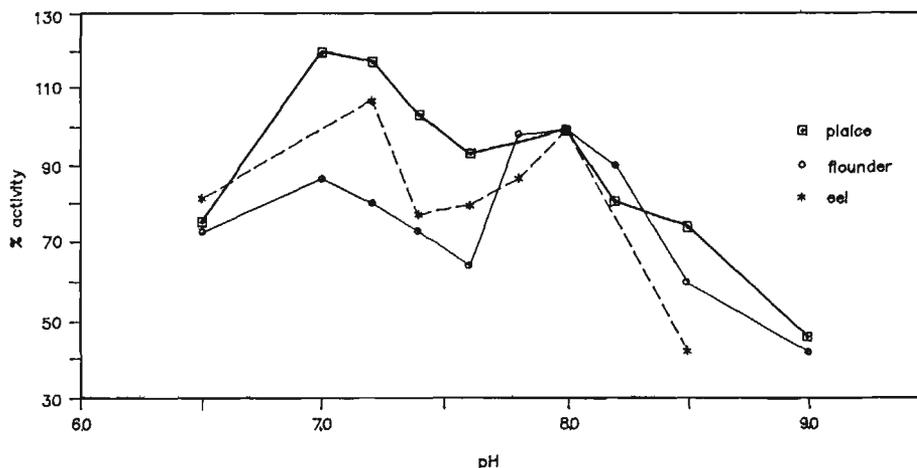
Figure 6. – pH dependency of 7 EROD activity in liver of plaice, flounder and eel. Activities are expressed as percentage of activity at pH 8.0 with our standard buffer.
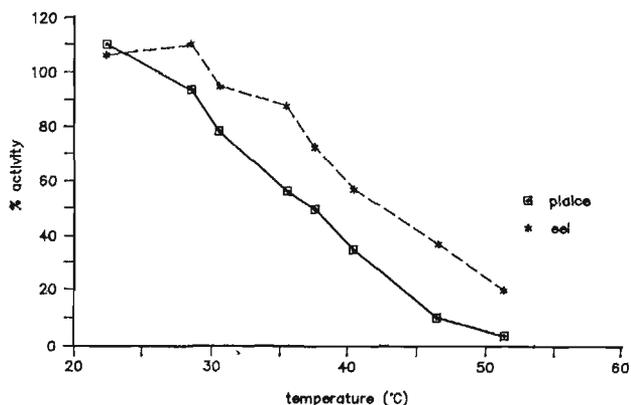

Figure 7. – Temperature effect on EROD activity in liver from plaice and eel. Residual activities were measured after the reaction mixture was subjected to different temperatures for 15 minutes. These activities are expressed as the percentage of activity at 22°C without incubation.

Figure 8. – *In vitro* inhibition of EROD activity by metyrapone on liver PEG microsomes from plaice and flounder. Results are expressed as the percentage remaining activity. Incubation with inhibitor was performed during 15 minutes before the assay.

Figure 9. – *In vitro* inhibition of EROD activity by antibodies on liver PEG microsomes from plaice and flounder. Results are expressed as the percentage remaining activity. Because of the ionic requirement of antibodies, activities were assayed in buffer (Tris 0.1 M, pH 8, NaCl 0.16 M).

during a time period sufficient to obtain good kinetic measurements (*fig.* 7). But when temperature increased, sizeable activity loss was readily apparent, making it no longer possible to obtain good results. This activity loss can be attributed in part to the degradation of the cytochrome P-450 system, which is known to be very unstable as well as to a drop in saturation of the oxygen dissolved in the reaction solution. Accordingly, it would appear that performance of the assay at room temperature (19 to 22°C) provides for a good compromise between the stability of the enzyme and its activation by temperature.

Finally, two inhibitors were tested on the microsomal fractions obtained as indicated above. Metyrapone, a known inhibitor of MFO activities, was
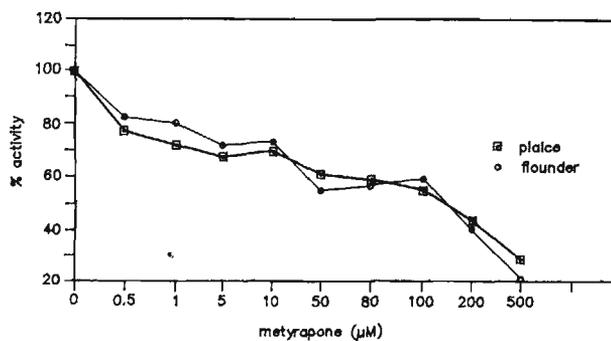
effective in inhibiting EROD activity (*fig.* 8 A). Inhibition was also obtained with rabbit anti-LM4 antibody (*fig.* 8 B). Such cross-reactivity between an antibody directed at the EROD isoform of a mammal (rat P-450 C) and the EROD isoform of a fish (scup P-450 E) has already been observed (Klotz *et al.*, 1986). These results demonstrate that EROD activity was actually measured in the fish species.

However, not the slightest EROD activity could be detected in the various invertebrates studied, regardless of the species and organs tested (hepatopancreas, gonads, muscle, whole animal).

DISCUSSION AND CONCLUSION

EROD activity is one of the biochemical parameters most often mentioned for inclusion in the battery of tests proposed for the implementation of biological monitoring programs. Numerous studies have in fact shown that induction of EROD activity is related to contamination level (experimentally) of fish (Melancon and Lech, 1983) as well as to environmental contamination level (Stegeman *et al.*, 1988, Addison and Payne, 1986, Payne *et al.*, 1988) with respect to some organic pollutants. Moreover, the direct assay of this activity is technically very easy and rapid to perform compared to that of other induced MFO activities. This simplicity allows routine use of the EROD activity assay and, particularly, in terms of this study, the facility of adaptation to the microplate technique.

The microplate technique itself has many advantages. In addition to reproducibility of results and a good sensitivity identical to that of the classical cuvette technique, its greatest advantage is the rapidity of measurements, allowing multiple simultaneous assays and thus long kinetic studies of interest for measuring low activities and avoiding interruption of the acetone reaction. Finally, it is possible to automate the technique, distribution of reagents and measurement and processing of data. The direct assay is preferable to immunochemical techniques such as the enzyme-linked immunosorbent assay (ELISA) that determine the isoform quantity of cytochrome P-450 catalyzing EROD activity. In particular, the direct technique makes it possible to eliminate numerous steps in the assay such as antigen coating, washings and antigen-antibody reactions, so that EROD activity can be determined in several minutes instead of several hours as for the ELISA method. Moreover, response is identical, as indicated by the very good correlation of results with the two methods (Stegeman *et al.*, 1987; Kloepper-Sams and Stegeman, 1989).

Furthermore, the use of immunochemical techniques, is limited by the specificity of antibodies. Although interspecific crossed assays have been developed (Stegeman *et al.*, 1987; Stegeman *et al.*, 1988) based on apparent similarities between the isoforms

of different species, use of the direct technique for measuring catalytic activity (Stegeman and Kloepper-Sams, 1987) makes it possible to get around the problem of species without preliminary antibody preparation and the fastidiousness of the ELISA method.

The application of our method is of particular interest for a biological monitoring system in which a large number of samples must be handled. Elimination of the long and tedious ultracentrifugation step not only speeds up the assaying procedure but also makes it unnecessary to acquire expensive and technologically sophisticated equipment.

In the context of biological monitoring relative, in particular, to PAH and PCB, the direct EROD activity assay using the microplate reader technique is a very interesting tool that combines the advantages of simplicity and rapidity essential to such surveillance conditions.

REFERENCES

Addison R. F., J. F. Payne, 1986. Assessment of hepatic mixed function oxidase induction in winter flounder (*Pseudopleuronectes americanus*) as a marine petroleum pollution monitoring technique. *Can. Tech. Rep. Fish. Aquat. Sci.*, 1505.

Bradford M., 1976. A rapid method for quantitation of protein utilizing the principle of Dye Binding. *Anal. Biochem.*, **72**, 248-264.

Burke M. D., R. T. Mayer, 1974. Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by B-methylcholanthrene. *Drug. Metabol. Dispos.*, **2**, 583-588.

Galgani F., G. Bocquené, 1989. A method for routine detection of organophosphates and carbamates in sea water. *Environ. Technol. Lett.*, **10**, 311-322.

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.

Klotz A. V., J. J. Stegeman, 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. V., J. J. Stegeman, C. Walsh, 1984. An alternative 7-ethoxyresorufin O-deethylase activity assay: a continuous visible spectrophotometric method for measurement of cytochrome P-450 monooxygenase activity. *Anal. Biochem.*, **140**, 138-145.

Klotz A., J. Stegeman, B. Woodin, E. Snowberger, P. Thomas, 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.

Kloepper-Sams P. J., J. J. Stegeman, 1989. The temporal relationships between P-450 E protein content, catalytic activity, and on RNA levels in the teleost (*Fundulus*

heteroclitus) following treatment with B-naphtoflavone. *Arch. Biochem. Biophys.*, 260, 525-535.

Melancon M. J., 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.

Payne J. F., J. Kiceniuk, L. L. Fancey, U. Williams, G. L. Fletcher, A. Rahimtula, B. Fowler, 1988. What is a safe level of polycyclic aromatic hydrocarbons for fish: subchronic toxicity study on winter flounder (*Pseudopleuronectes americanus*). *Can. J. Fish. Aquat. Sci.*, 45, 1983-1993.

Stegeman J. J., P. J. Kloepper-Sams, 1987. cytochrome P-450 isozymes and monooxygenase activity in aquatic animals. *Environ. Health. Perspect.*, 71, 87-95.

Stegeman J. J., F. Y. Teng, E. A. Snowberger, 1987. Induced cytochrome P-450 in winter flounder (*Pseudopleuronectes americanus*) from coastal Massachusetts evaluated by catalytic assay and monoclonal antibody probes. *Can. J. Fish. Aquat. Sci.*, 44, 1270-1277.

Stegeman J. J., B. R. Wooxin, A. Goksoyr, 1988. Apparent cytochrome P-450 induction as an indication of exposure to environmental chemicals in flounder (*Platichthys flesus*). *Mar. Ecol.-Prog. Ser.*, 46, 55-60.

Van der Hoeven T. A., 1981. Isolation of hepatic microsomes by polyethylene glycol 6,000 fractionation of the postmitochondrial fraction. *Anal. Biochem.*, 115, 398-402.

Williams D., D. Buhler, 1983. Comparative properties of purified cytochrome P-448 from B-naphtoflavone treated rats and rainbow trout. *Comp. Biochem. Physiol.*, 75 C, 25-32.