EROD MEASUREMENTS AND TOTAL CYTOCHROME P450 CONTENTS IN LIVERS OF DAB (*LIMANDA LIMANDA*) FROM THE SOUTHERN NORTH SEA

ENIT SCHOLTENS

July 9th 1992

RIJKSUNIVERSITEIT GRONINGEN

NEDERLANDS INSTITUUT VOOR ONDERZOEK DER ZEE, TEXEL

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SUMMARY

EROD activities in the microsomal- and S9 fraction and contents of total cytochrome P450 (ΣP450) were measured in livers of dab (Limanda limanda), caught at 15 different sites in the southern North Sea.
The three types of measurements were compared with each other: a good correlation (r=0.949) was found between measurements of EROD activities in the microsomal fraction and measurements of EROD activities in the S9 fraction. The correlation between EROD values in the microsomal fraction and the ΣP450 concentrations in the microsomal fraction were considerably less (r=0.580).
The EROD values from stations 4, 6 and 7 were significantly higher than the values from the other stations. These stations had stratified water columns and lower temperatures of bottom water, which might explain the high EROD values. No clear pattern was observed for the ΣP450 data.
The protein assay (Biorad-kit) was optimized.

INTRODUCTION

About 100,000 chemicals are identified by the European Inventory of Existing Chemical Substances (EINECS). For 99.99% of these chemicals, adequate information on sources, concentrations in the environment and effects on ecosystems is lacking (van Leeuwen 1991). Since the aquatic environment acts as a sink for most of the chemicals entering the biosphere, the need for research on the impact of these chemicals on aquatic ecosystems is very high. Of particular concern are the halogenated aromatic microcontaminants, such as polychlorinated biphenyls (PCBs),polychlorinated dibenzo furans (PCDF), polychlorinated dibenzodioxins (PCDDs) and various pesticides, because of their toxicological characteristics -they already show toxic effects at extremely low environmental concentrations- and their ability to accumulate in the environment. In appendix 1 a model of pathways of PCBs in the environment is shown. This same kind of pathway is applicable to other halogenated microcontaminants.
In the past twenty years a great number of investigations have been done in order to get a better understanding of the uptake of these toxicants by marine organisms, the way they are being accumulated and metabolized and their impact on certain physiological processes.
Biochemical methods are being developed for the detection of specific and early effects of these contaminants on the ecosystem. At least two different systems have been identified to be useful early warning indicators: the cytochrome P450 dependent monooxygenase system, also named the MO system, which can be induced by various organic micropollutants, and the metallothioneins (MTs), which can be induced by certain metals (Haux and Förlin 1988).
In this report a study on the induction of the cytochrome P450 enzyme system in the liver of dab (Limanda limanda) by halogenated aromatic compounds from different parts of the North Sea is described. Dab was chosen because its basic biology is well described, the migratory range of dab is small in comparison with the spatial scales over which monitoring is to be conducted and the dab is available throughout large parts of the North Sea. Additional information on the occurence of halogenated aromatic compounds in fish, the cytochrome P450 system and the induction of this system by these pollutants is given below.

The occurence of halogenated aromatic compounds in fish

The uptake of halogenated aromatic compounds by fish can occur directly from the surrounding water, from solution via surfaces in contact with the ambient (interstitial) seawater, such as gills and skin, or they can enter the body by ingestion of contaminated food. Once present in the interior of an organism, they are transported via the bloodstream. The distribution of the contaminants among different organs and tissues in fish is shown to be closely linked to lipid contents (Boon 1985). In general,

lipophilic compounds tend to accumulate in lipid-rich tissues such as the liver. In figure 1 a model with possible routes for lipophilic chemicals is shown.

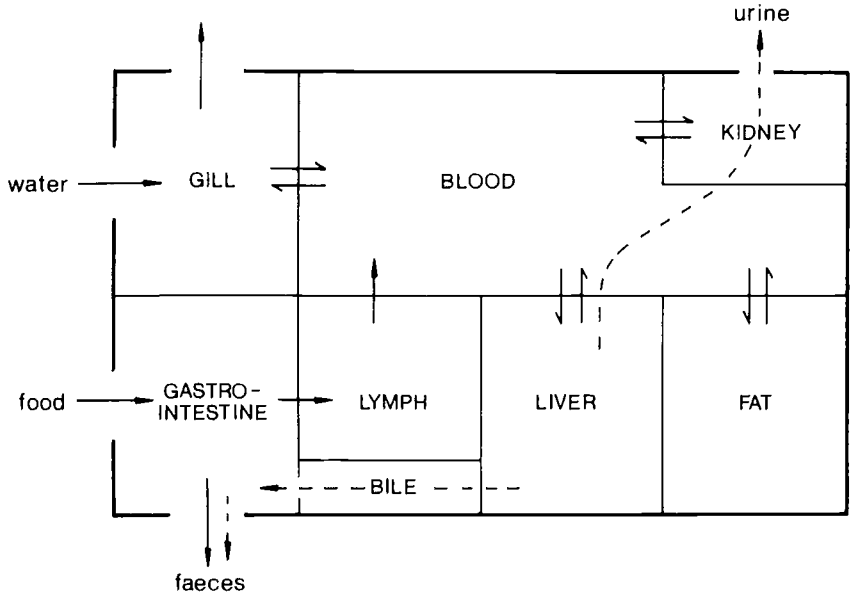

figure 1 fish model: possible routes for uptake and excretion of lipophilic chemicals. ——— original compound, ------ metabolites (from Bruggeman 1982)

Knickmeyer and Steinhart (1990) analysed PCBs in livers of male flatfishes (dabs, rough dabs and plaice) in the North Sea. In figure 2 the observed concentrations of PCBs at different locations in the North Sea are shown. These results correspond well with the input pattern of rivers to the North Sea (Knickmeyer and Steinhart 1990).

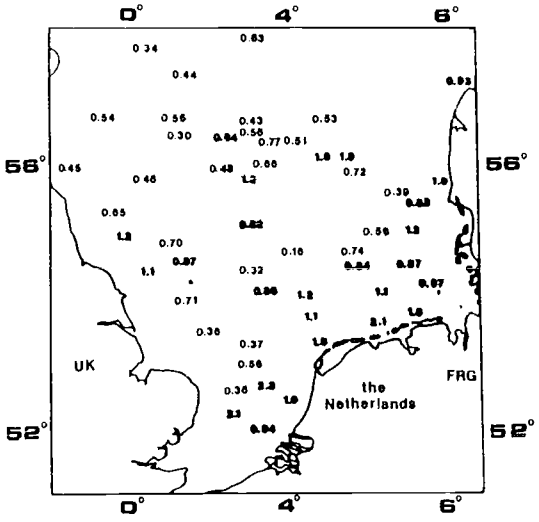

figure 2 concentrations of PCBs in livers of male pleuronectiformes (flatfishes) from the North Sea. Concentration µg/g n-hexane extractable lipid (from: Knickmeyer and Steinhart 1990)

In a limited way the halogenated aromatic micropollutants can be biotransformed (Bruggeman et al.1983, Varanasi et al. 1987). Biotransformation often leads to detoxification and a higher water solubility and therefore an increased elimination rate of the compounds. However, biotransformation can also lead to more toxic compounds. Biotransformation processess can be divided into 'phase I' and 'phase II' metabolism. The first phase (nonsynthetic phase) involves a change in the molecular structure of the substrate and can lead to reactive, more toxic intermediates. The most important enzyme system responsible for oxidative 'phase I' metabolism is the cytochrome P450 dependent monooxygenase system. Phase II (synthetic phase) metabolism involves the conjugation of a substrate to endogenous compounds and can also lead to more reactive conjugates (Boon et al. 1992a, Kleinow et al. 1987).
In figure 3 the role of biotransformation in the fate, effects and disposition of environmental pollutants in fish is shown.

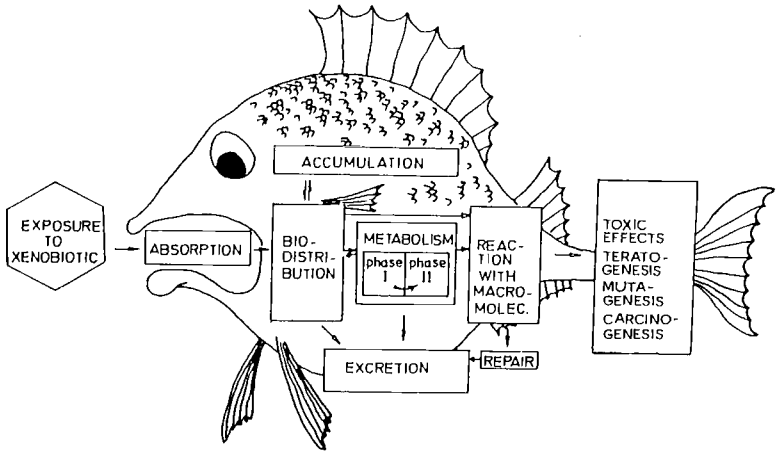

figure 3 the role of biotransformation in the fate, effects and disposition of environmental pollutants in fish (from: Goksøyr 1987)

The cytochrome P450 system

The name cytochrome P450 was established by Omura and Sato (1964). It was identified as a heme-containing protein with a characteristic difference spectrum maximum at approximately 450 nm, when it was in reduced form and bound to carbon monoxide.
The cytochrome P-450 dependent monooxygenase system, which has been found in most species and tissues, is located mainly in the smooth endoplasmatic reticulum. This multi-component membrane-bound electron transport chain receives reducing equivalents from NADPH (or in some cases NADH), which are then passed on to the flavoprotein NADPH-cytochrome P450 reductase and subsequently directed to clusters of cytochrome P450 (in case of NADH as

electron donor this occurs via cytochrome b_5). In combination with molecular oxygen and a suitable organic substrate, P450 forms a trimolecular complex, the substrate is oxygenated and a molecule of water is formed (Boon et al. 1992b, Goksoyr and Förlin 1992). In figure 4 an overview of this reaction mechanism is shown.

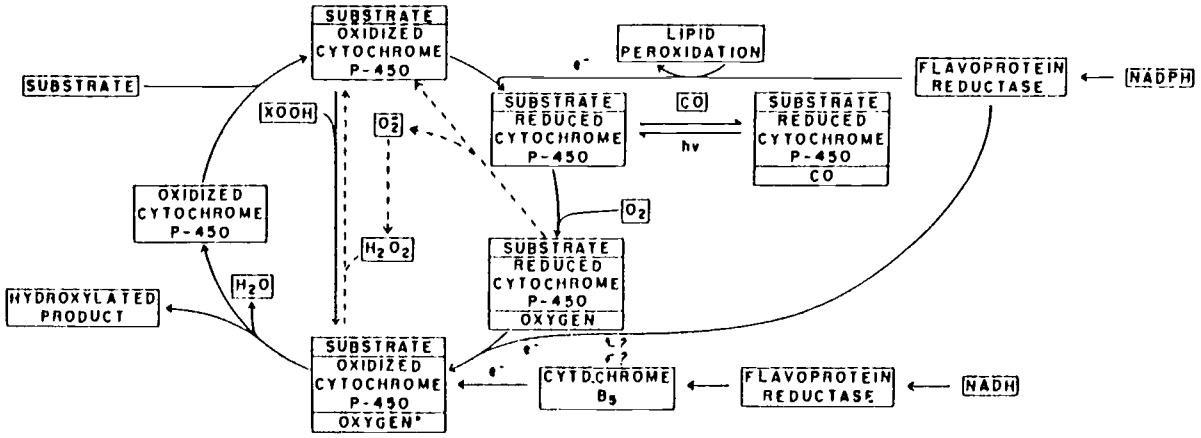

figure 4 reaction mechanism of the cytochrome P-450 dependent microsomal monooxygenase system (from Hodgson and Dauterman 1982)

The cytochrome P450s belong to a superfamily of structurally and functionally related hemoproteins. The superfamily consists of at least 150 different cytochrome P450 isoenzymes, belonging to 27 gene families (Nebert et al. 1991).
The cytochrome P450 system has different physiological functions, such as the biosynthesis of bile acids and prostaglandins, the regulation of steroid and thyroid hormones and the oxidation of fatty acids. The P450 enzymes are also able to metabolize the group of halogenated aromatic compounds mentioned above as well as other contaminants.

Some of the cytochrome P450 isoenzymes are expressed constitutively, which means that they are constantly present in the cell and can not be induced. The expression of other isoenzymes are strongly influenced by hormonal or chemical stimuli. They are called inducible isoenzymes. PCBs, DDT, alkanes, toluene and benzo(a)pyrene are just a few examples of chemicals that can induce P450 isoenzymes (for more examples see appendix 2) (Goksøyr 1987). Inducers can be divided into two types: PB-type inducers and 3-MC type inducers. The first type includes phenobarbital (PB), after which this type is named, and non-planar PCBs. The 3-MC type includes 3-methylcholanthrene (3-MC), planar PCBs, polychlorinated dibenzo furans, polchlorinated dibenzo dioxins, naphtoflavone and methylated xanthines. In contrast to mammals and birds, fish only show 3-MC type

induction. Induction is usually defined as *de novo* synthesis of protein. Normally, this is mediated by an inducer binding to a receptor, and this complex interacts with a genomic regulatory element to stimulate the transcription of a gene (Haux and Förlin 1988). The induction of specific cytochrome P450 isoenzymes is therefore usually accompanied by increases in certain monooxygenase activities. Sometimes the total amount of cytochrome P450 in the cell will increase after treatment with an inducer, but there are also indications that the synthesis of some (constitutive) forms may be repressed by the induction of others (Nebert 1979).

In monitoring the aquatic environment, the P450 induction response in fish has been evaluated as a convenient, 'early warning' signal of organic contaminants (for reviews, see Payne et al. 1987, Haux and Förlin 1988 and Goksoyr and Förlin 1992). The induction of the isoenzyme P450IA1, which is inducible by 3-MC type inducers, in fish has been used in a lot of studies. The activity of this enzyme can be measured by the use of different substrates, such as ethoxyresorufin-O-deethylase (EROD), 7-ethoxycoumarin-O-deethylase (ECOD) and aryl hydrocarbon hydroxylase (AHH) (Haux and Förlin 1988, Boon et al. 1992b). EROD and AHH measurements have been broadly applied to studies of effects of pollutants on fish both in the laboratory (e.g by: Boon et al. 1992a, Leaver et al. 1988 and Spies et al. 1982) and in the field (e.g by: Stegeman et al. 1988, Galgani et al. 1991 and Addison and Edwards 1988). From these studies it has become clear that P450IA1 mediated monooxygenase activities are often elevated in fish treated with inducers, such as PCBs, and in fish from polluted waters. In figure 5 an example of a dose-response relationship, found in a study by Janz and Metcalfe (1991), is given.

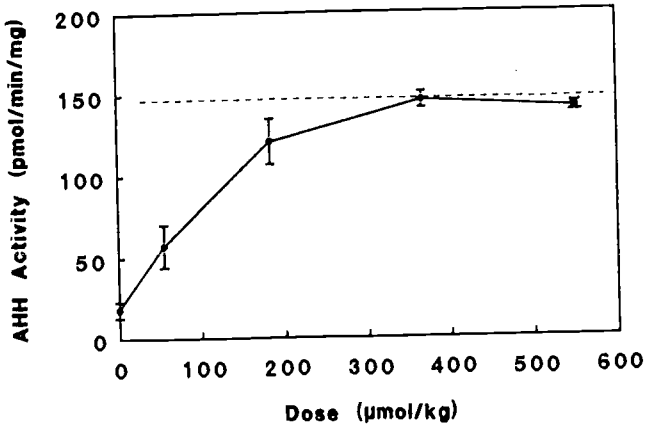

figure 5 dose-response relationship for induction of aryl hydrocarbon hydroxylase by ß-naphtoflavone in rainbow trout. Each point represents the mean ± SE of two to three fish. The dotted line represents the maximal AHH response (146 pmol/min/mg protein) (from: Janz and Metcalfe 1991)

The induction of P450 isoenzymes can also be measured with immunochemical techniques, in which the amount of protein crossreacting with a specific antibody is measured (Stegeman et al. 1987, Goksøyr 1991, Goksøyr et al. 1991).

There are a number of factors, beside the induction by chemicals, that might influence the activity of P450 enzymes:
1. Inhibition by chemicals, like alpha naphtoflavone and cadmium (Förlin et al. 1986).
2. Age and development: Förlin (1980) observed an age-related increase of certain mono-oxygenase activities in 0.5- and 1.5-year old juvenile rainbow trout treated with 3-MC or a PCB-mixture.
3. Sex and sexual maturity: In fish and mammals, the male cytochrome P450 levels and monooxygenase levels are typically higher than the female levels. These differences are most pronounced during the reproductive period of the annual cycle (Goksøyr 1987).
4. Temperature: Stegeman (1979) found that fish acclimated to colder temperatures exhibit higher enzyme activities than those acclimated to warmer temperatures when assayed at a standard incubation temperature. In contrast, Jiminez and Burtis (1989) found higher EROD activity levels at higher acclimation temperatures. Low temperatures seem to influence the induction process by increasing the time necessary to reach the maximal enzyme activity (Egaas and Varanasi 1982, Andersson and Koivussaari 1985).
5. Species: Constitutive enzyme activities, cytochrome P450 content and inducibility of the monooxygenase activities can differ considerably among fish species.
There is a range of other environmental as well as physiological factors that might influence the activities of the cytochrome P450 dependent monooxygenase system, such as light, moisture, altitude, disease, diet, ionizing radiation, and other stress factors like handling, noise, malnutrition, starvation etc. (Goksøyr 1987).
The factors mentioned above, can be responsible for a great variability in enzyme activity found in biomonitoring programs. Therefore these factors should be considered when cytochrome P450 assays are going to be used for biomonitoring purposes in fish.

Scope of this study

This study is part of the Integrated North Sea Program (INP), a project which tries to connect increased environmental concentrations of contaminants with the occurrence of certain changes in biochemical pathways, genetic damage and physiological responses in fishes. In this program the hepatic cytochrome P450 dependent monooxygenase system is used as a biological effect parameter. In livers from dab (Limanda limanda), from different parts of the North Sea, the EROD-activity of isoenzyme P450IA1 was measured in the S9-fraction and in the microsomal fraction.

The total amount of cytochrome P450 enzymes in these livers was measured.
The objective was to compare the three measurements with each other and to compare the values from the different sampling stations.
For the author of this M. Sc. thesis the study is part of a biology doctoral program of the University of Groningen, The Netherlands.

METHODS AND MATERIALS

Collection of the fishes

At fifteen different locations (numbered station 2 to 17, except nr. 16) dabs (Limanda limanda) were captured by beam trawling (see figure 6).

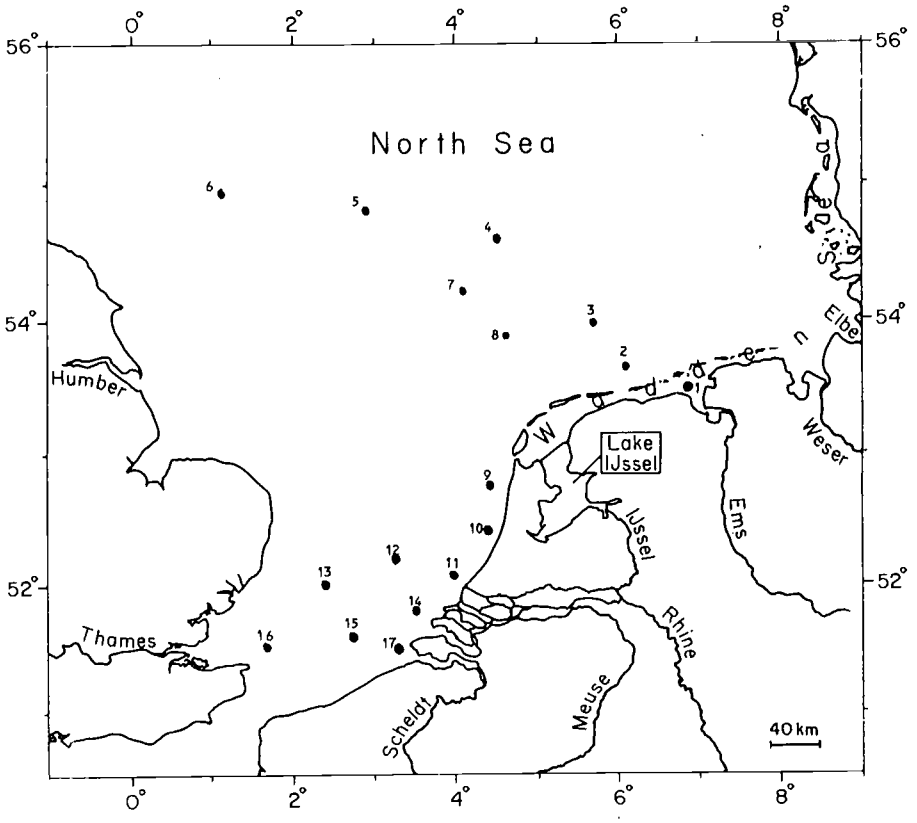

figure 6 locations of dab (Limanda limanda) sampling stations 1-17 in the North Sea (also see table 1 for information on precise locations of the stations)

At stations 1 and 16 no fish was caught.
Only male fishes with a length between 15 and 20 cm. were used in order to minimize the biological variables (EROD activity in female dabs vary within the reproductive cycle and fishes from different ages may show different EROD activities). The intention was to catch 25 fishes per station in 5 hauls (5*5). This only succeeded for station 3. At the other stations the 25 fishes came from more than 5 hauls or less fishes were caught. In table 1 the amount of fishes caught per station is shown. Each fish was numbered, like XLLY, which means; station X, Limanda limanda (dab) and fish number Y of this station. On deck the fishes were screened for outside diseases. Fishes that showed signs of a disease were discarded.

Conductivity, temperature, depth (CTD) and bottom salinity measurements were done at each station. In table 1 specific information about the sampling stations is given.

table 1 information about the sampling stations

STATION NR.	DATE	POSITION	DEPTH (m)	BOTTOM TEMP. (C)	STRATI-FICATION	BOTTOM SALINITY (X10-3)	NR . OF FISH CAUGHT
2	13-09-91	53 34'N;6 05'E	20	18.2	no	32	25
3	12-09-91	53 58'N;5 47'E	35	18	no	33.8	25
4	11-09-91	54 45'N;4 17'E	50	10.5	yes,at 40m	34.5	25
5	29-08-91	54 50'N;2 40'E	22	17	no	34.5	25
6	28-08-91	55 00'N;1 00'E	65	9.5	yes, at 20&45m	*)	25
7	10-09-91	54 27'N;4 00'E	46	12.5	yes,at 32m	34.5	25
8	27-08-91	53 45'N;4 30'E	40	*)	no	*)	25
9	26+30-08-91	52 50'N;4 30'E	24	19	no	33.4	24
10	02-09-91	52 32'N;4 31'E	20	*)	no	*)	4
11	03-09-91	52 05'N;4 05'E	20	19.4	no	31.7	24
12	04-09-91	52 08'N;3 19'E	32.5	18.5	no	34.8	25
13	05-09-91	52 02'N;2 20'E	46	17.8	no	34.8	14
14	09-09-91	51 49'N;3 36'E	21.5	19.4	no	33.5	25
15	06+07-09-91	51 40'N;2 41'E	36	18.4	no	34.8	15
17	08-09-91	51 32'N;3 11'E	21.5	19.6	no	34	15

*) means that the values are not available

Preparation of the livers

The fishes were anaesthesized by a blow on the head. After cutting off the tail, blood samples were taken for measurement of haemoglobin concentration, haematocrit and M.C.H.C. (Mean Corpuscular Haemoglobin Concentration) (data of haematology are not further reported here). The fishes were killed by cutting the spine. The body cavities of the fishes were opened. Bile was taken out for analyses of PAH-metabolites with a 1 ml syringe and was put into a -20°C freezer (data of these analyses are not noticed in this report). The livers were removed as quickly as possible and flushed with ice-cold 1.15% KCl to remove blood, remnants of bile and other contaminants. The livers were cut into three pieces.
One piece was put into a 2 ml Nalgene vial and directly frozen in liquid nitrogen (-196°C). This piece was stored in a -80°C freezer and after the cruise used for measurements of protein content, EROD-activity and ΣP450 in the microsomal fraction (see page 13, 14 and 15).
The second piece was frozen in a -20°C freezer for DNA-damage measurements (data will not be reported here).
The third piece was put into a 15 ml plastic tube and 2 ml of homogenization buffer (see appendix 16) was added. The livers were homogenized with an Ultra-Turrax, and homogenized with a Potter-Elvehjem. The tubes were kept on ice during homogenization

to prevent denaturation of the enzymes in the sample. The homogenates were put into 2 ml Safe-Lock Eppendorf micro test tubes and were centrifuged for 15 minutes at 13000g in an Eppendorf centrifuge (model 5415 C), placed in a 4°C refrigerator.
The supernatant, called the S9-fraction, was used for protein analyses, EROD activity measurements and ElISA analyses.
After the cruise the samples in the Nalgene vials were put into a -85°C freezer at the NIOZ.

Production of the microsomes

The liver samples were taken out of the freezer and defrosted on ice in about 20 minutes. From each station five pieces of livers from 5 different fishes were put together ("pooled") in a 15 ml plastic tube. Those 5 fishes were the nrs. 1-5, 6-10, etc. from each station. When a station, for example, only had 24 fishes, the last "pool" consisted of 4 fishes (nrs. 21-24). For station 9 the pieces of liver from fishes 09LL1to3 were missing. Therefore the first "pool" of this station consisted of fishes 09LL4and5.
Cold (4-5°C) homogenizationbuffer (see appendix 16), about three times the liver volume, was added to the plastic tube. During this procedure the tubes were kept on ice. The livers were homogenized with an Ultra-Thurrax and homogenized with a Potter-Elvehjem. The homogenized samples were poored into 14 ml threaded polycarbonate centrifuge tubes with a screw cap and homogenizationbuffer was added until the tubes were filled. Six of those tubes with homogenates were placed in a cold (4-5°C) Ultra-centrifuge angle rotor. Centrifugation took place in a Prepsin 50 ultra-centrifuge at 11000 rpm during 15 minutes. After centrifugation, the contents of the centrifugation tubes consisted of three layers. From bottom to top: the pellet, a clear supernatant and a thin layer of turbid supernatant. With a 15 ml syringe, only the clear supernatant was removed and put into clean 14 ml centrifuge tubes. Homogenizationbuffer was added until the tubes were filled. Centrifugation was done at 35000 rpm during 60 minutes. Supernatants were poored off. 0.75 ml of resuspendation buffer (see appendix 16) was added to the centrifuge tubes. The pellets, containing the microsomes, were resuspended with a Potter bar and poored into a 5 ml Potter tube. Another 0.75 ml of resuspendation buffer was added to the centrifuge tubes in order to remove the last remainders of the pellets. The samples in the Pottertube were homogenized. Two times 0.5 ml of the homogenate was put into 2 ml Nalgene tubes and quickly frozen in liquid nitrogen. The tubes were stored at -85°C.

Protein assay in the microsomal fraction

The analysis for the protein content was carried out with the protein assay kit II from BIO-RAD. This dye-binding assay is

based on the differential color change of a dye in response to various concentrations of protein. The dye is a mixture of phosphoric acid (55%) and methanol (15%), and should be stored at 4-5°C.
A BIO-RAD micro-plate reader (model 3550), which was directed by a personal computer, was used for quantification.
The procedure was carried out at roomtemperature (20°C). The analyses of all dilution steps of the standard range and the samples were carried out in triplicate.
For the standard curve a solution of 0.5 mg/ml bovine serum albumin (BSA) was made. This solution was used for the series of standards, shown in table 2. Of these standard solutions 320 µl was placed in wells of a BIO-RAD micro-plate.
The homogenates, containing the microsomes were defrosted on ice. They were diluted by adding 10 µl sample to 5 ml nanopure H_2O and mixing thoroughly with a Vortex mixer. From each diluted sample, which was kept on ice, 320 µl was put (in triplicate) into wells of the micro-plate. For the blanks 320 µl nanopure H_2O was

table 2 the amounts of BSA-solution and nanopure H_2O used for the standard curve

STANDARD NR.	AMOUNT OF BSA SOLUTION (ul)	AMOUNT OF H2O (ul)	TOTAL AMOUNT BSA (ug/well)
1	22.5	3577.5	1
2	56.25	3543.75	2.5
3	90	3510	4
4	123.75	3476.25	5.5
5	146.25	3453.75	6.5
6	168.75	3431.25	7.5
7	191.25	3408.75	8.5
8	225	3375	10

added to three wells. In appendix 3 the arrangement of the standards, blanks and samples in the micro-plate is shown. To all wells, 80 µl pure dye was added, using a 50-300 µl Digital Multichannel Pipette (from Titertek). The micro-plate was shaken in the BIO-RAD plate reader for three times five seconds. The mixture was then incubated for 5 minutes. After shaking for about five seconds, OD_{595} was measured versus a reagent blank.
The standard curve was calculated by the computerprogram. If the correlation coefficient of the curve was less than 0.99, the standardcurve was rejected. Initially a correlation coefficient of less than 0.99 was found most of the times. Therefore optimization of the procedure was undertaken with the help of Martin Eggens (personal communication).
Changes in the original method are described in section I of the Results.
The protein contents were calculated by the computer program and expressed as mg protein/ml microsomal sample.

EROD-activity measurements in the microsomal fraction

The method of Eggens (1989) with some modifications was used for the ethoxyresorufin-O-deethylase (EROD) measurements. During the EROD-activity test ethoxyresorufin is metabolized to resorufin by cytochrome P450, using oxygen and NADPH. Ethoxyresorufin and resorufin have a different excitation- and emission wavelength, therefore the reaction can be followed by fluorescence measurements.
The standard curve was obtained by making the series of standard mentioned in table 3. These solutions (see appendix 16) were preincubated at roomtemperature before use.
The amounts of solutions mentioned in table 3 were put into the first two colums of the micro-plate (shown in appendix 4). The plate was put into the BIO-RAD plate-reader in order to shake the samples three times for 5 seconds. The fluorescence was measured with a fluorescence plate-reader (Titertek Fluoroskan II, excitation filter: 538 nm and emission filter: 584 nm). The commands were given with the computer program EIA on a personal computer.

table 3 the amounts of resorufin, ethoxy resorufin-O-deethylase, albumin and phosphate buffer used for the standard curve

STANDARD NR.	AMOUNT OF STOCK RESORUFIN (ul)	AMOUNT OF EROD SOLUTION (ul)	AMOUNT OF 0.1% ALBUMIN (ul)	AMOUNT OF PHOSPHATE-BUFFER (ul)
1	0	300	25	10
2	25	275	25	10
3	50	250	25	10
4	75	225	25	10
5	100	300	25	10
6	150	150	25	10
7	300	0	25	10

The standard curve was calculated by the computer. A curve, with a correlation coefficient of 0.999 was used for the calculations of all the EROD activity measurements.
The microsomal samples were defrosted on ice. Dilutions were made by adding 40 μl sample to 120 μl 0.1% albumin solution (fourfold dilution). A reference sample was used to check each measurement. This sample was diluted 20 times by adding 10 μl sample to 190 μl P-buffer. From each diluted sample 25 μl was added two times in fourfold to the micro-plate (as shown in appendix 5). 300 μl of EROD-solution was added. In one of four corresponding wells and in two of the reference sample wells 10 μl P-buffer was pipetted. To the other wells 10 μl of NADPH (nicotinamide-adenine dinucleotide phosphate, reduced, 5mM) was added using a Digital Multichannel Pipette 5-50 μl (from Titertek). The enzymatic reaction was followed for 8 minutes by reading the plate every 2 minutes with the fluorescence plate-reader.

Since no temperature control was present on the plate-reader, the assay was carried out at room temperature (± 20°C).
The reaction kinetics were calculated with the use of the computer program. The EROD activity values were calculated by multiplying the MWK's, which were calculated by the computer, with the dilution factor and dividing this by the protein concentration of each sample. The values are expressed as Units per mg protein. One Unit is 1 nmol resorufin formed per minute.

Total cytochrome P-450 assay

The content of total cytochrome P450 present in the microsomes were measured by a double beam spectrophotometer from Perkin Elmer (PE Lambda 6UV/Vis) with a PECSS commandsystem.
The assay is based on the fact that the cytochrome P450 isoenzymes constitute a complex with carbon monoxide, which has an absorption maximum at 450 nm.
For each sample was calculated how much of the sample and of P-buffer (see appendix 16) had to be added to the cuvets in order to achieve a protein concentration of 2 mg/ml, in the case that 300 µl was the total volume of solutions in the cuvets. In some cases a protein concentration of 1.5 mg/ml had to be used because of the lack of a sufficient amount of sample.
Two cuvets were filled with nanopure water to make a background correction. 300 µl of sample and P-buffer were placed in two cuvets, one being the sample cuvet and the other being the reference cuvet. A piece of parafilm was placed on top of the cuvets. The cuvets were overturned three times to homogenize sample and buffer. The cuvets were put into the spectrophotometer. A blank scan was made from 500 to 400 nm after one minute. The measurements were done at 25°C. Carbon monoxide was bubbled through the sample cuvet during 40 seconds (about 2-5 bubbles per second). To both cuvets 12,5 µl sodium dithionite solution (70 mg/ml) was added to reduce cytochrome P450. Again a piece of parafilm was placed on top of the cuvets and the cuvets were overturned three times. The cuvets were placed in the spectrofotometer and incubated for 6 minutes (after 5 minutes the cuvets were overturned three times).
The height of the P-450 peak was calculated by substracting the lowest value in the 490-500 nm region (were CO-binding does not show any spectral difference) from the highest value in the 450 nm region. The concentration of ΣP-450 was calculated by the formula:

$$[\Sigma P\text{-}450] = \{\text{peak height} / 91\} \times \{1000 / [\text{protein}]\}$$
$$\text{units} = \text{nmol P450 / mg protein}$$

For each sample the measurement was done twice and the average was calculated.

Protein assay in the S9 fraction

On board of the research vessel "Pelagia", the protein assay in the S9 fraction had been carried out by Hedwig Sleiderink. The same Bio-Rad protein assay was used as for the microsomal fraction (mentioned above) and also the same instruments.

EROD-activity measurements in the S9 fraction

The EROD-activity measurements in the S9 fraction were also carried out on board by Hedwig Sleiderink. The same method and instruments were used as for the measurements in the microsomal fraction. Except for the fact that the fishes were not 'pooled': analysis were done for each individual fish. Afterwards the data of five fishes were averaged in order to be able to compare them with the EROD results in the microsomal fraction.

Statistics

For the comparison of the data from the different stations, statistical analyses were performed using a Student's t-distribution test and a studentized maximum modulus distribution test (on a Mackintosh computer using the program STATVIEW)(Sokal and Rohlf 1969). For more details see appendix 9.
The correlations between the different measurements were obtained by plotting the log-transformed data from EROD in the microsomal fraction against log-transformed data from EROD in the S9 fraction. Also EROD values were plotted against ΣP450 data after log-transformation (with the use of the computerprogram SYSTAT/SYGRAPH).

Chemicals

The Bio-Rad protein assay dye reagent concentrate was obtained from Bio-Rad laboratories GmbH.
D.T.T (threo-1,4-dimercapto-2,3 butandiol, 99%) was produced by Aldrich Chemie, Steinheim, Germany.
The used albumin bovine (96-99%) and glycerol (99%) came from Sigma Chemicals Co., St. Louis, U.S.A.
From J.T. Baker Chemicals B.V., Deventer, Holland the following products were used: sodium dihydrogen phosphate, potassium chloride, E.D.T.A., sodium hydroxide, methanol, potassium monohydrogen phosphate, sodium dithionite.
The carbon monoxide was produced by Hoekloos, Amsterdam, Holland and the NADPH by Boehringer Mannheim B.V., Almere, Holland.

RESULTS

This results section constists of two parts: the results of the optimization of the protein assay and the results of the EROD and ΣP450 measurements.

I OPTIMIZATION OF THE PROTEIN ASSAY

Changes in the original procedure of the protein assay are mentioned below.
A 400μg/ml BSA solution was made in order to prepare the standard curve. From this solution the series of standards, shown in table 4, was made.

table 4 amounts of BSA-solution and nanopure H_2O used for the standard curve

STANDARD NR.	AMOUNT OF BSA SOLUTION (ul)	AMOUNT OF H2O (ul)	TOTAL AMOUNT BSA (ug/well)
1	5	45	2
2	10	40	4
3	20	30	8
4	30	20	12
5	40	10	16
6	50	0	20

From each standard solution, 50 μl was put in threefold into the micro-plate wells. For the blanks, 50 μl H_2O was added in threefold to the wells. In appendix 6 the changed arrangement of the standards, blanks and samples in the micro-plate is given. The samples were defrosted on ice. Dilution took place by adding 25 μl sample to 2475 μl nanopure water in order to get a 100-fold dilution. If this dilution resulted in absorption values outside the range of values of the standard range, a different dilution was used for re-analyses of the sample. For example 25 μl sample in 3725 μl water for a 150-fold dilution.
From each diluted sample 50 μl was placed in triplicate in micro-plate wells.
The dye was diluted three times. 300 μl of diluted dye was placed in the wells, using a Digital Multichannel Pipette (from Titertek).
The plate was shaken three times during 5 seconds. After incubation during 5 minutes OD_{595} was measured.
The standard curves obtained had better correlation coefficients. However, the lowest protein concentration of the standard range was showing lower values than expected. Therefore standard nr.1 of table 4 was eliminated from the range and a new range, shown in table 5 was adopted:

With this series of standards, curves with high correlation coefficients (>0.99) were obtained. An example of a curve is shown in appendix 7.
All samples were measured two times in triplicate and the difference between the two average values was not allowed to be more than 5%.

table 5 the amount of BSA solution and nanopure H_2O used for the standard curve

STANDARD NR.	AMOUNT OF BSA SOLUTION (ul)	AMOUNT OF H2O (ul)	TOTAL AMOUNT BSA (ug/well)
1	10	40	4
2	20	30	8
3	30	20	12
4	35	15	14
5	40	10	16
6	50	0	20

II RESULTS OF THE EROD AND ΣP450 MEASUREMENTS

The overall results from the EROD measurements in the microsomal and the S9 fraction and the data from the ΣP450 assay are given in appendix 8.
In table 6 and figure 7 the averages from these data for each station are shown.

table 6 Limanda limanda. EROD activity (Units/mg protein) in the microsomal and S9 fraction and liver microsomal cytochrome P450 content (ΣP450) (nmol/mg microsomal protein) and standard deviations for different sampling stations in the North Sea

STATION	n	EROD micr. (st.dev.)	EROD S9 (st.dev.)	Cyt. P450 (st.dev)
2	5	0.141 (0.030)	0.057 (0.017)	0.423 (0.044)
3	5	0.174 (0.052)	0.038 (0.009)	0.451 (0.113)
4	5	1.256 (0.329)	0.793 (0.064)	0.572 (0.153)
5	5	0.237 (0.081)	0.109 (0.041)	0.464 (0.128)
6	5	1.223 (0.256)	0.723 (0.216)	0.478 (0.040)
7	5	0.899 (0.210)	0.639 (0.230)	0.499 (0.055)
8	5	0.172 (0.042)	0.116 (0.023)	0.326 (0.053)
9	5	0.120 (0.041)	0.065 (0.028)	0.382 (0.116)
10	1	0.452	0.147	*
11	5	0.268 (0.058)	0.237 (0.080)	0.309 (0.125)
12	5	0.188 (0.058)	0.110 (0.030)	0.306 (0.054)
13	3	0.319 (0.159)	0.145 (0.050)	0.354 (0.098)
14	5	0.127 (0.057)	0.045 (0.017)	0.250 (0.072)
15	3	0.264 (0.080)	0.135 (0.050)	0.371 (0.071)
17	3	0.087 (0.042)	0.065 (0.014)	0.230 (0.115)

* no value

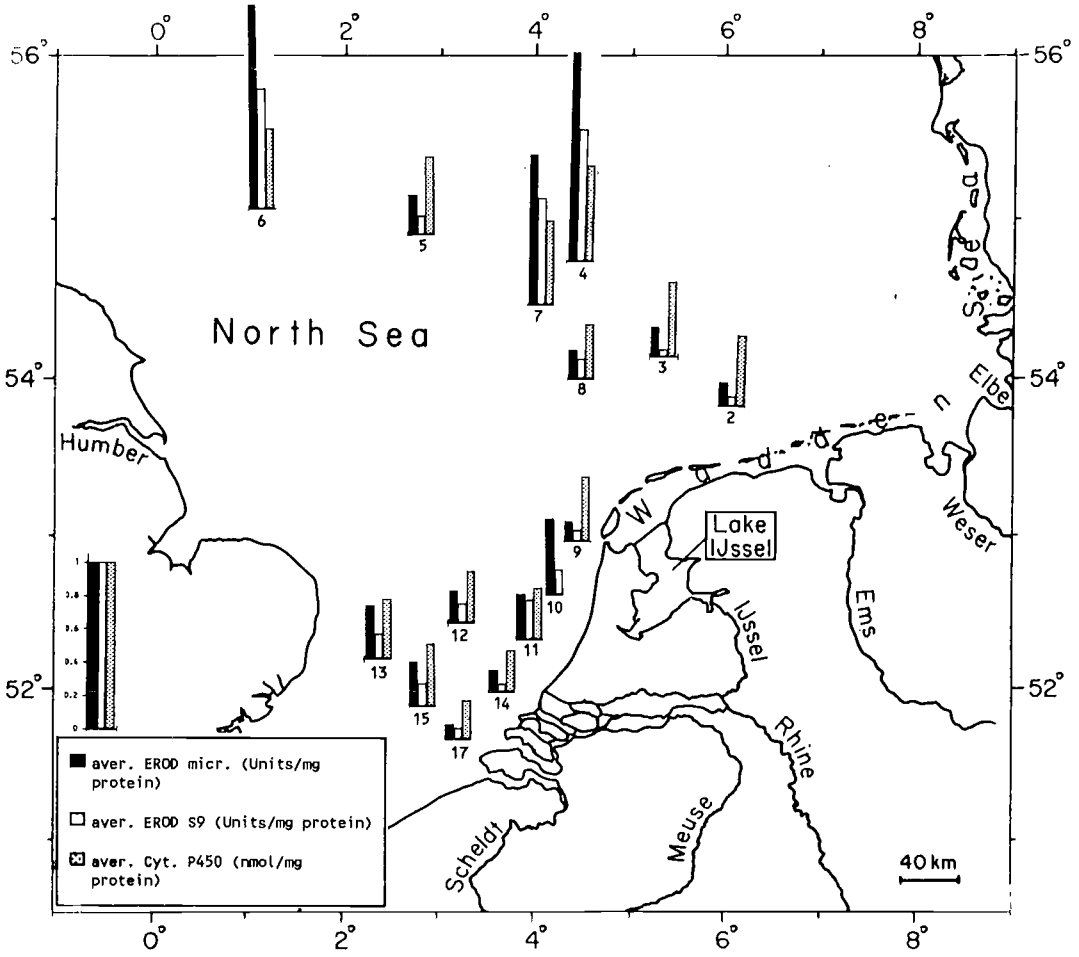

figure 7 Limanda limanda. EROD activities (Units/mg protein) of the microsomal and S9 fraction and concentrations of cytochrome P450 (nmol/mg microsomal protein). For each station average values are shown.

In the statistical analyses station 10 was eliminated due to an insufficient amount of data (n=1).

EROD values in the microsomal fraction
Stations 4, 6 and 7 did not differ significantly from each other, but all had significant higher EROD values compared to the other stations. The other stations were not significantly different from each other. In appendix 9 the statistical analyses are given.

EROD values in the S9 fraction
Stations 4, 6 and 7 did not differ significantly from each other, but did all have significant higher EROD values than the other stations. Station 11 was significantly higher than stations 2, 3, 5, 8, 9, 12, 14 and 17. EROD values of station 3 were

significantly lower than at the other stations except station 2, 9 and 14.
In appendix 10 the statistical analyses are given.

ΣP450 values
Cytochrome P450 contents of stations 8, 12, 13 and 14 were significantly lower than the values of stations 3, 6 and 7. The other values did not differ significantly from each other. In appendix 11 figures from the statistical analysis are given.

EROD values from microsomal fraction versus S9 fraction
The raw data from the EROD measurements in the microsomal fraction versus the data in the S9 fraction showed a correlation coefficient of 0.949. When log-transformed a correlation coefficient of 0.916 was found. Pools with less than 5 fishes were excluded because of statistical reasons. In figure 8a and 8b the correlations together with the 95% confidence intervals are shown. EROD values in the microsomal fraction were always higher than in the S9 fraction, but the factor varied from a factor 1.1 for stations 11 up to a factor 4.6 for station 3 (mean=2.03, σ_{n-1}=0.86).

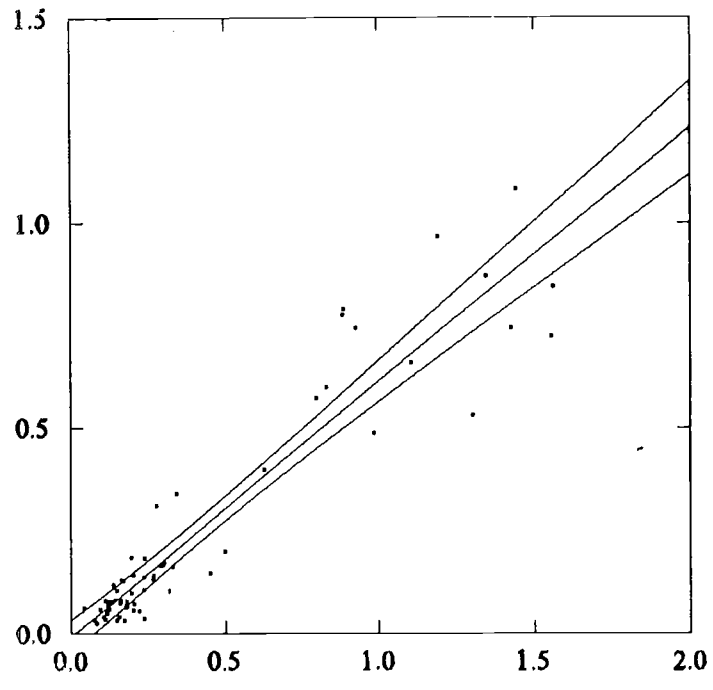

fig. 8a

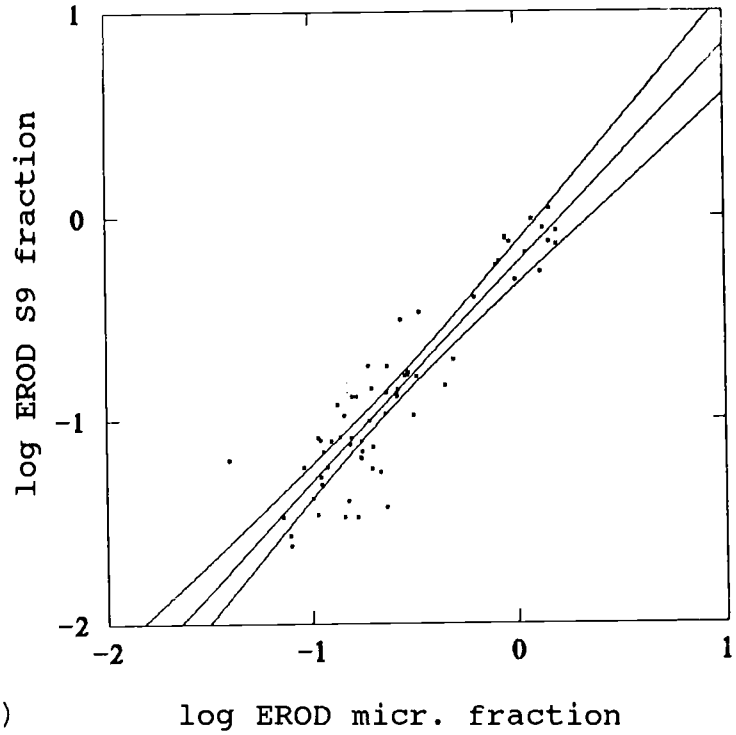

fig. 8b

figure 8 a:EROD values in the microsomal fraction versus EROD values in the S9 fraction (Units/mg protein, r=0.949)
b:log-transformed EROD values in the microsomal fraction versus log-transformed EROD values in the S9 fraction (Units/mg protein, r=0.916)

EROD values versus ΣP450 contents
Data from EROD activities in the microsomal fraction versus data from ΣP450 contents show a correlation coefficient of 0.580 (see figure 9a). Log-transformion of these data show a correlation coefficient of 0.568 (see figure 9b). Pools from less than 5 fishes were not used for all pools compared should have the same sample size.

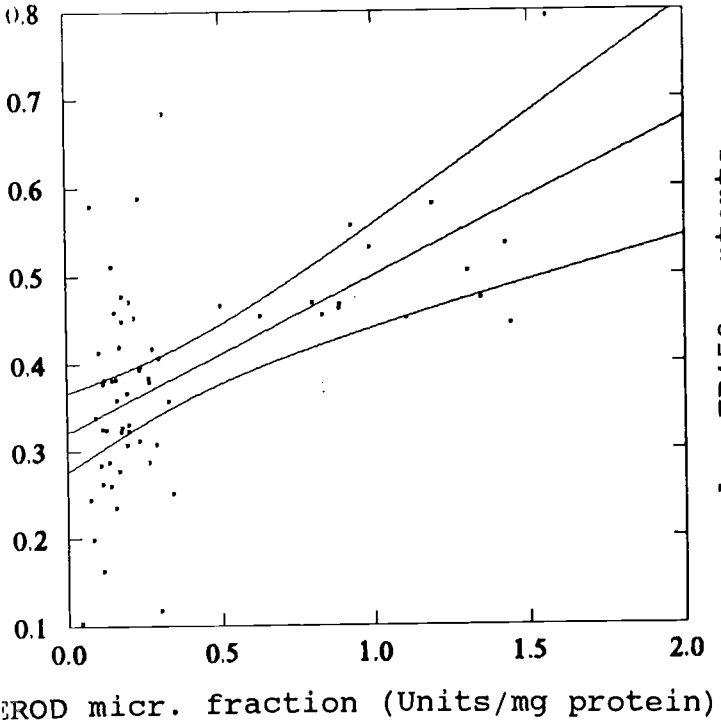

fig. 9a

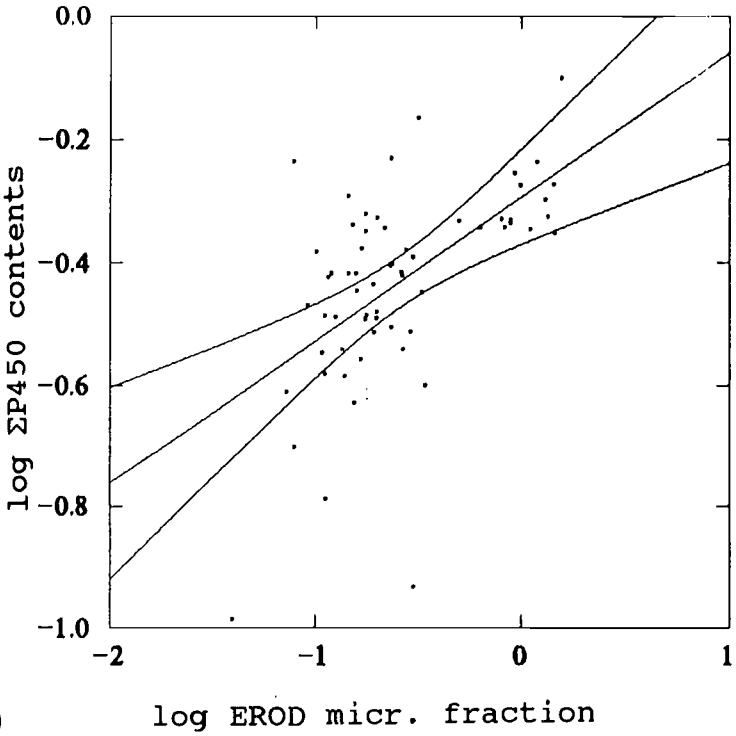

fig. 9b

figure 9 a:EROD values (Units/mg protein) of the microsomal fraction versus ΣP450 contents (nmol/mg microsomal protein), r=0.580
b:log-transformed EROD values of the microsomal fraction versus log-transformed ΣP450 contents, r=0.568

DISCUSSION

Better correlations coefficients of the standard curves, obtained after modification of the protein assay, were due to a combination of the changes that were undertaken. Diluting the dye before adding it to the wells might have improved the way the dye reacted with the BSA (personal conversation with Martin Eggens). Furthermore the amounts of BSA/well were higher in the standard solutions after modification of the assay. This also might have influenced the reaction of the dye with the BSA.

The theory of getting higher cytochrome P450 enzyme activities in polluted areas in comparison to reference areas is supported by studies of Galgani et al. (1991), who found a correlation between EROD activity and PCBs and HCH contents in plaice from the northwest part of France (appendix 12a). Addison and Edwards (1988) found that EROD activities in flounder followed an observed pollution gradient in Langesundfjord, Norway. Sulaiman et al. (1991) observed EROD activities in flounder from polluted sites (Forth estuary, Scotland) that were 9-fold higher than the EROD activities from the reference site. Studies in the North Sea have shown that hepatic monooxygenase activities increase along a possible pollution gradient (Lange et al. unpublished). An observed pollution gradient in the North Sea and measurements of EROD activities and concentrations of Cytochrome P450 are shown in figure 10.

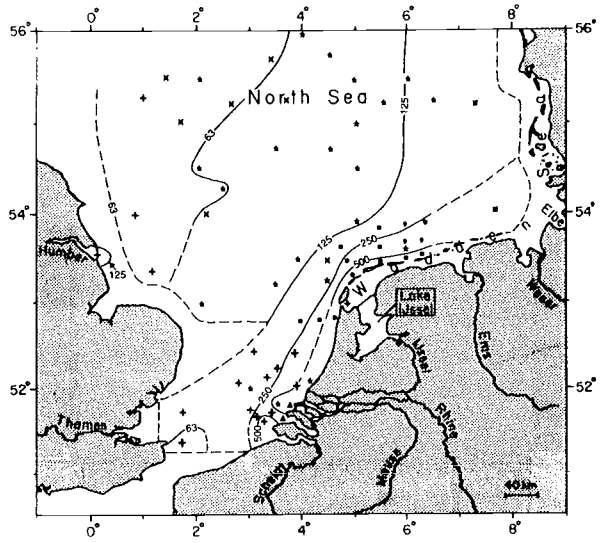

fig. 10a

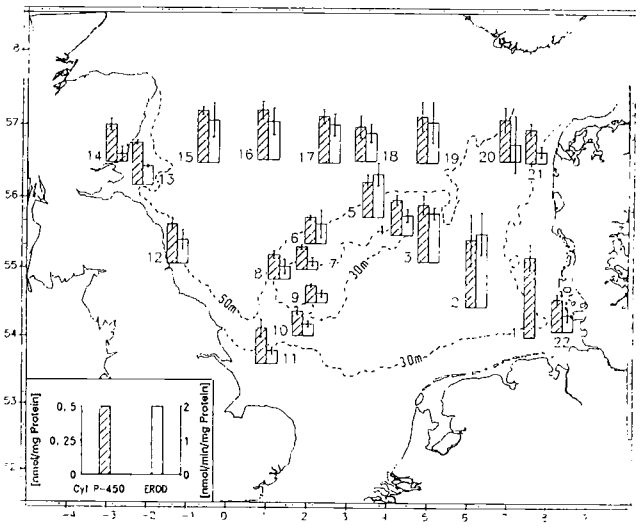

fig. 10b

figure 10 a: isolines of concentrations of 2,2',3,4,4',5' hexachlorobiphenyl (CB-138) in the benthic polychaete worm Nephtys spp. in ng.g^{-1}pentane extractable lipid in the North Sea (J.P. Boon, unpublished)
b: Limanda limanda. Concentrations of Cytochrome P450 (nmol/mg protein) and EROD activity (nmol resorufin/ min/mg protein). Averages and standard deviations are shown (Lange et al., unpublished)

A study from Sleiderink (unpublished) showed an increase of EROD activity along a pollution gradient in the North Sea in dab (Limanda limanda) (shown in appendix 12b). The present study does not include data of toxicant concentrations, since the analyses of PCBs in the muscle tissues of the same fishes used for the EROD/ΣP450 assays are not completed yet. Therefore a direct comparison of EROD activities and PCB concentrations in the dabs is not possible. When the EROD results are compared with the pollution gradient shown in figure 10a, with figure 10b, appendix 12a/b and figure 2, it becomes clear that the observed EROD values in this study (table 6 and figure 7) do not correspond with values which were expected; the values do not follow the pollution gradient. Stations 4, 6 and 7 have much higher EROD values than the other stations. Table 1 shows that at these stations the water column was stratified, resulting in lower bottom temperatures (7-9 °C). Therefore possible explanations for these results might be found in the different physical and chemical properties of stratified water columns, such as low bottom temperatures.

Temperature plays an important role in the activity of the hepatic mixed-function oxidase system of fishes. Because body temperatures of poikilotherms correspond closely to habitat temperature, organisms require mechanisms (e.g. temperature compensation) for maintaining their physiological and metabolic activities despite fluctuating environmental temperatures (Jimenez and Burtis 1989). Temperature-induced changes in cytochrome P450 dependent reactions have been described in thermally acclimated fish under laboratory conditions. Andersson and Koivusaari (1986) showed that the ECOD activity and B(a)P hydroxylase activity of cytochrome P450 were higher in liver cells from cold-acclimated (4 °C) trout than in warm-acclimated (17°C) trout at all assay temperatures used (appendix 13a). Stegeman (1979) found higher levels of B(a)P hydroxylase and NADPH-cyt.c reductase activities in fishes (Fundulus heteroclitus) acclimated to 6.5 °C than in similar fishes acclimated to 16.5 °C. All samples were assayed at 25°C. Furthermore Blanck et al. (1989) obtained similar results in the field situation: rainbow trout adapted to declining water temperature showed a minor decrease in ECOD and BPH activities in liver microsomes when measured at ambient temperature. However, if the measurements were performed at constant temperature (18°C), the apparent monooxygenase activity increased about threefold. In contrast to the studies just mentioned, Jimenez and Burtis (1989) observed different results. Significantly higher EROD levels were found in bluegill sunfish at higher acclimation temperatures if the fishes were injected with B(a)P (appendix 13b). All measurements were done at 30 °C incubation temperature. Higher levels of hepatic EROD activity have also been reported both in laboratory bluegills acclimated to higher temperatures and in wild fish collected during the summer (Jimenez et al. 1988). Low temperature also influences the process of monooxygenase induction by increasing the time necessary to reach the maximal enzyme activity (Egaas and Varanasi 1982, Andersson and Koivusaari 1985).

The bottom temperatures of stations 4, 6 and 7 (respectively 10.5, 9.5 and 12.5 °C) in the present study were lower than the temperatures of the other stations (17-19.6 °C). Since exceptionally high levels of activities were found at stations 4, 6 and 7, this study seem to support the results of the studies from Andersson and Koivusaari (1986), Stegeman (1979) and Blanck et al. (1989). Since no increasing EROD levels were found along the possible pollution gradient (figure 10a), it looks as if in this case temperature might have had much more influence on EROD levels than the inducing pollutants, so that induction could not be observed anymore. However, care has to be taken with the comparison of the results of the present study with the results of the studies mentioned above; different fish species and different activity assays were used in these studies. Different species might show different response mechanisms and different activity assays might give different responses to temperature changes.
When the averages from EROD values in the microsomal and S9 fraction are plotted against bottom temperature (shown in appendix 17), there seems to be a tendency of increasing EROD activity with decreasing temperature. Due to an insufficient amount of data, a correlation between EROD activity and temperature can not be proven statistically.

Microsomal content of total cytochrome P450 determined spectrally reflects the sum of all iso-forms present and does not reveal the isoenzyme composition nor any feature pertaining to a specific isoenzyme (Stegeman and Kloepper-Sams 1987). EROD activity, however, specifically indicates the activity of the isoenzyme P450IA1. This could be the reason why in this study no good correlation was found between EROD activity values (in microsomal and S9 fraction) and contents of total cytochrome P450 (see figure 9). An immunoassay, using specific antibodies against cytochrome P450IA1, might give a better idea of the amount of P450IA1 enzymes present (Goksøyr 1991). An ELISA assay was done on board of the ship with the S9 fractions of the same livers as used in the present study, but the definitive results are not available at this moment. The preliminary results are shown in appendix 14. Data from stations 2 and 4 are missing. Although no statistical tests are carried out with these results, it seems obvious that similar results as the EROD values are obtained with the ELISA assay: stations 6 and 7 having higher values than the other stations.
The samples used for the total cytochrome P450 assay were also used for the protein assay, which means that these samples were defrosted and frozen again before the cytochrome P450 assay was carried out. Cytochrome P450 enzymes which are denaturated show a peak at 420 nm. Since variable heights of peaks at 420 nm were observed, it is possible that this defrosting had impact on the ΣP450 values. Furthermore, remainders of blood can partially be held responsible for the peak at 420 nm.

Because of their high abundance, wide distribution, easily visible gross disease and relatively small migration range, the

dab became an important target species in pollution monitoring programmes in the North Sea. Rijnsdorp et al. (1991) however, did a tagging experiment with dabs and suggested that at any time the dab population at a sampling site is a temporary aggregation of fish originating from a large area. Dabs which were tagged and released in the German Bight, Terschelling and the southern coast of the Netherlands were found back at locations far from the release position (appendix 15). In the beginning of the spawning period, which runs from January till September with a broad peak from February to April, the adult dabs leave the immediate coastal waters and concentrate in the southeastern North Sea near Helgoland, west of the Horns Rif and around the Doggerbank area with local concentrations in the central North Sea. It is suggested that fishes caught during the spawning period may originate from individuals that are widely dispersed over a large area of the southern North Sea (Rijnsdorp et al. 1991). Therefore it might be advisable not to catch dabs for monitoring purposes in the spawning period. Since dabs also show migration patterns in the beginning of the winter, in order to avoid the cool temperatures of the shallow areas (Rijnsdorp et al. 1991), the best part of the year for monitoring programs with dab might be the autumn. The measurements of the present study were carried out in August and September, which is the end of the spawning period. It is therefore possible that fishes caught at some stations were at that moment migrating to different areas. It is not sure in which way this might have influenced the results.

In the present study, average EROD activities in the microsomal fraction were found to be 1.1 up to 4.6 times higher than in the S9 fraction. Eggens et al. (in press) also found higher EROD activities (about 4 times) in the microsomal fraction. This is due to the amount of aspecific cytocolic proteins which are removed during the ultra centrifugation step in the isolation of the microsomes. This gives an increase of the EROD activity based on microsomal protein content.

Finally, it must be stated that other factors might have influenced the EROD and ΣP450 results. Differences in salinity and nutrition between the stations, inhibiting compounds and dab population differences are just a few examples of these factors. More research has to be done on the influence of environmental variables, especially temperature and migration patterns of dabs, on the monooxygenase enzyme activities. Otherwise it is possible that dabs are not as suitable as a biomonitoring species in the North Sea as they are supposed to be.

CONCLUSIONS

Several conclusions can be drawn from the results of this study:
- Better correlation coefficients of standard curves were obtained after optimization of the protein assay.
- The levels of EROD activity in the microsomal fraction as well as in the S9 fraction do not correspond with the expected pollution gradient of environmental contaminants known to induce the P450IA1 isoenzyme.
- EROD activities at stratified stations, with lower bottom temperatures, are significantly higher than at stations without stratification.
- There is a good correlation (r=0.949) between EROD activities in the microsomal fraction and the activities in the S9 fraction.
- Not a good correlation of EROD activities with contents of total cytochrome P450 contents was found (r=0.580).
- More research needs to be done to determine the influence of environmental variables, such as temperature, on EROD activities in the field situation.

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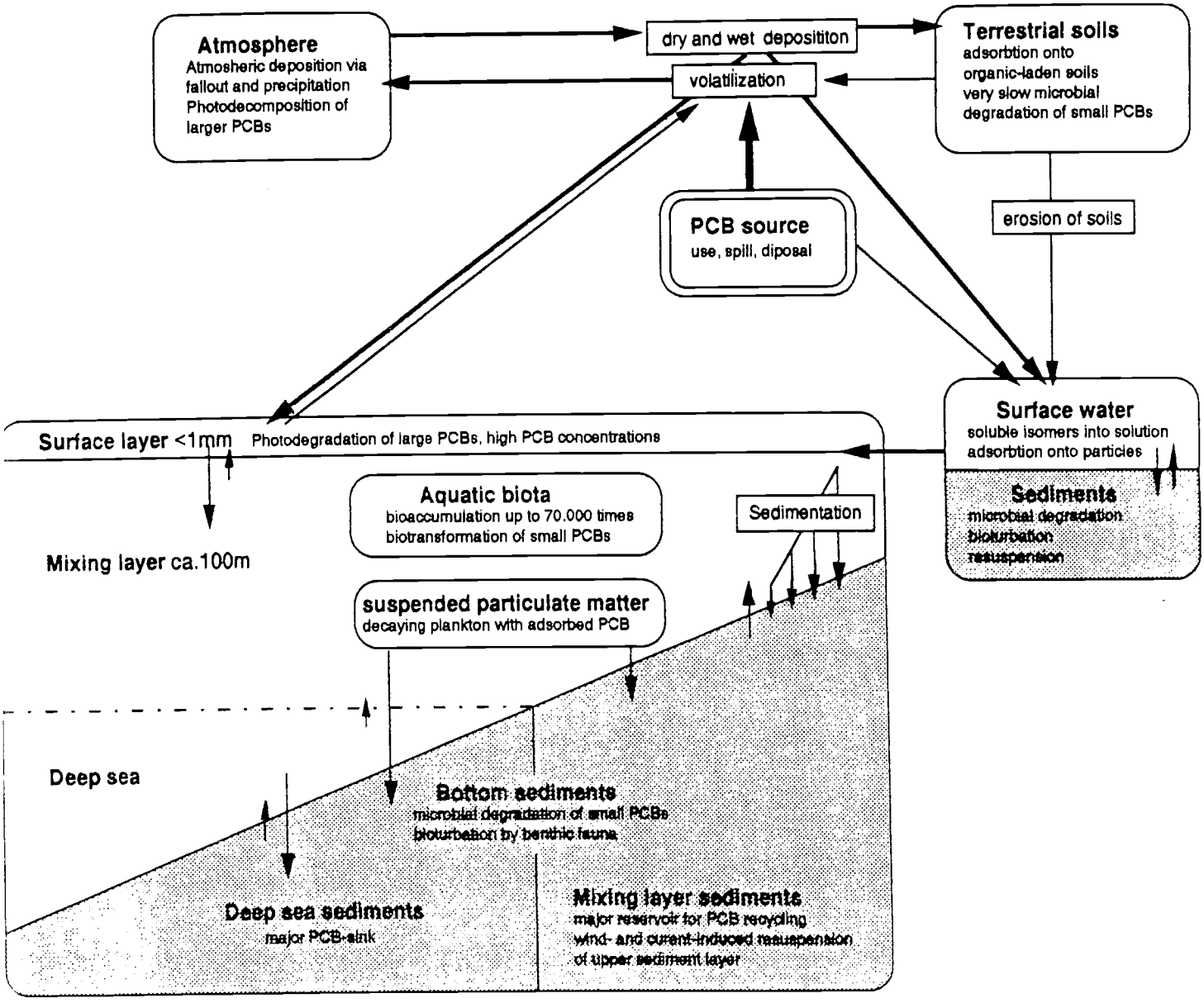
APPENDIX 1



Pathways of PCBs in the environment (from: Casal and Schweizer 1991)

APPENDIX 2

Examples of xenobiotics that are inducers of the mammalian (A) and fish (B) cytochrome P450 system (derived from Goksøyr 1987).

A

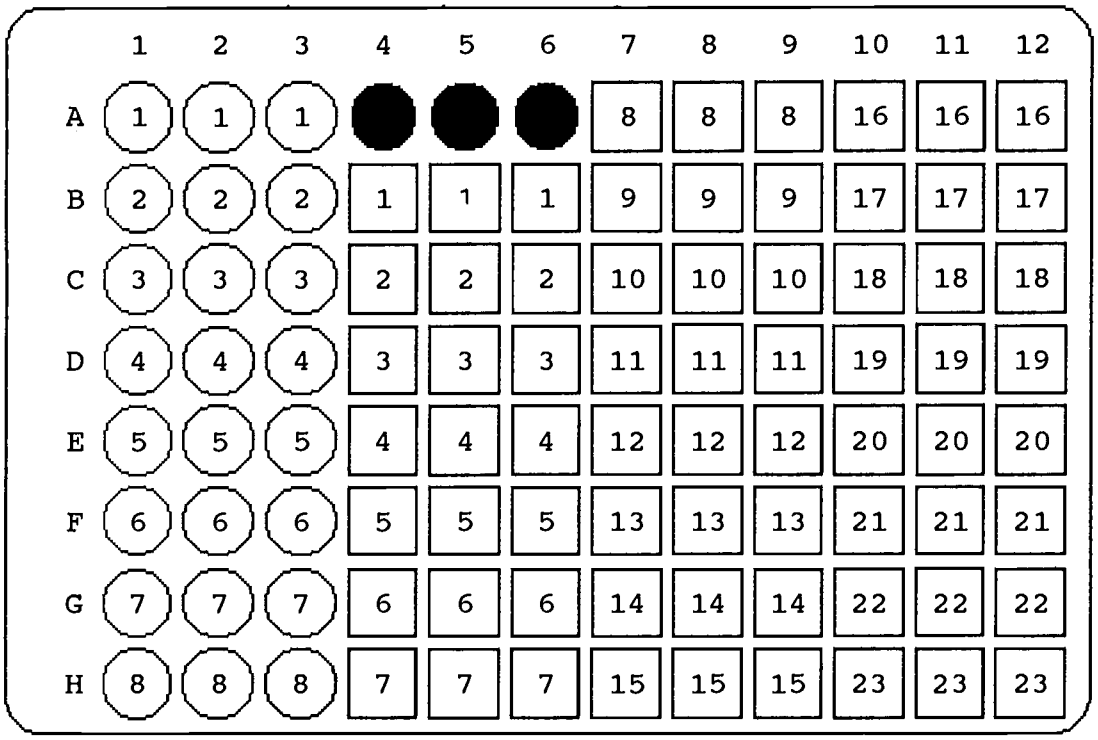
Benzo(a)pyrene
3-Methylcholanthrene
Benzo(a)anthracene
7,12-Dimethylbenzo(a)anthracene
ß-Naphthoflavone
Phenobarbital
Phenylbutazone
Aminopyrine
Chlorpromazine
Tolbutamide
Chlorcyclizine
Probenecide
DDT
Benzene
Caffeine
Pregnenolone-16αcarbonitrile
Alkanes
Kepone
Mirex
Methadone
Griseofulvin
Methylated aromatic hydrocarbons
Ethoxyquin
Ellipticine
Ethanol
Isosafrole
Piperonyl butoxide
Ethinylestradiol
2-Acetylaminofluorene
Polyhalogenated biphenyls (PCB and PBB)
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)
Styrene
trans-Stilbene oxide
Chlorinated antibacterials
Rifampicin
Terpenes and sesquiterpenes
Lindane
Theophylline
Synthetic pyrethroids
Acetone
Clofibrate
Toluene

B

Benzo(a)pyrene
3-Methylcholanthrene
ß-Naphthoflavone
Pyrene
Chrysene
7,12-Dimethylbenzo(a)anthracene
2,3,7,8-Tetrachlorodibenzo-p-dioxin
Polychlorinated biphenyls (PCB)
Phenoclor DPG
Butylated monochlorodiphenyl ethers
Hexabromobenzene
Butylated hydroxyanisole
Butylated hydroxytoluene
tert-Butylhydroxyquinone
Ethoxyquin
Cyclopropenoid fatty acids
Isosafrole

APPENDIX 3

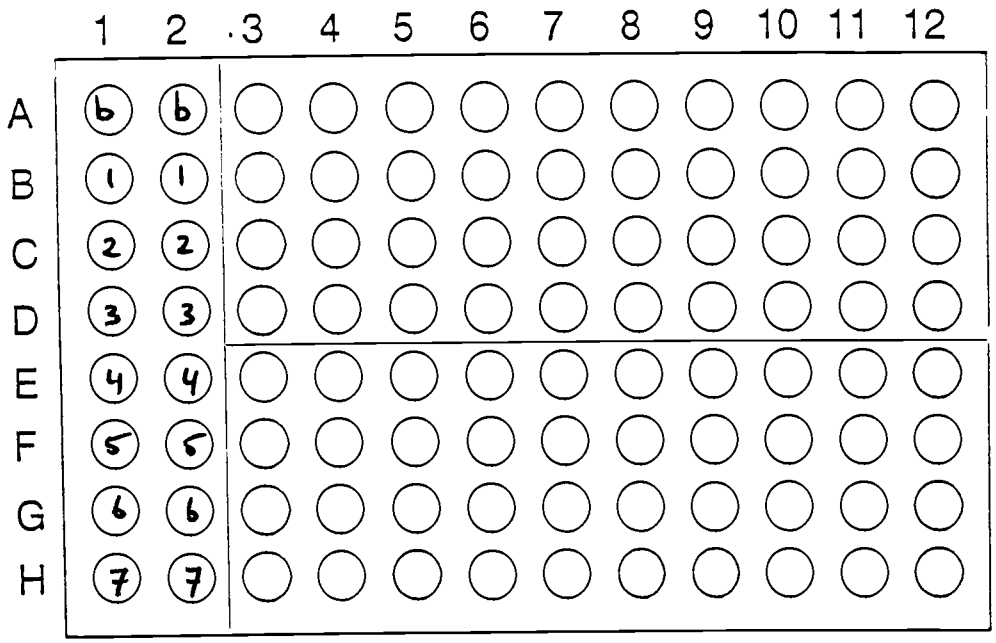
THE ARRANGEMENT OF THE BLANKS, STANDARDS AND SAMPLES IN THE WELLS OF THE MICRO-PLATE OF THE PROTEIN ASSAY



Wells A1,2,3 to H1,2,3 were filled with standard solutions 1 to 8 in threefold
Wells A4,5,6 were blanks
Wells B4,5,6 and C4,5,6 were filled with sample 1 (twofold)
Wells D4,5,6 and E4,5,6 were filled with sample 2 etc.

APPENDIX 4

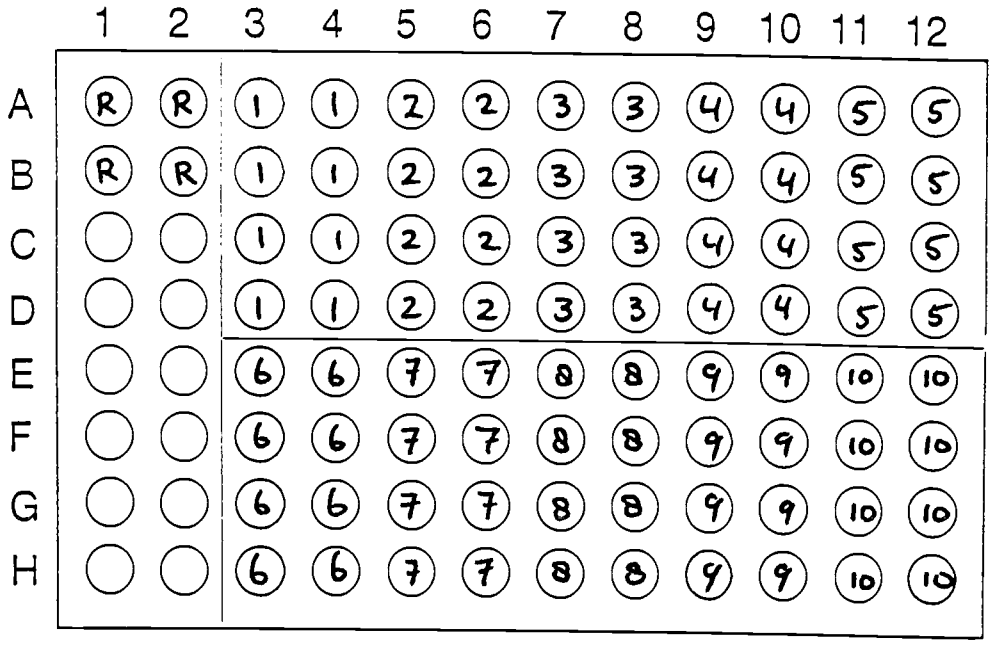
THE ARRANGEMENT OF THE STANDARD SOLUTIONS IN THE WELLS OF THE MICRO-PLATE OF THE EROD ASSAY



Wells A1&2 are blanks
Wells B1,2 to H1,2 are filled with standard solutions 1 to 7 (twofold)

APPENDIX 5

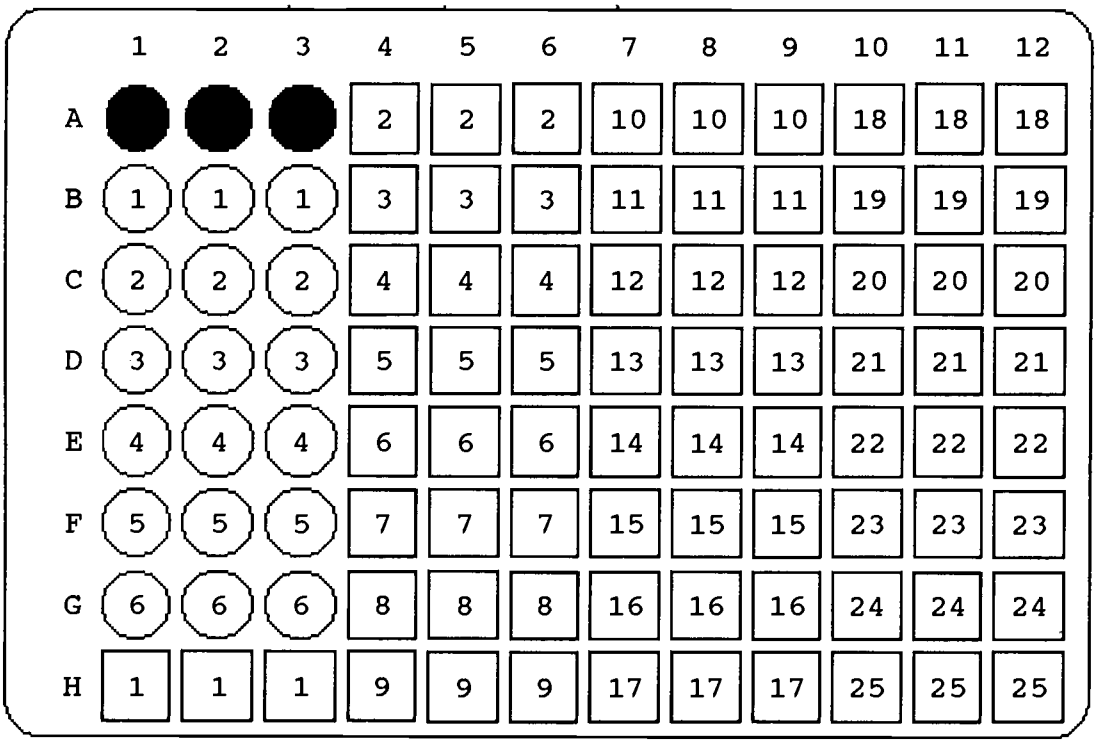
THE ARRANGEMENT OF THE SAMPLES AND REFERENCE SAMPLE IN THE MICROPLATE OF THE EROD ASSAY



Wells A1,2 are filled with reference sample, with NADPH
Wells B1,2 are filled with reference sample, no NADPH
A3,4 are filled with sample 1, no NADPH (twofold)
B3,4 to D3,4 are filled with sample 1, with NADPH (twofold)
A5,6 are filled with sample 2, no NADPH (twofold)
B5,6 to D5,6 are filled with sample 2, with NADPH (twofold)

APPENDIX 6

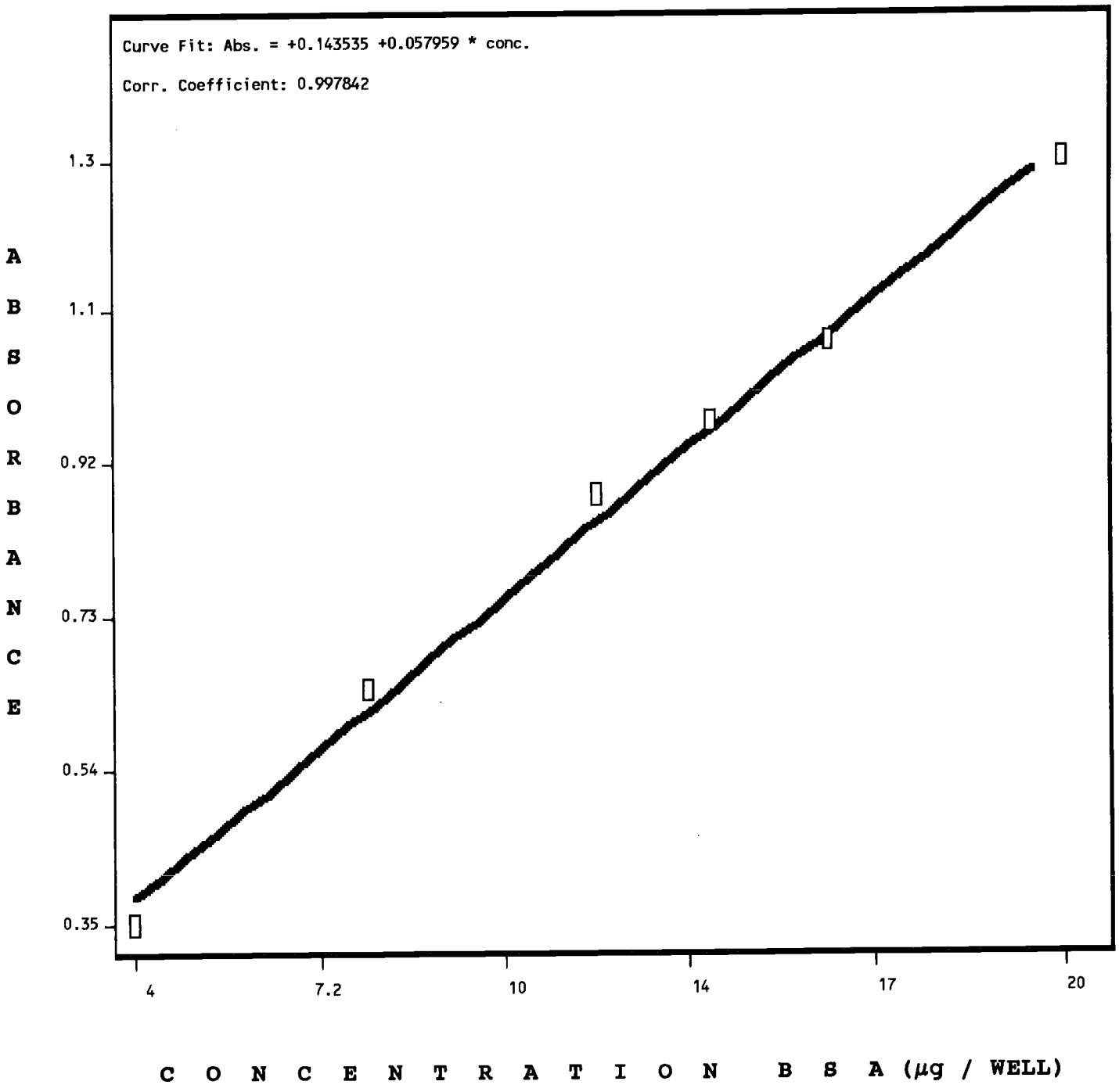
THE ARRANGEMENT OF BLANKS, STANDARDS AND SAMPLES IN THE WELLS OF THE MICRO-PLATE OF THE PROTEIN ASSAY, AFTER OPTIMALISATION



Wells A 1,2,3 were blanks
Wells B 1,2,3 to G 1,2,3 were filled with standard solutions 1 to 6 in threefold
Wells H 1,2,3 and A 4,5,6 were filled with sample 1 (twofold)
Wells B 4,5,6 and C 4,5,6 were filled with sample 2 etc.

APPENDIX 7

EXAMPLE OF A STANDARD CURVE OBTAINED IN THE PROTEIN ASSAY, AFTER OPTIMALISATION OF THE PROCEDURE



APPENDIX 8
Results from EROD and total P450 measurements

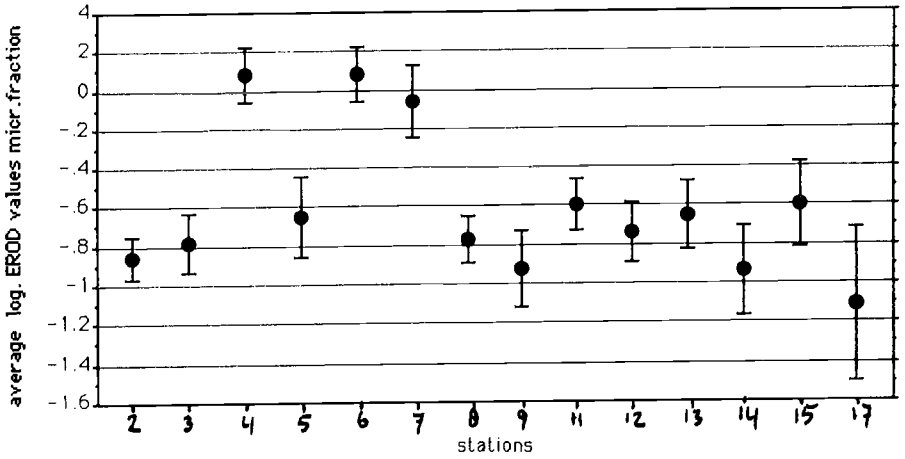
NR. OF DABS	n	EROD MICR. FRACTION (Units/mg protein)	EROD S9 FRACTION (Units/mg protein)	TOTAL P450 CONTENT (nmol /mg protein)
2LL1-5	5	0.101	0.041	0.414
2LL6-10	5	0.151	0.040	0.458
2LL11-15	5	0.119	0.058	0.382
2LL16-20	5	0.176	0.064	0.477
2LL21-25	5	0.157	0.081	0.382
3LL1-5	5	0.233	0.037	0.588
3LL6-10	5	0.216	0.055	0.453
3LL11-15	5	0.167	0.033	0.419
3LL16-20	5	0.145	0.033	0.511
3LL21-25	5	0.107	0.034	0.284
4LL1-5	5	1.556	0.722	*
4LL6-10	5	1.347	0.870	0.473
4LL11-15	5	0.927	0.742	0.556
4LL16-20	5	0.889	0.789	0.467
4LL21-25	5	1.561	0.844	0.793
5LL1-5	5	0.315	0.105	0.684
5LL6-10	5	0.115	0.069	0.377
5LL12-16	5	0.200	0.073	0.471
5LL17-21	5	0.296	0.166	0.406
5LL22-26	5	0.261	0.133	0.384
6LL1-5	5	1.106	0.659	0.451
6LL6-10	5	0.833	0.598	0.455
6LL11-15	5	1.305	0.531	0.504
6LL16-20	5	1.427	0.743	0.535
6LL21-25	5	1.442	1.083	0.444
7LL1-5	5	0.988	0.486	0.531
7LL6-10	5	0.630	0.397	0.454
7LL11-15	5	0.801	0.573	0.469
7LL16-21	5	1.192	0.966	0.581
7LL22-26	5	0.885	0.775	0.462
8LL1-5	5	0.197	0.142	0.331
8LL6-10	5	0.232	0.107	0.393
8LL11-15	5	0.159	0.130	0.359
8LL16-20	5	0.138	0.082	0.261
8LL21-25	5	0.134	0.118	0.288
9LL4+5	2	0.108	0.064	0.285
9LL6-10	5	0.144	0.104	0.381
9LL1-15	5	0.177	0.070	0.327
9LL16-20	5	0.078	0.027	0.580
9LL21-24	4	0.091	0.058	0.339
10LL1-4	4	0.452	0.147	*
11LL1-5	5	0.191	0.184	0.366
11LL6-10	5	0.275	0.310	0.417
11LL11-15	5	0.236	0.183	0.396
11LL16-20	5	0.341	0.338	0.251
11LL21-24	4	0.299	0.171	0.117
12LL1-5	5	0.154	0.075	0.235
12LL6-10	5	0.264	0.130	0.379
12LL11-15	5	0.233	0.136	0.312
12LL16-20	5	0.166	0.129	0.277
12LL21-25	5	0.125	0.078	0.325
13LL1-5	5	0.193	0.098	0.307
13LL6-10	5	0.265	0.140	0.288
13LL11-14	4	0.498	0.198	0.466
14LL1-5	5	0.112	0.048	0.163
14LL6-10	5	0.079	0.024	0.199
14LL11-15	5	0.072	0.033	0.245
14LL16-20	5	0.198	0.057	0.323
14LL21-25	5	0.174	0.065	0.322

* values not available

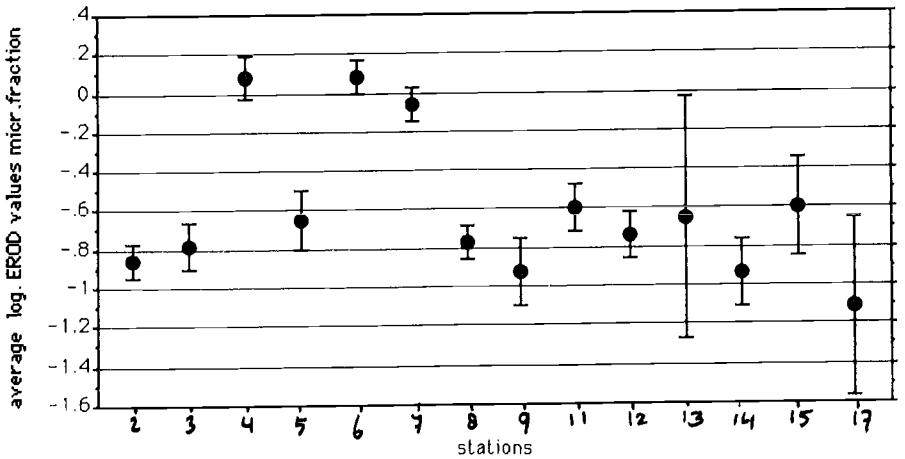
NR. OF DABS		EROD MICR. FRACTION (Units/mg protein)	EROD S9 FRACTION (Units/mg protein)	TOTAL P450 CONTENT (nmol/mg protein)
13LL1-5	5	0.193	0.098	0.307
13LL6-10	5	0.265	0.140	0.288
13LL11-14	4	0.498	0.198	0.466
14LL1-5	5	0.112	0.048	0.163
14LL6-10	5	0.079	0.024	0.199
14LL11-15	5	0.072	0.033	0.245
14LL16-20	5	0.198	0.057	0.323
14LL21-25	5	0.174	0.065	0.322
15LL1-5	5	0.329	0.163	0.357
15LL6-10	5	0.289	0.165	0.308
15LL11-15	5	0.175	0.078	0.448
17LL1-5	5	0.111	0.079	0.262
17LL6-10	5	0.111	0.052	0.326
17LL11-15	5	0.039	0.063	0.103

APPENDIX 9

Results of the Student's t-distribution test. De data were log-transformed, the standard errors were calculated and for each station the Student t's and studentized maximum modulus m's were determined (statistical tables, Sokal and Rohlf (1969)). Calculations for the scattergrams, shown below, were carried out with the following formulas:
t-distribution: log average EROD + t x standard error / $2^{-\frac{1}{2}}$
maximum modulus: log average EROD + m x standard error / $2^{-\frac{1}{2}}$



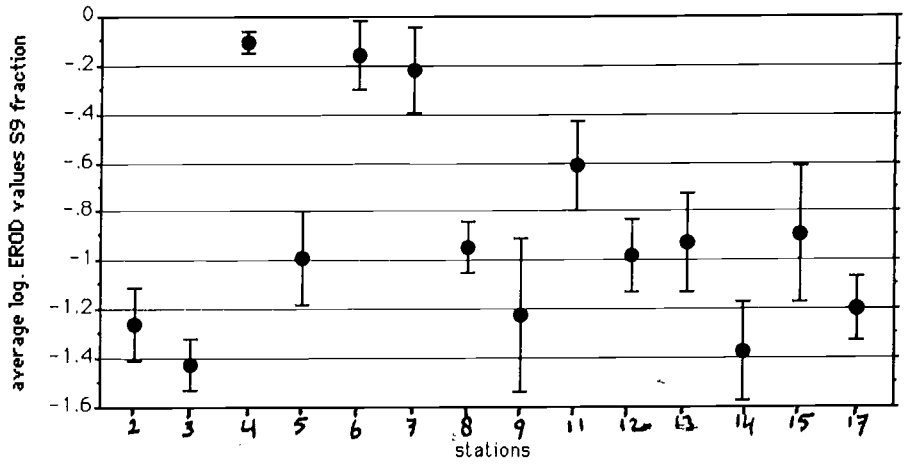
Scattergram with average EROD values (microsomal fraction) after log-transformation and ranges of maximum modulus values. **IN CASE THE RANGES OF TWO STATIONS DO NOT OVERLAP, THERE IS A SIGNIFICANT DIFFERENCE BETWEEN THE VALUES**



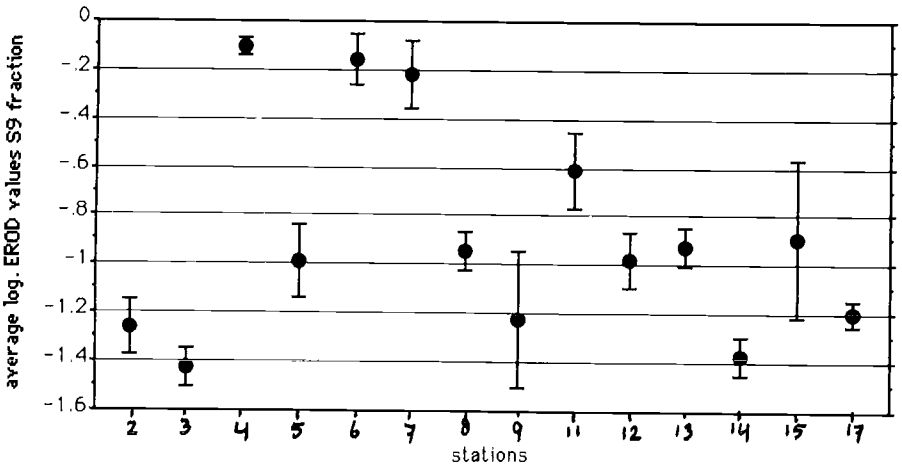
Scattergram with average EROD values (microsomal fraction) after log-transformation and ranges of Student's t-values. **IN CASE THE RANGES OF TWO STATIONS DO OVERLAP, THERE IS NOT A SIGNIFICANT DIFFERENCE BETWEEN THE VALUES**

APPENDIX 10

Results of the Student's t-distribution test (EROD S9 fraction). For method see appendix 9.



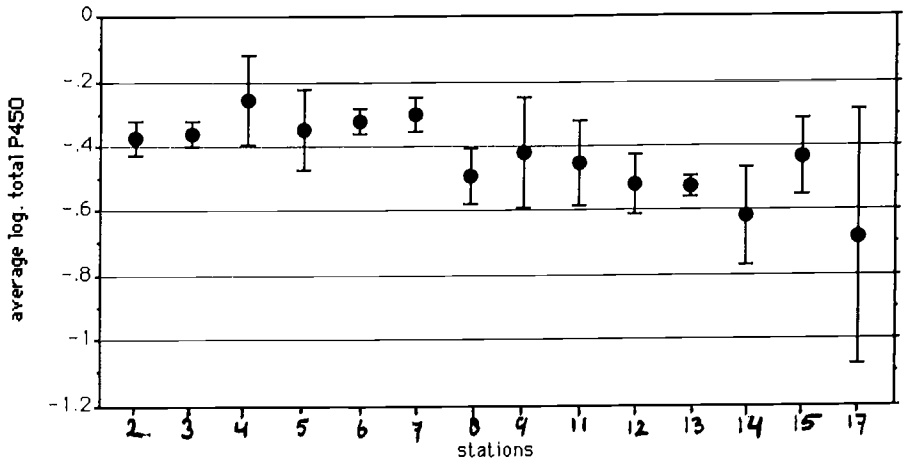
Scattergram with average EROD values (S9 fraction) after log-transformation and ranges of maximum modulus values. **IN CASE THE RANGES OF TWO STATIONS DO NOT OVERLAP, THERE IS A SIGNIFICANT DIFFERENCE BETWEEN THE VALUES**



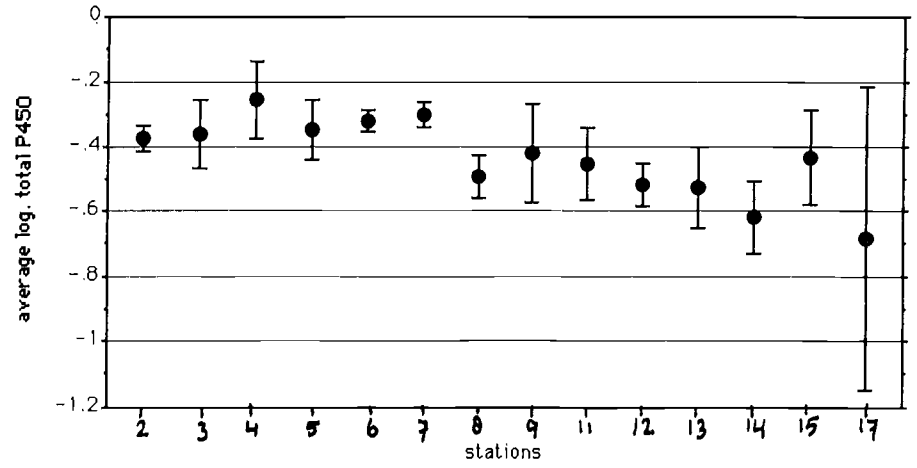
Scattergram with average EROD values (S9 fraction) after log-transformation and ranges of Student's t-values. **IN CASE THE RANGES OF TWO STATIONS DO OVERLAP, THERE IS NOT A SIGNIFICANT DIFFERENCE BETWEEN THE VALUES**

APPENDIX 11

Results of the Student's t-distribution test (Cytochrome P450 contents). For method see appendix 9.

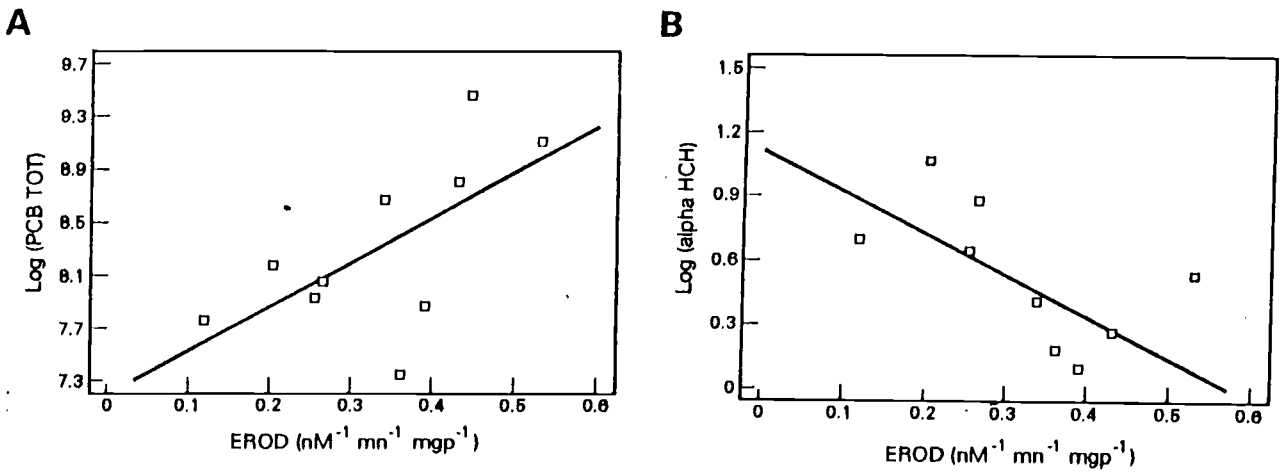


Scattergram with average Cytochrome P450 contents after log-transformation and ranges of maximum modulus values. **IN CASE THE RANGES OF TWO STATIONS DO NOT OVERLAP, THERE IS A SIGNIFICANT DIFFERENCE BETWEEN THE VALUES**

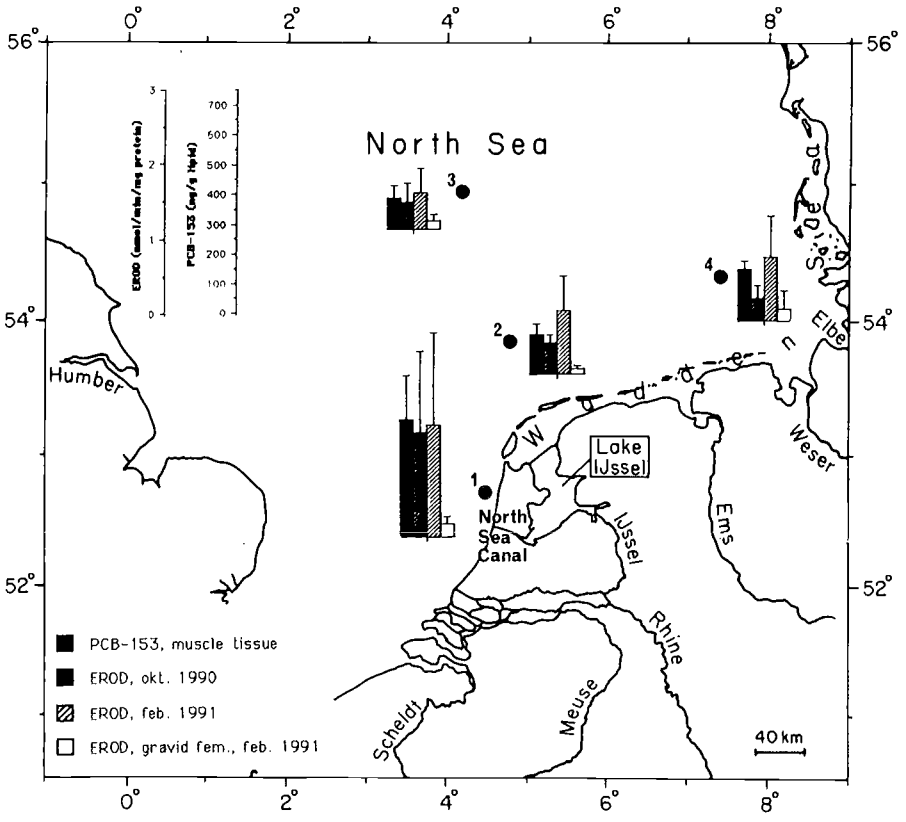


Scattergram with average Cytochrome P450 contents after log-transformation and ranges of Student's t-values. **IN CASE THE RANGES OF TWO STATIONS DO OVERLAP, THERE IS NOT A SIGNIFICANT DIFFERENCE BETWEEN THE VALUES**

APPENDIX 12



12a: correlation between PCB (A) or HCH (B) concentrations and EROD activities in plaice. Correlation coefficients were 0.62 and -0.69 for (A) and (B) respectively (N=10, $p \leq 0.05$). From: Galgani _et_ al. (1991).



12b: EROD activities (nmol/min/mg protein) in liver and PCB-153 concentration (ng/g lipid) in muscle tissue of dab (_Limanda limanda_) from the Southern North Sea (Sleiderink, H.M., J.P Boon and J.M Everaarts)

APPENDIX 13

13a: (from Andersson and Koivusaari (1986))

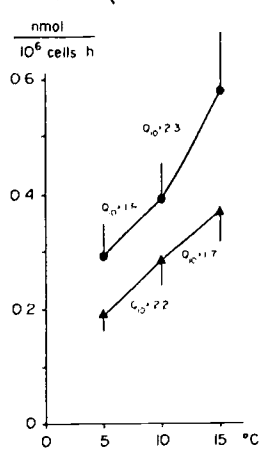


Fig. 2.

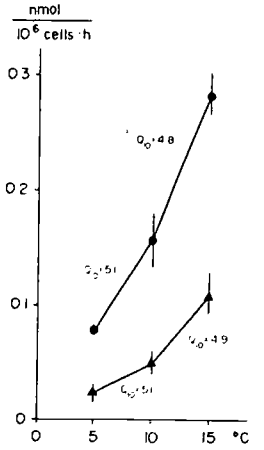


Fig. 3.

Fig. 2. The rate of 7-ethoxycoumarin deethylation in isolated liver cells from cold-acclimated (●——●) or warm-acclimated (▲——▲) fish. Incubations were performed with 5×10^6 cells per ml for 1 h and contained 0.2 mM 7-ethoxycoumarin. Values are means ± SE of 4 to 6 animals.

Fig. 3. The rate of benzo(*a*)pyrene hydroxylation in isolated liver cells from cold-acclimated (●——●) or warm-acclimated (▲——▲) fish. Incubations were performed with 5×10^6 cells per ml and contained 80 μM benzo(*a*)pyrene. Values are means ± SE of 4 animals. Warm-acclimated groups are statistically different from cold-acclimated groups, collectively tested ($P<0.017$).

13b: (from Jimenez and Burtis (1989))

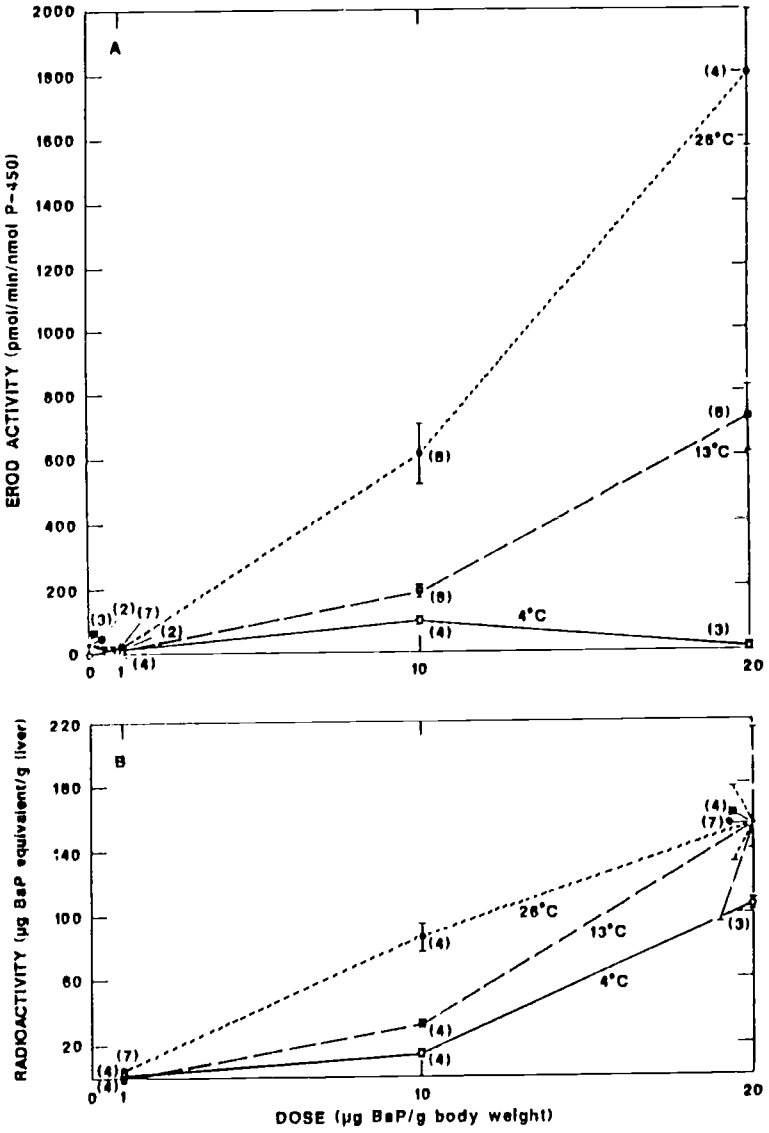
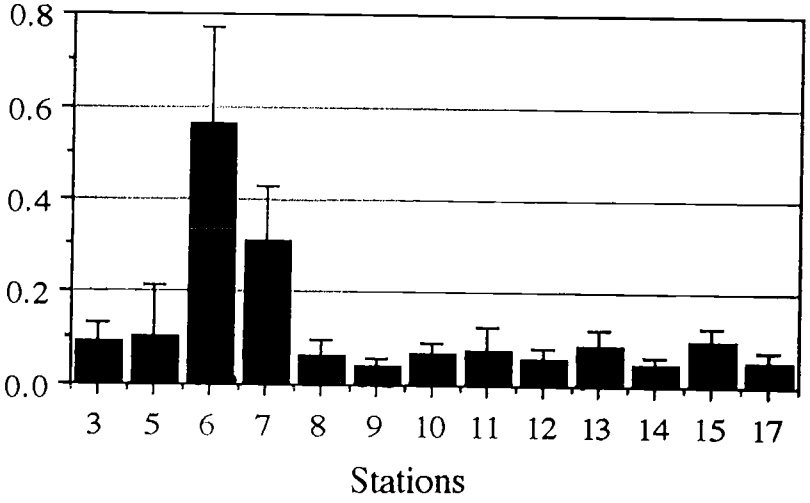


Fig. 3. The effects of temperature [4°C (solid line with open circles), 13°C (dashed line with solid squares), 26 C (dotted line closed circles)] and dose (1, 10 or 20 μg BaP/g body weight) on (A) EROD activity and (B) accumulation of ^{3}H-BaP by fish livers in unfed bluegills 5 days after injection. The mean ± SE and the number of observations are indicated. Each observation represent the pool of four livers.

APPENDIX 14

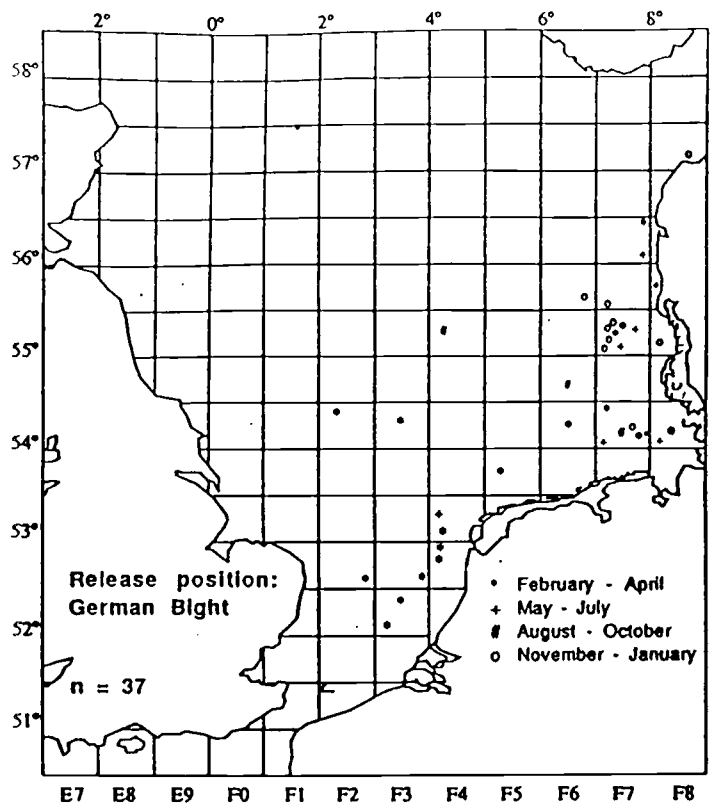
P450 1A1 ELISA
(Abs 490 nm)
Dabliver S9-fract.
(preliminary data)



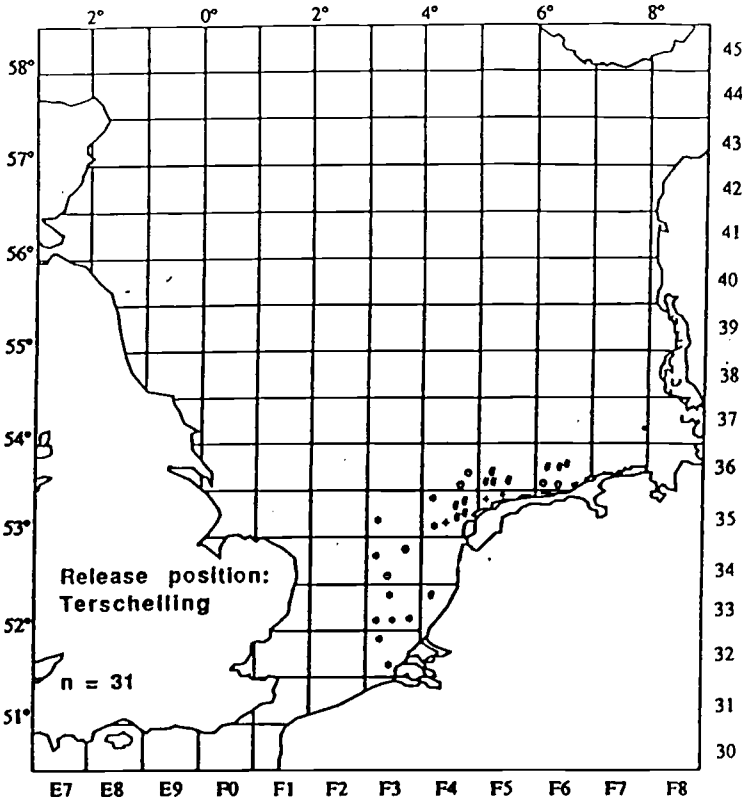
P450IA1 ELISA, carried out on board of the ship by Anders Goksøyr and Jonny Beyer, preliminary results

APPENDIX 15

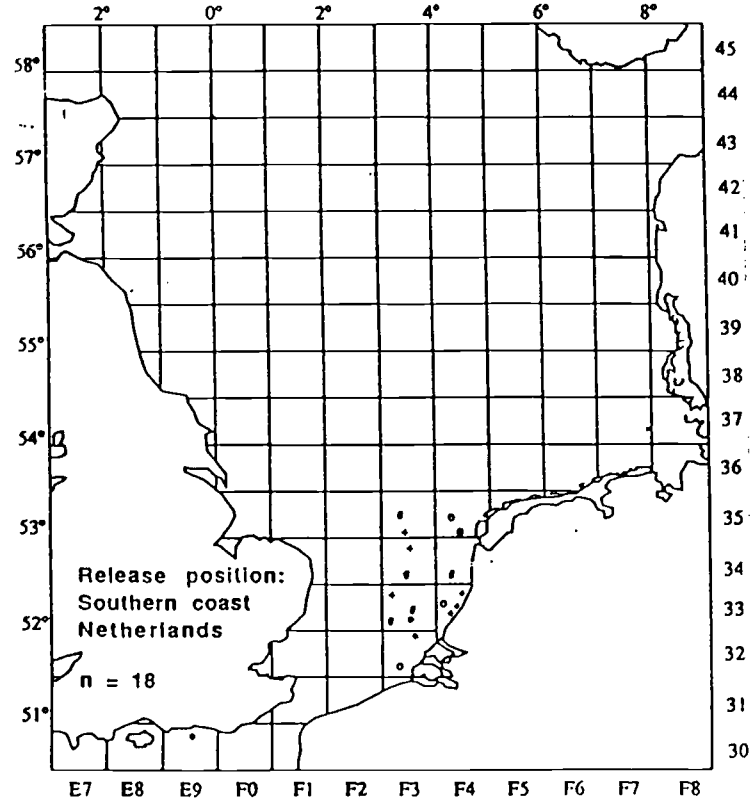
1)



2)



3)



Results of tagging experiments from Damm et al. (1991). 1) dabs were tagged in December in the German Bight and migrated in all directions without a clear preference. 2) dabs were tagged in July north of the Frisian Islands and showed a clear migration into the Southern Bight during spawning. 3) dabs were tagged along the southern coast of Holland were mainly recaptured in the southern Bight.

APPENDIX 16

BUFFERS AND SOLUTIONS

Homogenisation buffer I (used for preparation of the livers)

0.1 M sodium dihydrogen phosphate
0.1 M potassium chloride
1 mM EDTA (ethylen diamin tetraacetic acid)
10% glycerol
pH=7.4

Homogenisation buffer II (used for production microsomes)

0.1 M sodium dihydrogen phosphate
0.15 M potassium chloride
20% glycerol
1 mM EDTA
1 mM DTT (dithiotreitol)
pH=7.4

Resuspendation buffer (used for production microsomes)

0.1 M sodium dihydrogen phosphate
20% glycerol
1 mM EDTA
1 mM DTT
pH=7.4

Phosphate buffer (P-buffer) (used for EROD- and ΣP450 assays)

0.1 M potassium monohydrogen phosphate
0.1 M sodium dihydrogen phosphate
pH=7.4

EROD solution (used for EROD assay)

665 μl stock ethoxyresorufin (in ethanol) in 200 ml P-buffer (=0.5nM), bewaren in donker

RESORUFIN solution (used for EROD assay)

- 700 μl resorufin stock solution in 50 ml EROD solution
- measurement of the OD_{572}: 0.030 absorbance units
- calculation of resorufin in the solution by the formula:
  $[resorufin] = E/\epsilon.l$
  (E= extinction, ϵ= extinctioncoefficient (73 mM^{-1}), l= cuvet-length (1 cm))
- the concentration of resorufin was 0.030/73.1 = 0.41 μM
- bewaren in donker

0.1% albumin solution

250 mg bovine serum albumin (BSA) in 250 ml P-buffer

APPENDIX 17

Temperature (°C) plotted against average EROD values in the microsomal fraction (black squares) and S9 fraction (white squares)