BIOMARKER-DIRECTED FRACTIONATION OF TOTAL EXTRACTS FROM AQUATIC ENVIRONMENTAL ABIOTIC MATRIXES

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Introduction

During the years of 1915 to 1980 a pulp mill industry was situated in the inner part of the Örserum bay (area 365,000 m^2, average depth 2.7 m) on the Swedish East Coast. The aquatic environment in this area is highly contaminated by polychlorinated biphenyls (PCBs) due to leakage from the process of recycling PCB containing self–copying paper[1]. In order to remove this significant point source of PCBs in the Baltic Sea the inner part of the Örserum bay will be dredged. The water will be separated from the dredged sediment and transferred back into the bay, subsequently the sediment masses will be placed in a covered dumpsite on land.

Recently, bioavailable compounds collected by means of semi-permeable membrane devices (SPMDs), bottom sediment and settling particulate matters (SPMs) were collected from the Örserum bay and extracted. The total extracts were fractionated by using an automated high performance liquid chromatography (HPLC) system. Three fractions were isolated and were mainly composed of 1) aliphatic/monocyclic aromatic compounds (MACs), 2) dicyclic aromatic compounds (DACs) and 3) polycyclic aromatic compounds (PACs). Newly fertilized rainbow trout (*Oncorhyncus mykiss*) eggs were exposed to the fractions at different dosages by using the novel nanoinjection technique[2]. The most potent fraction regarding induction of ethoxyresorufin *O*-deethylase (EROD) activity in the liver of *O. mykiss* larvae was the PAC-fraction, despite the fact that the DAC-fraction contains the most known potent inducers, non-*ortho* PCBs [3].

Furthermore, extracts from sediments and SPMs collected in the central Baltic Sea, in lakes in Sweden, in the Norwegian trench and in dialysates from SPMDs exposed in Lincoln Creek, Wisconsin, USA have also been shown to be potent inducers of EROD activity[4-7]. In addition, despite the fact that it was concluded that the major induction of EROD activity in these extracts was caused by PACs, chemical analysis data regarding the assumed most potent polycyclic aromatic hydrocarbons (PAHs) could only to a limited degree explain the observed induction.

In this study we investigate if bioavailable toxic compounds and compounds with ability to induce EROD activity are released into the water column of the Örserum bay during the dredging process. In addition, given the investigations mentioned above, it seems likely that other, today not identified toxic PACs, are present in the aquatic environment due to anthropogenic activities. Therefore, our overall aim is to present our strategy by utilizing the biomarker-directed fractionation approach in order to screen for the most toxic compound/s in the PAC-fraction.

Methods and Material

Investigation of bioavailable compounds in decanted water from sediment

Bottom sediment (n = 30) from the Örserum bay was collected using a gravity corer and pooled,

whereupon the water was decanted after sediment particles had settled. During the exposure experiment both water and room temperatures were 10°C, the light cycle was controlled by an astronomic clock following the light cycle for the time of the year, early September. Exposure of juvenile *O. mykiss* was performed in glass aquariums (volume 8 L, pH 8.0-8.2, O_2 saturation 51%-96%) containing different dilutions of the obtained decanted water, that was gently aerated with air bubbels in order to prevent loss of volatile compounds. Six exposure groups including one control with 10 juvenile *O. mykiss* (average weight = 3.6 g; average length = 7.1 cm) in each aquarium were exposed for 113 - 120 hours, whereupon the fishes were sacrificed, and their livers were dissected without damaging the gall-bladder. Each liver was homogenized in 300 µL 0.25 M sucrose using a Potter-Elvehjem homogeniser and the individual homogenates were immediately frozen in liquid N_2 and then stored at -140°C pending analysis of EROD activity[8] and measurement of protein concentration[9].

Extraction and clean up

Figure 1 summarizes the procedure from extraction to chemical fractionation of bottom sediment from the Örserum bay. Pooled bottom sediment (n = 10, depth 0-10 cm) collected with a gravity corer (type: Limnos) from the inner part of the Örserum bay was extracted wet using toluene (*p.a.* grade) in a Soxhlet apparatus (50 h) coupled to a Dean-Stark trap to remove water from the sample. A procedural blank, included throughout the extraction and fractionation steps, was treated in a way identical to that of the sediment sample. The sediment extract was volume reduced in a Rotavapor (low-pressure evaporation) and the obtained extracted organic matter (EOM) was dissolved in *n*-hexane (LiChrosolv®) and cleaned up on a silica gel column by using *n*-hexane as eluent. After extraction, the dry weight of the extracted sediment was determined. In order to remove sulfur from the eluate it was treated with elemental Cu and ultrasonic waves (1 h), whereupon the eluate was left over night in the dark at room temperature for complete reaction.

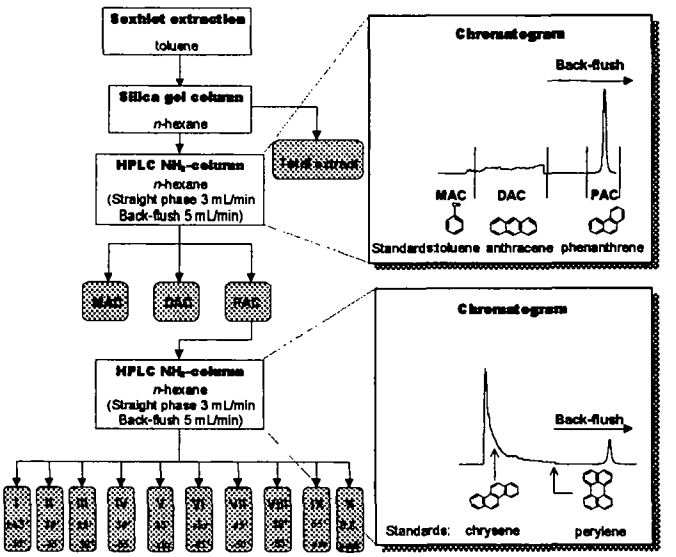

Figure 1. Schematic summary of the extraction and fractionation steps of a pooled (n = 10) bottom sediment sample collected in the Örserum bay. A procedural blank was treated identically with the sediment sample. Grey boxes illustrate the total extract and the 13 different isolated fractions exposed to newly fertilized *O. mykiss* eggs using the nanoinjection technique.

The solution was then filtered through glass wool in a Pasteur pipette to remove the Cu granulates. A final clean-up of the eluate was accomplished on a silica gel column with a small amount of sodium sulfate on top, using the *n*-hexane as eluent. An aliquot of the obtained eluate (total extract) was also collected for exposure of the newly fertilized *O. mykiss* eggs using the nanoinjection technique.

Fractionation

The silica gel eluate was fractionated by using an automated HPLC system, previously described by Zebühr *et al.*[10]. The NH_2-column (Waters, μBondapak™ 7.8 x 300 mm) when used in straight phase mode separates compounds according to their number of aromatic rings. By utilizing the observed retention times in straight phase mode at a flow rate of 3 mL/min for toluene, anthracene and phenanthrene three fractions of the eluate were isolated. The isolated fractions elute in the following order and are mainly composed of 1) MACs, 2) DACs and 3) PACs. Isolation of the PAC-fraction was accomplished by collection of the observed UV-detected peak when the NH_2-column was back-flushed with a flow rate of 5 mL/min. Subsequently an aliquot of the isolated PAC-fraction was further fractionated into 10 fractions in the automated HPLC system. The five first isolated fractions were collected during 5 minutes intervals between the first UV-detected peak and the observed complete elution time for chrysene. The following four fractions were collected during 5 minutes intervals until the observed complete elution time for perylene. By back-flushing the NH_2-column with a flow rate of 5 mL/min the 10^{th} fraction was isolated.

Nanoinjection

All the isolated fractions (13) and the total extract (1) including the isolated fractions from the procedural blank (13+1) were dissolved in triolein as carrier pending exposure to newly fertilized *O. mykiss* eggs using the nanoinjection technique[2]. When *O. mykiss* larvae have developed to about 60% of yolk-consumption the liver from each individual will be dissected and EROD activity will be analyzed.

Results and Discussion

Investigation of bioavailable compounds in decanted water from sediment

During the exposure no fishes died, nor could any adverse behavioural effects be observed. A dose-response effect regarding induction of EROD activity was observed between the different exposure groups (Fig.2). It is therefore concluded that bioavailable compounds with ability to induce EROD activity will be released in the Örsreum bay during the dredging process.

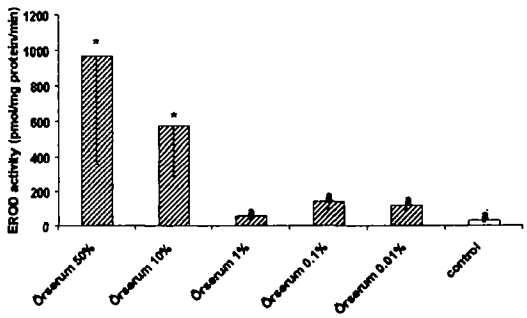

Figure 2. Average EROD activity and 95% confidence interval in liver from *O.mykiss* (n = 10) *exposed to different dilutions of the decanted water* from bottom sediment in the Örserum bay.
*: EROD activity is statistically significant higher (Kruskal-Wallis, $p<0.05$) as compared to the control.
a: No statistical significance difference regarding induction of EROD activity between these exposure groups.

Biomarker-directed fractionation and further perspectives

When the induction of the EROD activity in livers from *O. mykiss* larvae has been elucidated the most potent fraction/s will be further fractionated using preparative capillary gas chromatography (PCGC)[11]. By this method, compounds can be separated and collected at certain time intervals using a preparative trapping device. Six fractions can be obtained from each cycle and the procedure will be repeated until the amount of fractionated sample is equivalent to the fractions that was isolated using the HPLC system. Subsequently, *O. mykiss* eggs will be exposed to the isolated fractions using the nanoinjection technique. Exposure to newly fertilized *O. mykiss* eggs using the nanoinjection technique has proven to be a reproducible and very sensitive tool for mimic maternal exposure and thus investigations of early life stage toxicity[12]. Moreover, since this is an *in vivo* method using intact vertebrates we are able to investigate induction of EROD activity as a result of metabolism as well as a number of other relevant biological endpoints of high relevance, *e.g.* DNA adducts, endocrine disruption and histopathology.

Acknowledgements

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