In vitro CYP1A induction by 3,3'4,4'5 pentachlorobiphenyl (PCB#126) and 2,3,7,8 tetrachlorodibenzodioxin (TCDD) in flounder hepatocytes (*Platychthis flesus*).

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INTRODUCTION

The Flounder (*Platychthis flesus*) is a flatfish species that prefers shallow, coastal, waters of relatively low salinity. It is an abundant species around the Dutch coast, which, because of riverine outflows, receives significant amounts of contaminants[1]. The flounder is therefore considered a relevant species for ecotoxicological research in the Netherlands.
Ethoxyresorufin-O-deethylation (EROD) is an enzyme activity that is linked to the cytochromeP450 1A enzyme system[2]. In many species this enzyme is very sensitive to induction by compounds with a dioxin-like mode of action. These include: many dioxins and PCBs and polycyclic aromatic hydrocarbons (PAHs) like benzo(a)pyrene[2]. Consequently CYP1A activity is the most widely used biomarker for exposure to these compounds[3].
This paper describes the preliminary results of experiments, which have been done to develop an *in vitro* system for flatfish species like the flounder. The purpose of such a system could be to assess the induction potential of polyhalogenated aromatic hydrocarbons (PHAHs) and PAHs in different flatfish species. In addition such an *in vitro* system might also be of use in a study of the biotransformation and bioactivation of these compounds in a selected species. The future goal of this research will be: determination of species sensitivity. The technique will also be applied to other (flat)fish species in the near future.

EXPERIMENTAL

Isolation of hepatocytes
Three Flounders, 1 female and 2 males, 25-33cm in length, from the Dutch Wadden-Sea were acclimated to the laboratory for several months. Flounders were killed and livers excised. Livers were perfused via the largest hepatic vein with calcium free Hanks buffer for approximately 30 minutes. The osmolarity of Hanks buffer was increased to 380mOsm with NaCL in order to approach flounder serum osmolarity. The perfusion was stopped when the internal structure of the liver appeared to be sufficiently degraded. Hepatocytes were then dispersed, sieved through nylon netting, washed two times with Hanks buffer and finally dispersed in Wiliams-E medium supplemented with 25 mM Hepes, 10 mM

$NaHCO_3$, 25.6 mM NaCL 2.4 mM $MgCL_2$, gentamycine, 1 µM insulin, 10 µM hydrocortison and 2 mM L-glutamin. Total yield of the isolation was ±500*10E6 hepatocytes. Viability, as assessed with trypan blue exclusion, was ±90%. In general hepatocytes were present in small clusters of 3 to 6 cells. Also small numbers (<10%) of erythrocytes and other cell types were present. The Williams-E medium was dosed with either 0.3% DMSO or respective concentrations of PCB#126 or TCDD in this amount of DMSO. Cells were plated in 24 wells tissue culture plates (Greiner); 1 ml, 1.5*10E6 cells/well. Medium was not changed throughout the entire experiment. Hepatocytes were incubated in air at 14°C, similar to the water temperature in which the fish were kept. The dose range was 1 to 100 nM for TCDD and 3 to 300 nM for PCB#126. Cells were exposed for either 24 or 48 hours.

EROD and MTT-assay

Both assays were optimized for time and substrate concentration of the incubation.

EROD activity was assayed as described by Burke *et al*[4], with some modifications. Ethoxyresorufine and dicumarol (5 µM and 10 µM final concentration) in Williams-E (250µl) were added to the wells, followed by an incubation for 15 minutes at 14°C. This incubation was stopped with 1 ml of icecold methanol. Fluorescence was read after 15 minutes at room temperature on a Millipore Cytofluor 2300 plate reader.

The cleavage of the tetrazolium salt MTT (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide) was measured by adding MTT in williams-E (250µl) to a final concentration of 1 mg/ml). After 90 minutes at 14°C the contents of the wells were transferred to eppendorf vials at 4°C and quickly centrifuged. The pellet with the purple formazan product was then solubilized in 0.4 ml isopropanol, centrifuged again, and 0.3 ml was transferred to a 96 well plate to be read on a Bio-Rad microplate reader at 595nm. A detailed description of the method has been published earlier[5]

Protein was determined in a seperate plate according to Bradford[6]

RESULTS AND DISCUSSION

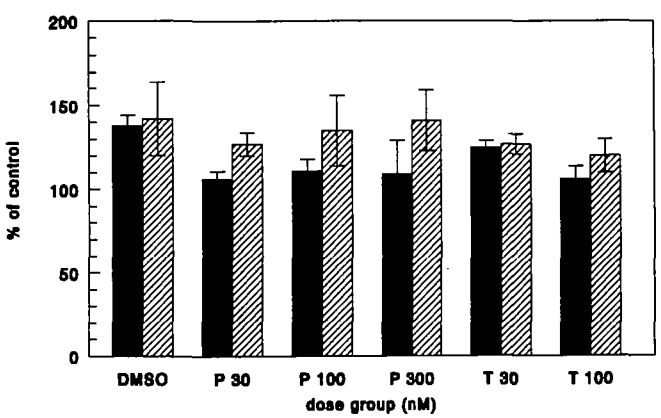

Figure 1 MTT activity of hepatocytes exposed to DMSO, or various concentrations of PCB#126 (P) or TCDD (T), for 24 (▬), or 48 (▨) hours. Control are DMSO treated hepatocytes, measured 4 hours after isolation. Error bar indicates sd of 4 determinations.

MTT

The MTT activities of hepatocytes dosed with DMSO or with PCB and TCDD concentrations of 30 nM or higher are represented in figure 1. It can be concluded that there is no toxic effect of the tested compounds at these concentrations. Moreover for all dose groups a slight increase in time of the MTT activity is observed. This is an indication that the viability of the flounder hepatocytes was not decreased during the experimental time. This is in accordance with the observation that in the course of the experiment more hepatocytes attached to the culture plates. After 24 and 48 hours approximately 50% and 80% of the hepatocytes had attached to form a monolayer. Unattached cells had mostly formed clusters of 10-40 cells.

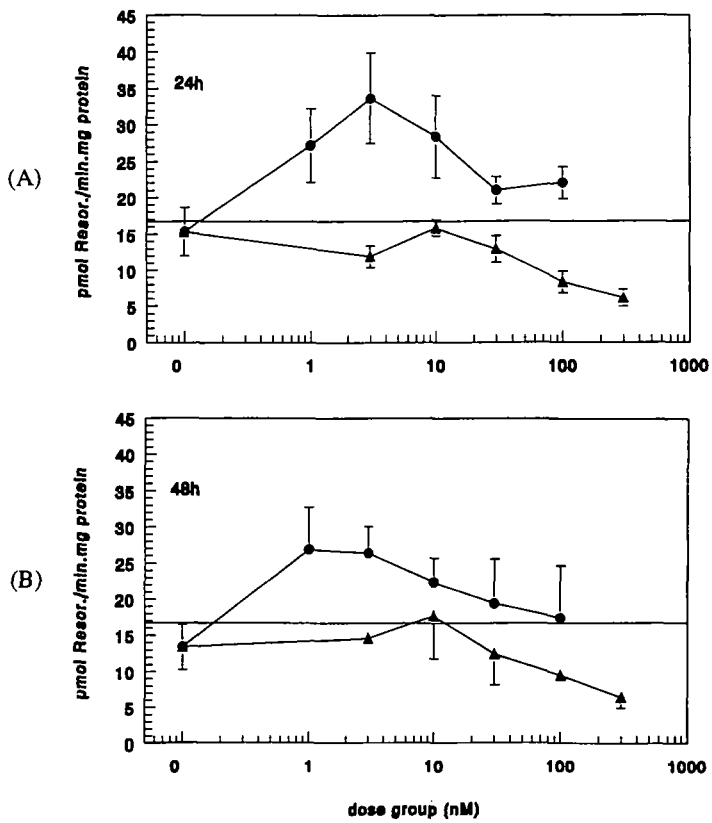

Figure 2 EROD activity of hepatocytes exposed to DMSO, PCB#126 (—▲—), or TCDD (—●—) for 24 (A), or 48 (B) hours. Horizontal line indicates EROD activity of DMSO treated hepatocytes measured 4 hours after isolation. Error bar indicates sd of 4 measurements.

CYP1A activity measured as EROD

EROD activity was induced relative to the control at all the TCDD concentrations tested. A maximum of twofold elevation of the control level was found after one day, at 3 nM TCDD (figure 2A). In contrast, PCB#126 did not induce EROD activity at any of the concentrations used. Moreover, the two highest concentrations of PCB#126 produced a

marked decrease in EROD activity, as compared to the control. The trend in EROD activity after 48 hours was similar to that after 24 hours (figure 2B).
This low ar absent induction of CYP1A is surprising because both compounds are known to be potent *in vivo* inducers in mammals, birds and fish.[2,7,8] A tenfold hepatic EROD induction by PCB#126 was reported from a dose respons study in the flounder, at a tissue concentration of 3.1 ng/g lipid. At higher dose levels the induction was lower, but still 3-7 times above control value[7]. In another *in vivo* experiment with flounder a maximum 27-fold average induction was determined after exposure to TCDD[8].
This discrepancy between the in vivo and the in vitro data could be caused by the individual sensitivities of the three flounders used; it could also be caused by the hepatocyte system used. The fact that MTT activity did not decrease, and that control EROD activity showed only a slight decrease in time, indicates a good viability of the flounder hepatocytes in these experiments. However, The role of some unknown factors, which might be important for induction, e.g. medium composition, temperature, extracellular matrix, can not be excluded based on these results. another possibility could be a maximum induction within the first 24 hours, followed by a decrease in enzyme activity. This has been reported earlier from studies with trout hepatocytes induced by ß-naphtoflavon[9]. However, another study with trout hepatocytes induced by ß-naphtoflavon or TCDD found maximum EROD induction after 48 hours or later[10].
The inhibition of EROD activity at high concentrations of PCB#126 could be caused by substrate inhibition of the enzyme. *In vitro* experiments with hepatic microsomes from flounders showed that a concentration of 500 nM of PCB126 produced a 25% inhibition of EROD activity[7].

CONCLUSIONS

The described technique yields viable flounder hepatocytes, that can maintain MTT and EROD activity for at least 48 hours in culture.
TCDD caused at most twofold induction of EROD activity at a concentration of 3 nM.
For an unknown reason PCB#126 caused no induction but only a decrease in EROD activity at concentrations of 100 nM or higher.
Similar studies will be performed with hepatocytes from other flatfish species.

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