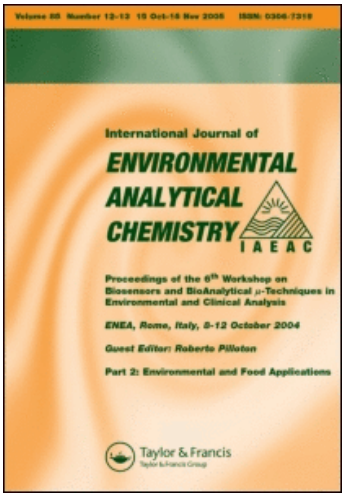

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The Determination of Polycyclic Aromatic Hydrocarbons in Indigenous and Transplanted Mussels (*Mytilys Edulis* L.) Along the Dutch Coast

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Dedicated to Professor W. Haerdi on the occasion of his 60th birthday

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A method, originally developed to investigate the pollution of Dutch coastal water with metals and PCBs,[1] was modified for the determination of the pollution with polycyclic aromatic hydrocarbons (PAH).

The method is based on active biological monitoring with mussels (*Mytilus edulis* L.). Its usefulness has already been demonstrated.[2] In the present study a method for the determination of PAH in mussels has been developed. The method is based on the hydrolysis of tissue with 4 M sodium hydroxide, extraction with hexane, clean-up with 10% deactivated aluminium oxide and quantitative determination with Reversed Phase High Performance Liquid Chromatography (RP-HPLC) and fluorescence detection.

The accumulation plateau of most of the PAH studied has not been reached after 60 days. Gradients of pollution were found, and at least one significant source near IJmuiden was detected.

KEY WORDS: PAH, reversed phase liquid chromatography, fluorescence biological monitoring.

1. INTRODUCTION

Xenobiotic compounds, compounds which are normally not present in organisms, may be actively metabolised by the organisms, or more often simply accumulate in the fatty tissue.

When bioaccumulation is matched by an equal elimination, the accumulation plateau is reached.

Monitoring uses the results of chemical analyses to compare environmental contaminations at different places or at one place at different times. The purpose of monitoring is to locate pollution and to study the change in concentration of pollutants with time and place.

Unpolluted areas are used in order to calculate the base-line levels. In the study reported here pollution was determined using Active Biological Monitoring (A.B.M.). With this type of monitoring, concentrations of xenobiotics are determined in tissue of organisms which are transferred from a relatively clean area to the area (probably polluted) under investigation.

The main advantages of the ABM method are the facts that: fluctuating contaminant concentrations are integrated over the duration of the exposure, and that direct information concerning biological effect on organism and exposure times are known.

In this study, the mussel *Mytilus edulis* (L.) was used: this organism has a pumping and filtering mechanism capable of concentrating compounds, including xenobiotic compounds, from water.

The study necessitated development of a method to analyse the PAH contents in the mussels. Hydrolysis with sodium hydroxide was used to destruct the mussel tissues.[3] This method is relatively cheap, and does not affect the PAH. Destruction with enzymes[3] was used initially, but found to be relatively expensive and time consuming. Separation of PAH from residual tissue can be achieved by extraction via steam distillation.[4] However, in several experiments low recovery of PAH was found with this technique: it later was evident that the PAH's tend to adsorb to residual fatty tissue. For this reason another extraction with hexane[5] was preferred. PAH are soluble in this solvent and its boiling point is relatively low, loss of PAH during extraction is therefore low.

Extracts were purified on a 10% deactivated aluminium oxide column[5] prior to analysis.

Other clean-up using partially deactivated silicagel,[7,8] silica SEP-PAK Cartridges (Millipore Waters, Etten-Leur, The Netherlands), or with liquid–liquid extraction[9] give unacceptable results.

Reversed phase liquid chromatography[10] was used for analysis of PAH. Because PAH fluorescence strongly, use of a fluorescence detector is preferred.

2. EXPERIMENTAL

2.1 Apparatus

The LC system was composed of a Waters (Etten-Leur, The Netherlands) model M-45 solvent delivery module, a Valco 7000 psi injector (Houston, TX, USA), and a LKB (Zoetermeer, The Netherlands) 113000 Ultrograrad solvent programmer. The column effluent was monitored with a Schoeffel (Rotterdam, The Netherlands) FS970 LC Fluorometer. The data was recorded with a Kipp & Zn. (Delft, The Netherlands) Fluorescence Spectrophotometer.

2.2 Stationary phase and column

The column was a laboratory-packed 250 × 5 mm I.D. stainless-steel column. The stationary phase consisted of 7 μm Lichrosorb RP-18 (Merck, Darmstadt, FRG).

2.3 Materials

Water was distilled via a quartz column, sodium hydroxide and sodium sulphate were purchased from Baker (Deventer, The Netherlands, Analytical Reagent), hexane from Mallinckrodt (Neunkirchen, FRG, nanograde), Alumina Woelm B Super I 04571 from Woelm (Eschwege, FRG), dichloromethane from Merck (Darmstadt, FRG, p.a.) and methanol from Rathburn (Walkerburn, Scotland, HPLC grade). The standards we used for qualitative and quantitative analyses were purchased from: Chemical Service (West Chester, USA) (anthracene (ANT), fluoranthene (FLU) and pyrene (PYR)), Schuchardt (Munich, FRG) (benz(e)pyrene (B(e)P) and perylene (PER)), TNO-OCI (Utrecht, The Netherlands) (benz(k)fluoranthene (B(k)F)), Eastman (Rochester, USA) (Benz(a)pyrene (B(a)P)) and

Aldrich-Europe (Beerse, Belgium) (coronene (COR)). Two internal standards were used to calculate recoveries and to quantify PAH-contents, 3,6-dimethylphenanthrene (DIM) (Analabs, North Haven, USA) and benz(b)chrysene (B(b)C) (Pierce Chemical, Rockford, USA).

For ABM mussels from the west coast of Ireland (Killry Harbour) were put and placed in cages and exposed at sites along the coast of the Netherlands. In this study, samples of Irish mussels exposed for 60 days at 13 such sites and samples of indigenous mussels from six sites were used. The sites were selected to facilitate the detection of any pollution gradient along the coast. Samples were additionally exposed for 10, 25 and 60 days at two locations to follow the accumulation in time.

To investigate the dependence of pollution on depth, samples were taken at several depths at one location. Unexposed Irish mussels and Dutch mussels for consumption were analysed as controls.

2.4 Procedures

2.4.1 Destruction of mussel tissue Homogenized mussel tissue was hydrolysed with 4 M sodium hydroxide. Five grams of tissue were added to 10 ml of 4 M sodium hydroxide in 25 ml flasks. 100 μl of a 3,6-dimethylphenanthrene solution (approx. 5 mg l^{-1}) and a benz(b)chrysene solution (approx. 0.5 mg l^{-1}) were added to the flasks, which were then shaken for three hours at 65 °C.

To determine recoveries, a standard solution containing 6 mg l^{-1} ANT, 1 mg l^{-1} FLU, 1 mg l^{-1} PYR, 5 mg l^{-1} B(e)P, 2.5 mg l^{-1} PER, 0.1 mg l B(k)F, 4.5 mg l^{-1} B(a)P and 10 mg l^{-1} COR was additionally added to homogenized tissue of unexposed Irish mussels. The recoveries of the components of this standard solution were used to correct the amounts detected in the mussel extracts. Linearity of detection was demonstrated in duplicate experiments in which 5 different volumes of this standard mixture were so used.

2.4.2 Extraction of PAH After hydrolysis, the sample was transferred quantitatively to a 100 ml separating funnel. Fifteen millilitres of hexane was added, and the separating funnel was shaken for two hours. After separation the hexane layer was collected, and reduced in volume to 1 ml in a stream of nitrogen.

2.4.3 Clean-up of the extract Ten percent deactivated basic aluminium oxide was used to clean-up the extract prior to analysis.

The aluminium oxide was activated at 800 °C for 4 hours, and then deactivated by adding 10% w/w water.

Just before the clean-up, a glass column (i.d. 1.5 cm, minimum volume 50 ml) fitted with a teflon tap (i.d. 2.5 mm), was filled sequentially with a piece of glasswool on the bottom, 2 grams of 10% deactivated aluminium oxide and 1 gram of sodium sulphate (washed with hexane and heated overnight at 300 °C).

The column was then washed with 20 ml of dichloromethane (dried with sodium sulphate) followed by 20 ml of hexane (dried with sodium sulphate): the mussel extract was then transferred quantitatively to the column, and eluted with a total amount of 25 ml hexane. The effluent was reduced to dryness under a stream of nitrogen, and then dissolved in 1 ml of methanol.

2.4.4 Analysis The mobile phase consisted of a gradient mixture of 90 v/v% methanol in water (A) and methanol (B).

The column was initially equilibrated with solvent A at a flow rate of 1.2 ml min^{-1}. A 100 μl aliquot of the sample was injected, and the solvent B was immediately initiated.

Peaks were identified by retention time and specific fluorescence (emission, excitation and synchronous scans of fractionated eluate from the end of the LC-line). The PAH were quantified by comparison of peak heights with those of the two internal standards.

3. RESULTS AND DISCUSSION

The recoveries (mean percentages and standard deviations) and the detection limits ($\mu g\, kg^{-1}$ wet mussel tissue) of the PAH investigated are given in Table 1.

It is assumed that the recovery of compounds accumulated by biological active organisms is comparable to that of the same compounds added in solution to tissue extracts. Further investigation with radioactivity labelled PAH to verify this assumption is recommended.

Figure 1 shows a typical chromatogram for the determination of PAH in mussel tissue. The peaks are identified in the caption.

Table 1 Mean (% *X*) and Standard Deviation (% S.D.) of the recoveries of PAH and internal standards, and detection limits (D.L., $\mu g\,kg^{-1}$ wet mussel tissue) of the PAH

PAH	*ANT*	*FLU*	*PYR*	*B(e)P*	*PER*	*B(k)F*	*B(a)P*	*COR*	*DIM*	*B(b)C*
% *X*	92	96	94	98	95	92	96	98	94	82
% S.D.	3	4	2	1	3	3	2	1	2	5
$n=6$										
D.L.	2	1	0.4	2	5	1	0.8	10	—	—

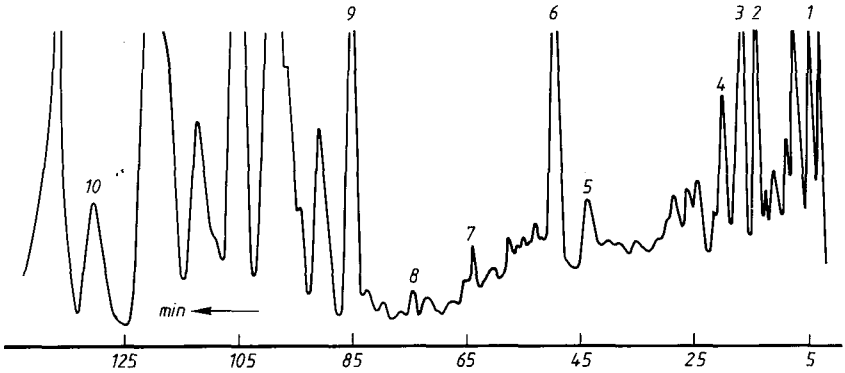

Figure 1 Typical chromatogram for the analysis of PAH in mussels. The peaks are : (1) anthracene, (2) fluoranthene, (3) pyrene, (4) 3,6-dimethylphenanthrene, (5) benz(e)pyrene, (6) perylene, (7) benz(k)fluoranthene, (8) benz(a)pyrene, (9) benz(b)chrysene and (10) coronene.

A typical calibration plot (for benz(a)pyrene) is presented in Figure 2: the other PAH showed similar curves. The gradients of the lines were used to calculate response factors.

A typical verification of peak identification (for that of anthracene) fluorescence by spectrophotometry is presented in Figure 3.

The PAH-concentrations found in tissue of Irish mussels exposed for 60 days and in the indigenous mussels are presented in Figure 4. The figures represent the mean values of the duplicates, and have been calculated using DIM as internal standard (calculations using B(b)C gave very similar results).

The calculated variation coefficients for the 21 samples analysed vary per compound: for example that for B(a)P is 11% using B(b)C

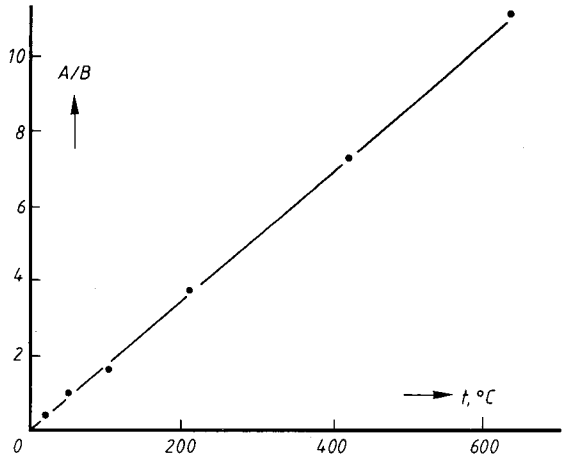

Figure 2 Calibration curve for benz(a)pyrene. A = peak height of benz(a)pyrene, B = peak height of the internal standard 3,6-dimethylchrysene, C = the standard concentration of benz(a)pyrene.

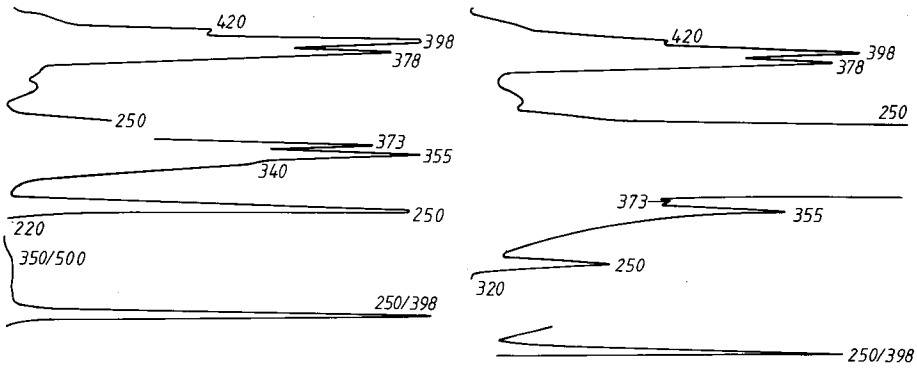

Figure 3 Verification of anthracene by emission, excitation and synchronous scanning fluorescence spectrophotometry.

or 8% using DIM, and that for ANT is 4% and 3%, respectively: values for the other compounds varied between 3% and 10%.

Figure 4 demonstrates the existence of pollution gradients in the

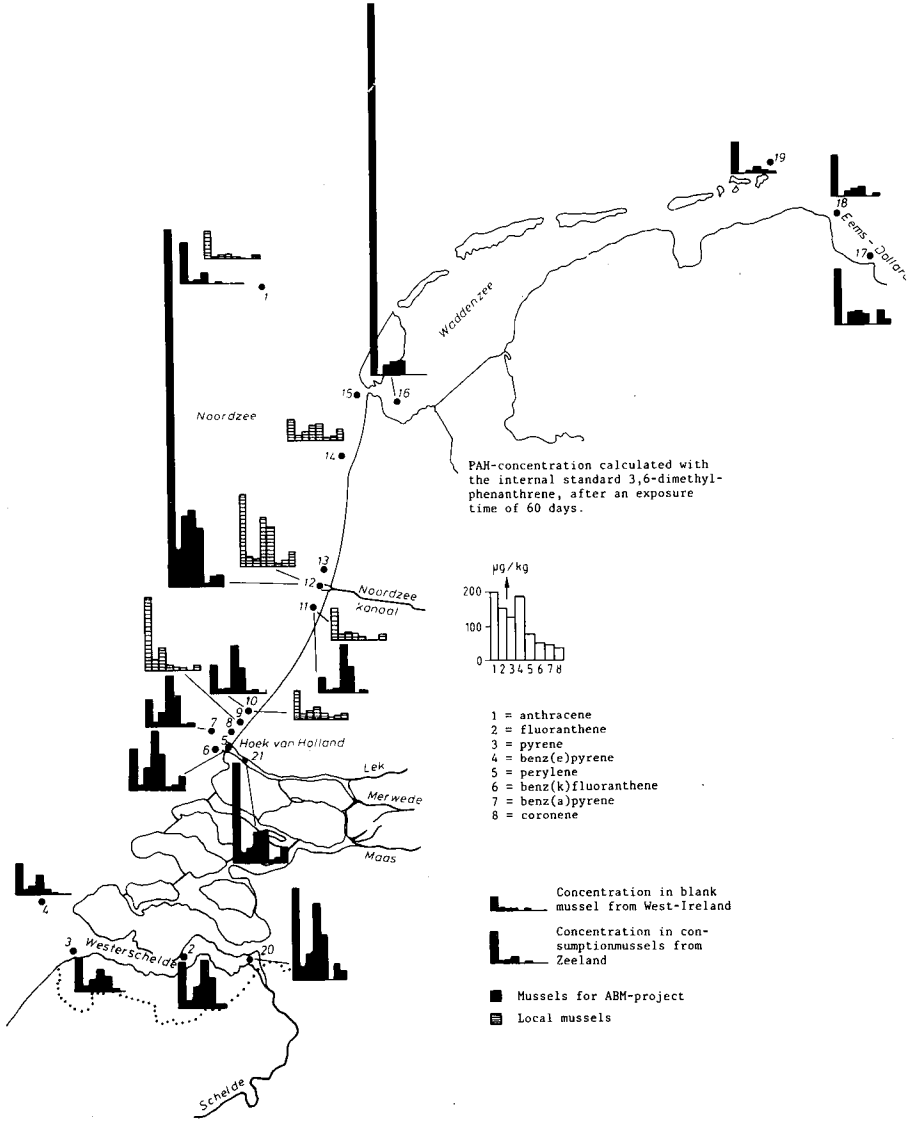

Figure 4 Average concentrations of PAH in mussels.

Westerschelde (sites 20, 2, 3 and 4), the Nieuwe Waterweg (sites 21, 5, 6, 7, 8, 9 and 10), the Eems-Dollard (sites 17, 18 and 19).

The generally high PAH concentrations at location 12, and the high ANT concentration at location 16 (verified in the duplicate analysis) are the most noticeable features of the data presented in Figure 4.

The PAH contents of indigenous mussels were not always comparable with those found in transplanted Irish mussels. It should, however, be noted that most of the ABM exposures took place in December 1979–January 1980, whilst the indigenous mussels were sampled in June 1980.

Figure 5 shows the accumulation curves for the 8 PAH found at location 17: those for location 12 were similar, except for the ANT (much higher at location 12) and FLU (undetectable at location 17).

After 60 days the accumulation plateau had not been reached in many cases. The apparent decrease in ANT content at location 17 may be due to a faulty analysis. However, if the results are correct the data may reflect a low half-life for elimination: Dunn and Stich[11] and Neff and Anderson[12] found the half-life for elimination of B(a)P from mussels to be approximately 15 days: that for ANT will probably be much shorter.

Table 2 presents the PAH-content in mussels exposed at 3 different depths: the PAH-contents increase with increasing depth.

4. CONCLUSIONS

—Hydrolysis of mussel tissue with sodium hydroxide, extraction of the PAH with hexane followed by clean-up with 10% deactivated aluminium oxide gave satisfactory results for the analysis of PAH in mussels. The PAH were easily determined, quantitatively as well as qualitatively, using fluorescence detection.

—Active Biological Monitoring with mussels seems to give useful results concerning pollution of Dutch coastal water.

—Gradients of pollution, and at least one significant source near IJmuiden, were detected. ANT concentrations are relatively high at several locations. Application of pattern recognition techniques to the composition of the PAH content of the mussels might

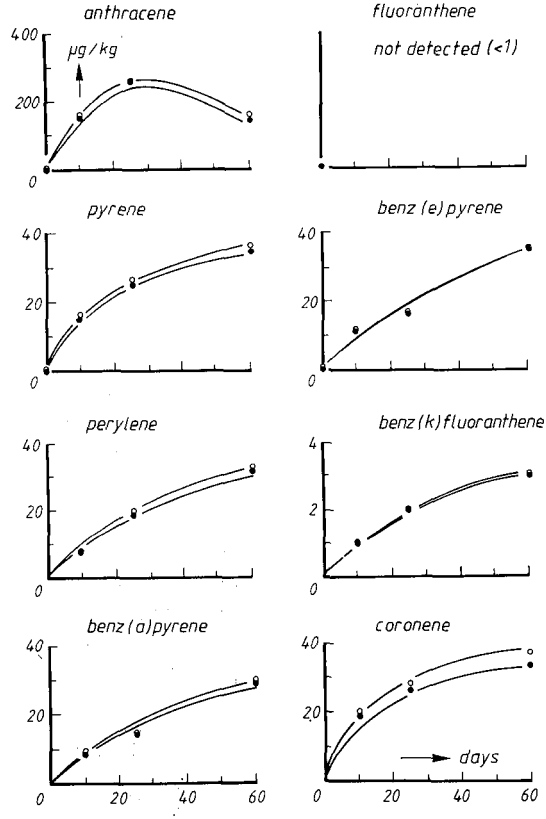

Figure 5 Accumulation of PAH ($\mu g\,kg^{-1}$ wet weight) in mussels exposed at location 17: PAH contents are calculated with 3,6-dimethylchrysene (●) and benz(b)chrysene (○) as internal standards.

Table 2 The dependence of PAH content ($\mu g\,kg^{-1}$ wet weight), calculated with 3,6-dimethyl phenanthrene as internal standard in mussels, exposed at location 21. Exposure time is 60 days

Depth	*ANT*	*FLU*	*PYR*	*B(e)P*	*PER*	*B(k)F*	*B(a)P*	*COR*
1 m	149	8	15	5	<5	1	<0.8	<10
3 m	187	21	22	19	8	1	<0.8	14
7 m	243	23	38	25	26	2	2	24

reveal that the chromatograms contain much more information than is suggested by a histogram representation.

—Recommendations for further chemical investigation can only be made when more data on the bio-accumulation and -elimination are available. Dispersion and destruction of PAH in the environment and in organisms, and the effects of season, fat-content and the dimensions of the mussels on the concentration of PAH in the environment should be further examined.

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