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Biological effects of contaminants: Microplate method for measurement of ethoxyresorufin-O-deethylase (EROD) in fish

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1 INTRODUCTION

Interest in the use of mixed function oxidase (MFO) as a monitoring tool for measuring the effects of pollutants derives from basic research carried out over the past twenty years (see 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 activity in wild fish and its activity increases dramatically, apparently to enhance the degradation and clearance of offending compounds. This suggests that the activity of the MFO system in naturally contaminated organisms might be a measure of the degree of chemical contamination. There have been a number of field studies in which elevated MFO activity in fish was found to be associated with contamination by hydrocarbons (Payne *et al.*, 1987).

The MFO system requires molecular O_2 and NADPH and involves a co-binding protein: cytochrome P-450. In marine fish, two of the model reactions of the MFO-system, aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin-O-deethylase (EROD) (see Figure 1), have been studied most intensively (Spies *et al.*, 1982; Payne *et al.*, 1987; Ellenton *et al.*, 1985; Luxon *et al.*, 1987). Both AHH and EROD, of which EROD is the more specific, are catalyzed by the cytochrome P-450IA sub-family (Nebert and Gonzalez, 1987). In mammals, the cytochrome P-450IA sub-family contains two isozymes, namely, cytochrome P-450IA1 and cytochrome P-450IA2, but in fish only the former appears to be inducible by contaminants (Nebert and Gonzalez, 1987). The cytochrome P-450IA1 isozyme in fish can be induced by polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzodioxins (PCDDs), and certain polychlorinated biphenyls (PCBs) that have a planar configuration. This has been reported many times in laboratory studies (Jimenez *et al.*, 1988), as well as in mesocosms (Addison and Edwards, 1988; Stegeman *et al.*, 1988) and field studies (Spies *et al.*, 1982; Luxon *et al.*, 1987; Addison and Edwards, 1988). This makes the measurement of EROD activity a good means of evaluating fish response to PAH contamination. It has recently been shown that specific isoforms of this protein are involved in the metabolism of xenobiotics. Therefore, an increase in this specific isoform is good evidence of the induction of the MFO system by contaminants. Ethoxyresorufin-O-deethylase (EROD) activity is a specific assay for the xenobiotically inducible form of cytochrome P-450, thus making measurement of this activity in fish liver a good means of evaluating fish response to PAH contamination.

The method described here has been adapted from the techniques described by Burke and Mayer (1974) and Klotz and Stegeman (Klotz *et al.*, 1985) for routine measurements of EROD in flatfish. The method uses microplate technology and is suitable for use in the field on research vessels and in the laboratory.

2 TEST METHOD

2.1 Species Selection

The requirements for a species to be considered as a suitable candidate organism for monitoring using EROD measurements are as follows:

- the species must be readily available and widely distributed, and its life history should be reasonably well described;

- it should be robust and amenable to laboratory culture and experiment, yet its MFO system should be sensitive to inducers and show a dose-response relationship of MFO activity to the degree of contamination;

- it must be large enough so that samples of specific tissues or organs can be obtained from individuals for analysis for both EROD activity and chemical constituents;

- a sufficient number of fish must be obtainable of a similar year class and sex to allow for statistical comparisons; and

- ideally, it should be a bottom dweller, since this is one of the interfaces at which petroleum accumulates in the sea, and should be a fish which does not migrate or has a limited migration pattern.

On the basis of these criteria, flatfish (Pleuronectidae, Soleidae) are the most appropriate organisms for testing.

2.2 Preservation of Samples

Measurements are ideally performed on 10-12 fresh livers. An alternative is to store the freshly dissected liver in liquid nitrogen or to store the fresh homogenates in liquid nitrogen or at -85°C in buffer containing 20 % glycerol. The samples can then be stored for days (up to 100 days) without change in activity.

2.3 Preparation of Homogenates

All manipulations must be performed at 4 °C.

Tissues are homogenized (1:5, w:v) in 50 mM TRIS buffer (containing 150 mM KCl, 1 mM EDTA, and 1 mM dithiothrietol (DTT)) at pH 8.0 using a Teflon and glass Potter Elvehjem homogenizer. For field measurements, the crude homogenate is centrifuged at 10,000 × **g** for 15 minutes and the supernatant (PMS = Post Mitochondrial Supernatant) is used for EROD measurements. For laboratory experiments, assays must be performed on microsomes. Subsequently, the 10,000 × **g** supernatant (PMS) is centrifuged at 100,000 × **g** for one hour using an ultra-centrifugation apparatus. The pellet is then resuspended in phosphate buffer (0.1M, pH 7.4) in order to obtain a microsome concentration of approximately 5 - 15 mg protein per ml. This microsomal suspension is used for the EROD measurement.

2.4 Protein Determination

Assays are performed (Bradford, 1976) by adding 100 μl of standard protein solution (bovine serum albumin, Sigma) or 100 μl of sample solution to 5 ml of protein reagent (Biorad Laboratories). After 2-minutes incubation, the absorbance of samples is read at 595 nm and compared to a standard curve using bovine serum albumin.

2.5 Ethoxyresorufin-O-deethylase (EROD)

All standards and substrates must be kept in the dark and refrigerated to minimize degradation which will occur in hours. Ideally, assays must be performed at 20 °C; if not, the temperature of measurement must be stated in the results.

2.6 Determination

a) Fluorimetric method

EROD is determined by the procedure of Burke and Mayer (1974). The incubation mixture consists of 2 ml 0.1 M phosphate buffer at pH 7.6 and NADPH (Sigma) at a final concentration of 200 μM; and the enzyme preparation has a final concentration of approximately 1 mg protein ml^{-1}. Ethoxyresorufin (Sigma) is added at a final concentration of 2.5 μM. Ideally, assays must be performed over a period of 1 to 10 minutes during the linear phase of the reaction. Fluorescence is read at 585 nm (excitation at 510 nm) using a spectrofluorimeter and compared to a standard curve prepared with resorufin (Sigma Chemical Co.). Internal standards can be used for calibration of the assay. An example of the emission and excitation spectra of the fluorescence of ethoxyresorufin is shown in Figure 2, and a standard curve for resorufin is shown in Figure 3.

b) Spectrophotometric determination

EROD is determined by the procedure of Klotz and Stegeman (Klotz *et al.*, 1985). The incubation mixture consists of 0.1 M TRIS buffer at pH 8, containing 0.1 M NaCl and 2 μM 7-ethoxy-resorufin (dissolved in methanol and filtered), and from 100 to 1,000 μg protein of the enzyme preparation in a final volume of 1 ml. The reaction is initiated by the addition of 0.5 nM NADPH. Absorbance is read at 572 nm using a spectrophotometer and compared to a standard curve prepared with resorufin (Sigma) in the μM range (0.1 - 500 μM), using an extinction coefficient of 73 mM^{-1} cm^{-1} measured at 572 nm.

2.7 Expression of Results

All results are expressed as micro-, nano-, or pico-moles of substrate (ethoxyresorufin) deethylated (or μmoles resorufin liberated) for one minute per mg protein (μm min^{-1} mg $protein^{-1}$).

2.8 Routine Determination

Fast (up to 20 samples per hour) and routine determinations of both spectrofluorimetric and spectrophotometric reactions can be performed directly in a plate reader commonly used in immunoassays (Galgani and Bocquené, 1991).

Furthermore, there is a good correlation between the results obtained by previous methods using cuvette assays and results obtained using the microplate technique. This is clearly shown in

Figure 4, comparing the results from a 340 Turner apparatus with those from a Fluoroscan II microplate reader.

The various steps of such assays can be summarized as follows: the reagents, as described above, are added to the microplate cups (see Figure 5) to give a final working volume of 300 to 400 μl in each well. The reaction is started and measurement is carried out on a colorimetric or spectrofluorimetric microplate reader; all 96 wells on the microplate can be read in a few seconds.

This method enables the conduct of simultaneous assays of a large number of colorimetric, fluorimetric and enzymatic reactions. In addition, the reduced volume of the system enables its use directly on-board research vessels during scientific cruises or in shore-based facilities. Reactions are performed according to the general procedures described above, but final volumes are reduced to 380 μl.

The strategy for field measurements is to prepare in advance as many reagents and solutions as possible. Most, such as those required for protein determinations, are stable and will withstand freezing and thawing if contained in plastic bottles. It is usually not possible to prepare nucleotide co-enzyme solutions in advance. However, since (usually) only small amounts of these are needed, it is desirable to pre-weigh appropriate amounts and keep them (cooled and desiccated) in small vials.

Any weight determinations are particularly difficult to perform accurately on-board ships because of the motion, unless a weighing table on gimbals is available. Fortunately, this assay is expressed in terms of protein content, which in turn is measured on board using only volumetric manipulations.

Safety deserves special attention, especially if the techniques are to be used in the field, where medical facilities are available only with difficulty.

The toxicology of ethoxyresorufin has not been investigated. Therefore, the use of disposable items is recommended whenever possible, as well as the use of safety items such as "propipettes" and gloves.

3 SOURCES OF ERROR

3.1 Sampling Conditions

Some variations of the MFO system and EROD activity occur under natural conditions. Some factors, such as season, sex and state of gonadal maturation affect the level of EROD activity. Therefore, to obtain maximum sensitivity, test measurements must be performed on samples with calibrated size, sex and maturation state. Ideally, samples should be immature, preferably males, and outside the reproductive season. In addition, both sample and reference populations should be examined at the same time, and sampling should not be performed during the migration period of the species considered, because that would affect the significance of the results.

3.2 Analytical Procedures

The major sources of variations in results have been identified as follows:

- contamination of liver by bile salts (section of liver without bile is needed);
- storage temperature (-85 °C);
- buffer changes (pH 7 - 8);
- replacement of potter by an ultraturrax for extraction (a soft homogenization is needed);
- homogenization time (1 minute);
- speed and length of centrifugation (at least 10,000 × g for 10 minutes);
- measurement temperatures (usually 20 °C and 37 °C).

4 INTERPRETATION OF RESULTS

From a monitoring point of view, one of the most valuable features of sensitive responses such as EROD induction is that they may define spatial and temporal boundaries of contamination from food or sediments.

An example of the data collected from a field experiment shows EROD induction in *Callionymus lyra* from the Bay of Seine (see Figure 6).

The data collected from field experiments show that the induction of EROD activity in flatfish is highly sensitive to the presence of petroleum hydrocarbons, at the ppm level, that occur in the water column or sediments. However, petroleum is a complex mixture and, because only some of its components are hepatic MFO inducers, the absolute sensitivity of the EROD response will vary with the composition of the oil. It is worth noting, however, that if PAHs are essentially absent, MFO induction may be low or undetectable (Payne *et al.*, 1987).

The only contaminants other than PAHs which are likely to be encountered in practice that are capable of inducing fish hepatic MFOs are PCBs. However, the PCB dose required to cause direct induction of hepatic MFO enzymes is so large that it is likely to arise only from major PCB contamination.

The time course of hepatic MFO induction seems to depend on the species; however, induction occurs within a few days in flatfish. The slower process in some species simply reflects the environmental temperatures and generally lower metabolic state at which these fish function. In practice, most petroleum spills continue for weeks or months. Therefore, the possibility of using EROD induction in fish must not be considered as confirming the obvious visible evidence of a major spill or blow out, but as indicating the general quality of an environment that is potentially threatened by relatively low-level "chronic" releases of petroleum. Such releases are, by their nature, more likely to be continuous, or at least frequent, though on a small scale, rather than being rare and spectacular. It seems that the flatfish hepatic MFO response would be relatively sensitive to these.

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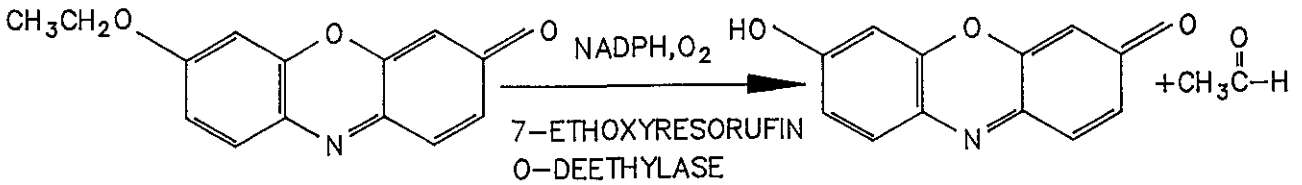


Figure 1. The ethoxyresorufin-O-deethylase reaction.

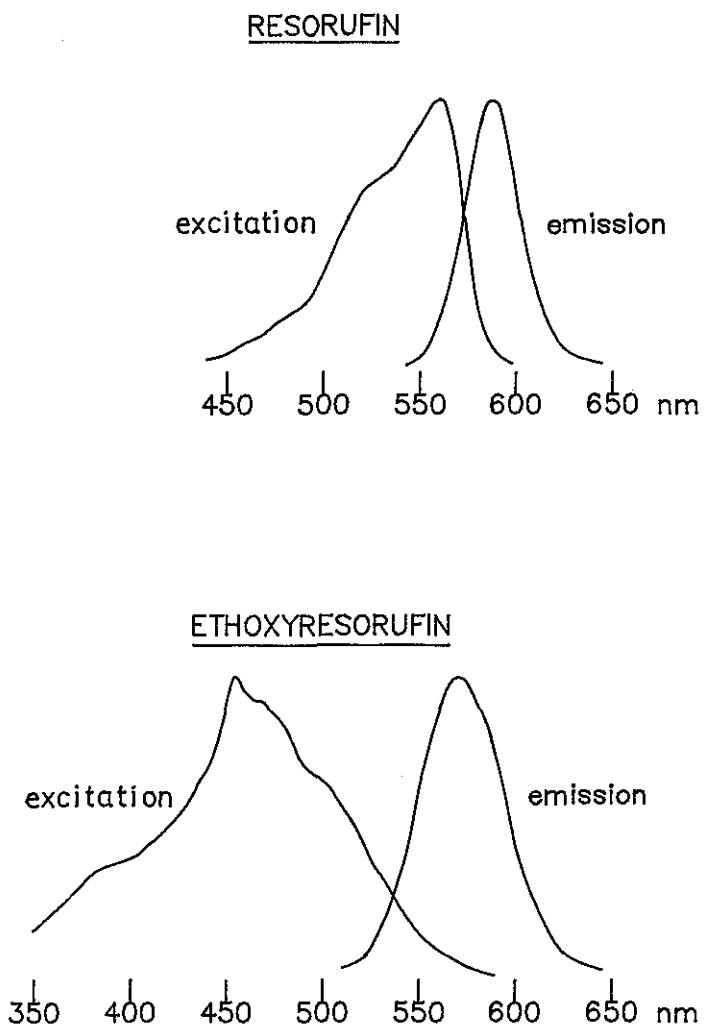
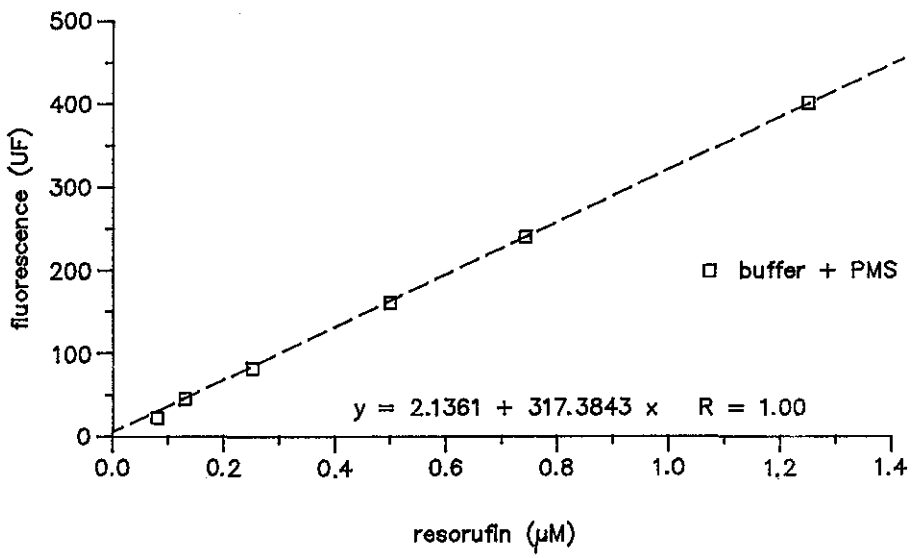


Figure 2. Emission and excitation spectra of the fluorescence of ethoxyresorufin and resorufin.



PMS: Post Mitochondrial Fraction (10,000 × g supernatant)

Figure 3. Standard curve for resorufin using a fluorimeter plate reader.

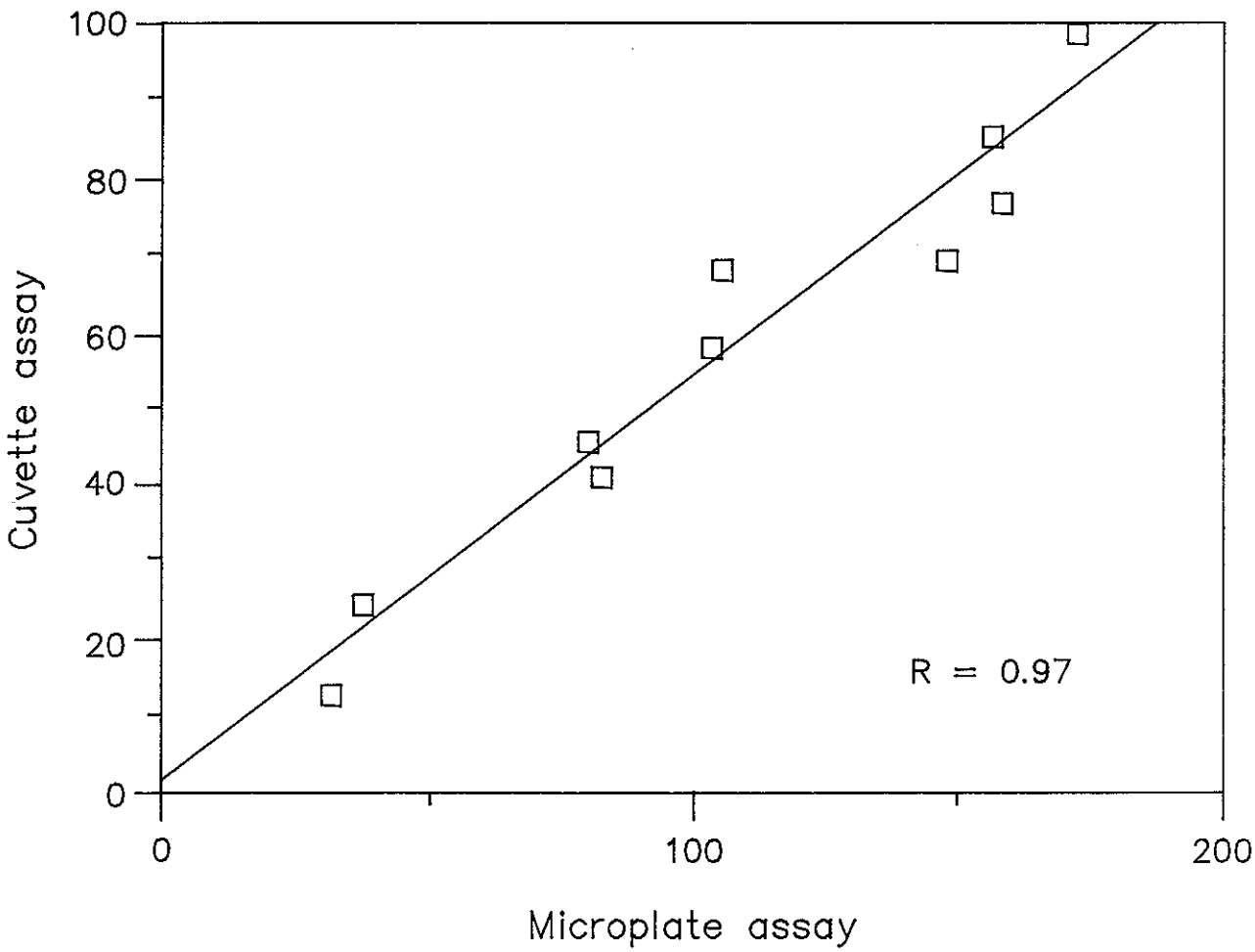


Figure 4. Relationship between spectrofluorimetric (340 Turner apparatus) and microplate (Fluoroscan II) determinations of EROD activity. The PMS fraction of different livers of plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*) were used for EROD measurements. Results are given in pmoles per min per mg protein.

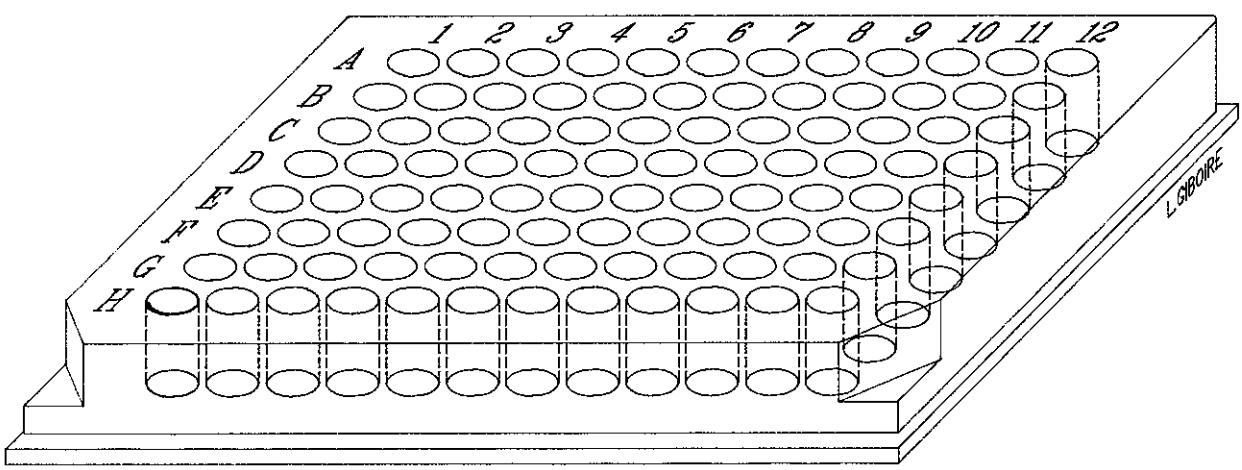


Figure 5. Microplate used for the routine determination of EROD activity. The plate (130 x 80 x 130 mm) contains 96 different wells; each reaction is performed in one well. Fluorescence measurement is performed using a Fluoroscan II apparatus (Labsystem Inc) that enables 96 different reactions to be followed simultaneously. Results are expressed in pmoles per min per mg protein. The microplate assay was found to be more sensitive because of the way in which excitation lighting and fluorescence reception were performed. For the cuvette reader, the light passes through the glass and may be reduced in intensity.

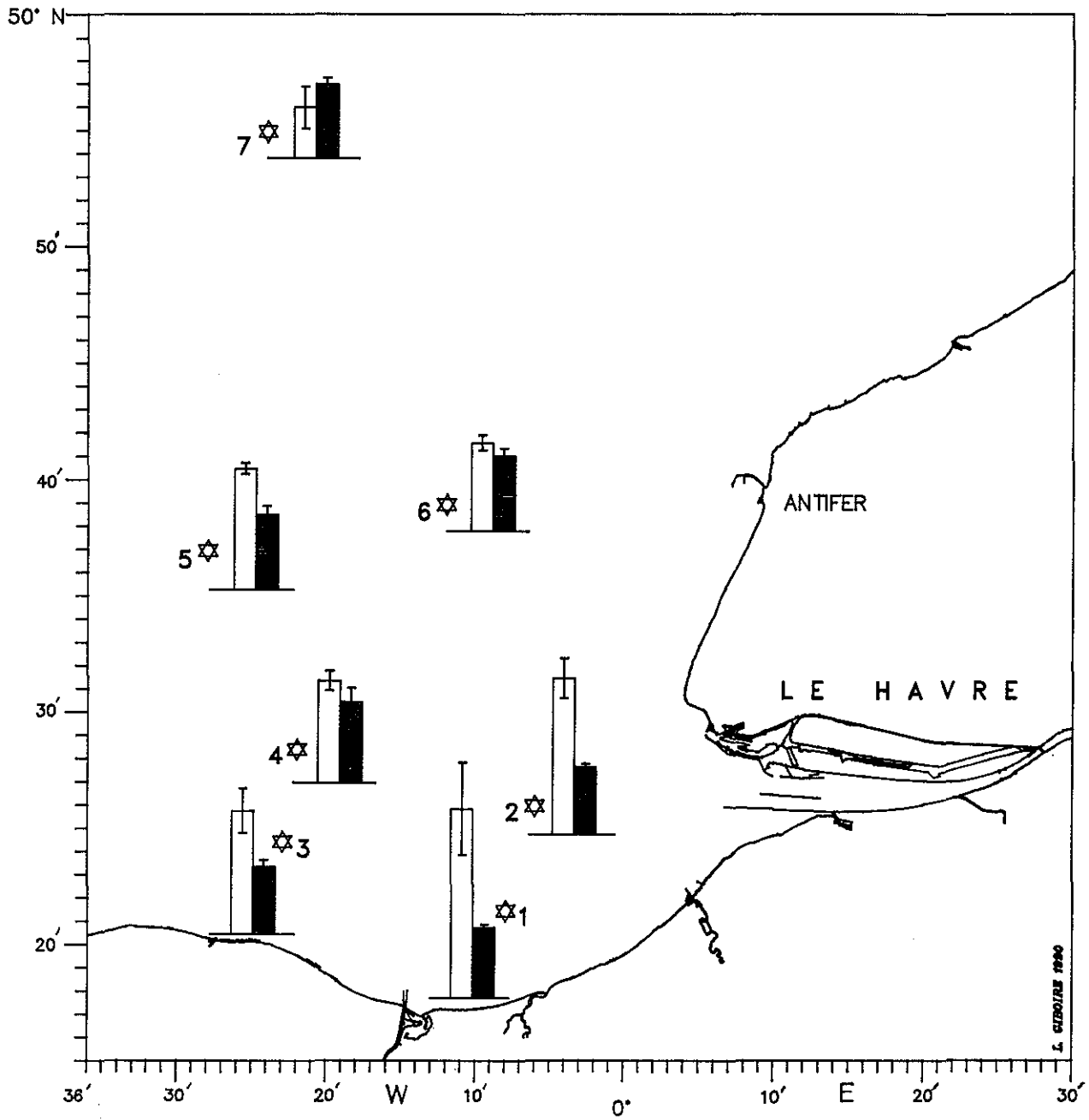


Figure 6. EROD activity ([ ]) and protein (■) contents in the liver of *Callionymus lyra* from the Bay of Seine (France). Measurements were performed on the PMS fraction of ten samples per station using the microplate method and the procedure described in the text. For EROD activity, 1 cm corresponds to 15 units (pmoles per min per mg protein). For protein content, 1 cm corresponds to 5 mg/ml soluble protein concentration of liver extracts.