ASSESSMENT OF ECOTOXICOLOGICAL RISKS OF ELEMENT LEACHING FROM PULVERIZED COAL ASHES

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Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. C.M. Karssen,
in het openbaar te verdedigen
op dinsdag 30 mei 1995
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

STELLINGEN

1. Van de uit poederkoolvliegas vrijkomende elementen, welke in marine bodem-organismen accumuleren, is in kwantitatieve zin arseen het belangrijkste element.
   - Dit proefschrift.

2. Daar bij arseen geen en bij seleen wel biomagnificatie optreedt, moet de conclusie zijn dat, bij het benodigde verdere onderzoek naar speciatie en biologische beschikbaarheid, seleen op de eerste plaats staat.
   - Dit proefschrift.

3. Het ontbreken van duidelijke effecten op de ontwikkeling van de glochidiën van Unio's, bij blootstelling aan relatief hoge seleenconcentraties, is, op grond van de over vissen bekende literatuurgegevens, een onverwacht resultaat geweest.
   - Dit proefschrift.

4. Het verdient aanbeveling nader te onderzoeken of het autoclaveren van voedings-oplossingen met Fe-EDTA de stabiliteit van het EDTA negatief beïnvloedt.
   - Dit proefschrift.

5. Reeds in 1980 wees Allen *et al.* op de rol van speciatie en de daarmee samenhangende biologische beschikbaarheid van elementen bij het opstellen van kwaliteitscriteria voor metaal concentraties in het aquatisch milieu. Ten onrechte wordt dit belangrijke gegeven vermeden bij het definiëren van criteria voor normstelling.
   Allen HE, Hall RH, Brisbin TD (1980) Environ Sci Technol 4:441-443.
   - Dit proefschrift.

6. Het positieve effect voor de kustvisserij enerzijds en de bescherming van de dijken en duinen anderzijds, welke beide op dit moment in de belangstelling staan, zou een stimulans moeten zijn voor meer onderzoek naar het gebruik van gebonden toepassingen van reststoffen in zogenaamde 'waste blocks'.
   - Dit proefschrift.

7. De toets met eendekroos is sterk verbeterd door de toepassing van beeldverwerking waardoor de methode eenvoudig en goed reproduceerbaar is geworden.
   - Dit proefschrift.

8. Binnen de recente discussie over de risico's van het gebruik van chloor verdient koelwaterchlorering voor aangroeibestrijding aanmerkelijk minder aandacht dan het gebruik van chloor in chemische produktieprocessen.

9. Het in de praktijk toepassen door overheid en waterbeheerder van de gezamenlijk door TNO, RIVM, KEMA en Delta Consult ontwikkelde MosselMonitor® voor waterkwaliteitsbewaking blijkt, tot nu toe, even uniek als het unieke karakter van dit bekroonde ontwerp.

10. Het verdient aanbeveling de zoetwater Unionidae, die een leeftijd kunnen bereiken van meer dan 50 jaar, wettelijk te beschermen en toe te voegen aan de registratielijst van de 'Wet op de Proefdiekunde'.

11. De tien jaar geleden gesignaleerde toekomstige problemen met de opslag van poederkoolvliegas vormen de basis voor dit proefschrift. Door de inspanning welke de E-sector heeft gepleegd in verbetering van de vliegaskwaliteit wordt op dit moment vrijwel alle vliegas toegepast. Dit heuglijke feit maakt de basis voor voortzetting van het onderzoek wel smaller.

H.A. Jenner
Assessment of ecotoxicological risks of element leaching from pulverized coal ashes.
Wageningen, 30 mei 1995

Behoed mij voor de dovende sintels,

verborgen onder de as.

Rabindranath Tagore,

Stray Birds, circa 1910

Voor Wilna

VOORWOORD

Het in dit proefschrift beschreven onderzoek is tot stand gekomen mede dankzij de inzet en het enthousiasme van een aantal mensen die ik hierbij graag wil bedanken.

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Het hier beschreven onderzoek is uitgevoerd bij KEMA Nederland BV (Arnhem) en TNO (Den Helder) en financieel mogelijk gemaakt door de Elektriciteitsproduktiebedrijven.

"Now let us take the tree, subject it to aerobic and anaerobic decomposition, mix its components thoroughly, bake at low heat for 200 million years, and determine the structure of the resulting mass, if one has mixed the debris from a variety of plants and added a variety of minerals, that is the problem facing the coal chemist"

Green *et al.* 1982

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

The present study has been carried out in the framework of the ecological assessment of coal combustion waste disposal. Special emphasis has been placed on marine, freshwater and terrestrial bioassays.

Electricity generation by burning fossil fuels such as coal, natural gas and oil implies acceptance of the production of residues in solid form and the formation of gaseous components. Coal firing results in large amounts of solid residues. In all coal fired power plants the fly ash is removed by fly ash precipitators. This means that solid residues are formed. In addition, flue gases in large power plants in The Netherlands are also desulphurized by means of a wet limestone process which produces effluents containing several elemental contaminants. In the Netherlands until recently, coal comprised about 50% of all fuels used. It is expected that this percentage will continue to rise slightly up to the year 2000. The total installation capacity in the Netherlands amounts to more than 15000 MW. Currently, this is comprised of 50% coal, <2% oil, 40% gas and 8% nuclear energy. The use of hydro-electric power and wind energy is still modest.

The allocation of fossil fuels is determined to a large extent by prices on the world markets. This explains the current large-scale use of coal for boiler burning. Forecasts indicate an increase in electricity demand of 1.1% per year to the year 2000. This will result in an additional growth of coal firing capacity of at least 5% - equivalent to an increase in coal use of approximately one million metric tonnes. In addition, natural gas will continue to be important and the import of relatively inexpensive electricity generated by hydropower will increase. Total current coal use is around 10 million metric tonnes per year. At an ash content of about 11%, the total amount of coal residues is nearly one million metric tonnes. In The Netherlands, only pulverized coal fired dry bottom boilers are installed.

COAL COMBUSTION

Today, coal firing is achieved by first grinding the coal into fine particles and, aided by preheated air in the boiler, spraying and burning the resultant pulverized coal. The combustion temperature is around 1600 °C. At this temperature most inorganic components have become gases or fluids. Temperatures in the boiler range between 1600 and 800 °C. Residence time of the coal particles in the flame is short, one to two seconds, after which there is a relatively rapid cooling down. The liquid particles are quenched and condensation of the gaseous components will occur on the outside. The complex physical and chemical reactions within the boiler produce a combustion product, pulverized coal (fuel) ash (PFA), whose composition of chemical properties is variable. The larger dust particles sink in the boiler, often coagulating into larger pieces, the so-called bottom ash. The amount of non-combustible material is about 11%. Of this, approximately 85% is PFA and 15% bottom ash. The PFA is carried to the electrostatic precipitators along with the combustion gases and separated.

A new development is the application of coal gasification. In this process, combustible gases (CO and H_2) are first removed from the coal by means of steam and high temperatures under reduced oxygen conditions. This mixture of gases is burned in the boiler. The residues are comprised of a heavily vitrified, slag-like product and flue gas. The ratios here, however, are approximately 15% flue gas and 85% slag, depending upon the type of process. The coal ashes can be distinguished as:

- Bottom ash. Bottom ash is created through the fusion of the larger particles in the boiler that sink to the bottom and, after being cooled by water and solidifying, are collected in a separate bunker
- Pulverized Fuel Ash (PFA). PFA is the precipitated fly ash. It is a fine greyish coloured, dust-like granular residue which is transported with the flue gases from the boiler to the electric precipitators. After precipitation the dry PFA is stored in bunkers and traded
- Fly Ash. This comprises 0.1 - 0.01% of the precipitated flue gas that, in principle, is emitted from the stack (fraction <10 µm). With the introduction of wet scrubbing of the flue gases for desulphurization, this last part of the flue gas automatically precipitates (>90%) in the water phase, ending up in the gypsum and/or in the waste water from the gypsum works.

On the basis of studies by Meij *et al.* (1983) and Meij (1989), a classification has been set up for the enrichment in concentration of macro-elements and micro or trace elements during the combustion process. A trace element in coal is defined as an element ranging up to about 1000 µg/g.

A three-level classification has been made:

Class I: not volatile during combustion, the concentration in the ashes (bottom ash, PFA and fly ash) will be alike

Class II: volatile, with a redistribution occurring on and in the different ash particles

Class III: very volatile, little or no condensation.

The classification in Table 1 is based on element behaviour during the combustion process. In Class **I**, from an environmental point of view, only Cr is an important element. In Class **II**, however, the other elements As, Mo, Cd, Cu, Ni, Pb, Zn are found. In Class **III**, the important elements are Se, B and Hg.

Coal contains all the elements originally present in the biota from which the coal formation took place (Carboniferous era: 255 - 203 million years ago). During coalification in a coal swamp all elements are concentrated 10^2 - 10^3 times. The concentration process consists of accumulated elements from the swamp biota but there is also an input from atmospheric dust and salts from lakes and seawater can also be expected (Swaine 1990).

Table 1. Classification of elements and trace-elements based on research at power stations in The Netherlands (after Meij 1992).

Class	**I**	I, Ca, Ce, Cr, Cs, Eu, Fe, K, La, Mg, Na, Rb, Sc, Sm, Sr, Si, Th, Ti
Class†	**IIa**	As, Cd, Pb, Sb, Ti, Zn
	IIb	Be, Co, Cu, Ge, Mo, Ni, P, U, V, W
	IIc	Ba, Mn, Rb and Sr
Class	**III**	B, Br, C, Cl, F, Hg, I, N, S, Se

†Class **II** is subdivided into three classes according to degrees of volatility

A second enrichment, by a factor of 10 occurs in combustion. Approximately 75% of the precipitated PFA is smaller than 40 μm. The finest fraction possesses the largest specific surface and contains the highest concentrations of Class II and III elements, partly due to condensation processes. Some physical properties of pulverized fly ash (PFA) are shown in Table 2.

In dry form PFA is a fine light gray to dark gray colored powder. Dry, it looks on cement powder and moistened it resembles a fine clay. The physical and chemical properties of PFA are determined to a large extent by the properties of the coal fired, as reflected by their 50 million year evolution. The texture of PFA closely resembles that of sandy loam. Chemically, PFA is made up primarily of aluminium silicates, iron oxides and alkali metals.

Table 2. Physical properties of pulverized fly ash (PFA), according to Bolt (1983); Hodgson (1983); Weststrate (1985).

Specific Density	2000 - 2400 kg/m^3
Dumping Density	800 - 1000 kg/m^3
Grain size	75 % < 40 μm (comparable to light loam/sand loam)
Specific Surface	3000 - 4000 cm^2/g
Capillary Rise	*c.* 2 m
Porosity	35 - 40 % (optimum compression)

The dominating crystalline phases in the different coal ashes are mullite ($3Al_2.2SiO_2$), α-quartz (SiO_2) and the iron oxides magnetite and hematite (Liem *et al.* 1983). A division can be made between macro-elements and a large number of micro-elements or trace elements. Table 3 provides an overview of the ranges and concentrations of elements in PFA produced in the Netherlands. For comparison purposes, the soil ranges for the Netherlands are given. In studying the table, it is clear that the macro-elements agree well with the values for conservation areas in the Netherlands. For PFA, concentrations of Al and Si are higher, but it should be borne in mind that conservation areas in the Netherlands are situated on poor soil, which contains few clay minerals such as Al and Si.

For the micro-elements, it is evident that the distribution from combustion over the three coal residual flows, seen as differences in concentrations, can vary greatly. Of the three classes of elements, the anionic elements, As, B, Sb, Se, Mo, W, V and, to a lesser extent, Cr, leach out fairly well, leading to relatively high concentrations in the leachate. The order for the leaching capability of the anionic elements is Se > Mo >> B = W > V > Sb > As > Cr (Van der Sloot *et al.* 1984). This leaching behaviour and ranking is caused by the fact that all elements are bound on the surface of the fly ash particles. Se appeared to be bound for 80 - 100% to the surface of the ash particles. Also, Se is found in substantial quantities in the ash. The cationic elements are normally strongly bound in the matrix of the fly ash particle, with subsequent low leaching behaviour. The reader should bear in mind that well known toxic elements such as Cd and Hg are seldom found in coal ashes and are of minor importance in the studies carried out.

NEW BURNING TECHNIQUES

New techniques, such as desulphurization, have been applied to clean flue gases and also improved burners to reduce NOx emission. These new burners produce a PFA that deviates from the physical and chemical properties of the conventional PFA. An increasing number of boilers are being rebuilt with these low NOx burners, thus increasing the production of low NOx fly ash. The chemical composition of low NOx ash and "conventional" ash is approximately the same (Meij 1992). The most important physical differences between PFA and low NOx PFA (Bolt *et al.* 1988) are:

- size distribution; the fraction <10 μm is smaller with low NOx ash
- low NOx flue gas has a larger specific surface. This means that porosity has to be greater
- scanning electron microscope photos show a more coagulated morphology of the fly ash.

Also, leachability of elements is of the same order, compared with conventional fly ash. In summary the differences are found mainly in physical properties.

Table 3. Average composition of coal combusted in the Netherlands together with the concentrations in the bottom ash, the collected PFA and emitted fly ash (assuming an ash content of the coal of 11%) modified after Meij (1992) and in the top soil (10 cm) of conservation areas in the Netherlands, after Edelman (1983) and Lexmond and Edelman (1992).

	COAL	BOTTOM ASH	PFA collected	FLY ASH emitted	TOP SOIL concentration range
macro elements, concentration in %:					
Al	1,81	16,45	16,45	16,45	0,05 - 9,0
Ca	0,25	2,27	2,27	2,27	< 0,01 - 0,65
Cl	0,07	0,01	0,01	0,06	-
Fe	0,59	5,36	5,36	6,44	0,08 - 3,9
K	0,17	1,55	1,55	1,55	0,27 - 2,18
Mg	0,10	0,91	0,91	0,91	0,08 - 1,18
Na	0,05	0,45	0,45	0,91	0,02 - 0,66
P	0,01	0,05	0,09	0,36	< 0,01 - 0,65
Si	3,13	28,45	28,45	28,45	0,03 - 0,05
Ti	0,07	0,64	0,64	0,64	0,03 - 0,48
micro elements, concentration in µg/1:					
As	7	0,6	64	318	1,6 - 28
B	35	< 100	159	318	-
Ba	350	1591	3182	5727	90 - 468
Be	4	18	36	76	-
Br	8	0,7	4	36	2,1 - 142
Cd	0,15	0,1	1	12	< 0,05 - 1,8
Ce	24	218	218	218	1,5 - 8,1
Co	7	45	64	134	0,3 - 16
Cr	20	182	182	182	11 - 117
Cs	1	9	9	9	0,57 - 9,7
Cu	20	109	182	418	0,83 - 50
Eu	0,4	4	4	4	0,08 - 1,7
F	70	6	64	1909	-
Ge	4	-	36	91	-
Hg	0,2	0,2	0,9	1,8	0,02 - 0,51
La	10	91	91	91	1,4 - 49
Mn	80	727	727	1309	19 - 769
Mo	3	14	27	95	0,1 - 40
Ni	15	68	136	477	< 0,5 - 47
Pb	15	55	136	818	3,2 - 200
Rb	15	136	136	191	8,9 - 152
Sb	1,5	3	14	82	0,3 - 3,0
Sc	4	36	36	36	0,53 - 16
Se	3	0,3	14	191	-
Sm	3	27	27	27	0,28 - 8,2
Sr	120	1091	1091	2182	-
Th	5	45	45	45	0,23 - 13
Tl	0,5	< 4	5	23	-
U	1,5	7	14	29	< 0,1 - 7,7
V	35	191	318	955	4,1 - 126
W	1,5	8	14	41	0,5 - 83
Zn	25	23	227	1591	6,4 - 189

APPLICATION AND DEPOSITION OF PFA

In The Netherlands the re-utilization percentage of PFA has been high. In 1992, about 95% of the total production was used. The expectation at that time, however, was that this percentage could not continue to be achieved, especially due to the increase in low NOx PFA. In 1992, of the 800.10^3 metric tonnes of produced PFA, 630.10^3 metric tonnes was supplied to the cement industry, 46.10^3 metric tonnes was used for asphalt fillers and another 124.10^3 went into synthetic gravel for use in the concrete industry. In Figure 1 the use of coal residues is presented according to information provided by the "Vliegasunie" (1993). In other countries, PFA that is not directly useable is employed for reclamation and embankment works (Denmark, Japan), or for "landfill" projects (U.K.), in order to close existing gaps in the landscape created by mining activities. Additionally in the U.K. PFA was dumped in the North Sea for many years (Bamber 1980; 1984; Eagle *et al.* 1979). Readily available solutions are chosen in the U.S.A, including slurry transport to lagoons (Adriano *et al.* 1980; Cherry *et al.* 1984). A separate application is found in agriculture, in which water repellant soils are improved by mixing PFA in the top layer (Hodgsen & Holliday 1966; Roberts 1966; Hodgson & Townsend 1973; Chang *et al.* 1977; Bestebroer & Janssen-Mommen 1992). The use of fly ash/bottom ash, possibly in combination with desulphurization products, in concrete frameworks for building artificial reefs is an option that is not yet often chosen (Woodhead *et al.* 1985). Similar experiments with concrete "blocks" are currently being conducted in the U.K. As far as the situation in the Netherlands is concerned, a solution will have to be found for the non-useable part of the PFA/low NOx PFA. Dumping on land, in freshwater and/or seawater is, for the time being, unacceptable. Compared to other countries in the world The Netherlands is absolute leader in the reuse of combustion residues today.

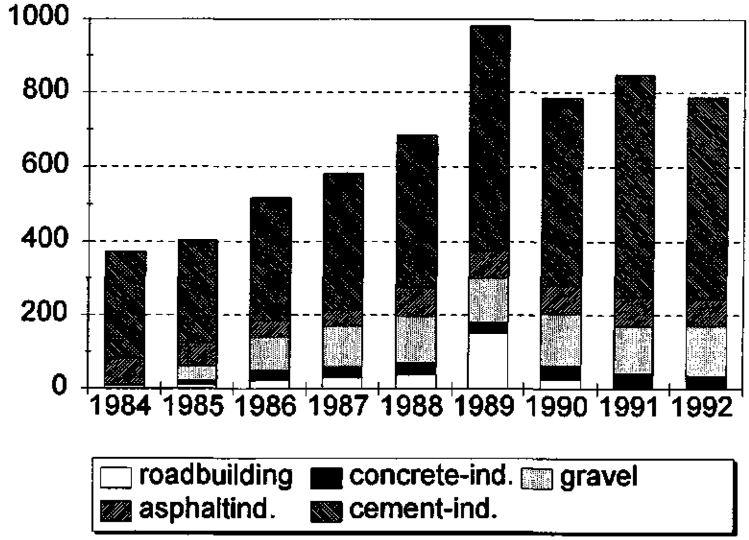

Figure 1. Histogram of the distribution of coal residues with regard to their application during the past decade.

ECOTOXICOLOGICAL HAZARDS OF COAL COMBUSTION

Open storage on land of PFA, or dumping in the aquatic environment, can lead to the leaching of elements (Theis 1975). Leaching from land disposal was demonstrated by Hjelmar (1990) in large-scale lysimeter tests with neutral/alkaline PFA at which the major trace elements were: As, Cr, Mo, Se and V. For minimizing the environmental impact it was proposed to implement a "controlled or reduced leaching" disposal strategy by using top layers of clay and sand. Warren and Dudas (1988) studied the adsorption and co-precipitation of trace elements and macro elements and concluded that nearly all elements mobilized in PFA leaching will be associated with the newly formed secondary minerals, *i.e.* As and Fe minerals, which can alter the bioavailability of the elements in question. In the aquatic environment the disposal of wet sluiced PFA can smother benthic invertebrates (Cherry *et al.* 1984; Dvorak *et al.* 1978) and elements can be accumulated to potential toxic concentrations (Guthrie & Cherry 1979a; 1979b). Hatcher *et al.* (1992) have studied lake sediments and biota near a coal ash basin on the shoreline of Lake Erie. In the sediments As and Co was more concentrated (except in spring). In fish the elements As, Se, Ni and Cr were higher compared to the reference sites. In the case of dumping in the aquatic environment, the PFA forms a substratum and is directly available for transferring elements to benthic invertebrates like the freshwater Unionidae. Along the indirect route the biota comes into contact with the leachates, for example, by means of the drainage system of a dump. In the case of artificial reefs build up by "waste blocks" a favourable effect was found on fisheries due to concentration of fish around the reef (Woodhead *et al.* 1985). Biomass collected from "waste blocks" and controls showed no increase in element concentration over two years of exposure. The number of ecotoxicological studies with non-stabilized and stabilized PFA, as mentioned above however, are scarce and the impact on existing benthic invertebrate populations is virtually insufficient.

Concentration levels in tissues may increase by element uptake referring to the term accumulation or bio-concentration: During accumulation, concentration levels in an organism increase in time (growth and/or age) since accumulation exceeds elimination (elimination = secretion + metabolism). Bio-accumulation is a broader term in the sense that it usually includes not only bio-concentration but also any uptake of toxic substances through consumption of one organism. Bio-magnification is the resultant total accumulation through enrichment via the food chain of at least two or more trophic levels. In this study the uptake of elements in organisms is expressed as the difference between exposed and control tissue levels. This supports the more actual picture, excluding the problems with bioavailability which arise by direct comparison of sediment pore water and tissue levels.

In principle, all elements occurring in the natural environment can be found in PFA. Originally, the elements were concentrated in the plants before coalification by natural accumulation processes as mentioned earlier. As a result of these processes there was an accumulation particularly of the essential elements. Non-essential elements such as Cd and Hg are only found in very low concentrations in plants. Their concentrations could be a contribution from atmospheric and/or a waterborne input during the coalification process. Literature on leaching of elements and their chemical approach is available on a rather large scale but to what extend PFA forms an ecotoxicological hazard is not yet investigated in detail.

RESEARCH OBJECTIVES AND APPROACH

The environmental engineering restrictions that are being considered by the government, will demand a major investment in the arrangement of temporary and/or permanent storage facilities. More background information is needed on the actual environmental risks of PFA deposition in the face of restrictive legislation.

The main objective of this study was to assess the effects on representive organisms, after exposure to PFA or leachates of PFA. The studies dealt primarily with toxic effects and focused on the impact of PFA on single species and groups of related species including their acute effects, bioconcentration and ultimate body burden. Emphasis was placed on reproductive effects in this study. Crawling behaviour of mussels was also studied to reflection to the physical differences of PFA from other substrates. A newly developed device was therefore used for valve movement monitoring. A phytomonitoring system with duckweed was developed for assessing effects on yield, using image processing.

The results are presented in three parts according to the environmental compartments concerned *i.e.* marine, freshwater and terrestrial. In Part 1, marine studies with benthic invertebrates were carried out in model ecosystems with different compositions of PFA and Waddensea sediment. In Part 2, the freshwater studies were carried out in flow chambers using the painters mussel *Unio pictorum*. Besides behavioural studies with PFA specific research was carried out with selenium on body burden and effects on reproduction. Selenium is a prominent constituent of PFA. In Part 3 research is described on the monitoring of leachates of PFA with duckweed. A separate chapter deals with growth, mortality and accumulation in plants and worms exposed to coal gasification slag.

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DESCRIPTION OF THE EXPERIMENTS, AND PRINCIPLES OF TEST METHODS

This chapter provides background description on the materials, methods and species used in the different experiments.

EXPERIMENTS IN THE MARINE ENVIRONMENT

The experiments were focused on benthic macro-invertebrates. The following species were used: the cockle *Cerastoderma edule* (L.), a suspension feeder that burrows just under the surface and possesses two short siphons; the balthic tellin *Macoma balthica* (L.), a deposit and suspension feeder, burrows relatively deeply (5 - 7cm) in the sediment and possesses two long, thin siphons for collecting sedimentary particles and plankton; the lugworm *Arenicola marina* (L.), a polychaete and a direct deposit feeder. *A. marina* burrows in the substrate, living in a U-shaped burrow which is open at one side. It is capable of living in anaerobic sediments if its burrow is well aerated; the rag-worm *Nereis virens* Sars, a polychaete worm which builds semi-permanent tubes. The mature worms are predators, using their extendable proboscises with jaws. *N. virens* is considered to be a well-adjusted animal that can meet new situations as they arise (Ricketts & Calvin 1939).

The experiments, as described in Chapter 3 and 5 were carried out by TNO-MW Den Helder at their experimental site in Den Helder harbour (Photo 1). Large circular tanks (2.2 m^3), called model ecosystems (mesocosms), were used. The bottoms of the tanks were covered with *c.* 25 cm of sediments of different compositions. The depth of the seawater above the sediments was *c.* 45 cm and this level was maintained throughout the exposure. Seawater was pumped continuously from the harbour through the mesocosms (30 litre/h). The water was not filtered and, therefore, contained all the necessary plankton and other food particles. The theoretical water replacement time was 50 h. Four different types of sediments were used: 100% pulverised fuel ash (PFA); 50% PFA and 50% sand from the Waddensea; 100% Waddensea sand as a control and, as a reference, a contaminated harbour sludge. To mimic dumping of PFA, a construction was built whereby 500 ml of PFA was dosed on a daily basis. Care was taken to ensure an even spread of PFA over the mesocosm in question. *C. edule* (chapter 5) were monitored after 230-days (September to April). These cockles originated from the previous experiment (chapter 3) which had ended in early December.

Photo 1.Mesocosms at TNO-MW Den Helder.

The experiments set out in Chapter 4 were carried out at a farm which specializes in breeding *Neridae* species (Topsy Bait) in Wilhelminadorp in Zeeland. A container system was constructed by special coated wooden panels (used in the making of concrete constructions) as shown in Figure 1 and Photo 2. The panels were joined with a single-component poly-urethane resin. Each container (100 x 35 x 35cm) housed a 10 cm layer of sediment covered by a 20 cm seawater column (225 l). Seawater was fed into the containers through PVC tubes at a flow rate of 3 l/min. All containers were aerated and in each of the four blocks of three (each block had an 800 l buffer tank for partial recirculation), 90 litres of water was replenished daily.

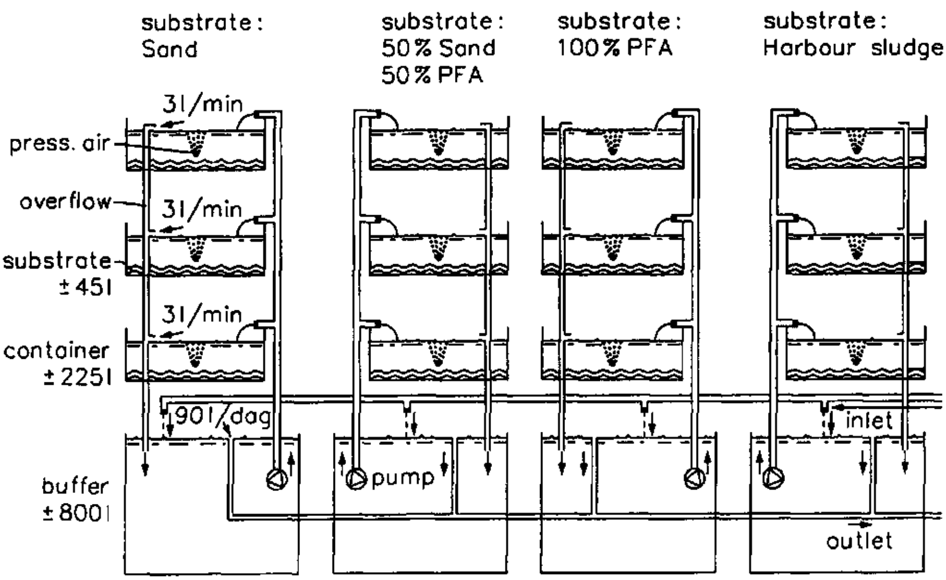

Figure 1. Outline of the experimental container setup for '*Nereis*'.

Photo 2. Container setup '*Nereis*' experiment.

EXPERIMENTS IN FRESHWATER

The accumulation and behavioural experiments with the freshwater mussel *Unio pictorum* were carried out in the KEMA Rhine laboratory situated directly beside the northern branch of the River Rhine. River water was pumped by means of a submersible pump to the laboratory where it was sieved (pore size 4 x 5 mm) before entering the laboratory for the removal of debris, empty *Dreissena polymorpha* shells, and small crayfish (*Orconectus limosus*). In the laboratory, the water passed through a lamellar filter for the removal of larger suspended matter, without reducing the plankton supply, and entered a buffer container in which a centrifugal pump pressurized the water to 2 bar. The lamellar filter was constructed of double polycarbonate plates fixed together in two blocks 50 cm wide, 70 cm long and 30 cm thick. Both blocks were placed at an angle of 55 degrees to the horizontal in order to ensure reliable operation. The water level in the storage container was checked with a level switch (Mobrey Sensall Ultrasonic Liquid Level Switch 005). The experiments were conducted in two blocks of 4 flow chambers, each chamber measuring 200 x 20 x 20 cm (l x w x h) and having its own water supply (Photo 3). The chambers and buffer container were made of resin-coated wooden panels similar to those used for the seawater containers in the *N. virens* experiments. Fine adjustments of the water flow were caried out by means of valves. Flow was continuous measured by pressure gauges (Validyne, model CD 18). A water flow rate of between 1 and 10 litre/min was possible, but the experiments described in the Chapters 7, 8 and 9 were all carried out with a flow rate of 1 - 5 l/min., which enables a refreshing rate of each flow chamber in 2 - 10 minutes. Oxygen concentrations were measured with an O_2 electrode (WTW OXI-91, Germany) and water temperature with a Pt 100 sensor via an AD-DA conversion card (Analoge Device) in a PC. The flow chambers were filled with Rhine sediment obtained in the vicinity of the laboratory and PFA from the Amer power station (batch A8850).

The mussel *Unio pictorum*

The mussel *Unio pictorum* L. (Photo 4) belongs to the family of *Unionidae,* a group of free living bivalves with a preference for muddy clay substrates (Jansen & de Vogel 1965; Lewis & Riebel 1984). The mussels are rather active and crawl or burrow quite a lot. They move by using their feet as anchors; the foot is protruded and the body then pulled after it (Trueman 1968). During these activities the valves are opened and closed regularly. In the winter, the mussels burrow themselves into the substrate and enter a form of hibernation (at least activity is much lower). Reproduction starts in spring. The fertilized eggs are retained in the outer gills and brooded in so-called marsupia until the larvae (glochidia) are large enough to be released (Harms 1907). The glochidia of *U. pictorum* attach themselves primarily to the gills of fish in this way distributed throughout the water body. According to the study of Comfort (1957) on the duration of life in molluscs *U. pictorum* can reach a remarkable lifespan of 13 - 15 years. He concludes that the normal maximum age for *Unio* and *Anodonta* species is probably not more than 20 - 30 years. Therefore sampling of large numbers and killing of the animals was avoided as far as possible.

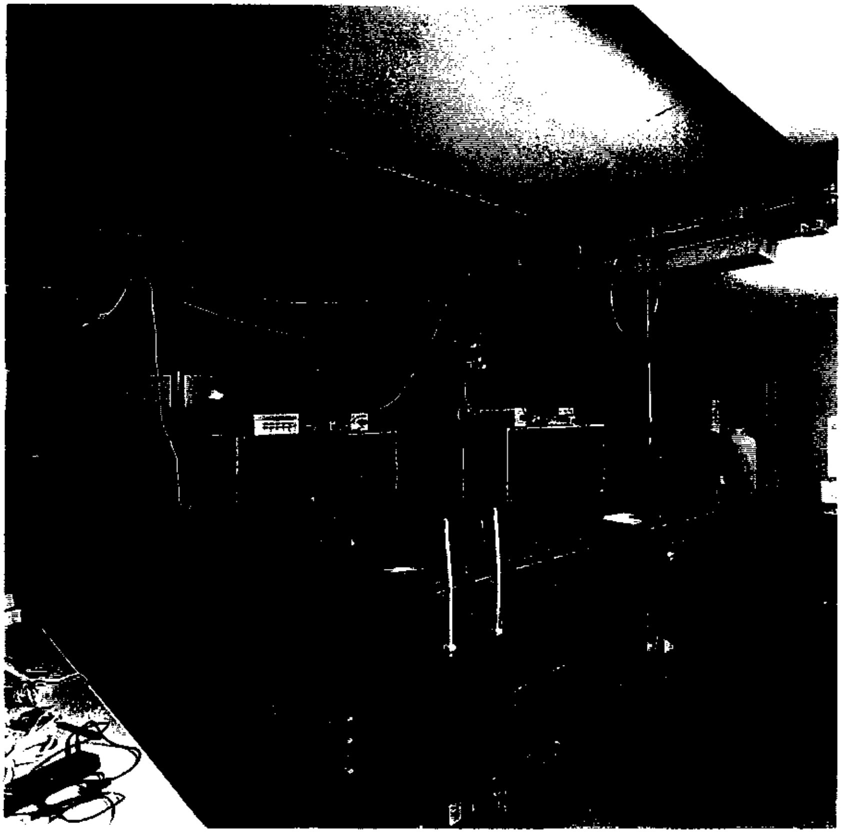

Photo 3. Photograph of the flow chambers at KEMA laboratory.

Life span is presumably highly dependent on growth rate which is correlated with water temperatures. *Margaritana margaritifera*, living in colder streams, is suspected of having by far the longest lifespan of any European species; ages of 60 to 100 years may well be attained.

Negus (1966) estimated the biomass of the standing crop of *U. pictorum* in the River Thames, at Reading, to be 382 kg/ha. In the River Linge tributary of the Rhine, the biomass is estimated to be two to four times higher (pers. observ.). The mussels are filter feeders: water containing food particles is sucked in via the inhalent siphon, sieved by the gills and released via the exhalent siphon. The necessary water current is generated by cilia on the gills and the flow is normally adjusted by either closing or opening the inhalent siphon but fine adjustments can be made with a lobate part of the mantle inside the exhalent siphon. The larger particles and indigestible fine sediments are packed in slime and released as pellets through the inhalent siphon. This activity causes rapid opening and closing of the valves. For the River Meuse, the total filtration capacity of the Unionid population was assessed as being 4,25 litres per m^2/h (Libois & Hallet-Libois 1987).

Valve movement detection systems

In recent years interest has been increasing in the development of biological warning systems for water quality surveillance. Chemical spills originating from ships and smaller releases from plants such as the 'Sandoz' accident (Kinzelbach & Friedrich 1990), can affect aquatic biota rapidly without immediately attracting the attention of the authorities. In a river system, a spill will pass downstream as a discrete water mass, affecting the biota while being gradually diluted. The existing physico-chemical analyses are often time consuming, restricted to a certain group of chemicals and involve high labour and other costs. Kramer and Botterweg (1990) reviewed current biological monitoring systems and concluded that while quite a number are present, relatively few have been tested and even fewer have reached a state of operational testing or commercial production.

Biological early warning systems have to comply with certain criteria in order to act as reliable alarm triggers for water pollution, (Koeman *et al.* 1978; Cairns 1979; Diamond *et al.* 1988):

- (semi) continuous measurement of behavioural or physiological patterns in organisms with automated detection of (short-term) changes in environmental conditions by evaluating the reaction of the organisms under observation

- reliable and fast response
- high sensitivity to a broad spectrum of pollutants
- easy handling of apparatus and organisms combined with a low maintenance requirement
- comprehensible and fully documented output.

Valve movement studies at KEMA have been carried out with *Unio pictorum* and *Dreissena polymorpha* on the effects of well known toxicants such as copper, cadmium and chlorine but also on the effect of high suspended solids (KEMA 1987a;1987b; Jenner *et al.* 1989; Kramer *et al.* 1989; Jenner *et al.* 1992). The overall conclusion is that bio-monitoring with early warning systems enables the detection of toxicants in a water body but that effects are strongly related to species dependant responsiveness. Bivalves for instance are extremely sensitive to TBTO (<6 μg/l) but in the case of cadmium 250 μg/l was needed to cause a response. High loads of suspended solids force the bivalves to close up, probably due to clogging of their sieve mechanism and a negative energy balance in pseudo faeces production. However, this ecological phenomenon, which is rather common at high river flows, has never been investigated properly.

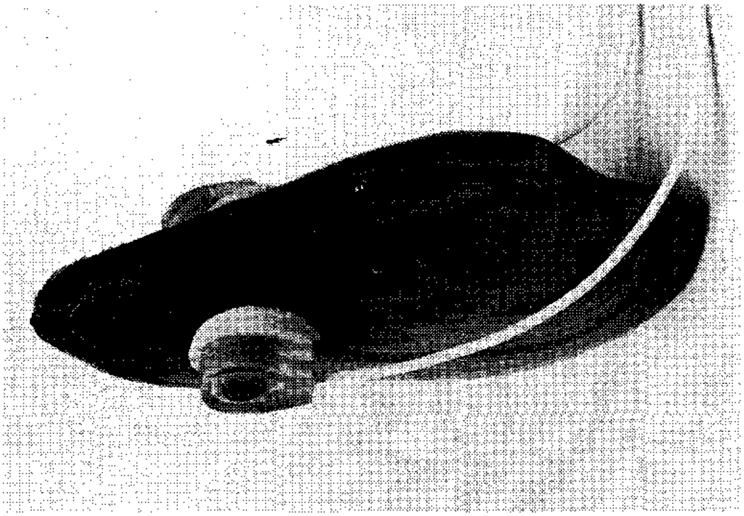

Photo 4. The mussel *Unio pictorum* with coils for valve movement registration cemented on the valves.

Biomonitoring cannot replace physico-chemical methods for the analysis and verification of the chemical or mixture of chemicals. But it surely forms a valuable supplement. Rapid early warning systems can be used for watching over process water quality at the intake point or outlet flows of plants. For antifouling purposes, such monitors can be used for the registration of the toxic action of hypochlorite and chlorine residuals in the cooling water of power stations (De Zwart *et al.* 1993). Studies with *Unio pictorum* (Photo 4; Fig. 2) have shown that valve movement behaviour can be measured adequately from these mussels (Jenner *et al.* 1992). The method is not restricted to mussels that use byssus threads for fixing themselves to solid substrates. Mussels and oysters can also be used. Oysters are perhaps an even better choice, due to their fully sessile existence, as they are cemented to their substrate throughout their lifetime.

Briefly, the following valve movements of *U. pictorum* can be distinguished:

- closure in the event of danger
- regular opening and closure during crawling and/or burrowing
- sudden opening during closure during faeces and pseudo-faeces production
- rapid joined closure movements (but not full closure) presumably to refresh the mantle cavity.

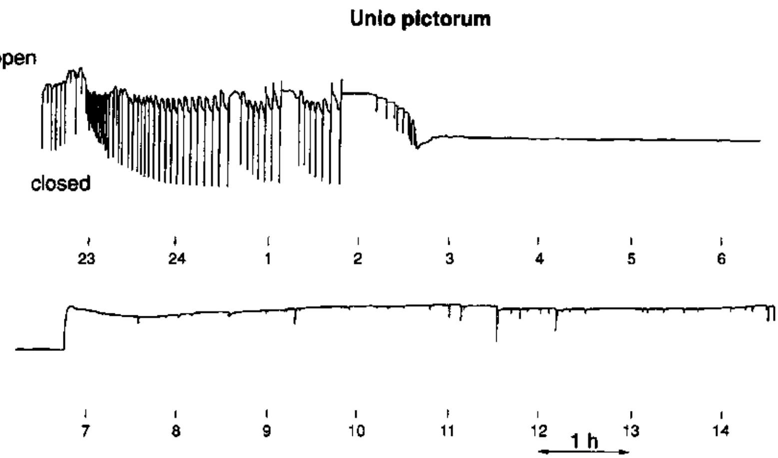

Figure 2. General activity pattern of *Unio pictorum*. The high activity indicated on the left is crawling behaviour. The pattern of single movements is due to faeces and pseudo-faeces production alternated with rest periods during which the mussels are nearly closed.

Amongst the types of bivalve behaviour used for bio-monitoring purposes have been the respiration rate of *Mytilus edulis* (Manley & Davenport 1979), the heart rhythm of *Scrobicularia plana* (Akberali & Black 1980), the burrowing activity of *Venerupis decussata* (Stephenson & Taylor 1975) and the pumping rate of *Mytilus edulis* (Redpath & Davenport 1988). The first records for valve movement date from the beginning of this century. Marceau (1909) used a mechanical system with sooted glass to register movement. Others used strain gauges to detect valve movement (Djangmah *et al.* 1979; Higgins 1980). The use of electro-magnetic induction was quite an improvement even though the mussels were still fixed in the first device built by Schuring and Geense (1972). This system was tested successfully in the laboratory by Slooff *et al.* (1983). KEMA further developed the electronic part, and pioneered the use of coaxal cables and miniature coils. At KEMA laboratories the monitor has been developed into a sensitive device for use as an early warning system (Jenner *et al.* 1989).

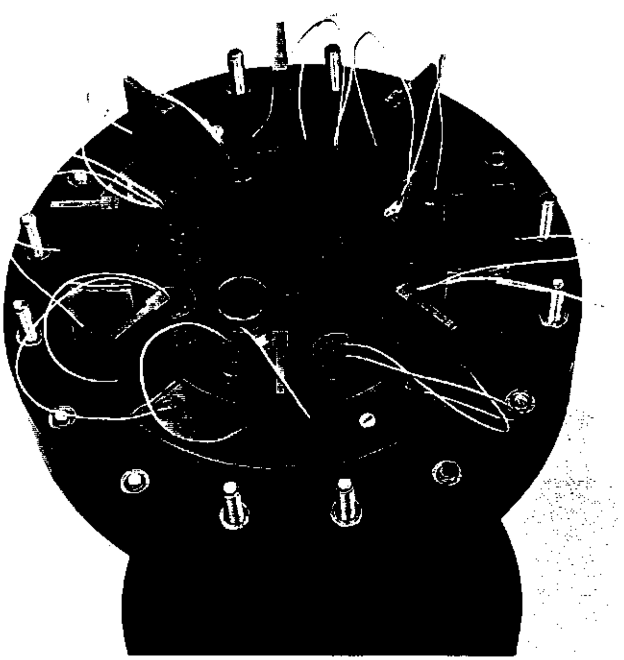

Photo 5. The stand-alone monitor for early warning purposes. Top view of the monitor without protection cap, with coils and vertical PVC plates for fixation of the mussels. In the center the cable connector and temperature sensor.

Once this part of the laboratory studies was completed a commercial design was developed in co-operation with Dr K.J.M. Kramer of the Laboratory for Marine Research, TNO-MW and Drs D. de Zwart of the National Institute of Public Health and Environmental Protection together with Delta Consult, an engineering company (Kapelle, Zeeland). The device was called 'MusselMonitor®' (Photo 5). The principle used for valve movement registration is inductive distance measurement with multiplexed HF-sensors at 500 kHz in combination with a computer system. Eight mussels are fixed on or connected to the device and monitored continuously. The 'MusselMonitor ®' can be connected by RS 232c to any device for automatic water sampling on the triggering of an alarm.

EXPERIMENTS WITH PLANTS

Lemna minor

Lemna spec. is one of the smallest angiosperms and is, according to Nasu and Kugimoto (1981), one of the plants most sensitive to heavy metal and other water pollutants. The advantages of using *Lemna* for phyto-monitoring studies are: its small size, high reproduction rate (cloning), aseptic culture and the high reproducability of the tests. This species was choosen on the basis of good earlier results obtained with Lemna minor by Adema and De Zwart (1984). All experiments with the duckweed *Lemna minor* were carried out in a culture cabinet in a temperature conditioned room. In contrast with ordinary growth experiments in which Erlenmeyer flasks are used. we used high petri dishes (diam. 9.5 cm, height 5 cm). The stock culture was kept in glass petri dishes but the experiments were conducted in disposable dishes cleaned in *c.* 0.1 N HNO_3 and rinsed with demineralized water prior to the experiments. The incubation time for all experiments was 14 days and each experiment was inoculated with 3 to 4 triplets (10 fronds) of *L. minor*. In addition to counting the number of fronds, image processing was used (PC Vision Plus framegrabber) with software developed at the Technical University of Delft (TIM; Difa Measuring Systems BV, Breda). The surface coverage parameter has a high resolution, is easy to use and its use is much less time consuming than counting by hand therefore, counting of the fronds is therefore omitted in 'leachate' tests (Photo 6).

Cyperus esculentus

The plant *Cyperus esculentus* (yellow nut sedge) is a nitrogen fixer which is capable of growing in both upland and wetland conditions. *C. esculentus* is commonly used for monitoring (Van Driel *et al.* 1983). The plants used originated from WES (U.S. Army Engineering Waterways Experiment Station) and obtained, with permission, from the stock culture of TNO-MW in Den Helder. The experiments in upland conditions were carried out using perforated flower pots with a diameter of 12 cm. The pots were placed in 2.8 cm high plant dishes and the water level was kept right up to the brim of the dish, using demineralized water. The plants in the wetland conditions were grown in closed flower pots, 13 cm in diameter and 12 cm high, which were filled with substrate to within 2 cm of the brim (volume 1.2 litres). The water level was kept up to the brim of the pot. After 10 weeks of exposure the plants were cut off at the point from which they started to grow (10 cm above the roots).

EXPERIMENTS WITH THE WORM *EISENIA*

The worm *Eisenia fetida* is a wellknown organism which is recommended as an indicator species in the EU/OECD procedure for ecotoxicological tests of chemicals (Stafford & Edwards 1985; Adema *et al.* 1988; Neuhauser *et al.* 1985; OECD 1984). Worms constitute the principal group of soil organisms, about 80% in terms of biomass. Worms improve soil structure (aeration and drainage) and the nutrient cycle, and contribute to the enrichment of the soil by organic matter. The work carried out by van Gestel (1991) with *E. andrei* and *Lumbricus rubellus* showed the influences of soil characteristics on toxicity. The development of a toxicity test based on reproduction amongst earthworms showed how valuable tests of these types are in the field of pesticide research. *E. fetida* was obtained from the National Institute of Public Health and Environment from a stock culture held by Dr. van Gestel. The worms were exposed to coal gasification slag in perspex cylinders which were 10 cm in diameter and 20 cm high. Fine meshed gauze was sealed over the bottom and the cylinders were placed in plant dishes. The worms were carefully washed with demineralized water and blotted with filter paper before weighing, in order to establish their weight.

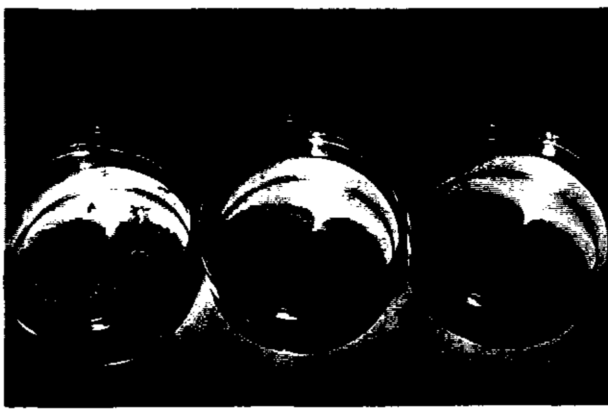

Photo 6.The effect of copper on *L. minor*, after 14 days. Note the yellow color of the fronds. On the upper left the control, upper middle 0.5 μM, upper right 1 μM, lower right 2.5 μM, lower middle 5 μM and on the lower right 10 μM.

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PART 1. MARINE BENTHOS STUDIES

Part one is made up by three chapters (chapter 3, 4 and 5) dealing with the effects of pulverized fuel ash (PFA) on a number of intertidal benthic invertebrates. Multi-species bioassays with arrays of ecologically important marine invertebrates, exposed under semi-natural conditions, is an increasingly utilised method for detecting the broad-scale effects of compounds released to the marine environment. With this aim in mind different sediment/PFA mixtures were tested. Containers of 2.2 m^3 and smaller ones of 0.25 m^3 were used of which the larger ones are called model ecosystems (mesocosms) to express their ability to mimic a part of the natural benthic community. The experiments described here are not as complex as the broad variation in realistic circumstances. They do provide more broad scale information about chronic effects than do shorter-term laboratory tests. In Chapter 3 and 4 mortality, growth and accumulation of elements is investigated for the species *Cerastoderma edule*, *Macoma balthica, Arenicola marina*, and *Nereis virens*. In Chapter 5, the chronic effects to *C. edule* were studied in which an attempt was made to find effects on reproduction and histo-pathological changes in tissues and cells.

Main objectives of part one are to address the following basic questions of marine PFA disposal:

- are benthic communities able to survive or to sustain in PFA substrates after and during disposal
- are the leached elements from the PFA accumulated in the exposed benthic organisms
- can disturbances be detected at the level of reproduction, histopathology and survival of the benthic bivalve *Cerastoderma edule*.

CHAPTER 3

THE ACCUMULATION OF METALS AND THEIR TOXICITY IN THE MARINE INTERTIDAL INVERTEBRATES *CERASTODERMA EDULE*, *MACOMA BALTHICA*, *ARENICOLA MARINA* EXPOSED TO PULVERIZED FUEL ASH IN MESOCOSMS

INTRODUCTION

Coal-fired generating stations produce two types of solid waste, bottom boiler ash and - pulverized fuel ash (PFA). The emitted fraction is called flyash. The PFA fraction is collected on electrostatic filters from the flue gases in so-called hoppers. Although in western Europe, Japan and the United States, a large proportion of PFA is utilized in the building industry, substantial amounts remain, creating a sizeable disposal problem (Bolt & Snel 1986; Cherry *et al.* 1984). In India and other rapidly developing countries, disposal or reuse is a fast growing problem. In 1985, The Netherlands produced some 6.10^5 tons of PFA, of which *c.* 90% was used for the production of cement and mass concrete, or as stabilization material in the building of roads. Production of PFA in The Netherlands is expected to rise to as high as 1.5 million tonnes per year by the year 2000, with a lesser percentage being reusable (Meij *et al.* 1986). An additional problem can be the application of low NOx burners in the boilers of new or re-equipped power stations in order to reduce NOx emissions and concentrations. The PFA produced by this process differs only slightly in chemical properties, but rather significantly in physical properties and consequently the application and amount of PFA used by industry can change. The question remains as to whether such a high percentage will be reusable in the coming decades. The PFA spheres contain a variety of heavy metals. A recent overview of metal concentrations in PFA for the U.S.A. is given by Norton *et al.* (1988).

This chapter is based on:
Jenner HA, Bowmer CT (1990) The accumulation of metals and their toxicity in the marine intertidal invertebrates *Cerastoderma edule*, *Macoma balthica*, *Arenicola marine* exposed to pulverised fuel ash in mesocosms. Environ Pollut 66:139-156.

The leachability of these metals depends mainly on their condensation temperature in the gaseous phase. Se, As and Hg, for instance, condense at a relatively low temperature on the outside of the PFA spheres (Page *et al.* 1979). After rapid initial leaching of the loosely bound elements under aqueous conditions, a slow, more long-term leaching is to be expected (Dudas 1981). The environmental effects of coal combustion, and specifically PFA disposal in aquatic systems, has been a research subject for several decades (Dvorak *et al.* 1977; Ryther *et al.* 1979; Bamber 1980; Hjelmar 1983; Brown & Ray 1983). This paper covers the following aspects of marine disposal:

1. The ability of dumping areas to support (living) macrofaunal communities after and during the disposal activity (carrying capacity);
2. The biomobility of heavy metals from PFA;
3. The possible effects on communities at the borders of dumping grounds and in adjacent areas.

The experimental work was carried out in mesocosms at TNO Den Helder, in two phases, a 90-day bioassay, using three target invertebrates (*Arenicola marina, Cerastoderma edule, Macoma balthica*) and covering the general aspects of mortality, and bioaccumulation. A longer, 140-day exposure was used to study the effects on reproduction in the cockle *C. edule*, and this will be subject of Chapter 5.

MATERIALS AND METHODS

The experiments with the species *Arenicola marina*, *Cerastoderma edule* and *Macoma balthica* were carried out in mesocosms of 2.2 m^3 (see chapter 2).

Sediment preparation

The PFA originated from the Amer 8 power station, Province of Brabant, The Netherlands. Approximately 4m^3 of a muddy-fine sand was collected from the Waddensea sand flats near to Den Helder. Five test sediments were prepared from these constituents, as indicated below.

- Mesocosm 1 (M1): A 27 cm layer of pure PFA was placed in one tank and is referred to below as the '100% PFA' treatment. This represents conditions at the centre of a marine dumping ground.

- Mesocosm 2 (M2): 50% PFA and 50% Waddensea sand were mixed together with the aid of a small concrete mixer, to create roughly 800 litre of mixed sediment. The mixture was placed directly in one of the mesocosm tanks to a depth of 27 cm. This is referred to below as the 50% PFA treatment.
- Mesocosm 3 (M3): This tank contained a 27 cm layer of Waddensea sand only, and, between 30 September 1987 and 19 November 1987 was dosed with a 500 ml volume of PFA on a daily basis. Dosing was intermittent. The position of the water inlet to M3 was periodically changed (weekly) to ensure an even spread of settling PFA over the whole tank.
- Mesocosm 4 (M4): A contaminated, harbour-dredged sediment (coarse hydrocyclone fraction) remaining from a previous experiment was used as a reference sediment. Its physical and chemical properties were well known and, above all, the biomobility of its most important contaminants was predictable, with the exceptions of As, Se and Sb. This tank contained 19 cm of sediment.
- Mesocosm 5 (M5): A 27 cm layer of Waddensea sand was placed in a fifth tank for use as a control mesocosm.

Buildup of test fauna and mesocosm functioning

Seawater (height 45 cm) was carefully introduced, so as not to disturb the sediment structure. The mesocosms were allowed to acclimatize for 48 h before the introduction of the test animals on 4 September 1987. The M3 test fauna were introduced on 15 September 1987. A total of 170 adult *A. marina* (*c.* 4 g weight), 200 adult *C. edule* and 125 adult *M. balthica* were placed in each mesocosm, except M3, which received 102 *A. marina*, 100 *C. edule* and 175 *M. balthica*. It should be noted that the mesocosms were supplied with coarsely filtered seawater via a large sand filter and contained 5 - 50 mg/l suspended matter, depending on tidal conditions at the intake area. The mesocosms were not supplied with any form of food or nutrients, apart from the phyto- and zooplankton that developed naturally in each system.

Monitoring and sampling

After 90 days, all test animals were sampled for chemical and biological analysis. It should be noted that in M3, which was dosed for *c.* 50 days, the final sampling took place at the end of the period of dosage, on 17 November 1987 and no *C. edule* were returned to this tank. Afterwards, 25 - 65 *C. edule* were returned to the tanks after measurement of the shell length and height. These were allowed to reburrow and remained in the 100% and 50% PFA tanks as well as the reference and control sediments for a further 140 days, in order to examine the status of gamete production. These animals were sampled finally in mid-April for histological and chemical analysis and results will be published in Chapter 5. Mortality was monitored in two ways, first by keeping count of the numbers of animals surfacing in each tank on every second day. Dead animals were immediately removed. However, live animals that surfaced were allowed to remain there until they either reburrowed or died. It should be noted that surface mortality was only a part of the total. All survivors and dead shells were counted (pairs only), at the end of the experiments.

Sample preparation for chemical analysis

Samples of all three test animals were collected for chemical analysis of bioaccumulated metals on a number of occasions. This included: samples of all three species before placement in the mesocosms 1, 2, 4 and 5 and after 90-days exposure; tissue samples of all three species in mesocosm 3 at the end of *c.* 50-days dosing, and tissue samples from *C. edule* in mesocosms 1, 2, 4 and 5 after 230-days exposure. *A. marina* were allowed to depurate their gut contents in seawater overnight, before being sacrificed, placed in HCL-rinsed glass-ware, homogenized and frozen for storage. Teflon liners were used in the plastic lids to prevent contamination. *M. balthica* and *C. edule* were separately removed from their shells, and fluid and tissue were homogenized and frozen for storage, in a similar manner as above. Oyster tissue (US/NBS -1566) was used as a standard for the analysis of Cr and Ni, while beef liver (US/NBS -1577) was used as a standard for the remaining six elements, i.e., As, Cd, Cu, Mo, Se and Zn.

Sediment samples were taken from mesocosms 1, 2, 4 and 5 at the end of the 90-day exposure with the aid of a 3 cm PVC core. Six cores from different parts of the mesocosm were mixed together to form one sample. Mesocosm 3 was similarly sampled at the end of dosing. All sediment samples were stored frozen until analysis. Standard river sediment (US/NBS -1645) was used as a standard for all elements.

Chemical analysis of sediment and tissue

The sediments were treated according to the Dutch standard NEN 6465 (NNI 1981). By this method, the samples were digested with *Aqua regia*. The elements As and Sb were analyzed by Electrothermic Atomisation-Atomic Absorption Spectrometry (ETA-AAS); the element Se was analyzed by Hydride Generation-Atomic Absorption Spectrometry (HG-AAS). The remaining five elements (Cd, Ni, Zn, Cu, Cr) were quantified by Inductive Coupled Plasma-Atomic Emission Spectrometry (ICP-AES). The dry weight of the samples was determined after oven-drying at 105 °C to constant weight.

Approximately 2g of tissue homogenate subsample was digested in an open system with a mixture of sulphuric acid and peroxide. The clear extract was analyzed, following dilution, for the metals chromium and nickel. This analysis was carried out by ETA-AAS. For the remaining six elements (As, Sb, Se, Cu, Cd and Zn), *c.* 0.5g was weighed into quartz ampulla and following closure (by melting) the encapsulated samples were irradiated with thermal neutrons at a flux of 1013 n/cm^2/s for three h. The irradiated tissue was digested with the aid of a sulphuric acid-peroxide mixture in a closed system followed by a bromide distillation. The distillate and residue were further separated with the aid of ion-ex changers, after which the different fractions were analyzed with a NaI well detector.

RESULTS

Salinity and temperature

Salinity was measured daily at the point of seawater intake. Values ranged from 24.9 - 30.2 g/l (Sal.), with an average of 27.8 g/l over the period of the 90-day bioassay. The meso cosm temperature in September 1987 remained largely above 17 °C, then dropped rapidly to *c.* 14 °C at the end of the month. October and the first 3 weeks of November showed a gradual decline from 14 °C to 10 °C.

Sediment

The PFA had, as expected, a high proportion of grains in the silt/clay (<63μm) fraction, i.e. 74%, with *c.* 17% very fine sand (63 - 125μm) and the remaining 8.5% consisting of fine sand (125 - 250 μm). The control sediment was of Waddensea origin and consisted predominately of very fine (30%) and fine (31%) sand, with only 12.7% silt clay. The contaminated reference sediment contained 16% fine sand, *c.* 40% very fine sand, and approximately 43% silt clay. The 50% PFA treatment was a mixture of the already described PFA and the control sediment, and thus possessed grain size characteristics of both. Table 1 presents this data and the organic contents of all sediments. The apparently organically rich PFA consisted entirely of carbon originating from partially burned coal, which had no nutritive value whatsoever. The water content of all five sediments was similar, and ranged from to 31 to 39%.

Table 1. The grainsize composition of the test sediments. The dry organic matter content of each Sediment is indicated in parenthesis.

	Grain size (μm)				
	<63	*63–80*	*80–125*	*125–160*	*>160*
M1 (2·2%)	73·0	7·6	10·3	4·2	4·9
M2 (4·8%)	34·6	7·4	26·6	20·1	11·3
M3 (3·1%)	17·0	7·3	32·9	30·1	12·7
M4 (5·1%)	42·7	15·5	24·4	10·1	7·3
M5 (2·8%)	17·7	7·8	36·3	29·1	9·1

Mortality

A relative estimate was made of the total mortality by assessing the surface mortality of the three test species during the 90-day bioassay. The results are presented in Table 2. *A. marina* showed high initial mortalities in the first 6 days of the bioassay which amounted to about 52%. As the control tank also suffered similar losses, at about 42%, it is assumed that the lack of sediment acclimation in combination with the trauma of bulk sampling, transport and dry holding prior to the test, was the cause. In calculating final mortality, these initial deaths have been excluded. After 90-days exposure, a clear trend was evident, only four worms remained alive in the 100% PFA tank, 30 worms in the 50% PFA, 42 worms in the dosed tank, and 53 worms in the control. After the first 60 days, no worms were seen to come to the surface at all, indicating severe stress, except in the 100% PFA tank. Here, due to burrowing difficulties five or six worms remained active on the surface for a further week and six surface deaths were observed in the first month of the bioassay. The vast majority of the deaths occurred unobserved below the sediment surface.

Table 2. The numbers of test animals exposed during the 90-day bioassay are presented, along with the numbers which surfaced and died, the number which died without surfacing, the number recovered at the end of the 90-day exposure, and the adjusted survival percentage for *Arenicola marina*, *Cerastoderma edule* and *Macoma balthica* in each mesocosm. The adjusted survival figures do not include animals which died during the first 6 days of the test (see text).

	M1 *100% PFA*	*M2* *50% PFA*	*M3* *Dosed PFA*	*M4* *Reference*	*M5* *Control*
Arenicola marina (90 day)					
Start	170	170	102	170	170
Surface mortality	89 (52%)	57 (34%)	0 (0%)	63 (37%)	71 (42%)
Subsurface mortal.	77	83	60	unknown	49
Survival[a]	4	30	42	unknown	53
Adjusted %	4·5%	26·5%	41·2%	unknown	54·5%
Cerastoderma edule					
Start	200	200	102	200	108
Surface mortality	40	50	35	14	15
Subsurface mortal.	17	7	37	unknown	7
Unaccounted for	21	0	0	unknown	0
Interim sampling[b]	20	20	0	20	20
Survival (2/12/88)	102 (56·7%)	123 (68·3%)	30 (29·4%)	unknown	65 (74%)
Macoma balthica					
Start	125				
Surface mortality	11				
Unaccounted for	14				
Interim sampling	20				
Survival	80 (80%)				

[a] After subtraction of initial mortality, see text.
[b] Interim sampling for histological preparation.

In the case of *C. edule*, considerably mortality occurred among individuals which surfaced. This happened usually within one week after appearance above the sediment. The survival recovery (at 90-days) was, as expected, highest in the control, at 74% followed by the 50% PFA treatment, at 68%, then the 100% PFA at 57%. However, the highest mortalities occurred in the tank with the PFA dosage, where only 29% of the cockles survived the 49-day dosage. It should be noted that 16 individuals surfaced before commencement of dosing and were removed. Towards the end of dosing the number of surfacing cockles increased and death followed within 24 h. The reference sediment was not examined for deaths because of the difficulty of adequate sampling of the extremely 'heavy' sticky substrate.

In the early weeks of the bioassay it became evident that a substantial wild population of *M. balthica* still survived in all mesocosms with Waddensea sand. This wild population could not be distinguished from the 125 - 175 introduced individuals. Hence mortality could not be followed in the three tanks where control sand was used in the treatment (M2, M3, M5). In the 100% PFA tank, however, 80% of the 125 introduced *M. balthica* survived exposure and 14 individuals could not be accounted for. It is interesting to note that in the dosed tank, where such high *C. edule* mortality was recorded, over 400 *M. balthica* were found alive in contrast to the 175 individuals at the start of the bioassay, indicating that no severe impact had occurred on the population.

Sediment metal concentrations

Analysis of the pure PFA (99.7% solid) yielded results which were very similar to those found in the sediment in M1 (100% PFA), although the latter was some 10% lower for most of the eight elements. The pure PFA duplicate analyses are presented in Table 3. After the 90-day exposure, the metal concentrations in the PFA sediments were generally not exceptional. Only As was found in higher concentrations (43 µg/g). In the reference sediment, Cd and Zn levels were higher than for the PFA. The concentrations of the different metals in the sediments are presented in Figure 1.

Table 3. Macro elements (%) and the heavy metal concentration tested (µg/g D.W.) of the used PFA (Amer 8 Powerstation) and the original metal concentrations of the harbour sludge (M4); destruction according NEN 6465.

Element	*PFA*	*Harbour sludge*
Al	15·2	
Ca	1·3	
Fe	4·8	
Mg	0·6	
P	0·18	
Zn	84	1050
As	48	27·7
Cr	58	206
Cu	110	112
Ni	63	46·4
Cd	0·97	10·8
Sb	8·4	—
Se	18·6	—

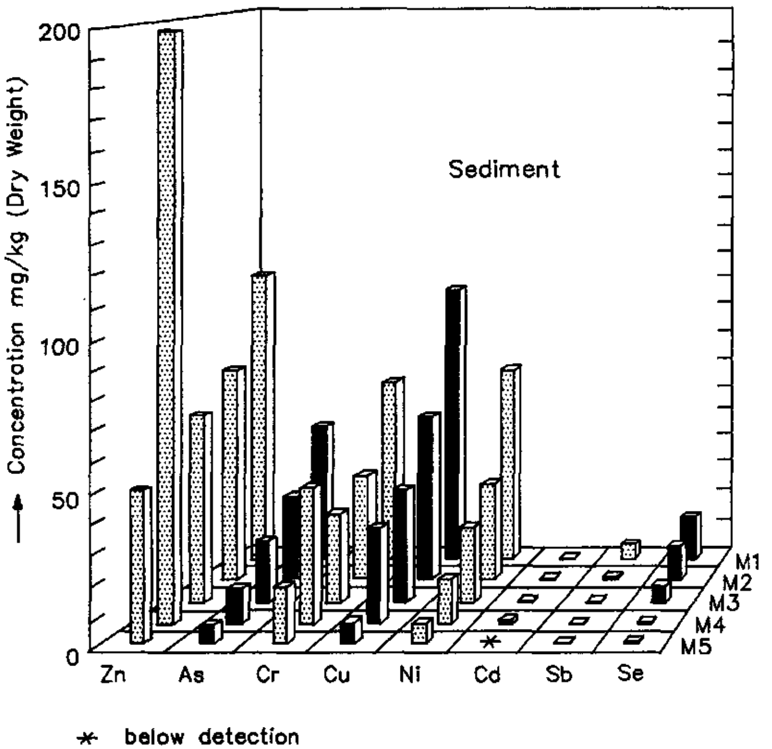

Figure 1. The concentrations (µg/g) of zinc, arsenic, chromium, copper, nickle, cadmium, antimony, and selenium in the test sediments are presented in 3D configuration (dry weight basis). The elements are on the x-axis, the respective concentrations on the y-axis and the test sediments on the z-axis.

Tissue metal concentrations

As expected, the metal concentrations measured by total body analyses varied considerably. The results for the 90-day exposure are presented in Figures 2, 3 and 4.

Arsenic. As was present in the pure PFA at a mean concentration of 47.5 µg/g and in the 100% PFA mesocosm 1 at 43 µg/g. The test *A. marina* had similar background tissue levels (45.5 µg/g) before exposure and accumulated As to 207 µg/g after 90 days in mesocosm 1. Mesocosm 2 (50% PFA) had 25 µg/g in the sediment, a little over the expected 50% of values in mesocosm 1. This was responsible for a spectacular increase of As in *A. marina*

(15x) to 665 µg/g after 90 days. On the other hand, *C. edule* showed no tendency to accumulated As and *M. balthica* accumulated to 150% and *c.* 300% of the respective sedimentary value in M1 and M2. The deeper burrowers, *A. marina* and *M. balthica*, both had higher As background levels and relative to *C. edule* showed increased accumulated levels in the M3 dosed PFA treatment.

Zinc. None of the three species showed any tendency to accumulate Zn, at least not in relation to the sedimentary levels present in the five mesocosms. *A. marina* showed concentrations of between 100 and 140 µg/g in all treatments, which, when compared to sediment levels as high as 195 µg/g in the reference sediment, and an initial tissue value of 90 µg/g suggest an ability to regulate Zn regardless of the current sediment chemistry. The same applies to *C.edule* and *M. balthica*. The initial tissue level in *M. balthica* was a massive 542 µg/g, compared to a sedimentary concentration of only 81 µg/g in the 100% PFA mesocosm 1. Whereas depuration of Zn might have been expected in *M. balthica* during the 90-day exposure, this was not the case. Zn concentrations at 90-days ranged from 536 (M3) to 766 µg/g (M5), this latter value being measured in the control *M. balthica* sample. There is clearly a metabolic requirement for high concentrations of Zn in this species.

Cadmium. Cd was one of the elements of primary concern in this study, being in general readily accumulatable and one of the most toxic. The M1, 100% PFA sediment contained *c.* 1 µg/g Cd: while the 50:50 PFA/sand, M2 treatment and the M3 dosed treatment had around half this value: the control Cd concentration was below the limit of detection. The contaminated reference sediment (M4) on the other hand showed a Cd concentration of over 2 µg/g. Bioaccumulation was highest (x7)[1] in *A. marina* from the 100% PFA mesocosm at 6.99 µg/g, while the M2 50% PFA and the M3 dosed PFA treated *A. marina* had accumulated 1.98 and 1.44 µg/g, respectively, over an initial tissue value of 1.01 µg/g. Strangely, the M4 reference sediment *A. marina* population did not accumulate Cd. This overall pattern can perhaps be explained by the presence of fresh organic matter in the sediments which may have rendered Cd less available.

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[1] Bioaccumulation factors, e.g x7, are always calculated in relation to background tissue levels measured at the start of the experiment.

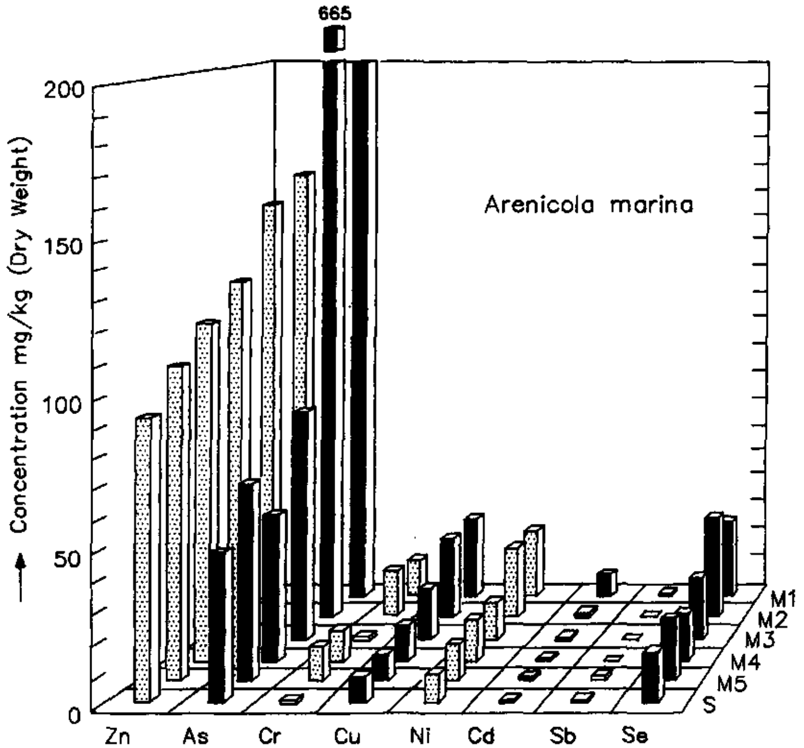

Figure 2. The concentrations (µg/g) of zinc, arsenic, chromium, copper, nickle, cadmium, antimony, and selenium in the tissues of *Arenicola marina* after 90 days exposure to the test sediments (dry total tissue basis) are presented in 3D configuration. The elements are shown on the x-axis, the respective concentration on the y-axis and the test sediments on the z-axis. S indicates the concentrations in the tissue at the start of the exposure.

Antimony. Sb concentrations remained extremely low in *C. edule* and *M. balthica* in relation to sedimentary values. Initial tissue concentrations were <0.01 µg/g and remained so in the M5 control mesocosm in both species, despite a small background concentration of 0.16 µg/g in the sediment. Sedimentary concentrations of 6.8 and 3.6 µg/g Sb in the M1 100% PFA and M2 50:50 PFA/sand treatment yielded less than 1 µg/g and less than 0.5 µg/g in *M. balthica* and *C. edule* tissue, respectively. *A. marina* had an initial concentration of 0.17 µg/g which increased to 0.80 and 2.01 µg/g in M1 and M2, respectively. With all three species, the highest accumulation occurred in M2.

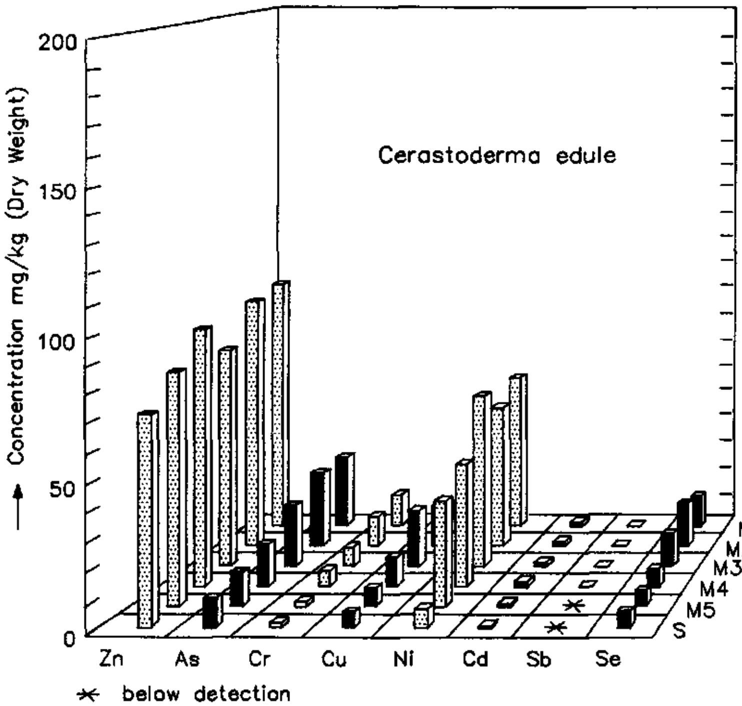

Figure 3. The concentrations (µg/g) of zinc, arsenic, chromium, copper, nickle, cadmium, antimony, and selenium in the tissues of *Cerastoderma edule* after 90- days exposure to the test sediments (dry total tissue basis, less shell) are presented in 3D configuration. The elements are shown on the x-axis, the respective concentration on the y-axis and the test sediments on the z-axis. S indicates the concentrations in the tissue at the start of the exposure.

Nickel. Ni concentrations in *A. marina* and *M. balthica*, were never raised to the values of the sediments in their respective exposures. Furthermore, they showed little increase over the initial tissue concentrations (<2x). Surprisingly, *C. edule* accumulated Ni in moderate concentrations. In M1, the tissue value (51.4 µg/g) almost reached the sediment concentration at 61.0 µg/g. In M2 (50% PFA) the 90-day tissue values exceeded those in the sediment and the initial tissue concentrations by 8 x. It is also noteworthy, M3 (the dosed PFA mesocosm) *C. edule* had a 90-day concentration of 55.5 µg/g. as compared to a sedimentary concentration in M3 of 24 µg/g and initial tissue concentrations of Ni at 5.4 µg/g, i.e., 10 x.

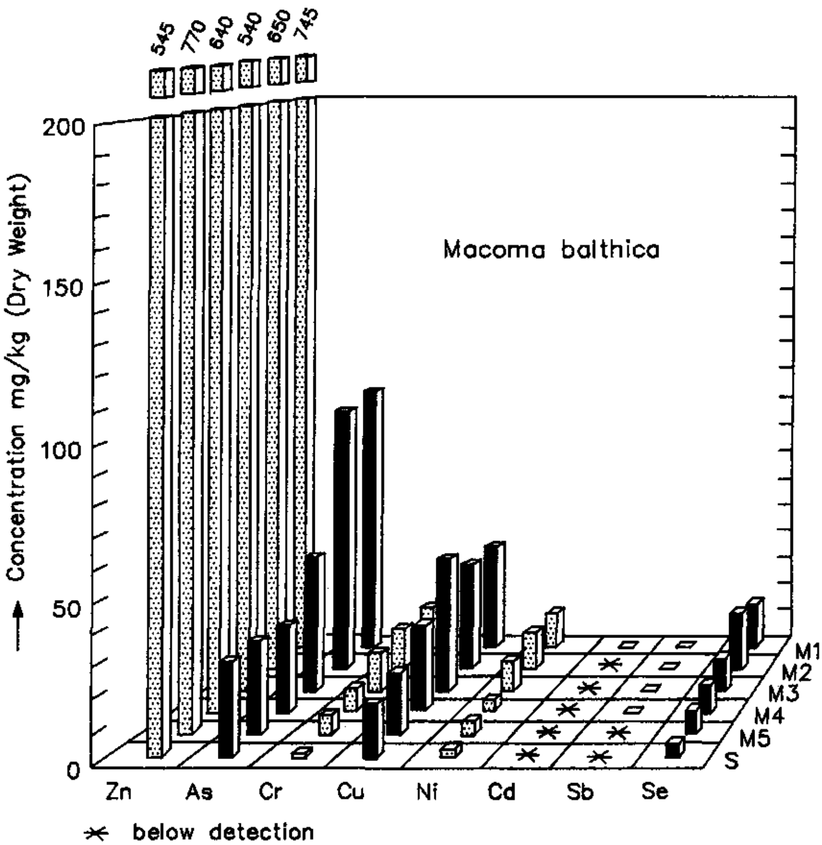

Figure 4. The concentrations (µg/g) of zinc, arsenic, chromium, copper, nickle, cadmium, antimony, and selenium in the tissues of *Macoma balthica* after 90- days exposure to the test sediments (dry total tissue basis, less shell) are presented in 3D configuration. The elements are shown on the x-axis, the respective concentration on the y-axis and the test sediments on the z-axis. S indicates the concentrations in the tissue at the start of the exposure.

Copper and *Chromium*. The tissue concentrations of Cu and Cr remained below respective background sedimentary concentrations in all three species. This may of course be due to the low bioavailability of these metals in all five sediments or, more probably, the ability of all three species to regulate these metals at sedimentary concentrations of between 4.3 (M5) and 87.0 µg/g (M1) for Cu, and 17.0 (M5) and 57.0 µg/g (M1) for Cr. Tissue levels were never seen to increase greatly above background tissue concentrations, the largest difference being seen in *M. balthica* M3 individuals where the 90-day concentration for Cu (38.1 µg/g) was 2.5 x higher than the initial tissue concentration (15.1 µg/g). With Cr, the background tissue concentration was very low at 0.5 -1 µg/g in all three species, and accumulation was observed to be highest in M2 *A. marina* (10.2 µg/g) M1 *C. edule* (6.2 µg/g) and M1 *M. balthica* (9.4 µg/g).

Selenium. Where Se is concerned, *C. edule* and *M. balthica* tissue levels remained mostly below the respective sediment concentrations in the individual mesocosms (<16.2 µg/g) and were elevated above background tissue concentrations by no more than × 4 in any one treatment. In *A. marina*, the initial tissue concentration of 12.7 µg/g was very similar to the highest sediment value in M1 at 16.2. Thus, concentrations of 23.3 and 30.8 µg/g hardly represent a spectacular accumulation of this element.

DISCUSSION AND CONCLUSIONS

A broad variation is found between the three species in both mortality and metal accumulation patterns. Mortality of the exposed species was initially high, but this may be related to collection stress. The exception is *M. balthica* which suffered less mortality and could be collected and/or caged well (Cain & Luoma 1985). Results show a clear mortality gradient between the mesocosms. Mortality among *M. balthica*, at 28% could only be detected in the 100% PFA. In the other (mixed) sediments, an indigenous and living population of clams obscured the number of dead animals (if there were any). The observed surface mortality for *A. marina* is high for all types of tested substrates, especially for the 100% PFA treatment, where the worms were clearly unable to burrow properly.

Metal concentrations in the two PFA sediments (100% and 50%) after 90-days of exposure showed about a 10% loss of the initial total concentrations. The greatest difference was found for Cu of about 23 µg/g. As expected, Cr concentrations remained equal to the initial PFA concentrations, as this metal is fixed in the matrix of the PFA spheres. Release of the anions As en Se was much lower (< 5%) than expected, given the known high leacheability of As and Se. Van der Sloot (1983) estimated the leachable fraction of Se and As to be in the order of 80 - 100% and 50 - 80% respectively.

Accumulation of As by *A. marina* was pronounced, especially in mesocosm M2 (50% PFA), indicating the relatively high bioavailability of As in this PFA-sand mixture. The worms from the 100% PFA treatment were, however, emaciated and had lost considerable weight (*c.* 50%) which may have disrupted their pattern of As accumulation. The shift in accumulation in M2 was not found for *C. edule* and *M. balthica*. *M. balthica* showed intermediate As levels, but the striking difference in accumulation between the M1 and M2 mesocosms is lacking, while the cockle showed the lowest accumulated levels.

A. marina has two available uptake routes within the sediment: 1. by ingesting sediment and accumulation through the gut wall, or 2. from the interstitial water by accumulation across the mucous layer and epidermal membranes, including the gills. In estuarine sediments, As is found as arsenate in the surface layer and as arsenite in the deeper (>10 cm) sediment (Lemmo *et al.* 1983). Riedel *et al.* (1987) found a rapid and high uptake of arsenic by *Nereis succinea* when this was added as As(V) to the sediment. In the anoxic sediment, As(V) was rapidly reduced to As(III) and associated with sedimentary solids. Ryther *et al.* (1979) showed that As in PFA is particle- bound to the glassy spheres, but is still bioavailable to deposit feeders. Adsorption of As(V) and As(III) by the fine silt in M2 (50% PFA) can thus be expected to occur. Where the interstitial water is concerned, Waslenchuk and Windom (1978) showed the low complexation of As-oxides by humic and fulvic acids; complexation was found specifically with dissolved, low molecular weight material.

The other anion, Selenium, showed a similar pattern, but lower levels of accumulation for all three species, again with a somewhat higher accumulation in mesocosm M2 (50% PFA). *M. balthica* showed a seven-fold increase in the accumulated Se in the M2 situation, from 2.2 to 14.2, with a sediment concentration of 8.3 µg/g. By contrast, Johns *et al.* (1988) reported mean levels of 3.1 to 6.7 µg/g accumulated in *M. balthica* living in natural sediment in San Fransisco Bay, with a mean concentration of as low as 0.35 µg/g Se. At pH >8 adsorption of Se to fine silt should be negligible (Bar-Yosef & Meek 1987) and Se accumulation in bivalves should have a linear relationship with Se concentration in seawater. Differences in accumulation in the mesocosm M2 should be attributed to speciation, with corresponding higher or lower bioavailability. For both As and Se it can be concluded that the observed higher accumulation in M2 (50% PFA) is presumably due to the different redox in M2 versus M1.

The cations Cu, Cr and Ni showed no unexpected accumulation patterns and abscence of influence of the organic fraction, as was found in mesocosm M2. One exception has to be made for the element Zn; as seen in Figure 4, Zn is accumulated to high levels. In the initial PFA total Zn concentrations was 83 - 86 µg/g. After 90 days, the concentration was still 81 µg/g, indicating that loss by leaching is low as was found for the other analyzed metals. In mesocosm M4, with the reference sediment which is moderately polluted by metals the zinc concentration is about 200 and about three times higher compared with mesocosm M2 (50% PFA). Accumulation by *A. marina* showed, however, a lower accumulation. It can be concluded that the bioavailability of Zn in the PFA exposed mesocosm is somewhat higher compared with the reference sediment. Compared to *A. marina* and *M. balthica*, *C. edule* showed an overall lower accumulation. *M. balthica* accumulated Zn to high levels between 500 and 770 µg/g (DW). These high background levels of Zn seem normal. Kaitala (1988) found levels in the same range (40 - 80 µg/g wet weight). *Mytilus edulis* showed Zn concentrations of <200 µg/g (DW), but Zn concentrations could raise to >20.000 µg/g (DW), in the kidneys in contaminated sediments. In uncontaminated sediments, concentrations were about 800 µg/g (DW) in the kidney (Lobel & Wright 1983; Lobel 1987).

In mesocosm 3 (M3) daily PFA doses of 500 ml were used to simulate a disposal site. From the results obtained, it is clear that *C. edule* suffers strongly from such repeated doses, with a mortality of 70%. *A. marina* can resist the treatment, more or less, with a mortality of about 60%, whilst. *M. balthica* experiences no mortality. In the present study, by far the highest mortalities of cockles were found in the dosed mesocosm (M3); where only 29% survived. In a sedimentation study, Turk and Risk (1981) found exactly the same results with *M. balthica* as reported here: light sedimentation with a marine sediment produced no apparent effects on the density of the population. Strong effects were, however, found in populations of *Corophium volutator* and *Mya arenaria*.

In common with the study of Bamber (1984) on disposal sites in the North Sea, where a sharp decline of species density was found towards the centre of the ash dump was found, the observed mortalities in M1 indicate severe detrimental effects. However, the missing factor in Bamber's study is data on the accumulation of metals. From the M2 results, it can be predicted that, at the periphery of dump sites, bioaccumulation of some metals, particularly As, may be substantial. While, in the experience of the present authors, bioaccumulation in mesocosm-test fauna is dependant on the water exchange rates, which are usually substantially lower than in the field, this does not take away from the usefulness of mesocosms as a predictive tool.

The effects of the physical properties of PFA as a substrate on the benthic fauna were studied by Bamber (1980), who concluded that there was a resemblance with natural mud. However, the mesocosm study tends to show that the physical properties and behaviour of PFA indeed cause disturbance, i.e. the mortality of a bivalve filter feeder due to small quantities of PFA in the water column and the inability of some deposit feeding polychaetes (as evidenced by the lugworm) to form burrows in 100% PFA substrates. Owing to these factors, i.e. bioaccumulation, increased turbidity and the unsuitability of PFA as a substrate, the conclusion of Bamber (1984) that 'Dumping over a wider area to reduce the degree of "sedimentation" at the sea-bed would minimize the prominent lethal effect of the dumping itself,' does not seem to be an attractive option.

It can be concluded that dumping of PFA as ash in a marine environment will lead to severe depletion of the invertebrate population *in situ*, with metal accumulation in the benthic fauna. Species composition are likely to change, with consequent shifts in predators. Recovery of such dumping sites, of PFA without organic materials, will take years, as was shown by Pfitzenmeyer (1970; in Bamber 1984) in a study in Chesapeake Bay with natural sedimentary material containing animals.

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THE ACCUMULATION OF METALS AND TOXIC EFFECTS IN *NEREIS VIRENS* EXPOSED TO PULVERIZED FUEL ASH

INTRODUCTION

Coal-fired electricity generation in The Netherlands is still increasing, as is the resultant production of solid waste products, i.e. bottom ash and pulverized fuel ash (PFA) or flyash. In 1985, The Netherlands produced some $0.6.10^6$ tons of PFA and this is expected to rise to $1.5.10^6$ tons by the year 2000. In modern boilers the trend is towards the installation of low NOx burners, which reduce atmospheric pollution, but will produce an altered type of PFA. If this new type of PFA is less suitable in current industrial applications (concrete, road stabilisation, cement and bricks) a storage and/or disposal problem will be created by the unusable material. The possible environmental consequences of PFA disposal in general are considered here.

The environmental effects of coal combustion residues are attributed largely to heavy metal leaching and, in aquatic systems this has been a topic of study for the last decade (Dvorak *et al.* 1977; Ryther *et al.* 1979; Bamber 1980; Brown & Ray 1983). PFA consists of glassy spheres posessing a variety of heavy metals and metaloids bound to their surfaces and contained within them. Their leachability depends on the type of PFA, i.e. burning conditions and coal type, as well as the chemical environment of the dump site (Page *et al.* 1979; Warren & Dudas 1988). In the marine environment the polychaete worm *Nereis virens* Sars is a common species in boreal-littoral and sublittoral zones.

This chapter is based on:
Jenner HA, Bowmer CT(1992) The accumulation of metals and toxic effects in *Nereis virens* exposed to pulverised fuel ash. Environmental Monitoring and Assessment 21:85-98.

Juvenile *N. virens*, which are generally assumed to be more sensitive to contaminants than adults (McGreer 1982), were used to study the physical and chemical acceptability of PFA as a sediment. High mortality rates were expected due to increased metal concentrations and the tight structure of the PFA sediment. Juvenile *N. virens* do not dig tunnels but creep just beneath the surface of the bottom. Adult worms generally have more than 40 segments and this stage was reached some 9 weeks after the start of the experiment.

The worms have a high tolerance to changes in temperature, oxygen and salinity (Sayles 1935; Smith 1957; Neuhoff 1979). The bioassay took place in flow through containers, of a format large enough not to restrict the normal burrowing activities of *N. virens*. The substrates used in the containers were 100% PFA, 50% PFA and 50% sand, a sand control and a reference of contaminated harbour-dredged material. The principle aim of the bioassay was to assess the possible consequences for the fauna of PFA incorporation into natural sediments, on or near a dumpsite.

MATERIALS AND METHODS

Juvenile *N. virens* were used in a container system in which the worms were exposed for 12 weeks in several mixture of PFA. The experimental container system was constructed as described in Chapter 2. Seawater was fed to the containers at a flow of 3 litre/min and dissolved oxygen content remained above 8 mg/l during the 12 weeks of exposure. The juvenile *N. virens* were not fed during the exposure, and were dependent on benthic diatoms for their nourishment; these grew rapidly in mats covering the bottom.

The PFA originated from the Amer 8 power station, located in the Province of Brabant, The Netherlands, while the reference sediment originated from the harbour of Rotterdam. The control sand was collected from the foreshore near to the location of the experimental site itself. Four test sediments were prepared: C1 contained 100% PFA; C2 contained a mixture of 50% sand and 50% PFA; C3 contained a reference sediment from the harbour of Rotterdam and finally, C4 housed the Control sediment of sand.

The study was carried out with the marine polychaete *Nereis virens* Sars at a commercial worm breeding farm in the Province of Zeeland from early April to the end of June. One hundred juvenile *N. virens* with an average weight of 200 mg (fresh weight) were placed in each container. After 4, 8 and 12 weeks one container of each substrate was sampled for all living worms. The worms were washed and weighed, then after three days of depuration in clean seawater to remove the gut contents, the worms were carefully blotted dry on filter paper and weighed again. The tissues were freeze-dried and stored for analysis. For elemental analysis the sediments and PFA were treated according to ASTM 3683 (ANSI/ASTM 1978) which gives an almost complete destruction. Analyses for metals in sediments and tissues were carried out by ICP-AES and AAS (Marquenie *et al.* 1988). In addition to total destruction of the sediment samples, a leaching method was performed to gain more information about their metal leaching capacity. The extraction was carried out according to EPA method (US-EPA 1980), using a liquid/solid ratio of 20 at a pH of 5, and constant stirring for 24 h. However, in contrast to the EPA method which called for leaching in acetic acid, 0.5 N nitric acid was used. Grain size distribution of the sediments was performed using a Particle size analyzer (Malvern 2600C). Before measurement the samples were sieved over 500 µm and 1 mm sieves. Measurements were carried out in ethanol suspensions, using a 300 mm lens at a measuring range of 5.8 to 564 µm.

RESULTS

The PFA (C1) and reference sediment (C3) both contained a high proportion (60%) of the silt/clay fraction (<53 µm), with 20% and 13% respectively of very fine sand (53 - 83 µm), and 20% and 13%, respectively, of fine sand (83 - 201 µm). In contrast, the grain size of the control sediment (C4) showed a distribution of 50% fine sand and contained only 18% silt/clay. The 50% PFA sediment (C2) was a mixture of the already described PFA and the control sediment, thus posessing grain size characteristics of both (Table 1). The organic content of the PFA (and thus partially of the mixed sediment C2) was, however, due to unburned coal particles, and offered no degradable organic matter as a possible food source for the test organisms.

Mortality

At the start of the experiment, 100 juvenile *N. virens* with an average wet weight of 200 mg were placed in each container. The pattern of survival of these animals after 4, 8 and 12 weeks is presented in Figure 1. It is evident that in all sediments a considerable mortality occurred within the first period of 4 weeks and 30 (C2) to 45% (C4) of the worms died within this period. This high initial mortality is further discussed below. However, at 8 and 12 weeks, little further mortality had occurred, some 40 to 50% of the introduced individuals survived. According to Friedman's method for randomized blocks (Sokal & Rohlf 1981), a significant lethal effect is not seen in the test sediments.

Table 1. The grain size composition (%) of the 4 sediments tested in the containers. The organic matter content (%) of each sediment is indicated next to the container number.

	grain size (μm)		<53	53–83	83–201	>201
C1:	100% PFA	(6.8%)	60	16	20	4
C2:	50/50% PFA	(5.5%)	50	12	18	20
C3:	Harbour Ref.	(4%)	60	13	13	14
C4:	Control	(5.6%)	18	6	24	52

Growth

The growth of *N. virens* measured as the mean individual wet weight showed considerable differences for each sediment type (Fig. 2). In the control (C4), the worms increased their weight tenfold to over 2g each. The worms in the other sediments grew far slower, the reference sediment (C3) being the next best at 1.8g. However, due to the nutrients in this sediment (4% organic matter), a better growth rate had been expected. In the fly ash exposures C1 and C2 sediment, growth was very poor by comparison, at 1.0g and 0.8g respectively.

Metal accumulation

The elements As, Cd, Cr, Cu, Ni and Zn were measured in the *N. virens* tissue after 0, 4, 8 and 12 weeks. Table 2 and the Figures 3 to 9 present the mean concentration of duplicate analyses of whole animals. It was expected that the concentrations of the elements would increase during the period of exposure without reaching equilibrium. The sedimentary values are included in the above figures. As can be seen in Figure 3, As concentrations in *N. virens* tissues decreased in C3 (harbour sediment) and remained more or less equal in C4 (control). However, in C1 (100% PFA) and C2 (50% PFA) an initial increase to 76 and 52 µg/g respectively was found up until week 8, after which an unexpected decrease was seen at week 12 (C1: 55 µg/g; C2: 46 µg/g). Cadmium was expected to be accumulated from the reference harbour sediment exposed *N. virens*. A background concentration in the sediment of 10.8 µg/g was present in this sediment but tissue concentrations remained low at 1.3 µg/g after 12 weeks, as can be seen from Figure 4.

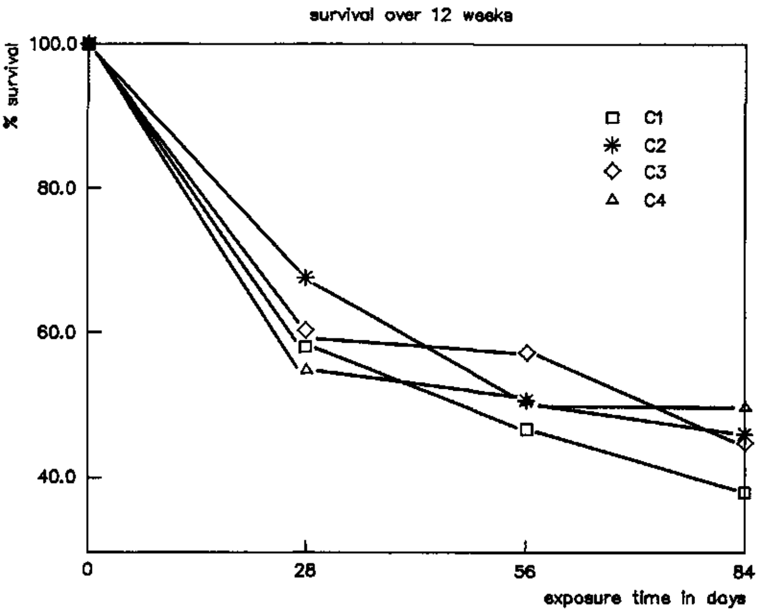

Figure 1. The % survival of *Nereis virens* exposed for 12 weeks to the test sediments: C1 = 100% PFA, C2 = 50% PFA/ 50% sand, C3 = Reference harbour dredged sediment and C4 = control sediment.

The PFA sediments contained a Cd concentration of little higher than the control, and the resultant bioaccumulation levels reflect this pattern. No great accumulation of chromium was observed, although the concentrations in the PFA (C1) and the harbour sediments (C3) were 156 and 197 µg/g respectively. The control *N. virens* levels ranged from 0.5 to 1.5 µg/g, while the C1 (100% PFA) and C2 (50% PFA) levels were never higher than 3 µg/g (Fig. 5).

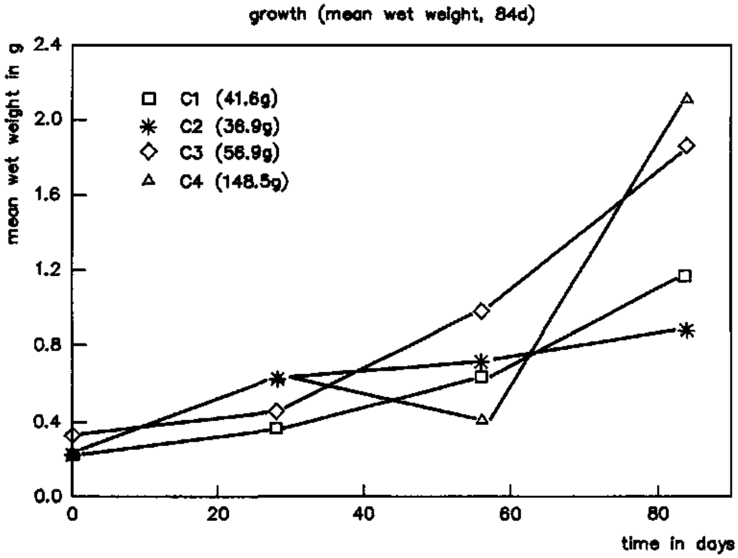

Figure 2. The growth of *Nereis virens*, expressed as mean wet weight (g) during 84-days (12 weeks) exposure to the test sediments: C1 = 100% PFA, C2 = 50% PFA/ 50% sand, C3 = Reference harbour dredged sediment and C4 = control sediment. Within parenthesis is shown the combined biomass of *N. virens* at 4, 8 and 12 weeks sampling for each type of sediment.

The concentrations of copper (Fig. 6) in the tissues were about 8 µg/g at the start of the experiment, a situation reflected by the control (C4). After 4 weeks the concentration increased in the worms exposed to the PFA (C1 and C2) and reference (C3) sediments to between 19 and 28 µg/g (threefold).

Table 2. Metal concentration in *Nereis virens*. Mean concentration of duplo measurements (µg/g freeze-dry-weight) of total body analyses after 0, 4, 8, and 12 weeks. C1 = 100% PKVA, C2 = 50% PFA/ 50% sand, C3 = Reference harbour dredged sediment, C4 = control.

	weeks	As	Cd	Cr	Cu	Ni	Se	Zn
beginning	0	27.2	0.2	0.8	8	1.7	1.4	214
C1	4	50.7	0.7	1.9	28.7	5.1	4.2	172
	8	76.1	0.8	2.8	24.5	4.8	6.7	129
	12	55.6	0.4	3.0	18.1	3.3	10.7	99
C2	4	31.2	0.5	1.9	19.0	4.1	4.2	149
	8	51.8	0.5	2.8	19.2	4.2	8.9	128
	12	46.5	0.4	3.0	10.2	3.9	8.9	127
C3	4	22.2	1.4	0.8	27.1	5.9	1.7	232
	8	9.2	1.2	0.7	16.5	1.6	1.5	137
	12	9.9	1.3	1.6	21.3	6.2	2.1	133
C4	4	23.3	0.1	0.4	6.2	2.9	0.9	120
	8	27.5	0.2	0.5	8.6	1.1	1.1	94
	12	19.4	0.2	1.6	5.9	1.6	1.0	75

For nickel accumulation was negligible, despite a sedimentary concentration of 130 in C1, 70 in C2 and 47 µg/g in C3 sediments. This pattern, as shown in Figure 7 is remarkably similar to that of Cr. Selenium accumulates in *N. virens* (Fig. 8). Even after 12 weeks in C1 (100% PFA) no equilibrium was reached (14 µg/g), indicating that accumulation from sediments with a high percentage of flyash would most likely continue for longer periods than 12 weeks, which in terms of accumulation equilibrium is relatively long. In both reference and control sediments, no accumulation of Se was seen. Zinc was found at relatively high concentrations 886 µg/g in the reference sediment (C3) but no accumulation was found in the worms. The tissue concentrations (Fig. 9) produced by all four test sediments ranged from ca. 75 to 232 µg/g. After the 12 weeks exposure the sediments were sampled for leaching tests to determine the degree of leaching of the elements, and for complete destruction to determine the differences in concentration between start and end. The results are presented in Table 3A and 3B. It should be noticed that As and Se concentrations were higher after 12 weeks compared with the start, indicating the mobilisation of both elements in seawater. Zn is leachable to high concentrations, and although start concentrations are missing for the C4 sediment it is expected that Zn is mobilized to high levels.

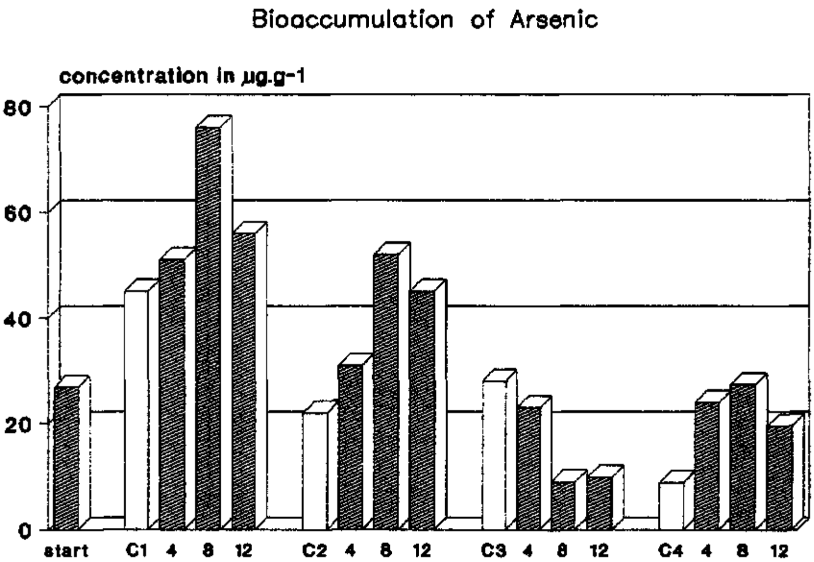

Figure 3-6. The concentration of As, Cd, Cr and Cu in the test sediments (C1-C4 sed.) and accumulation in *Nereis virens* tissue after 0, 4, 8 and 12 weeks. Start indicates the initial concentration (week 0) in *N. virens*. C1 = 100% PFA, C2 = 50% PFA/ 50% sand, C3 = Reference harbour dredged sediment and C4 = control sediment.The white bars represent the element concentration in the sediment.

DISCUSSION AND CONCLUSIONS

The study was carried out with juvenile *N. virens* to gain a more realistic picture of the colonisation of 'fresh' PFA sediments. It was expected (McGreer 1982) that juveniles would have difficulty in maintaining themselves due to the elevated metal concentrations. Benthic diatom growth was heavy, and although no measurements are available, lack of food does not seem to have been the cause of the initial mortality in all four sediments. In mesocosm experiments with cockles, baltic tellins and lugworms (Jenner & Bowmer 1990), comparable high initial mortality was found. The cause is presumably stress by the handling of the organisms. The population density of 100 individuals per m^2 was not too high, as Blake (1979) reported densities of 50 mature individuals with a mean weight of 8 g in natural sediments. For the nereid *N. diversicolor* numbers of 300 per m^2 were found by Chambers and Milne (1975). In the present experiment, growth of *N. virens* was apparently normal in the control situation with a tenfold increase to over 2 g each individual after 84 days. Growth in 100% PFA, 50% PFA and harbour sludge was retarded compared with the control, which can be seen as a chronic effect, probably not caused by elevated metal concentrations in the sediment but by less optimal physical conditions of the sediment, *i.e.* the high silt/clay fraction.

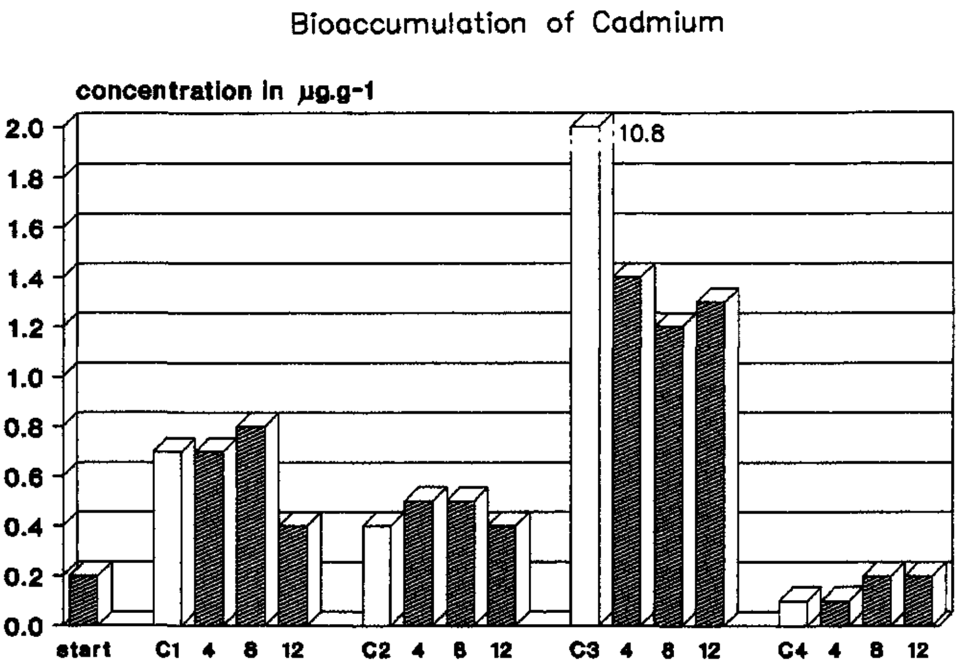

Figure 4. (For caption see Figure 3)

The heavy metals showed sharp contrasts in their uptake patterns: Ni, and Cd hardly accumulated at all in *N. virens*, while As, Cu and Se were moderately accumulated. The behaviour (leaching and accumulation) of Ni, Cr and Cd point to a low bioavailability within the experimental framework for the polychaete worm, which appears to have been able to strictly regulate these metals. The leaching at pH 5 is low indicating that Cr is strongly bound in the matrix of the PFA spheres. The same conclusion can be made for Ni. The Cd concentration in the leachate of the harbour sediment was relatively high and should in theory lead to accumulation but that did not occur, presumably because real Cd concentrations in the interstitial water are much lower. The leaching technique used here (pH 5, S:L = 1:20) did not accurately reflect the availability of the metals in the sedimentary interstitial waters, a conclusion also supported by Ray *et al.* (1981).

The accumulation of As in *N. virens* increased until week 8 in the flyash exposed worms (both duplicates), after which concentrations declined. As the growth rate of the worms was more or less linear in C1 and C2 over the whole experiment, this is not considered to be an important factor in altering the uptake patterns of As. The expected high accumulation of As, due to the relative high concentration of 45 μg/g in the PFA does not occur. One possibility is that bioavailability changed with time under an altered redox regime. Research by Rhyter *et al.* (1979) and Riedel *et al.* (1987) showed that As is bound at the surface of the PFA spheres in a biologically (relatively) available form. This As species (As(V)) may have been accumulated rapidly in the beginning of the exposure in C1 and C2, but reduction to As(III) with time in the sediment may have decreased uptake into tissue and allowed for a slight depuration. This contention is, however, difficult to confirm out of the current experimental data and remains theoretical.

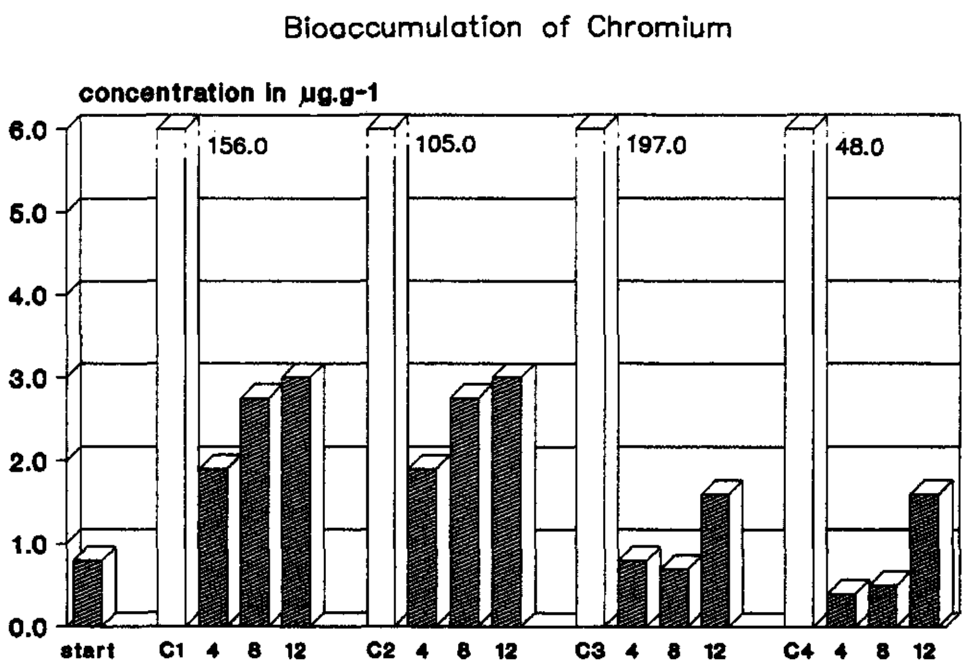

Figure 5. (For caption see Figure 3)

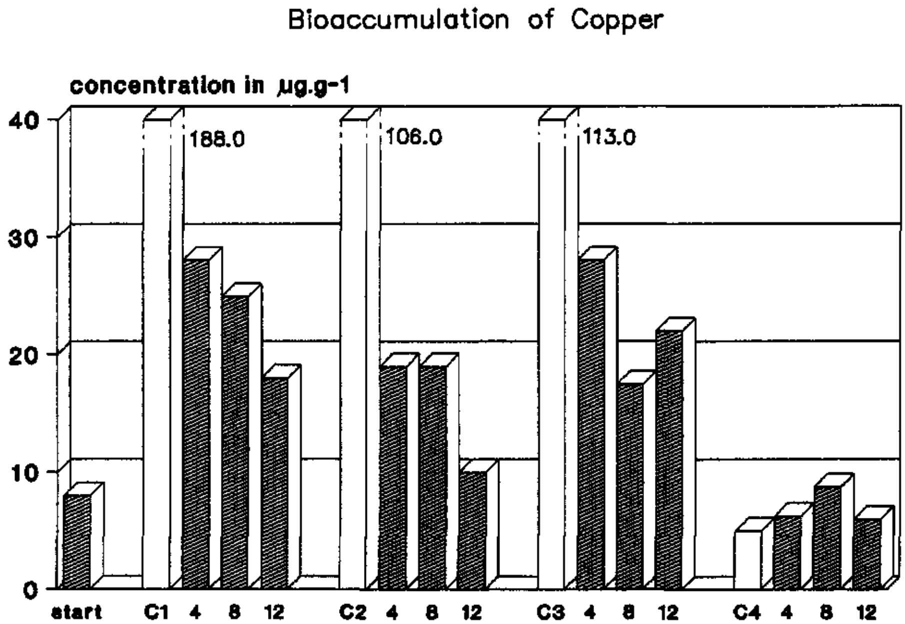

Figure 6. (For captions see Figure 3)

N. virens accumulated Se at a steady rate during the experiment in 100% PFA, and leveled off after 8 weeks in the 50% PFA/sand mixture. The present result agrees with data for lugworms and mussels in a related mesocosm study (Jenner & Bowmer 1990). Initial concentrations in *N. virens* tissues were <1.5 µg/g and increased to a level of 9 µg/g (C2: 50% PFA) and >18 µg/g at which equilibrium is not reached after 12 weeks exposure in C1 (100% PFA). This result, in combination with the relatively high leached concentration in the laboratory tests with PFA, indicate an actual leaching of Se in a form which is bioavailable. Selenium has the potential to cause teratogenic effects and under certain circumstances can biomagnify in the foodchain (Gillespie & Bauman 1986; Biddinger & Gloss 1984). Copper accumulation shows a slight increase after 4 weeks, followed by a decline in the following weeks. This pattern is probably caused by a change in bioavailability of Cu due to complexation of the free Cu ions. The route of Cu-uptake by *N. virens* is expected to shift with time from direct ion uptake out of the pore water by the gills towards a more indirect uptake via nutrition.

Bioaccumulation of Nickel

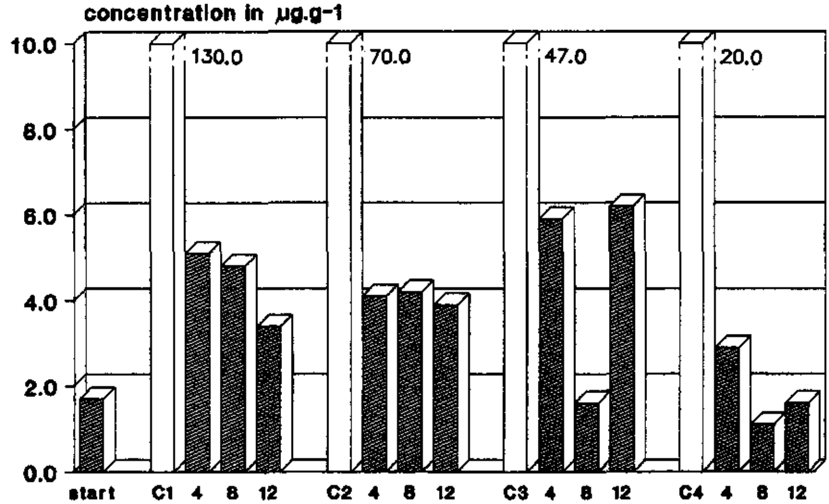

Figure 7-9. The concentration of Ni Se and Zn in the test sediments (C1-C4 sed.) and accumulation in *Nereis virens* tissue after 0, 4, 8 and 12 weeks. Start indicates the initial concentration (week 0) in *N. virens*: C1 = 100% PFA, C2 = 50% PFA/ 50% sand, C3 = Reference harbour dredged sediment and C4 = control sediment. The white bars represent the element concentration in the sediment.

Bioaccumulation of Selenium

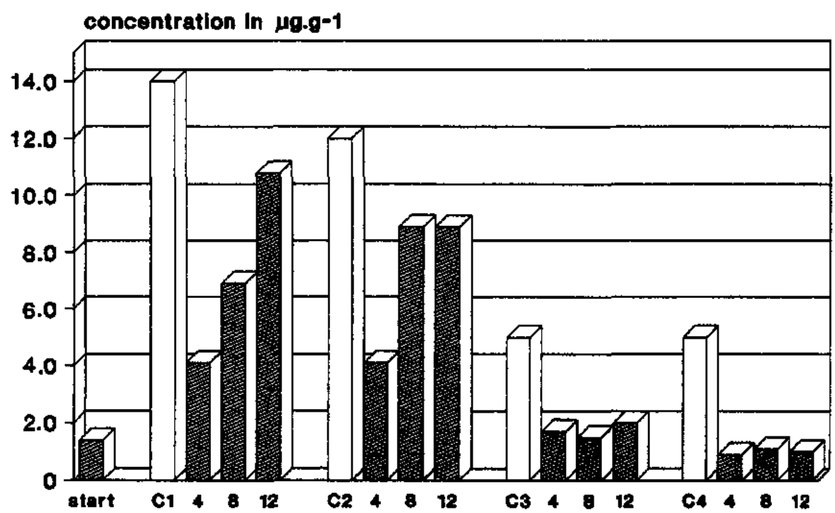

Figure 8. (For caption see Figure 7)

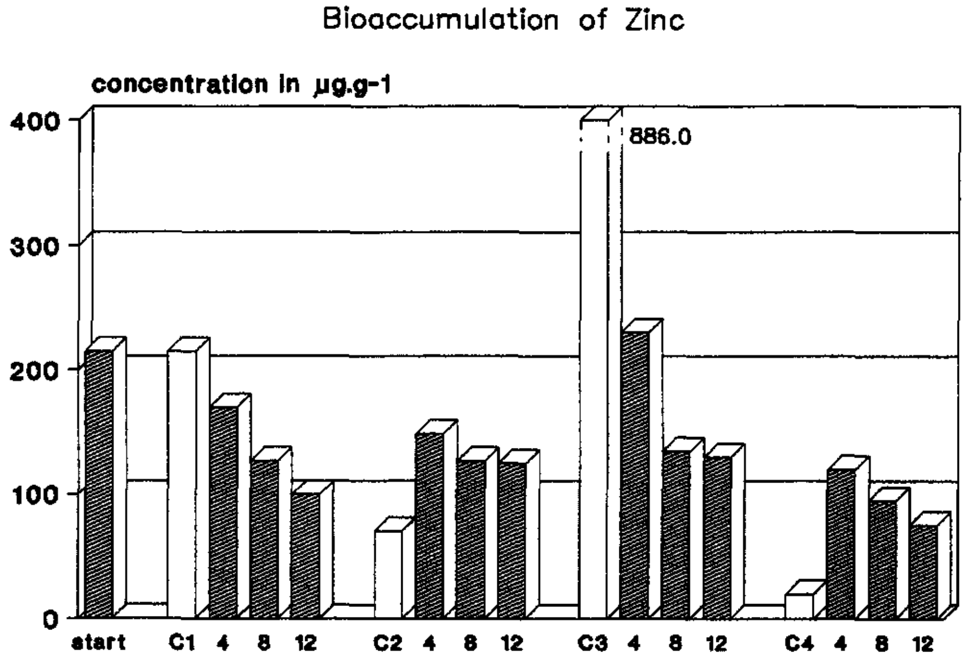

Figure 9. (For caption see Figure 7)

Table 3 A. Metal concentrations (µg/g) of the different substrates at the start and after 12 weeks. C1: 100% PFA; C2: 50-50% PFA; C3: Reference; C4: Control.

	As	Cd	Cr	Cu	Ni	Se	Zn
C1 0 wk	45	–	155	190	136	15	132
C1 12 wk	32	0.7	157	185	123	13	112
C2 12 wk	22	0.4	105	106	70	12	66
C3 0 wk	28	10.8	206	112	46.4	-	1050
C3 12 wk	19	8.9	188	114	48	<5	680
C4 0 wk	8.9	<0.1	48	<5	<20	<5	<20

Table 3 B. Metal concentrations (μg/l) of the lecheates of the different sediments after 12 weeks.

	As	Cd	Cr	Cu	Ni	Se	Zn
C1 0 wk	195	5.2	21	18.2	70	236	66
C1 12 wk	287	2.1	11	8.1	23	273	54
C2 12 wk	97	1.0	<5	12.8	<5	78	43
C3 12 wk	18.5	111	<5	41	121	<5	5160

At the beginning of the experiment more free Cu ions will exist in the water phase which may have been responsible for a rapid initial accumulation. With time this amount is reduced by complexation with organic matter (Marquenie 1985; Zorkin 1986). This change in availability may explain a steadily lower Cu concentration towards the end of the 12 weeks exposure to 100% PFA.

N. virens appears to have been able to regulate Zn exceptionally well to accumulated levels of 75 to 175 μg/g. The leacheate tests for the harbour sediments tentatively suggest an availability of circa 700 μg/g. The decline in concentration with time in all four exposures may be a natural process, as described by Bryan (1976) and Bryan and Hummerstone (1971), who found a good homeostasis with Zn. However, according to Gillet (1987) a difference of a factor 2 is normal within a season. Our concentration data are in good agreement with the data of Rhyter *et al.* (1979). In a comparable experiment with Zn-concentrations in the PFA of 170 μg/g accumulation was in the order of 220 μg/g.

This study leads to the following conclusions:

1. Mortality of *N. virens* was largely restricted to the initial period of the exposures, and no significant differences were observed over the whole experiment. Growth, on the other hand, was reduced in the PFA and harbour sediment exposures, although the direct influence of metal contamination in this remain unproven.
2. Selenium originating from PFA accumulates slowly but consistently in *N. virens*. Exposure times have to be tripled to 36 weeks or longer in order to gain a better insight in selenium accumulation and effects.

3. The other metals: As, Cd, Cr, Ni, Zn, and Cu - were only accumulated to little morte than background sedimentary levels. Accumulation was rarely more than 3x the start tissue concentration. However, this is not typical of benthic invertebrate in general, as many species appear to regulate or accumulate metals in an entirely different way.
4. Care should be taken when assessing potential environmental dammage on the basis of sediment chemistry alone. The chemical analysis technique used should be carefully examined in the light of sedimentary bioavailability used bioaccumulation data where possible.

ACKNOWLEDGEMENTS

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CHAPTER 5

THE DETECTION OF CHRONIC BIOLOGICAL EFFECTS IN THE MARINE INTER-TIDAL BIVALVE *CERASTODERMA EDULE*, IN MODEL ECOSYSTEM STUDIES WITH PULVERIZED FUEL ASH: REPRODUCTION AND HISTOPATHOLOGY

INTRODUCTION

At present in The Netherlands, coal based electricity generation amounts to circa 40% of the total output, an increase of 30% compared with the 1980s. The production of pulverized fuel ash (PFA), more commonly known as fly-ash, has consequently increased. PFA contains significant quantities of a broad range of elements including heavy metals, whose concentrations can vary considerably, depending on the origin of the coal and the type of combustion process (Meij 1992). The 'quality' of the resultant PFA may thus vary, rendering some types unsuitable for reusage on environmental grounds. The application of 'low NOx' burners in order to reduce NOx emissions (oxides of nitrogen forming greenhouse gasses), has led to changes in physical and chemical properties of the PFA. The question remains as to whether the same proportion of the PFA production and especially the 'low Nox' type PFA can be reused in the coming decades. If this is not the case, then a storage and disposal problem will be created, a situation which already exists in many European countries. PFA poses a threat to the environment due to the leaching of a variety of elements both from the surface and from within its constituent particles; this has been a topic of research for the last two decades (Dvorak *et al.* 1977; Ryther *et al.* 1979; Bamber 1980; 1984; Brown & Ray 1983). PFA consists of glassy siliceous spheres containing many other elements.

This chapter is based on:
Bowmer CT, Jenner HA, Foekema E, Van der Meer M (1993) The detection of chronic biological effects in the marine intertidal bivalve *Cerastoderma edule*, in model ecosystem studies with pulverised fuel ash: reproduction and histopathology. Environ Pollut 85:191-204.

A rough distinction can be made between cations (e.g. Cu, Ni, Cr and Zn) which occur mainly within the matrix of the particles and anions (e.g. As, Se, F, B, Mo) which occur adsorbed upon the outside of the PFA spheres due to their lower vapour pressure at the time of formation. The fate of elements in the combustion process from coal to precipitated fly-ash is described by Meij (1992). The anions will tend to leach more rapidly under aqueous conditions, especially As, Se, Mo and B (Van der Sloot *et al.* 1983; Jenner & Mommen 1989).

There has been a shift away from the chemical monitoring of environmental problems towards a more integrated ecotoxicological approach in recent decades. Furthermore, where vast quantities of solid materials are concerned, there is a need to go beyond purely laboratory based toxicological research in order to predict the possible environmental consequences, in this case, as a result of PFA storage and disposal. It should be stressed that the dumping of PFA at sea is prohibited in The Netherlands. This study addresses itself rather to the international situation, where PFA dumping at sea is still common (Eagle *et al.* 1979).

Multispecies bioassays with arrays of ecologically important marine invertebrates, exposed under semi-natural conditions, are an increasingly utilised method for detecting the broad-scale effects of a substances when released to the marine environment. While they are not as complex as mesocosm experiments, which attempt to faithfully model a particular environment and its governing processes, they do provide more broad scale information about chronic effects than do shorter-term laboratory tests. For the sake of convenience, the terms model ecosystem or model ecosystem bioassay has been adopted throughout this paper.

This paper reports on the second phase of a study carried out in an outdoor bioassay system at TNO Den Helder (Jenner & Bowmer 1990) and concerns the detection of chronic effects in the filter-feeding bivalve mollusc *Cerastoderma edule* as a result of PFA exposure. Effects within individual organisms can be detected at various levels of organisation, ranging from the purely biochemical, through the early stages of sub-cellular pathology where lysosomes become destabilised, to more complex effects such as metabolic disturbance of growth, reproduction and ultimately survival (see Sindermann 1980; Bayne *et al.* 1980; 1988). This study focusses on measuring disturbances at the level of reproduction, histopathology and survival.

MATERIAL AND METHODS

Sediment of the model ecosystems

Five circular 2.2 m^2 model ecosystems (height, 80 cm; diameter, 180 cm) were filled with the test sediments in late September 1987 and the material was allowed to settle for 1 week. Test sediments and their preparation are described in Jenner & Bowmer (1990), as is the experimental setup. The exposed test species were: 200 *Cerastoderma edule*, 200 *Macoma balthica* and 170 *Arenicola marina*. The latter two species are not further considered here (see Jenner & Bowmer 1990). The same notation of test sediments described in Jenner & Bowmer (1990) is used again: M1: 100% PFA; M2: 50% sand and 50% PFA; M4: reference (contaminated harbour-dredged sediment); M5, Control (Waddensea sediment). It should be noted that M3 as reported in the above paper was not used in this present study. The initial exposure lasted 90 days, *i.e.*, until early December, when samples of all three species were taken to assess mortality, growth and in the case of the cockle, histopathological condition. For *C. edule,* the wet weight, dry weight and ash-free dry weight were determined and the shell length and width was measured to the nearest 0.01 mm with a digital callipers. Tissue homogenates were analyzed by AAS and NAA (see Jenner & Bowmer 1990). Samples of *C. edule* were fixed in neutral buffered formalin for histological sectioning. After the 90-days sampling was complete, the remaining *C. edule* (25 - 50) were returned to the model ecosystems for a further 140 days, giving a 230-days exposure. The aim was to examine reproductive maturation the following April (1988), at which time a further histopathological analysis was carried out. Mortality during the second phase of the experiment was not monitored regularly. The individuals which were returned to the tanks after the 90-days sampling were recovered after 230 days by digging and sieving the top 10 cm layer of sediment and the absolute numbers of living cockles were recorded.

Test species

The cockle *C. edule* is a burrowing, filter feeding, Cardiid bivalve with a strong shell and short siphons, occupying the upper 3 to 4 cm sediment layer. While greatly influenced by the overlying water-column, cockles are considered by the present authors to be in close physical contact (foot, mantle and siphons) with the upper (oxidised) sediment layers, where contaminant release takes place. Along with the cockle, the baltic tellin *Macoma balthica* and the lugworm *Arenicola marina* were used in these exposures. Bioaccumulation in these latter two species is reported elsewhere (Jenner & Bowmer 1990), and is treated only incidentally here. These latter two species represent slightly different microniches, *M. balthica* being a deeper burrowing, surface deposit feeding bivalve, and *A. marina* being a deep burrowing, direct deposit feeder.

Histological preparation

C. edule were sampled to examine for cytological condition and pathological abnormalities after 90 and 230 days. They were fixed in neutral buffered formalin (NBF) on the day of collection. *C. edule* had one adductor muscle severed with a scalpel before fixation to allow adequate infiltration of the tissue. The NBF was replaced after 3 weeks to ensure continued preservation and no tissues were further processed until 3 months after fixation. After this period, they were removed from their shells, the tissue being cut into two portions along the horizontal plane. The tissues were placed in standard histological processing cassettes and rinsed in running tap water for several hours to remove grit, mucus etc. They were then processed using a vacuum impregnation processor on an 8h cycle and blocked out on the cassettes. All wax blocks were sectioned in duplicate (approx. 200 µm apart) at 4 µm on a rotary microtome. The mounted sections were stained with Ehrlichs haematoxylin and eosin (HE) on an automatic slide stainer.

Reproduction

Histological material from *C. edule* exposed for 230 days in the model ecosystems to the 100% PFA, 50% PFA and 50% sand, the reference sediment and the control sand were examined by light microscopy to assess the relative reproductive maturity and development.

Oocyte diameter frequency

One hundred primary oocytes from each female in the four samples were measured along their longest axis and at 90° to this, with an ocular micrometer graticule at x400 magnification. The mean of the two values was graphed in histogram form for all 100 oocytes. Subsequently, oocyte diameters for all sampled females in a particular model ecosystem were pooled and regraphed. These composite graphs allowed comparison of the oocyte size frequency and state of development between the different PFA treatments, reference and control.

Gonad packing density or relative volume

A Weibel ocular microscope graticule (Briarty 1975) was used to assess the packing of the eggs in the gonads, sometimes known as the relative volume. This is a point counting method often used with the light microscope. When killed in formalin the bivalve foot relaxes, releasing muscular pressure on the nearby viscera and interspersed gonads. Thus, with identical histological preparation, the packing density of the oocytes in the gonads should be indicative of the volume of a tissue produced in relation to the space available. Five fields (42 points each) were measured per individual female, and the % oocyte surface area was calculated in relation to empty space. Qualitative observations of gonad morphology and condition are also presented.

Maturity index

The reproductive stage of maturation of each mussel was determined from the histological slides according to general morphological and germ cell developmental criteria as given by Seed (1969). The scores (1, resting gonads, 2, early proliferation, 3, maturation, 4, ripe, and 5 spawning) of the individuals were averaged to provide an indication of the reproductive status of the whole population. A mean score of 4 indicates that the population is fully ripe prior to spawning, while a score of 1 indicates the absence of gametogenic proliferation. An average score of higher than 4 indicates that spawning has already commenced.

Histopathological screening

All duplicate slides were examined microscopically and some 10 tissues per individual mussel were screened using a protocol for determining the cytological/pathological condition of a range of key tissues. See Bowmer *et al.* (1991, *Dreissena polymorpha*, a freshwater mussel) and Table 1 for further information, also Bright and Ellis (1989, *Cereantheopsis americanus*, a burrowing marine anemone) and Peters and Yevich (1989, *Macoma charlottensis*, a burrowing tellenid bivalve). In assessing condition, it is assumed that 'normal' is definable (in this case cytological normality), an assumption upon which medical science is founded. The emphasis in the present analysis is on identifying normality and the extent of cytological deviations from this position, as defined by a set of criteria peculiar to the cytology and morphology of each tissue or organ. The authors fully accept that analysis of a standard section containing a limited range of tissues for purely cytological parameters alone provides only a partial diagnostic picture.

The cytological quality of the following tissues and organs was assessed on a simple scale of 1 to 5: hepatopancreas, stomach, style-sac, intestine, nerve ganglia, kidney, gill, adductor muscle, foot and haemal connective tissue in general. A full survey of invertebrate parasites was also completed using the histological material.

Table 1. The assessment criteria for histopathological condition, based on a scale of 1 to 5, five being normal.

	QUALITY	DEFINITION
Score 5	Excellent: the 'normal' ideal tissue condition	Apparently fully functional tissue or organ; Epithelia, brush borders, basement membranes and supporting connective tissue are regular in cytology and intact, showing an expected degree of haemocyte infiltration, and no inflammations, small cystomas, etc.
Score 4	Good: still healthy	The tissues are generally regular in cytology and only occasional small scale anomalies are present, i.e. a localised *light* inflammation, or other small irregularity.
Score 3	Moderate: the majority of tissue still functions	A *moderate* condition with obvious disruption of the tissues is present (<50% of the observed area if well represented in section), this can be a substantial inflammation, the presence of multiple cystomas, hyperplasia of the tissue, atrophy, etc.
Score 2	Poor: the majority of the tissue is damaged	The majority of the tissue shows severe inflammation, hyper/neoplasia, is atrophied, wasted, or otherwise *severely* abnormal. In the case of an organ, there are substantial morphological abnormalities.
Score 1	Very poor	This score is only rarely given, as little or no normal tissue remains; the tissue or organ can be assumed to have ceased to function. Massive wasting and degeneration are evident.

RESULTS

Mortality

Cerastoderma edule came to the surface of the sediment when under stress, where they were recorded every second or third day. Death usually followed within 1 - 5 days after surfacing. Figure 1 presents the pattern of mortality of *C. edule* during the entire model ecosystem experiment. Few if any *C. edule* were found to have died and remained buried. In the Reference (M4) and Control (M5) sediment, a gradual mortality occurred among the populations with survival rates of 93% and 87%, respectively. Mortality had more or less ceased in these model ecosystems after 45 days. The 100% PFA (M1) sediment produced a sharper pattern of mortality, with no levelling off of the curve in Figure 1 after 90 days. Some 57% survived the entire 230-days exposure. The 50% PFA (M2) exposed individuals showed a generally low mortality until the 60-days mark when a sudden increase occurred, leaving only 75% alive after 90 days and 57.5% after 230 days.

Growth

The growth and flesh quality of *C. edule* was monitored by measuring mean shell lengths and widths as well as mean wet weights (less shell) and % ash-free dry weights at 0, 90 and 230 days. As can be seen in Table 2, growth was slow in all cases and standard deviations of both length and width are both relatively large in this respect at ca. 10%. The control cockles showed an increase of ca. 0.3 mm in length (l) and 0.5 mm in width (w) to December (90-days), followed by a further increase of 1.4 (l) and 1.0 mm (w) to April (230-days). However, few of the other exposed cockles showed an interpretable net growth during the 230-days experiment. Apparent decreases in the growth of the population exposed to the 50% PFA mixture and the reference sediment may have been due to differential mortality of older size classes. It should be noted that the observed increases are slight in relation to the population variation; these are adult cockles which have passed through the phase of rapid shell growth.

The pattern of wet weight (less shell) is clearer; from a mean start weight of 1.70 g, a slight increase (+0.16 g) was recorded to December and a further increase to April (+0.14 g) in the control population. The Reference (M4) cockles remained the same and both the 100% PFA group and the 50% PFA exposed animals declined to a mean wet weight of ca. 1.3 g after 230 days.

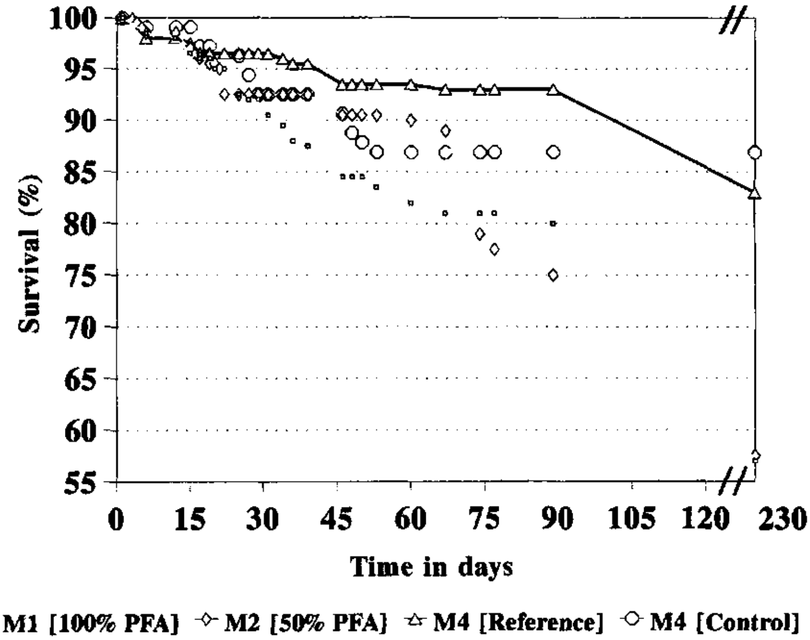

Figure 1. *Cerastoderma edule*: mortality during the 230-days exposure to PFA sediments, among surfacing individuals.

Bioaccumulation of heavy metals and metalloids

The patterns of heavy metal and metalloid bioaccumulation observed in *C. edule*, *A. marina* and *M. balthica* during this experiment have been presented in detail in Jenner and Bowmer (1990) and the uptake to 90-days will only be briefly dealt with here. Table 3 shows the concentrations of As, Cu, Zn, Ni, Cd, Sb, Cr, and Se accumulated by *C. edule* after 90 and 230-days exposure. It is assumed that this species accumulated most of the named elements to equilibrium well within 90 days.

Cadmium showed little sign of accumulation between 0 and 90-days. Arsenic barely doubled in cockles from the 100% (M1) and 50% PFA (M2) exposures between 0 and 90 days. These accumulation levels remained remarkably similar at 230-days in all model ecosystems, and showed no further increases. The element Sb was below the limit of analytical detection in the start sample, and showed a definite accumulation of unknown significance to ca. 1 µg/g in the 100% and 50% PFA exposed animals after 90 days. This concentration approximately doubled to 2 µg/g after 230-days exposure to the 50% PFA and 50% sand mixture only. No Sb accumulation was seen in the control.

Chromium was accumulated to ca. 10x the start tissue concentration (0.5 µg/g) at 90-days in the 100% and 50% PFA, and to 4x this value in the reference sediment population. The accumulation pattern was the same at 230-days with the exception of the 50% PFA individuals, which had reached ca. 12 µg/g.

Selenium levels were not measured at 230-days, but were present at ca. 1.4 µg/g in the cockles at the start. At 90-days, increases of 3 to 4x were recorded in both PFA sediments.

Table 2. Mean wet weight, shell length and width of *C. edule* at 0, 90 and 230-days are shown as measures of growth. Due to sampling procedures the wet weights s.d. is not available for 90 and 230-days.

0 days	90 days	230 days
Start sample.	**100% PFA**	
Weight: 1.7 ± 0.4	Weight: 1.56	Weight: 1.30
Length: 27.8 ± 1.6	Length: 27.8 ± 2.1	Length: 29.0 ± 2.1
Height: 18.2 ± 1.3	Height: 18.2 ± 1.5	Height: 19.0 ± 1.5
	50 PFA and Sand mixture	
	Weight: 1.62	Weight: 1.34
	Length: 29.1 ± 2.3	Length: 28.5 ± 1.9
	Height: 19.2 ± 1.7	Height: 18.9 ± 1.5
	Reference sediment	
	Weight: 1.71	Weight 1.98
	Length: 28.3 ± 2.1	Length 28.3 ± 1.7
	Height: 18.1 ± 1.5	Height 18.8 ± 1.4
	Control sediment	
	Weight: 1.86	Weight 2.20
	Length: 28.1 ± 3.0	Length: 29.4 ± 1.4
	Height: 18.6 ± 2.3	Height: 19.6 ± 1.4

Table 3. The start concentrations (day 0) of As, Cu, Zn, Ni, Cd, Sb, Cr and Se and those accumulated by *C. edule* after 90 and 230-days exposure (mg kg^{-1}) are shown. Se concentrations after 230-days are missing. M1 = 100% PFA; M2 = 50 % PFA and 50% Waddensea sand; M4 = reference contaminated harbour-dregded sediment; M5 = control sediment of Waddensea sand.

	Day 0	Day 90				Day 230			
		M 1	M 2	M 4	M 5	M 1	M 2	M 4	M 5
As	9.5	17.8	21.8	13.8	10.4	18.3	20.9	12.5	10.6
Cu	4.4	8.8	10.6	7.6	6.1	13.8	20.4	10.6	17.2
Zn	71.3	80.1	74.5	83.6	76.2	118.5	82.2	132.7	142.2
Ni	5.4	51.4	44.0	39.4	36.5	79.7	87.5	76.6	52.8
Cd	0.18	0.43	0.38	0.64	0.29	1.63	1.58	0.42	0.61
Sb	<0.01	0.34	0.37	0.09	<0.01	0.38	0.69	<0.01	<0.01
Cr	0.5	6.2	4.8	4.8	2.0	6.1	12.2	3.1	1.6
Se	1.4	7.7	9.8	1.9	2.0	-	-	-	-

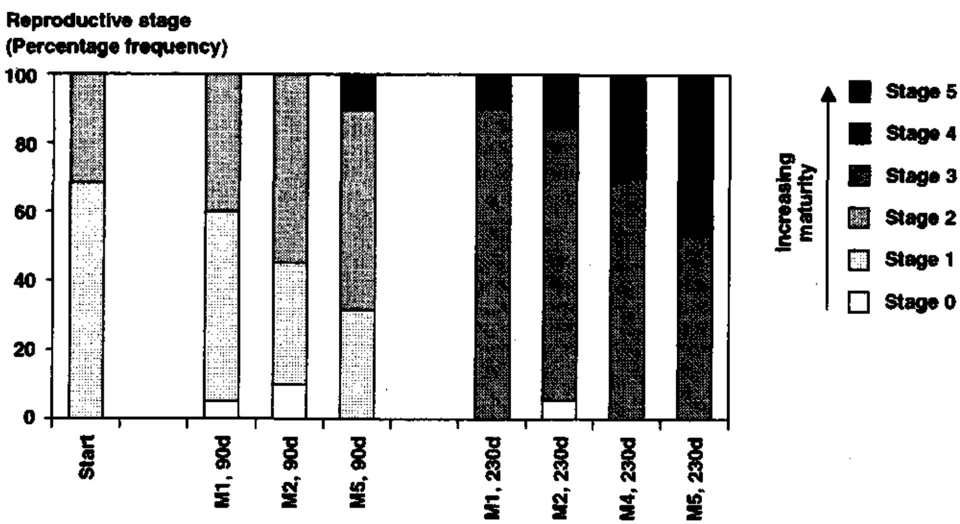

Figure 2. *Cerastoderma edule*: the stage of reproductive maturity expressed as procentage frequency in the populations sampled. M1 = 100% PFA, M2 = 50% PFA and 50% sand, M4 = contaminated harbour dredged reference sediment, M5 = clean control.

Zinc showed no uptake at 90-days in any model ecosystem population, but had doubled from ca. 70 µg/g to in excess of 150 µg/g in all model ecosystems including the control (50% PFA excepted).

Nickel increased from <10 µg/g at the start to ca. 50 µg./g at 90-days in all treatments. At 230-days, further increases to ca. 75 - 100 µg/g were recorded in all populations except those from the control. A similar pattern was observed for Cu, although the general concentrations were lower than for Ni.

Reproduction

Sex ratio and stage of maturity

Table 4 shows the sex of all the cockles examined. Clearly, in September at the start of the experiment and in December after 90d exposure, many individuals were so underdeveloped as to be un-sexable. However, given that *C. edule* has a 1:1 sex ratio normally and roughly half of the populations were identified as females, it is to be expected that most or all of the un-sexable individuals were males. These stage 1 individuals had not yet undergone the mitotic divisions necessary to produce identifiable spermatocytes. In April, all individuals were sexable with the exception of one parasitically castrated individual; The sex ratio after 230-days exposure was 7:3, 3.5:6.5, 4.2:5.8 and 3.5:6.5 males to females in the control, 100% PFA, 50% PFA and harbour dredged reference sediment respectively. In small samples of invertebrates, such differences are to be expected (Bowmer 1982). However, the suggestion in the data of a higher differential mortality among the males cannot be ruled out.

The stage of maturity was morphologically determined for each individual according to a simple five step scheme. Many such schemes have been developed, including very detailed ones such as that of Lammens (1967) for *M. balthica* which identified 3 immature stages, and 8 stages for maturing males and a further 8 for females. Following common practise, the stage of each individual was averaged for the population to provide a maturity index in the present study. As can be seen from Figure 2, the gonad development apparently followed its natural seasonal cycle, the stage of morphological development having advanced considerably in all three samples, increasing from a mean gonad index of 1.4 - 2.0 in December to 3.0 to 3.7 in April the following year (on a morphological scale of 0 to 5). This

December control sample appeared to have a higher mean level of maturity (2.0) compared to the rest caused by a larger proportion of individuals in stage 2 and two individuals which still retained some gametes from the previous year, placing them in stage 5.

The control samples clearly showed the greatest morphological development in April (mean 3.65), some 50% having arrived at the fully ripe stage 4 or the spawning stage 5, showing gonads filled with spermatozoa or fully ripe vittelogenic oocytes. On the other hand, the 100% and 50% PFA exposed individuals were mainly at stage 3, (means of 3.00 and 3.10 respectively) where the males possessed a mixture of all spermatogenic stages (spermatogonia, spermatids, spermatocytes and spermatozoa) arranged in 'strings'. The gonad follicles were as yet incompletely expanded. Females possessed a mixture of vitellogenic and previtellogenic types, with only some individuals showing fully ripe gonads.

Oocyte diameter frequency

The oocyte (egg) diameter frequency i.e., the diameter measurement of ca. 100 eggs as measured per individual female cockle from the histological slides, is presented in Figure 3. The histograms for each individual have been pooled for each sample (exposure) to provide a composite view. From these it is evident that *C. edule* oocytes reach a size of 50-70 µm just prior to maturity (formalin preserved material).

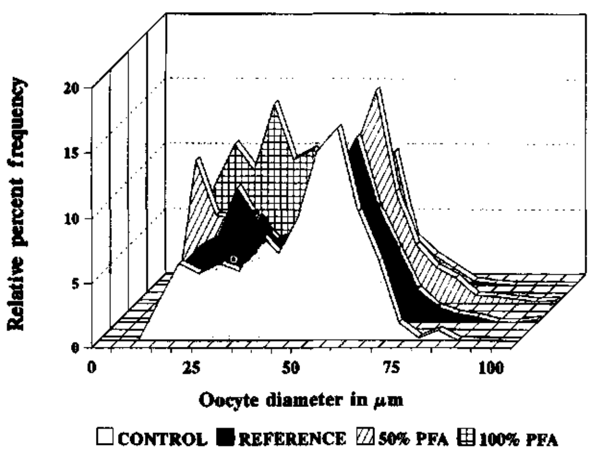

Figure 3. *Cerastoderma edule*: The relative percent frequency of oocyte diameters. The histograms comprise ca. 150 measured oocytes from each female; this data has been pooled for each PFA exposed population to provide a measure of maturity.

Furthermore, the control individuals have a clear peak of mature oocytes with a mode of 55 μm diameter. This peak is entirely absent in the 100% PFA exposed individuals which were evidently far less developed, in agreement with the maturity index above. The 50% PFA and reference sediment exposed individuals both showed clear peaks of mature oocytes, but had relatively more immature cells. An oocyte diameter of 40 μm was arbitrarily chosen as the limit below which eggs were unlikely to be ripe in time for spawning. The percentage of 'unripe' eggs was 61%, 40.4%, 40.9% and 3 4% in the 100% PFA, 50% PFA, Reference and control sediments respectively.

Gonad packing density/relative volume

As shown above in Table 4, the sexes are separate in *C. edule*. The reproductive system is morphologically similar in both sexes. The male and female gonads do not form a discrete organ, the round or oval follicles being spread diffusely throughout the visceral mass, surrounding the digestive gland and the central spirals of the gut. Additionally, the small individual follicle sacs are found extensively among the muscle blocks in the area below the foot.

The diffuse nature of the gonadial material makes it difficult to dissect out this organ or perform measurements on it as a whole; therefore the assessment of absolute numbers of oocytes produced was not attempted in this study. As outlined in the Materials and Methods, a random point counting method (Wiebel graticule) applied to histological slides of the tissue was employed to at least gain some idea as to how much relative effort was put into the production of oocytes (the males were not similarly examined) in the histologically sectioned material. The results are presented in Table 5. The gonads are made up of surprisingly large amounts of fluid-filled space both within and between the sacs. Some of the spaces stain purple in histological section (HE), suggesting the presence of mucous. On average, 'empty' blood spaces and mucous makes up ca. 60% of the relative volume of the gonad. This varied little between the exposed populations, or between individuals. The 100% PFA, 50% PFA and reference sediment exposed populations did show a tendency towards more connective tissue (muscle and membranous connective tissue) within the gonad area. Generally however, the relative volume of oocytes and oogonial foci was very similar. The Input into gonadial product appears to have been similar in all the test populations. However, this method only focuses on the follicles and not on the packing of the gonad area as a whole. Thus, all four populations appeared to have an equal reproductive input in terms of female gamete material, but the state of development of that material differed widely. The 100% PFA exposed population possessed less well developed/matured eggs than did the other populations.

Histopathology

The histopathological analysis is based on the quality of selected individual tissues and focusses primarily on the condition of the digestive gland, the major centre of metabolic activity in bivalve molluscs. Table 6 shows the mean scores of the 10 tissues and organs analyzed on a scale of 1 to 5 as indicated in the materials and methods section and Table 1. The operative part of the scale for each tissue is between 3 and 5 when mean population or sample scores are being considered; the data is not normally distributed.

Table 4. The sex ratios and and maturity index (± sd) of *C. edule* is presented for all model ecosystem exposures to PFA, reference sediment and control sediment. M1 = 100% PFA; M2 = 50 % PFA and 50% Waddensea sand; M4 = reference contaminated harbour-dregded sediment; M 5 = control sediment of Waddensea sand.

Cerastoderma edule			
Start September '87			
(1.32 ± 0.46)			
♂ : 1			
♀ : 10			
? : 8			
Control Dec. '87	100% PFA Dec. '87	50% PFA Dec. '87	
(2.00 ± 0.76)	(1.35 ± 0.57)	(1.45 ± 0.67)	
♂ : 2	♂ : 0	♂ : 2	
♀ : 12	♀ : 8	♀ : 11	
? : 5	? : 12	? : 7	
CONTROL April '88	100% PFA April '88	50% PFA April '88	Reference April '88
(3.65 ± 0.76)	(3.10 ± 0.30)	(3.00 ± 0.79)	(3.30 ± 0.46)
♂ : 12	♂ : 7	♂ : 8	♂ : 7
♀ : 5	♀ : 13	♀ : 11	♀ : 13
? : 0	? : 0	? : 1 (castrated)	? : 0

Conditions scoring 1 or 2 are severe enough to be relatively rare in a sampled population, as the individuals may rapidly succumb and die. The specific scores for the individual tissues and organs are detailed below.

Start

At the start of the experiment, a score of 4.0 ± 0.8 was recorded for the digestive gland. This indicates a good condition, i.e. the hepatopancreas was found to be of the expected size in relation to the size of the animal and the tubules and branches were relatively

Table 5. The relative volumes of female *C. edule* gonadial tissue are shown as a percentage of the total volume. The relative gonal (oocyte) volume is a measure of the anaimals ability to produce oocytes. The data is based on a mean of five random point counts per individual, presented as a population mean with sd.

	Oocytes	Oogonia	Connective tissue	Muscle	Empty space	Mucous
100% PFA	23.7 ± 4.1	5.8 ± 3.6	10.5 ± 3.8	4.0 ± 3.4	30.2 ± 6.0	27.0 ± 5.0
50% PFA	25.7 ± 5.0	3.6 ± 1.6	9.5 ± 3.4	3.6 ± 1.6	32.8 ±10.5	26.4 ± 10.0
Reference	28.5 ± 4.0	4.6 ± 4.2	8.7 ± 4.0	1.5 ± 1.6	25.8 ±10.5	31.0 ± 1.5
Control	25.7 ± 1.2	3.7 ± 6.0	7.2 ± 3.8	0.6 ± 0.5	20.0 ± 8.9	42.5 ± 8.6

closely packed together. The average score reflects a light to moderate infiltration of the blood spaces by granulocytes and lymphocytes in the majority of individuals. Tubule degeneration was rare. One individual showed degeneration of the entire hepatopancreas, with haemocytes and cellular debris in the tubule and branch lumina as far as the stomach. This may be related to the presence of fungal hyphae in some tubules. The stomach, style-sac, intestine, nerve ganglia, kidney, and gills showed population scores of greater than 4.5 on average, indicating a good to excellent condition, with normal epithelia and little or no signs of inflammation of degeneration. The muscular organs, i.e. the foot and large adductor showed normal muscle blocks and surrounding epithelia.

The connective tissues of the haemal spaces, i.e surrounding the intestine and hepatopancreas as well as the gonads, scored 3.6 ± 1.0 which can only be considered as moderate.

Furthermore the large standard deviation indicates that in some individuals these tissues were in quite poor condition. Inflammatory responses were present in the central connective tissues surrounding particularly the gonads, accompanied by the occasional small to medium haemocytoma (<250µm diameter, see Lowe & Moore, 1979). This condition is considered to be the result of resorption of the remaining gametes in the gonads following spawning some months earlier.

Table 6. The average histopathological condition of *C. edule* tissues is presented. Individual tissues are scored on a scale of 1 to 5 (5 being normal). While such data are not strictly speaking mathematically normal, a mean and standard deviation are sufficient to illustrate trends.

Tissue / organ	Start	Control	100% PFA	50% PFA	Control	100% PFA	50% PFA
	0d	90d			230d		
Hepato-pancreas*	4.0 ± 0.8	4.2 ± 0.9	3.5 ± 0.6	3.6 ± 0.5	4.4 ± 0.7	4.3 ± 0.6	4.2 ± 1.1
Stomach	4.6 ± 0.8	4.8 ± 0.6	4.6 ± 0.7	4.9 ± 0.3	4.8 ± 0.6	4.6 ± 0.8	4.8 ± 0.6
Style-sac	4.8 ± 0.4	4.6 ± 0.5	4.6 ± 0.6	4.2 ± 0.8	4.6 ± 0.7	4.7 ± 0.6	4.8 ± 0.4
Intestine*	4.3 ± 0.7	4.4 ± 0.7	4.6 ± 0.5	4.1 ± 0.8	4.3 ± 0.6	4.5 ± 0.8	4.8 ± 0.4
Nerve ganglia	4.8 ± 0.4	5.0 ± 0.0	4.7 ± 0.6	4.8 ± 0.6	5.0 ± 0.0	4.8 ± 0.4	5.0 ± 0.0
Kidney*	4.7 ± 0.6	4.5 ± 0.7	4.3 ± 0.7	4.6 ± 0.7	4.6 ± 0.7	4.5 ± 0.5	4.5 ± 0.8
Gill*	4.9 ± 0.3	4.7 ± 0.5	3.9 ± 0.9	4.3 ± 0.8	4.7 ± 0.5	4.5 ± 0.5	4.8 ± 0.4
Adductor	5.0 ± 0.0	4.9 ± 0.2	5.0 ± 0.0	5.0 ± 0.0	4.8 ± 0.6	5.0 ± 0.0	5.0 ± 0.0
Foot	4.9 ± 0.5	4.8 ± 0.6	4.8 ± 0.6	4.8 ± 0.4	4.8 ± 0.5	4.8 ± 0.5	5.0 ± 0.0
Haemal connective tissue*	3.6 ± 1.0	4.6 ± 0.5	4.2 ± 0.8	4.6 ± 0.6	4.6 ± 0.8	4.4 ± 0.8	4.1 ± 1.2

90-days exposure

The data from the samples taken in December 1987 need to be interpreted in terms of seasonal factors. In winter, light is much reduced, water temperature declines rapidly and the production of phytoplankton in the model ecosystems is minimal in parallel with a reduced metabolism on the part of the test organisms. Thus, occasional signs of malnutrition are not unusual. The hepatopancreas scored 4.2 ± 0.9 in the control individuals. The overall condition was somewhat more variable than at the start of the experiment, and localised hepatopancreas tubule degeneration was evident in some individuals with or without the usually associated infiltration/inflammation of the blood spaces and connective tissues, see Plates 1 and 3. Some individuals showed a thinning out of the tubule and branch structures lateral to the stomach.

All other tissues scored more than 4.5 on average with the exception of the intestine, which at 4.4 gave little cause for concern. The connective tissue surrounding the intestine and gonads was in excellent condition, and all inflammatory reactions had disappeared since the start of the experiment 90-days earlier. The gonad morphology itself remained unchanged.

The individuals exposed to 100% and 50% PFA for 90-days showed a reduced hepatopancreas condition of 3.5 ± 0.6 and 3.6 ± 0.5, respectively; many individuals showed distinct signs of malnutrition. The tubules and branches were frequently widely separated in large open blood spaces, indicating a considerable lack of function, see Plate 2. In some individuals, local degeneration of tubules and branches was observed. Inflammation was usually localised to these lesions. Small (30 - 100 µm) haemocytomas were evident in ca. 20% of all individuals from both samples.

The gills of the 100% PFA exposure showed a score of 3.9 ± 0.9, which, while not particularly low in itself, indicated considerable variation in this organ. Individuals showing other signs of apparent malnutrition (e.g. hepatopancreas) often presented wasting of the gill filaments with a lack of cellularity in the apical and lateral ciliated cells as well. Occasional abrasion of the filament tips was noticed. The kidney and haemal connective tissue showed slightly lowered scores in the 100% PFA exposure, while the gill, style-sac and intestine were also slightly less than optimum, though never lower than a mean of 4. Occasional ulceration of the intestine was observed, and this combined with the abrasion sometimes observed on the gills is hardly surprising when the nature of the PFA material itself is considered. Muscular organs such as the foot and the large adductor muscle, rarely showed any abnormalities of either the muscle bundles or the surrounding epithelia, and apart from occasional signs of malnutrition most individuals achieved scores of 4 or 5.

Plate 1. Normal hepatopancreas showing digestive tubules (DT) and digestive epithelia (DE). Note the close packing of the tubules (x 250, score 4).

Plate 2. Digestive tubules (DT) in a cockle under extreme food stress. The epithelium (DE) is almost totally degenerated and the tubules are shrunken and separated (x 250, score 2).

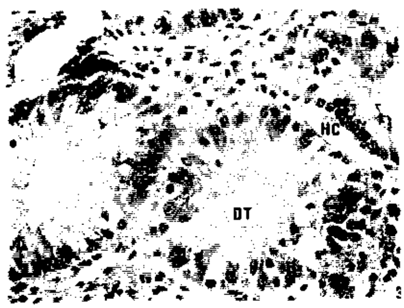

Plate 3. A close up of a digestive tubule (DT) showing the digestive cells surrounded by the haemolymph canals (HC) (x 400, Score 4).

230-days exposure to PFA

The samples taken after 230d exposure showed a generally good, pathology-free condition. Conditions observed in the 100% and 50% PFA exposures after 90-days were not present after 230-days exposure. The hepatopancreas scores for the 230-days control as well as the 100% and 50% PFA groups were all above 4.2 on average, while the other tissues examined were mostly above 4.5. Figure 4 A to C. show the frequency of the histopathological scores recorded for the digestive gland, intestine and haemal connective tissue respectively. It can be seen that after 90-days exposure, a relatively larger proportion of the digestive glands were in stage 3 than in any of the other exposed populations, indicating the presence of moderate lesions. The intestines appeared to be relatively variable where the frequency of each score was concerned, showing no apparent relationship to either the length of exposure, or the PFA treatment. Likewise, the haemal connective tissue appeared to show no PFA related trends in terms of the frequency of lesions with a particular severity.

Parasitology

The parasites listed in Table 7 were all observed in histological sections, and as a result, taxonomic identification was not attempted. Infestations of the metacercaria of trematode worms which often infect seabirds as their final host are relatively common in marine bivalves (Lauckner, 1983) and the 4 cases observed out of more than 300 cockles examined in detail are not unusual. Such infestations can cause severe deterioration in the health of the bivalve hosts, with a consequent lowering of histopathological population scores. The data has not been corrected for the 4 observed cases in this study. Intestinal worms (phylum unknown) are apparently common in cockles as evidenced by three of the samples following 230-days exposure, where from 4/20 to 7/20 individuals were affected. These incidences usually involved a single individual worm, and no direct histopathology was ever associated with their apparently benign presence. Solitary cysts of ca. 150µm in diameter were frequently found in the peripheral muscular tissues. These cysts stained brown - orange with HE and contained a trematode, worm-like organism. They were only ever observed as cysts, sometimes surrounded by connective tissue, but usually they elicited little host reaction. Dark purple cysts of a similar size were often observed in the pericardial area of the cockles, and like the chitin cysts were only ever observed as cysts. They sometimes occurred in groups but caused no apparent host reaction.

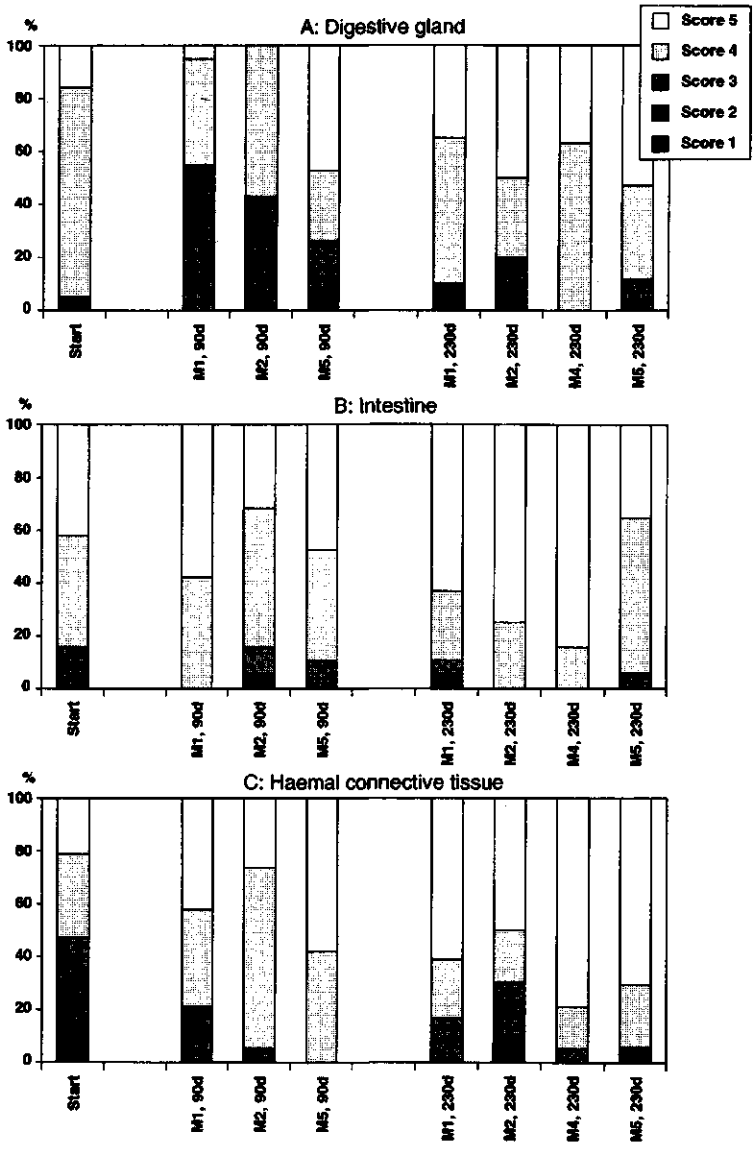

Figure 4. *Cerastoderma edule*: the frequency of occurrence of histopathological conditions in individuals is shown for three tissues of all the PFA exposed populations. Per sample *c.* 20 individuals were examined. It can be seen that after 90-days exposure, relatively more individuals had digestive glands with lowered scores (moderate lesions = score 3) when exposed to PFA then the control animals (See text for further details).

One individual from the start sample showed clear evidence of branched fungal hyphae in the digestive cells of the hepatopancreas. This was accompanied by severe oedema of the tubules and degeneration of whole branches. It was typified by an apparent stream of (digestive) cellular debris in the tubules, branches and stomach.

Copepod crustaceans were relatively common in December after 90-days exposure, being more prevalent in the 100% PFA sample (20%). The control at 230-days exposure showed an infestation rate of 45%. These infestations usually consisted of a solitary individual, more often than not, already dead and encapsulated in a haemocytoma and connective tissue envelope. They had only a slight influence on the overall histopathological scores. Finally, protozoans were often observed on the gills and in the mantle cavities of the cockles. These had an apparently commensal rather than a parasitic relationship with their hosts.

DISCUSSION

The individual elements of this study consisted of observations on growth, reproduction, histopathology and mortality in relation to PFA exposure and heavy metal accumulation. The results of these individual analyses are summarised in Table 8. The accumulation of the 8 metals examined after 90-days exposure was remarkably low (1 - 10x background tissue levels). No other contaminants of importance are considered to have been present in the model ecosystems. As Jenner and Bowmer (1990) noted, this species is clearly a good regulator of metals. They reported, that some metals were readily available in the exposure systems (same experiment), as evidenced by a spectacular uptake of As (665 μg/g in the 50% PFA exposure) in the lugworm *Arenicola marina* and by the apparently preferential uptake of Zn in *Macoma balthica* in all exposures (540 to 770 μg/g). The lugworm is however a direct deposit feeder, while *M. balthica* is an indirect surface deposit feeder, both are exposed to contaminants through its diet of sediment as well as through the ambient interstitial and burrow water concentrations of the metals. Conversely, the filter feeding cockle is not as closely dependent on the sediment; it survives largely on a diet of phytoplankton and is a relatively shallow burrower. It may thus be less directly exposed to the available metals leaching from the PFA sediments.

Table 7. The incidence of all the parasites found in the histological material of the samples examined for histopathology and reproduction is presented. No attempt has been made to identify these parasites beyond phylogenetic/Class.

	Trematode	Intestinal worm	Chitinous cyst	Purple cyst	Fungal hyphae	Copepod	Protozoans
Sept. 1987							
START	1 heavy infection	4	1	1	1 Severe hepatopancreas degeneration/ oedema	—	1
Dec. 1987							
100% PFA	—	—	2	—	—	5	—
50% PFA	—	—	2	—	—	1	1
Control	—	—	—	2	—	1	—
April 1988							
100% PFA	—	4 (Light)	1	—	—	—	3
50% PFA	2 (mode rate to heavy)	6	1	—	—	—	—
Control	1	7	1	1	—	9	—

However, despite this apparent lack of bioaccumulation in chronic exposures to PFA sediments, pathological and reproductive effects were observed. Ultimately, long-term mortality was substantially higher (43% in both PFA sediments as against ca. 12% in the control; 230-days exposure) indicating that the population stability had been negatively affected.

Cu and Ni appeared to be either poorly available, which is quite conceivable given the structure of PFA spheres, or to be regulated within the cockles at ca. 15% of the sedimentary concentrations. Except for Cd, none of the metals had accumulated in *C. edule* at higher levels after 230-days that at 90-days, i.e. when the animals were approaching reproductive maturation. Cd was some 3 times higher at 230-days (ca. 2 µg/g) than at 90- days in both the 100% and 50% PFA exposures.

The normal homeostatic response capability of *C. edule* to heavy metals may have been exceeded, resulting in the observed pattern of histopathological lesions (digestive gland) at 90-days exposure in 100% and 50% PFA. This would lend credence to the argument that the pressure to detoxify metals entering the body and not their accumulation level is crucial in determining the effects of contaminants on individuals and populations. The observed effects were compounded at 90-days by (normal) deteriorating winter conditions and consequent food scarcity, creating a situation of multiple stress.

Table 8. Summary of effects.

	Trematode	Intestinal worm	Chitinous cyst	Purple cyst	Fungal hyphae	Copepod	Protozoans
Sept. 1987							
START	1 heavy infection	4	1	1	1 Severe hepatopancreas degeneration/ oedema	—	1
Dec. 1987							
100% PFA	—	—	2	—	—	5	—
50% PFA	—	—	2	—	—	1	1
Control	—	—	—	2	—	1	—
April 1988							
100% PFA	—	4 (Light)	1	—	—	—	3
50% PFA	2 (mode rate to heavy)	6	1	—	—	—	—
Control	1	7	1	1	—	9	—

Uptake of metals from the PFA, while being one focus of the research is not the only factor influencing the condition of the test animals. The physical effects of unsuitable sediment on *C. edule* was also an important factor. The resemblance of PFA to natural mud as assumed by Bamber (1980) is too superficial.

Of importance in this study, is the fact that while the histopathological and reproductive effects appear relatively slight in relation to the overall variation eveident in the data, they correlate well to the observed mortality. This implies that the occurrence of relatively slight effects, in particularly digestive gland histopathology, is an indicator of mortality. In animals exposed to 50% and 100% PFA, there were occasional histopathological signs of malnutrition, evidenced by the loose packing of the hepatopancreas in the lateral regions adjacent to the two adductor muscles. This condition, although confirmed by the weight loss in both populations over 230-days exposure was not easily detectable with certainty in histological slides. The fact that sufficient food material was generally available in the model ecosystems (in the form of phytoplankton) is evidenced by the fact that both the control population and that exposed to the harbour dredged reference sediment increased in wet weight by nearly 30% during the 230-days exposure.

Lowe and Moore (1979) reported the occurrence of a non-neoplastic cellular condition in populations of *Mytilus edulis* from around the coast of the UK. These authors observed two types of (tumour-like) lesion, which they termed haemocytomas. One was related to the mussels defence mechanism reacting to the presence of parasites such as *Mytilicola intestinalis*. The other type of lesion, however, was not apparently parasite induced, and the authors considered that they may be caused by environmental pollution. Haemocytoma lesions were observed in the present study, and were occasionally surrounded by a layer of fusiform granulocytes. These types of lesion are typical of several bivalve species, and have been commonly observed in *Cerastoderma edule*, although they were not frequently observed in the present study. Lowe and Moore (1979) record instances of up to 27% in some populations.

Sunila (1986) examined *Mytilus edulis* which were collected 6 km from the outfall of a large titanium-oxide plant. The effluent included sulphuric acid, ferrous sulphate, titanium dioxide, Va, Mn, Zn, Cr, Ni and Co, with a pH of 1. An area of 3 km^2 was reported to be covered by TiO2 mud, while a further 120 km^2 was affected by ferric hydroxide flakes on the seabed. A control site some 70 km to the south was chosen for comparison. The sample from near the outfall showed 'spawning' stage of development, while the reference mussels showed little development as would be expected in winter.

- Male gonads of the mussels showed occasional cytolysis of ripe sperm; the follicles were filled with granulocytes. Female follicles contained anomalous oocytes, granulocytes and cellular debris.
- Digestive gland tubules were irregular; eosinophilic cell exudate filled the lumina, the cells were irregular, lacking a striated border. The columnar epithelium had become a metaplastic cuboidal or squamous epithelium.
- The intestine and stomach epithelium contained many macrophages and granulocytes, forming large foci on the basal lamina of the epithelium. The lumens of the digestive tract were filled with foreign (unidentified) material.

It should be stressed, that such extensive pathologies as observed by Sunila (1986) were seldom observed in the present study, even though a variety of metals were involved, and it is suggested that the conditions of exposure in the model ecosystems were far less extreme.

Berthou *et al.* (1987) conducted a survey of 5 locations in the aftermath of the Amoco Cadiz oil disaster, examining 15 oysters (combined *Ostrea edulis* and *Crassostrea gigas*) either monthly or quarterly for 7 years (ca. 4500 individuals). In March 1979, >1000 µg/g (dry-weight basis) total hydrocarbon was accumulated at one location; this had fallen to between 20 and 200 in 1985 with an apparent seasonal cycle. The background level at the clean location was ca. 20 µg/g. The pathological analysis of the oysters was based on a "lesion index", including the gut, gills, gonad and interstitial tissue. At the individual level, the scale runs from healthy (no lesions with a score of 0), to slight lesions (with a score of 1) and so on, for each individual tissue. These scores are summed per individual to give a lesion index and per population to give a mean population index. Additionally, a gonad index was measured as the perpendicular diameters across the gonad follicles. The pathologies observed during the first three months of the survey, i.e., directly after the oil disaster were accompanied by mortalities. The digestive tract had the highest incidence of lesions followed by the interstitial tissue and gills. The data is unfortunately presented as a yearly average, and seasonal trends are not given. The digestive gland lesions consisted of vacuolation or complete atrophy and destruction of the epithelium when the tissue index rose to 68 in the summer of 1978, this was apparently accompanied by leucocytosis of the digestive tract in general. Neoplastic tumours did not increase either during or after the worst years of oil pollution. Since 1982, the oysters examined appeared to have a normal index, despite still substantial oil loadings. This is in direct contrast to the findings of the present study, where the accumulation of metals was very slight, but chronic effects were none the less observed, leading to increased mortalites over 230- days exposure.

In a field study of a fjord-like inlet in British Columbia, Bright and Ellis (1989) sampled four stations at depths of ca. 150 m for the Tellenid bivalve *Macoma charlottensis*, on one occasion in June 1987. The fjord was the site of a copper mine. The authors described the detailed pathology observed, and used a qualitative scale to compare the 4 populations. This was based on the prevalence of general pathological conditions in each population, and agreed well with the assessment scheme used in the present study. Digestive tubule necrosis and haemocyte infiltration characterised some severe lesions, particularly from the sample point adjacent to the mine tailings disposal site. The gills nearly all exhibited oedematous sub-filamentary tissue; sometimes gill degeneration was severe. Inflammation and breakdown of the kidney tubule epithelium was also common.

In the present study with *C. edule* exposed to PFA sediments for 230 days, clear detrimental effects were found at various levels. Survival was reduced to below 60% in both PFA exposures as against nearly 90% in the control and reference sediment. This was connected to a substantial loss in wet weight over the experimental period. Signs of histopathological stress were seen in the digestive gland after the first 90-days exposure. Furthermore, reproduction in the surviving animals was impaired; oocyte maturation being delayed.

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CONCLUDING REMARKS TO PART ONE

Part one focuses on the effects of PFA as a substrate in simulated field situations in the marine environment. PFA contains a wide variety of elements of which the most easily leachable ones are the elements precipitated on the outer surface of the PFA spheres. Of those elements the anions As, Cr, Mo and Se and the cations Cu, Ni and Zn are of environmental interest into relation with their concentration in PFA.

In tidal flats and coastal sediments, the mobility of elements is mainly regulated by the organic matter in combination with the sulphur content (Salomons & Förstner 1984). Just below the sediment - water interface elements from sedimentated suspended matter can be mobilised by the decomposition of the organic fraction and the reductive dissolution of Fe and Mn (Paalman *et al.* 1991). Insoluble sulphide-metal complexes are formed in deeper, anoxic layers (See review Bourg 1988). However, PFA is lacking in both organic matter and sulphur and hardly any similarity exists between the biomobility of elements in tidal flats and freshly build up dumpsides of PFA. It is questionable whether even old dump sites will catch up with the same quantities of organic material as muddy tidal flats. Leaching chemistry at marine PFA dump sites will be quite different compared to tidal flat sediments. Monitoring the accumulation of elements with organisms *in situ* offers an appropriate way for comparing both sediment types.

In the experiments with mesocosms, described in Chapter 3 and 5, the release of elements after 90 days was in the order of 10%. Surprisingly the largest difference was found for Cu and not for As and Se (<5%) as expected. However, both anions should leach out rapidly from PFA (Van der Sloot 1985). According to Swartz *et al.* (1985) in a sediment toxicity study with cadmium, the toxicity for benthic organisms corresponded well with the concentrations in the interstitial water. Concentrations of macro elements in the pore water of PFA are determined mainly by precipitation/dissolution processes and, to a lesser extent, by adsorption/desorption processes. According to Salomons (1985), As and Cr are probably controlled by adsorption/desorption processes and mainly depending on the concentrations in the solid phase of PFA. Van der Hoek *et al.* (1994) found a Ca-phase controlling the leaching of Se and As. Sorption reversibility of As(V) appeared to be less than that of Se (IV) which may partially explain the lower availability of As in leachates.

Accumulation of elements by the tested benthic fauna showed a high accumulation of As and a lesser content of Se. It is known that rapid accumulation of As can occur in *Neridae* (Riedel *et al.* 1987). Of the cations, no accumulation of any importance was found. Only Zn is accumulated, which is in agreement with the findings of Lobel (1987). From the experiments with *N. virens* (chapter 4), it can be seen that the accumulation of Se in mixture of 50% PFA and 50% sand levels off after 12 weeks of exposure. In 100% PFA an equilibrium level is reached after 12 weeks and accumulation will continue. In a recent study by Kress (1993a) and Kress *et al.* (1993b) of PFA dumping in the Mediterranean Sea for the coast of Isreal at 1500 metres depth it was concluded that physical changes occurr to the sea floor substrate by non homogenous distribution of the fly-ash. No accumulation of elements was found in benthic organisms at the dump sites, however from laboratory experiments a slight increase of Cd and Cr was to be expected. This result was attributed to the aggregation of the fly-ash on the sea floor lowering the specific surface area of the fly-ash and subsequent leaching of elements.

The number of surviving animals in our experiments is a reflection of the physical effects of PFA as a substrate, instead of the acute toxicity by leached elements. The lugworm suffered heavily from the dense packing of PFA which prevents adequate burrowing by this species. In 100% PFA mortality was 96%, but also in the control sediment mortality was high (55%). The lugworm is an obligate deposit feeder whereas the bivalves filter directly from the water column or just over the top deposition layer. Therefore, malnutrition of *A. marina* in the PFA was also to be expected. *Neridae* feed on small prey from the sediment surface. The cockle showed a mortality of 40% and the ragworm showed a high mortality only in the beginning probably due to the handling of the tiny worms. In contrast the Baltic tellin showed only a minor mortality of 20% and, later on in the experiment a wild population even was found. It can be concluded that PFA has a, species related, effect on the digging capability and feeding methods. Any resemblance with mud, as concluded by Bamber (1980), is too short sighted and the dumping of PFA will lead to severe depletion of the invertebrate population.

In the prolonged experiment with cockles, over 230-days exposure, survival was reduced to below 60% in both PFA sediments as against nearly 90% in control and reference sediment. A substantial weight loss was found after the exposure period. Some signs of histopathological stress were seen in the digestive gland after the first period of 90 days. Reproduction was impaired and oocyte maturation was delayed. Severe histopathological deviations were not observed as, for instance, Sunila (1986) found in *Mytilus edulis*, collected 6 km from the outfall of a titanium-oxide plant. In this case *M. edulis* female follicles contained anomalous oocytes, granulocytes and cellular debris.

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PART 2. FRESHWATER BENTHOS STUDIES

In Part 2 (chapters 7, 8 and 9) experiments with the freshwater mussel *Unio pictorum* are described. The experiments were carried out in a laboratory at KEMA on the banks of the River Rhine which ensures the availability of large quantities of river water. Deposition of PFA and other waste products in the freshwater ecosystem in The Netherlands is legislatively out of the question at the moment. However, studies with this freshwater bivalve species, can extend our knowledge of the ecotoxicological risks of element leaching from PFA in freshwater.

In Chapter 7, the acceptance of a PFA substrate by *U. pictorum* is examined by growth and behaviour of the mussel. Growth is expressed as both the increase of fresh weight and of the length of individual mussels in PFA and compared with Rhine sediment. Behaviour in PFA and Rhine sediment is tested by recording the activity of the mussels (crawling) in their substrates with the laboratory version of the MusselMonitor® (see chapter 2).

In Chapter 8, cadmium uptake and elimination was studied in dosing experiments. The reason for using Cd was that, at the same time, a research programme was running with Cd and *Unionidae* at the University of Utrecht. In their laboratory, with aquaria without substrate and food, the behaviour was completely opposite to the behaviour observed in our flow chambers with Rhine sediment (chapter 7). The mussels in our experiments were open and active for almost all of the time while the mussels in the aquaria were nearly closed almost continuously and only showed activity for short periods (Herwig 1989). A large difference was expected in the accumulation and elimination of toxicants due to this difference in elementary behaviour. Therefore, the experiments on the accumulation of Cd were carried out in the flow chambers in which the mussels could crawl freely around and filter for food. The work was carried out in collaboration with the University of Utrecht. The studies by Hemelraad *et al.* (1986a; 1986b) with the mussels *U. pictorum*, *Anodonta cygnea* and *A. anatina* demonstrated a biphasic accumulation pattern for cadmium over a period of 16 weeks. During elimination Cd concentrations remained high in the kidney presumably due to redistribution. It was hypothesized that the kidney was the ultimate target organ and that other organs only accumulate Cd temporarily. Four experiments were carried out including a field experiment in which mussels were sampled from an unpolluted site in the North of Holland and exposed in the River IJssel, which was known at that time to be polluted with Cd, for nearly 15 weeks.

In Chapter 9, experiments are described with Se(IV) dosed at relatively high concentrations (50 µg/l) and with Se charged PFA substrates and Rhine sediments. Se leaches out of PFA, under oxic conditions, predominantly as Se(IV), however small amounts of Se(VI) have also been demonstrated (Van den Hoek *et al.* in press). It was therefore decided to use Se(IV) for the present studies. In the study by Porcella *et al.* (1991) on the assessment of the Se cycling and toxicity in aquatic ecosystems, Se was the focus for the following reasons: Se is an essential nutrient, but can become toxic at only slightly higher concentrations than the ambient in clean water; Se occurs in several oxidation states, each having a varying chemical and biological behaviour; Se is a trace element in fossil fuels and can cause reproductive problems in fish; ecological factors appear to play a large role in Se cycling and biological effects. Lemly (1993) even postulated that waterborne Se concentrations of 2 µg/l or greater (in 0.45 µm filtered samples) should be considered hazardous to the health and longterm survival of fish and wildlife populations due to the high potential for foodchain bio-accumulation, dietary toxicity, and reproductive effects.

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CHAPTER 7

SUBSTRATE ACCEPTATION BY *UNIO PICTORUM* OF PULVERIZED FUEL ASH; GROWTH AND BEHAVIOUR

INTRODUCTION

Dumping of large quantities of PFA in the aquatic environment gives rise to the concern of effects on the benthic community by element leaching. Apart from accumulation and possible toxic effects on the organisms a pragmatic question is: do benthic organisms accept the PFA as a substrate? Most benthic organisms use their substrate as a source for feeding and sheltering. The particles of PFA tend towards a dense setting due to their spherical form The structure of PFA in general resembles a sandy loam. PFA is not chemically inactive, as is shown by its pozzolanic characteristics and adsorption capacity (Pietersen 1993).

In the Rhine laboratory at KEMA, growth and behavioural studies were carried out with the painter mussel *Unio pictorum*. The mussel belongs to the family *Unionidae*, which are free living freshwater bivalves. *Anodonta anatina* is a closely related species which is often found together with *U. pictorum* in cross sampling sections of rivers. Both species crawl by extruding the foot into the substrate, using the tip of the foot as an anchor whereupon the body is pulled towards the tip of the foot (Trueman 1968). *U. pictorum* prefers the less muddy sediments and is located more towards the bank. *A. anatina* is found in the softer and more muddy sediments in the deeper parts. Both species prefer lotic conditions. *U. pictorum* lives partly buried in the sediment and crawls around in the top layer. The animals are active in summer time at 20 °C, but in winter the mussels show hardly any activity, and hide away deep in the sediment. The mussel is a filter feeder, *i.e* food particles are filtered out of the water by the gills.

This chapter is based on:
Jenner HA, Van Aerssen GHFM. Substrate acceptation by *Unio pictorum* of pulverized fuel ash; growth and behaviour.

Special grooves covered with cilia transport the food particles to the mouth at which a selection is made for digestion. Large or indigestible particles will be packed to a pellet with slime. These pellets are referred to as pseudo-faeces, and are forced out through the inhalent siphon. The digested food (faeces) leaves the body with the sieved water through the exhalent siphon. These activities cause a sudden valve closure. Both siphons are located at the anterior side of the mussel and are easy to observe between the valves. The water current in the mussel is maintained by cilia on the gills.

Monitoring the valve movement of bivalves goes back to the beginning of this century (Marceau 1909). Most of the older studies were focused on more ecological and physiological questions, for example, the existence of diurnal rhythms (Barnes 1955; Hoggarth & Trueman 1967; Salanki 1964; 1969). Only recently has the recording of the response by bivalves to aquatic toxicants been studied with different types of monitors (Jenner *et al.* 1989; 1992; Kramer *et al.* 1989; Borcherding 1992; Borcherding & Volpers 1994; Mouhabad & Pihan 1992).

In this chapter two items are studied:

- **growth** of the mussel *Unio pictorum* in a PFA substrate compared with the growth in Rhine sediment in a spring and summer period (Exp. 1 and 2)
- **valve movement behaviour** of *U. pictorum* in PFA substrate and sediment from the River Rhine (Exp. 3).

MATERIAL AND METHODS

U. pictorum (length 65 - 91 mm) were sampled in the river Linge, a tributary of the Rhine, at a location with densities of ca. 50 mussels/m^2 and acclimatized in a flow chamber at the KEMA laboratory for one week. *U. pictorum* is a common species in the Rhine, therefore sediment from the Rhine was used as a control. In both growth experiments, the length (mm) and the fresh weight (g) of the mussels were measured at the start and after the exposure period of 10 - 11 weeks. Each mussel was marked with a number. Four flow chambers were used for the growth experiment, 2 of which were filled with 15 cm of Rhine sediment and 2 with PFA, which was well stirred to avoid lumps in the substrate (see also chapter 2 for the general setup). The PFA used was obtained from a batch from the Amer power station, unit

8 (batch A-8850), which has been a standard, conventional PFA for many years. The PFA was rinsed three times with demineralized water in order to remove rapidly leachable elements. Therefore, it is assumed that changes in growth were not due to the initial direct effects of elements. Fifteen mussels were placed in each flow chamber. Dim light conditions were used during the experiments. The flow rate of the Rhine water was adjusted to 5 l/min in each flow chamber. Two growth periods were investigated: (Exp. 1) a 'spring' period (April 23 - July 03) and (Exp. 2) a 'summer' period (June 04 - August 20) in successive years. Water temperatures and velocities were measured daily, throughout the test periods, in order to ensure that no anoxic conditions and no differences in temperatures developed in and between the flow chambers.

The behaviour of mussels in Rhine sediment and PFA substrate (Exp. 3) was investigated with a laboratory version of the MusselMonitor®. The monitor was developed, at the KEMA, to a final laboratory version which was thereafter commercialised by Delta Consult, in co-operation with TNO and RIVM, into a 'stand alone' early warning system for aquatic toxicants (see chapter 2 for detailed information). The behaviour (= valve movement) registered by the monitor can be divided into separate responses:

- closure
- regular opening and closing during crawling and burrowing activities
- sudden closing and opening for cleansing the mantle cavity of (pseudo)faeces
- in the case of pollutants, *e.g.* chlorine addition, fast, short contractions can also be distinguished as the mussels try to cleanse the mantle cavity of the irritating action of toxic compounds.

In this experiment, 5 mussels which were able to crawl around freely in the substrate, were used. Activity was registered, and expressed as the number of times the valve opened and closed within a period of *ca.* 15 hours. This time scale of 15 hours was rather arbitrary and was borne out largely by the length of the data files on the disks. The test period was from August to September, over 6 weeks, during which only 420 hours (28 files) were registered and divided equally over the whole period.

RESULTS

Growth experiments (Exp. 1 and Exp. 2)

No mortality was observed in the mussels which had been transferred to either Rhine or PFA sediments. The water temperatures of periods 1 and 2 are presented in Figure 1. Oxygen concentrations were between 7 - 8 mg/l. The growth results of both periods are expressed as length to fresh weight for the individual mussels (Figs. 2 and 3). In Table 1, mean growth in weight and length and associated regression co-efficient (r^2) are presented. A linear relation in growth was assumed over the relatively short period of time (71 and 77 days). Growth was shown to be better in Rhine sediment than in PFA. Differences were found between the mean increase in fresh weight and length for each substrate during both periods. For Rhine sediment, the mean increase in weight and length in Experiment 1 was: 2.2 g and 1.7 mm, respectively, and in Experiment 2: 3.6 g and 2.6 mm, respectively. The lower water temperatures caused the differences in growth rate between Experiment 1 and Experiment 2. The variations in growth which occurred in one substrate were sometimes due to large differences in the growth of the individual mussels. On one occasion a negative growth was even found in Rhine sediment. In PFA substrates, the mean increase in weight and length in Experiment 1 was: 1.6 g and 1.0 mm, respectively, and in Experiment 2: 2.8 g and 1.6 mm, respectively. Differences in growth exist between PFA and Rhine sediment. The increases in weight and length in PFA are less than in Rhine sediment. Regression co-efficients are, however, still high, even for PFA in Experiment 2 with a r^2 of 0.70.

Table 1. Mean growth in fresh weight and length with the regression co-efficient r^2 at the start and at the end of the periods.

Exp. 1	weight (g)	length (mm)	r^2 (day 0)	r^2 (day 71)
PFA	1.6	1	0.89	0.87
Rhine	2.2	1.7	0.92	0.90

Exp. 2	weight (g)	length (mm)	r^2 (day 0)	r^2 (day 77)
PFA	2.8	1.6	0.83	0.70
Rhine	3.6	2.6	0.84	0.77

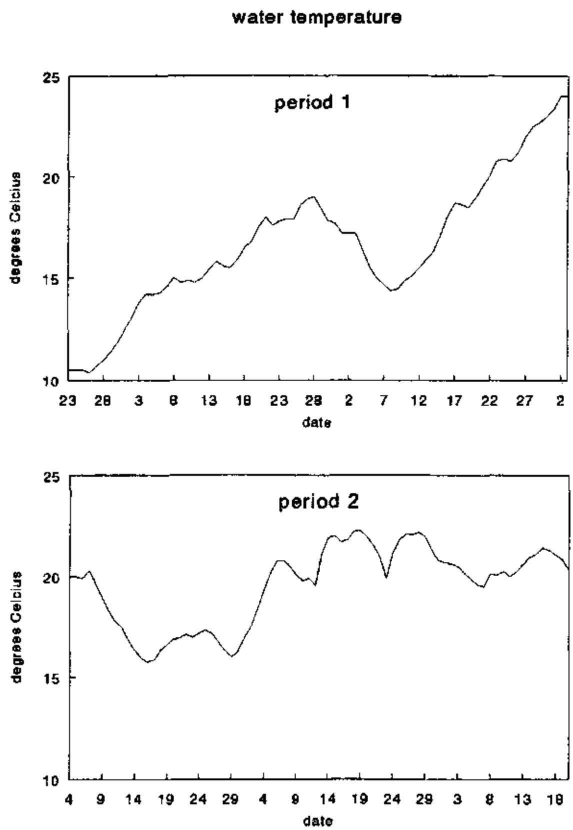

Figure 1. Water temperatures in Exp. 1 (April 23 - July 03) and Exp. 2 (June 04 - August 20).

Valve movement behaviour (Exp. 3)

The results of the valve movements are expressed as the average activity per 15 hours and presented as files in Figures 4 and 5. The mussels were more active at the start which can be attributed to the more solid character of PFA which hindered the mussels in their burrowing activity. A comparison of the first 120 h showed a difference in activity. After 120 hours no large differences could be seen, which was due to the sedimentation of suspended matter in the flow chambers by which crawling is improved. In file 23 the standard deviation was high in both situations, which means that low activity occurred together with extremely high activity. This could be attributed to a change in water quality causing some mussels to close and others to crawl more intensively.

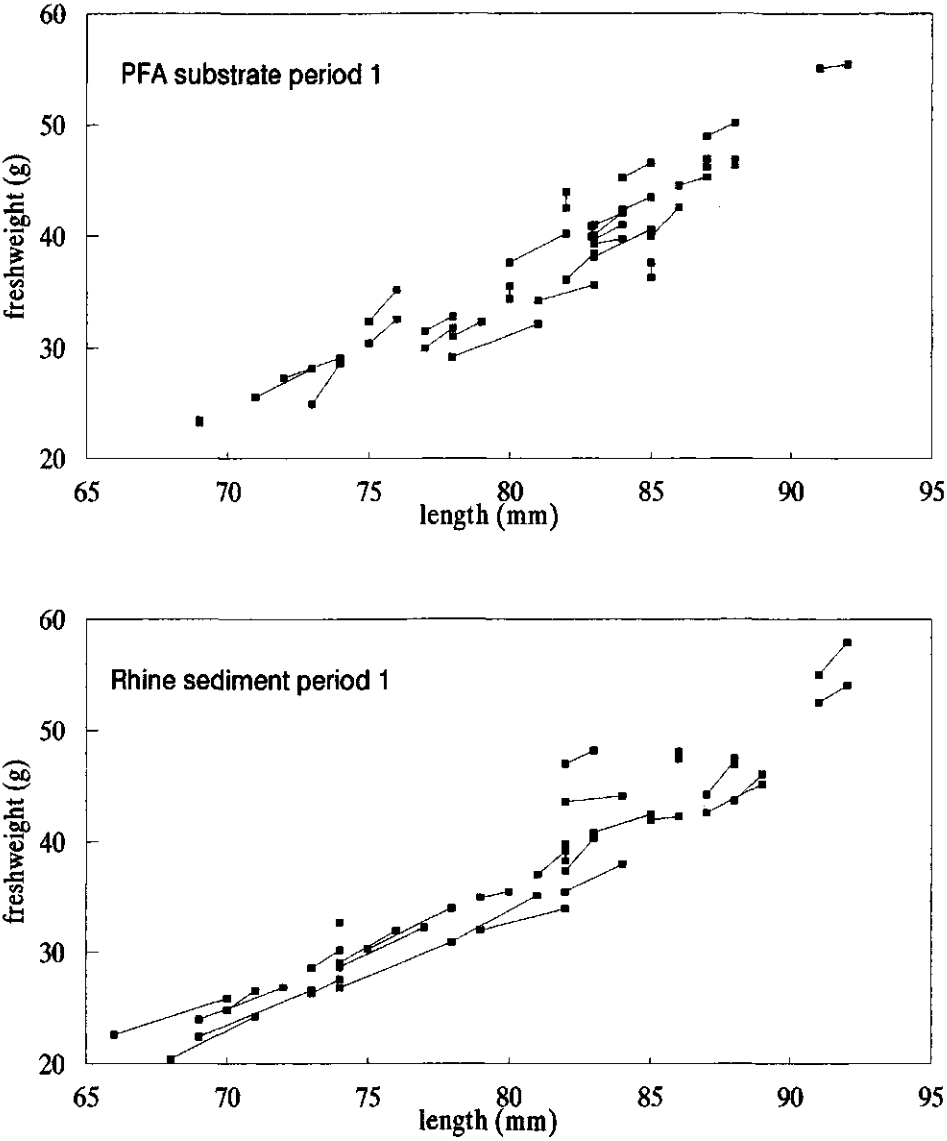

Figure 2. Growth of *U. pictorum* in Rhine sediment and PFA in Exp. 1 (April 23 - July 03).

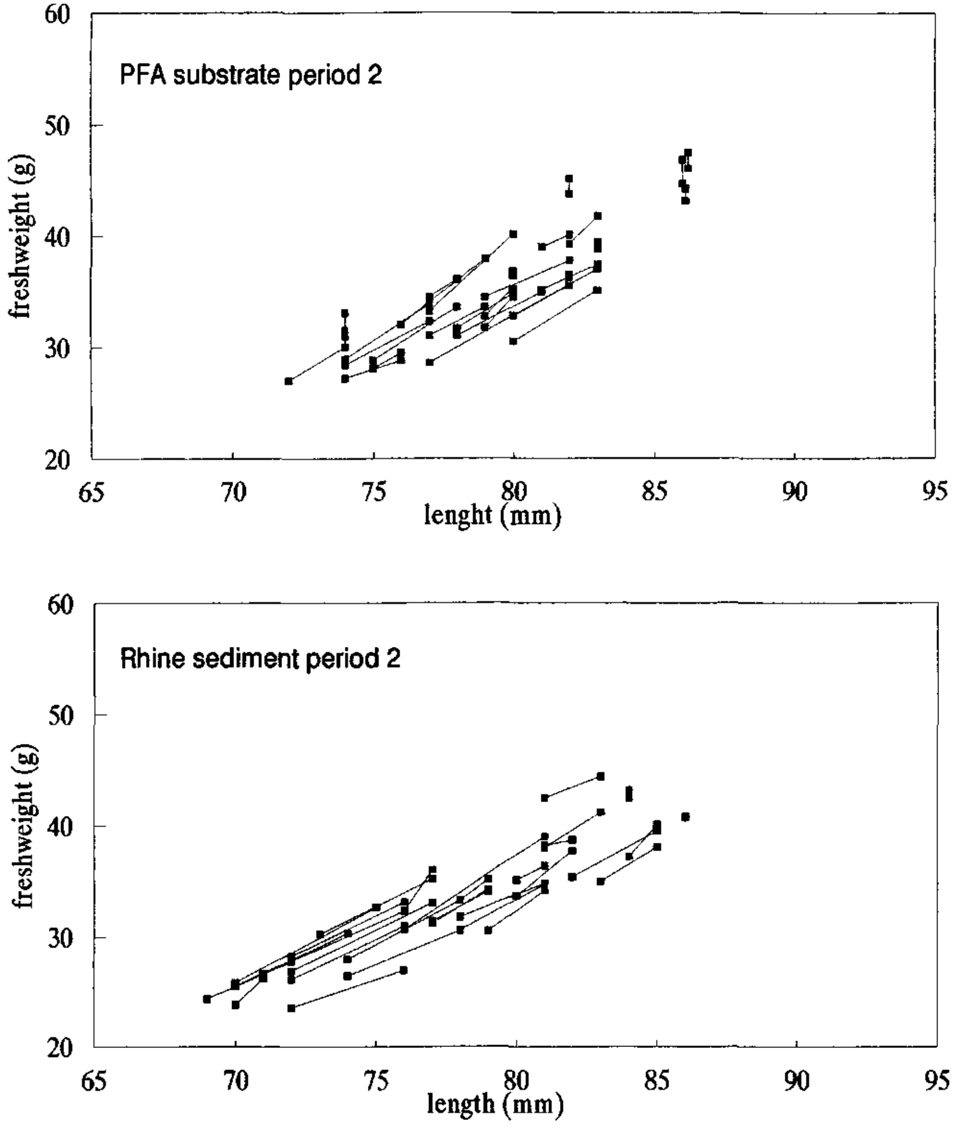

Figure 3. Growth of *U. pictorum* in Rhine sediment and PFA in Exp. 2 (June 04 - August 20).

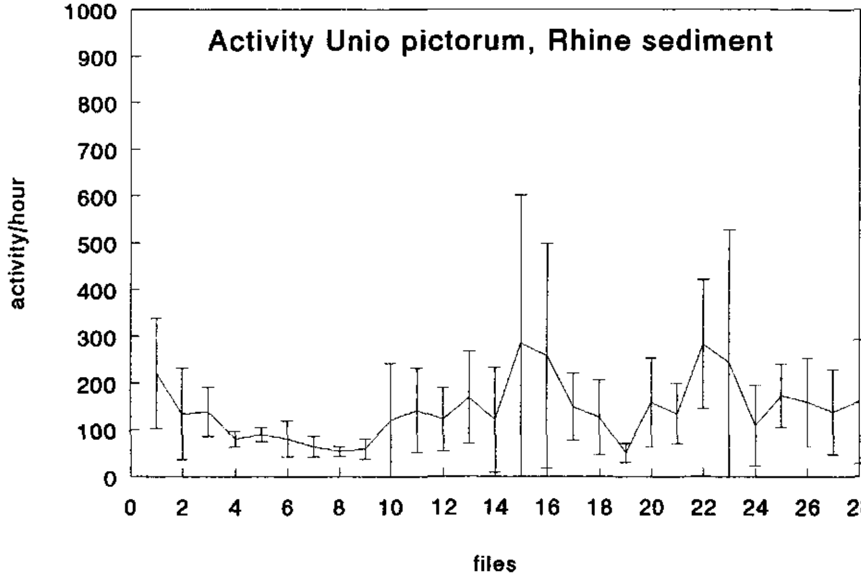

Figure 4. Valve movement behaviour of *U. pictorum* expressed as mean activity with S.D. over 15 h in Rhine sediment.

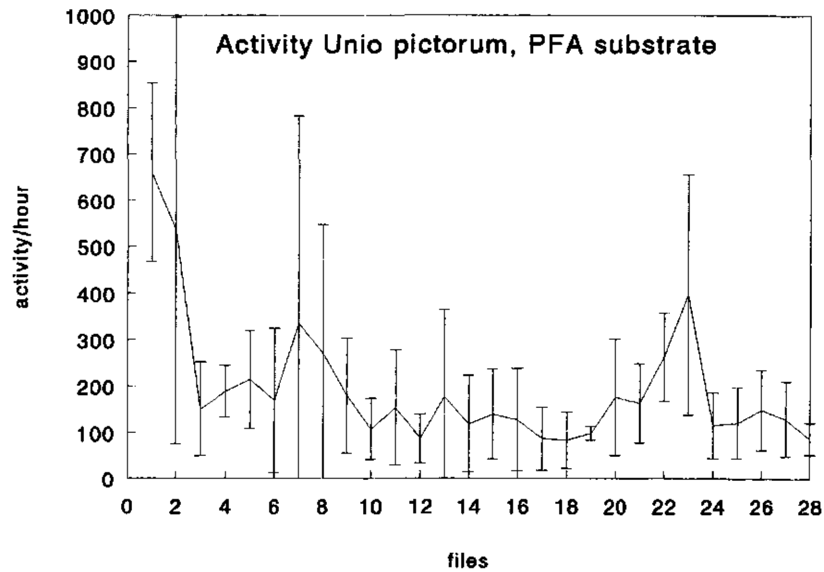

Figure 5. Valve movement behaviour of *U. pictorum* expressed as mean activity with S.D. over 15 h in PFA substrate.

DISCUSSION AND CONCLUSIONS

Results of the growth experiments show that there are differences between Rhine sediment and PFA, indicating a lesser acceptance of PFA as a substrate. This conclusion is supported by the differences in behaviour of the mussels in both substrates during the first period of 120 h. Within this period, activity (crawling and digging) was higher compared with Rhine sediment.

Some remarks have to be made in connection with these conclusions. These types of experiments with mussels in any event, restrict them in their absolutely natural decision possibilities, so growth and behaviour can be only compared relatively. In a study on growth and migration with the mussels *U. pictorum* and *A. Anatina* in the River Linge over 5 years, results showed that growth is rather variable (KEMA 1992). Yearly growth of smaller mussels (length *ca.* 50 mm) could be 22 mm while no growth was found at all in older mussels (length 90 mm). The average yearly growth in length was 4.1 mm for a group of *U. pictorum* within the length class (66 - 90 mm) used in our experiments.

The ability of both species to select their substrate is well developed, which is illustrated by the almost invisible border between the distribution of *U. pictorum* and *A. anatina* in their natural habitat. In the hypothetical situation of PFA being deposited in freshwater, it is doubtful whether young mussels would act as pioneer organisms. Perhaps the edges (mixing zone) around the deposition would be colonized, but even that seems questionable.

In summary, PFA is certainly not an ideal substrate due to its solidification in time. This is supported by the higher activity of the mussels in the first time period at the start of the behaviour experiment and the somewhat lower growth rate compared with Rhine sediment.

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CHAPTER 8

CADMIUM KINETICS IN FRESHWATER MUSSELS (UNIONIDAE) UNDER FIELD AND LABORATORY CONDITIONS

INTRODUCTION

It is well known that bivalves accumulate trace elements to levels far above those in their ambient water. Cadmium is one of the most extensively studied pollutants in the aquatic environment (Nriagu & Coker 1980; Nriagu *et al.* 1981). Laboratory studies by Hemelraad *et al.* (1986a; 1986b) on the freshwater mussels *Unio pictorum*, *Anodonta cygnea* and *Anodonta anatina* demonstrated a biphasic accumulation pattern for cadmium over a period of 16 weeks. After this period, cadmium concentrations increased markedly in *A. anatina*. In these semi-chronic, laboratory exposures, the animals were kept without substrate or food. The final order of cadmium concentrations, in decreasing order of magnitude was: kidney > gills > hepatopancreas > foot. During elimination, the concentrations of cadmium remain high in the kidney due to a redistribution. It was hypothesized that the kidney is the ultimate target organ and that other organs temporarily accumulate cadmium (Holwerda *et al.* 1988). Also Balogh and Salánki (1984) described a biphasic accumulation of cadmium in the kidney of *A. cygnea*: a non-linear and a linear phase in cadmium accumulation were distinguished, with the second phase starting after about 24 hours of cadmium exposure.

Much research on freshwater mussels is being carried out in the laboratory, using more or less semi-natural environments. The role of food, temperature, suspended matter and substrate (Graney *et al.* 1984) as found in natural water systems has so far been greatly neglected. Moreover, the mechanism of the elimination of cadmium in freshwater organisms is still not well understood. Bias and Karbe (1985) found a loosely bound fraction

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This chapter is based on:
Jenner HA, Hemelraad J, Marquenie JM, Noppert F (1991) Camium kinetics in freshwater clams (Unionidae) under field and laboratory conditions. Sci Total Environ 108:205-214.

(accumulated) of cadmium in the periostracum of *Dreissena polymorpha*, whereas no significant elimination could be detected from the soft tissues even after 50 days. This indicates a strong binding of cadmium in the soft tissues. Robinson and Ryan (1986) concluded, for the seawater species *Mercenaria*, that even after 64 days of depuration no loss of cadmium occurred. Scholz (1980), showed that during exposure of *Mytilus edulis* to a concentrations of 100 µg Cd/l, no equilibrium is reached within 20 days. After transferring the mussels to unspiked running seawater, elimination was found to be an exponential process with a half-life of between 14 and 29 days. In contrast, Viarengo *et al.* (1985) showed a minimal elimination of cadmium for *M. edulis*, whereas Borchard (1983; 1985) found a half-life for cadmium in *Mytilus* of between 96-190 days, with prolonged duration under the condition of decreasing food availability, presumably a result of metabolic processes.

The aim of this study is to integrate previous results from a comprehensive laboratory study of the unionids *U. pictorum*, *A. cygnea* and *A. anatina* and a field study with cadmium on *A. cygnea*. Semi-field experiments were carried out with the freshwater mussel *U. pictorum*, a common species in the River Rhine. *U. pictorum* is a free living bivalve that actively moves through and burrows in the top layer of the sediment. To simulate the natural habitat, artificial river beds (micro-streams) were designed through which water from the River Rhine flowed. Natural river sediment was used as a substrate.

MATERIALS AND METHODS

The semi-field experiment was carried out with adult species of *U. pictorum*, which were collected in the River Linge (The Netherlands). This small canalized river is connected to the River Rhine. Mussels were kept in the artificial flow-through micro-streams of the KEMA laboratories. Mussels of approximately equal size (80 mm) were selected for each experiment .

Micro-streams measured 200 x 20 x 20 cm (l x w x h) and were continuously supplied with water (1 l/min) from the River Rhine. In order to avoid intrusion of organisms and debris in the micro streams, the river water was first sieved (pore size, 4 x 5 mm) and then passed over a lamella separator. The materials used were PVC for the tubes and concrete wood piling for the micro-streams. The panels were joined with a one component poly-urethane resin.

Cadmium was applied as $CdCl_2$ from an acidified stock solution (pH 5.0) using a peristaltic pump (LKB Microperpex) to a final concentration of 55 µg/l. Cadmium concentrations in the micro-streams were monitored (each day in the first week and thereafter once a week) by direct measurements (dissolved and adsorbed to suspended matter). Cadmium was also determined in sediment at the beginning and at the end of the micro-streams. Cadmium was applied and mixed directly in a separate part of the stream just in front of the compartment containing the mussels to guarantee constant availability of cadmium. The water temperature (sensor type: PT 100), and the oxygen content (Triox-electrode, WTW, Germany) were regularly measured. Four separate experiments were performed:

Experiment 1

Mussels were exposed to a nominal cadmium concentration of 50 µg/l for 12 weeks, followed by an elimination phase from November 1986 to June 1987. Both control and exposed mussels were kept in streams with substratum from the River Rhine. The whole experiment lasted for 41 weeks for both controls and cadmium-exposed animals.

Experiment 2

During the summer of 1987, mussels were exposed to a nominal cadmium concentration of 50 µg/l for a period of 20 days in the presence of substratum, in order to investigate the influence of the season (e.g. temperature and food). Exposure was carried out between june 1987 and July 1987.

Experiment 3

Similar to Experiment 2, but without substratum, to study the influence of substratum. The experiment lasted from May 1987 to June 1987 (the exposure was for 20 days).

Experiment 4

Mussels were again exposed to 50 µg/l cadmium to investigate the influence of water temperature.

In order to study the kinetics of cadmium uptake under field conditions, *A. cygnea* was exposed in the River IJssel, a branch of the River Rhine, near Kampen for nearly 15 weeks. Mussels were collected from an unpolluted watershed, the Voorboezem at Wieringen (northern part of Holland). The mussels were randomized into test groups of 10 specimens.

Each individual was glued to a nylon string of 40 cm. allowing relative free movement in the top layer the river bottom.

The length of the mussels was between 80 and 100 mm. After each experiment the mussels were weighed, the length was determined and the kidney, gills, hepatopancreas and foot were dissected and lyophilized. Complete destruction of tissue was carried out in teflon coated pressure-destruction bombs (manufacturer: Berghof, Germany) with HNO_3 - HF - $HClO_4$. Internal standards were used originating from the Dutch Bureau of Standards. Depending on the cadmium concentrations, analyses were carried out either using inductively coupled plasma atomic-emission spectroscopy (ICP-AES) and/or furnace-atomic absorption spectroscopy (F-AAS + Zeeman correction). The chemical analyses were carried out according to KEMA procedures (Marquenie *et al.* 1988). All cadmium concentrations are expressed in µg/g freeze dry weight (FDW).

RESULTS

The cadmium flow from water to mussels and sediment was assessed by measuring the difference in concentrations in water, suspended matter and upper layer (3 cm.) of the sediment at the beginning and the end of the micro-streams. Over the 12 week period the mean cadmium emission to the water body was 66.4 µg/l. This value can be partitioned into 64.9 µg/l dissolved and 1.5 µg/l bound to suspended particulate matter (>.45 µm). At the outlet of the microstreams the cadmium concentration was 59.9 µg Cd/l (59.3 dissolved and 0.6 bound to suspended matter). Therefore, during the 5 minute residence time in the microstreams 6.5 µg Cd/l dissipates from water to sediment and biota. Of the mean input of 14 mg/l suspended matter 3.2 mg sedimented during the 5 min. residence time. This could play an important role in cadmium binding. The overall situation is retention of 10% in the micro stream and 90% of the cadmium passing out of the system. During the dosing time of 84 days the mussels are exposed to about 127.2 mg matter 127.2 mg cadmium in the waterphase and about 1.9 mg from suspended matter at a filtering rate of 24 l per day (Kryger & Riisgard 1988).

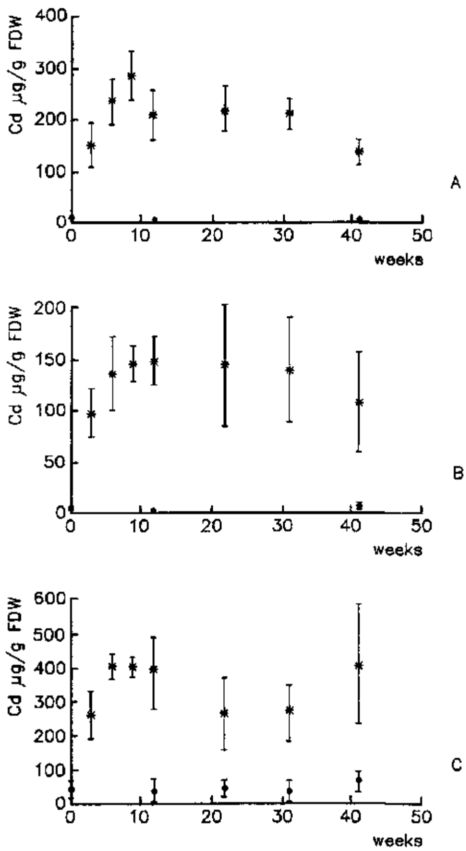

Figure 1. Cadmium concentration in *U. pictorum*, gills (1A); hepatopancreas (1B) and kidney (1C) during a dosing period of 12 weeks and the following elimination period of 29 weeks. Nominal cadmium concentration was 50 µg/l. (* = exposed mussels; • = controls; FDW= Freeze Dry Weight).

During 12 weeks of exposure the cadmium concentration in gills, hepatopancreas and kidneys increased considerably (Figs. 1A-C), the maximum concentrations reached in this period were 300, 150 and 400 µg/g respectively. While the concentrations in gills and hepato pancreas of the control mussels were low (<10µg/g), the kidney already contained a considerable background amount of cadmium (50 µg/g). Cadmium accumulation was particular rapid in the latter organ and a plateau was reached after 3 weeks of exposure. The elimination of cadmium progressed very slowly. In the kidney the concentration first decreased to 250 µg/g, then increased again to 400 µg/g after 29 weeks elimination. For gill tissue a slow but progressive elimination was observed, resulting in 150 µg/g after 29 weeks. For the hepatopancreas the elimination was much slower; no change was observed during the first 20 weeks elimination and after 29 weeks concentrations were lower with 30% to a level of 100 ug/g. At the end of 29 weeks of elimination, gill, hepatopancreas and kidney levels were lower by 50, 30 and 0 % respectively.

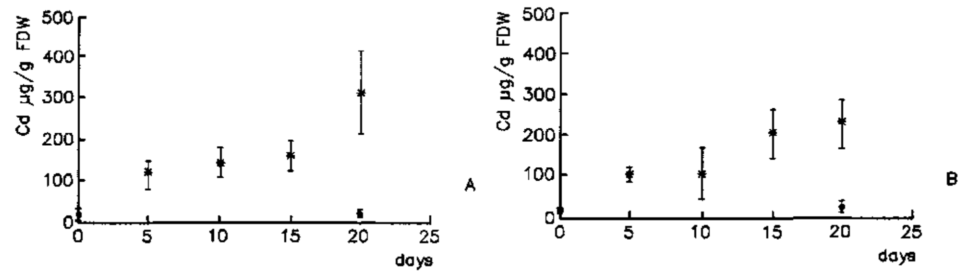

Figure 2. Cadmium accumulation in the kidney of *U. pictorum* during a 20 day dosing period with a nominal cadmium concentration of 50 µg/l1; 2A: without substrate, 2B: with substrate. (* = Cd exposed mussels; • = controls; FDW= Freeze Dry Weight).

The results of the short term cadmium uptake (Exp. 2) in the kidney are shown in Figure 2A and 2B. The pattern of cadmium accumulation is the same as for Experiment 1: an initial rapid increase in the first week with a plateau from day 5 - 10(15) and a second increase from day 10(15) to day 20. After 20 days, cadmium concentration in the kidneys of mussels without substrate did not differ significantly from those in mussels with substrate. Experiment 3 was a repeat of Experiment 2B, but at a higher water temperature to compare the influence of temperature on the accumulation rate and level. The influence of higher water temperatures and assumed subsequent higher physiological activity is reflected in an increase in cadmium concentration (Fig.3); at 20 days the mean concentration is approximately double that of Experiment 2B. The cadmium concentration at the 5- day sampling intervals showed no biphasic pattern as in Experiment 2A en 2B. Note the controls (0 days exposure) with relatively high cadmium concentrations.

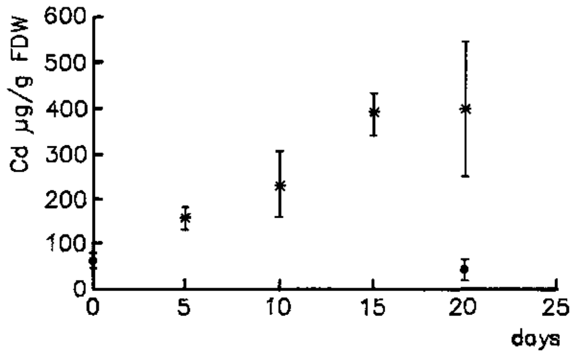

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Figure 3. Cadmium accumulation in the kidney of *U. pictorum* during a dosing period of 20 days with a nominal cadmium concentration of 50 µg/l. (* = exposed mussels; • = controls; FDW= Freeze Dry Weight).

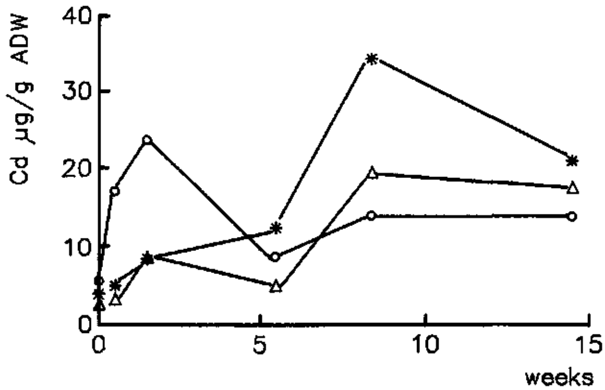

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Figure 4. Cadmium concentration in gills (*), hepatopancreas (△) and kidney (o) of the mussel *A. cygnea* transplanted from an unpolluted area to the river IJssel, a branch of the river Rhine. Exposure time 15 weeks. (ADW= Ash free Dry Weight).

The long-term exposure of *A. cygnea* in the field (Fig. 4) resulted in an uptake pattern similar to that observed for animals exposed in the laboratory. Concentrations in kidneys strongly increased the first 2 weeks and then declined ending to a constant level. Concentrations in hepatopancreas and gills showed a biphasic pattern, with a second uptake phase starting after 5 weeks of exposure. This phase relates to a remarkable doubling of cadmium concentrations (dissolved Cd) of 0.5 µg/l to 1.0 µg/l in the river water which started in the first week of August 1978 (Anonymous 1978).

DISCUSSION AND CONCLUSIONS

Bivalves accumulate cadmium, depending on environmental concentrations, to high levels. Bioaccumulation as continuous bioconcentration throughout the lifetime of the bivalve does not occur (Taylor 1983; Kay 1985). On the one hand, the LC (50; 96h) for the seawater mussel *M. edulis* is 1550 µg Cd/l (Amiard-Triquet 1986); on the other hand, natural populations of the freshwater mussel *D. polymorpha* were negatively affected at concentrations >0.1-5 µg/l (Van Urk & Marquenie 1989). The absence of acute or direct toxic effects, however, enables extensive study under both laboratory and field conditions, focused firstly on routes to target organs and intracellular binding mechanisms and, secondly on release mechanisms for accumulated cadmium and (bio)magnification studies.

The accumulation pattern found for the kidney shows a simple correlation with dosed concentrations and water temperature, although variability between individual mussels was high. Accumulation seems to be a rapid process with a dosing concentration and temperature dependent plateau at 3 to 6 weeks. These results are in accordance with field experiments and with George (1980; 1984), who described a relative simple accumulation pathway for cadmium which was proportional to external cadmium concentrations, linear with time and with no energy dependence. However they contradict the laboratory results of Hemelraad *et al.* (1986-b) for *A. cygnea* for which a biphasic pattern was found. The accumulation pattern was concentration-dependent. At 5 µg/l, accumulation was linear and at 25 µg/l it became biphasic. Perhaps there was an "over-shadowing" of the pattern in our (field)experiments due to the natural, relative rapid changes in parameters such as temperature, suspended matter and food availability. From the results of cadmium partitioning between the water phase and suspended matter, it may be concluded that the water phase is the most important source of cadmium for the mussels.

Graney *et al.* (1984) in experiments with *Corbicula fluminea* in artificial (laboratory) streams, showed a higher tissue accumulation without substrate. It was suggested by Graney *et al.* that in their study cadmium speciation played an important role in changing the availability of the cadmium for the clam *C. fluminea*. In our experiment (Exp. 2), with and without substrate, accumulation was not influenced by the presence or absence of substrate. A shift in accumulation was predicted, because valve movement activity is low for Unionids (long closure periods) by absence of substrate (Herwig 1989).

In our experiments, cadmium dosing and mixing with the river water was situated directly in front of the exposed mussels in order to avoid a change in bio-availability of the cadmium, although some cadmium binding with suspended matter could not be prevented. The kidney was the target organ with a cadmium concentration factor of 6000. Its low biomass, however makes it of minor importance to total body burden. The elimination from gills and hepato pancreas and maintenance of high levels in the kidney are in agreement with results from the laboratory studies of Holwerda *et al.* (1988). During elimination, all three organs lose about one third of their cadmium content and no further elimination occurs after a period of 29 wee-ks. Robinson and Ryan (1986) and Viarengo *et al.* (1985) found comparable results with the seawater mussels *M. mercenaria* and *M. edulis*. The accumulation of cadmium results from the intracellular complexation with available ligands and is controlled by lysosomal activity. After initial cadmium binding to available ligands, further stable cadmium binding to metal-lothionein-like proteins occurs (Nolan 1983; Viarengo *et al.* 1985; Hemelraad *et al.* 1986-a). Detoxification by binding to metal-binding-proteins cannot be the only factor in the high resistance of organisms to the toxic effects by cadmium (Kohlner & Riisgard 1982) because only 22% was bound to metal-binding-proteins. Lysosomal vesicles (George 1984) for sequestering cadmium and, as proposed by Hemelraad *et al.* (1989), specific Cd-lysosomes can be produced. Production of metal-binding-proteins and lysosomal vesicles may be res-ponsible for the plateau in the accumulation pattern found in this study.

Cadmium toxicity for mussels and mussels, can be explained as follows. The process of concentration (accumulation) to high levels is rapid without direct toxic (lethal) effects. Detoxification by metal-binding-proteins will require energy and resources which are drawn from the energy and nitrogen (protein) pool of the animal. A delayed effect will occur in which the population size diminishes due to the constant drain of energy and protein causing energy exhaustion (Van Urk & Marquenie 1989). So the mussel is subject to a rapid accumulation and a slow elimination, with the overall effect of a steady increase in the cadmium concentration and a consequent declining population size. One effect directly or indirectly caused by the concentrated cadmium is a change in the activity of the mussels. The mussels become less active with respect to opening/closure patterns of their valves, which is also found by Balogh & Salanki (1984) and Herwig (1989).

It can be concluded that from this semi-field experiment that the process of cadmium accumulation is rapid and almost irreversible, with the concentration plateaus dependent on the cadmium concentration in the ambient water and the water temperature. Unionidae proved to be rather resistant to cadmium toxicity, which may have evolved from the sediment-inhabiting way of life.

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CHAPTER 9

SELENIUM ACCUMULATION AND HISTOPATHOLOGICAL EFFECTS ON THE OOGENESIS OF *UNIO PICTORUM*

INTRODUCTION

The increased number of coal-fired power stations in the last decade has led to concern about the elements released through the use of coal as fuel in the power industry. Nearly all the elements of the periodic table are found in coal and its residues, including bottom ash and precipitated fly ash or pulverized fuel ash (PFA). Nearly 100% of the elements remaining in the fly ash is washed out during the Flue Gas Desulphurization (FGD). Such equipment is built into all coal firing units in The Netherlands nowadays. The two main existing routes for contaminants entering the environment due to coal firing are, firstly, direct effluents such as those from FGD installations and, secondly, indirect ones caused by leaching of coal residues or where flyash has been used in industrial applications. The emission from the stacks has become negligible in many Western European countries.

Especially anionic elements have a potential for rapid leaching from ashes (Van der Sloot *et al.* 1985). Selenium is one of the elements which is readily becoming more recognized as an ecotoxicological problem in aquatic systems (Raptis *et al.* 1983; Porcella 1987; Shepard 1987). However, long-term environmental impact studies on aquatic ecosystems are scarce (Chau 1986). Selenium is known to cause teratogenic effects in birds and fish as a result of bioconcentration and biomagnification (Hoffman & Heinz 1988; Lemly 1985). Concentrations of 9 - 12 µg/l Se in water lead to a concentration of 3 - 5 µg/g (dry-weight) in plankton. These concentrations are generally regarded as unacceptable in the prey organisms of fish. The ovaries of fish can subsequently accumulate concentrations of > 8 µg/g (D.W), causing

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teratogenic effects. There is no normal embryonal development and the larvae suffer mortality (Hilton *et al.* 1980; Gillespie & Baumann 1986). Sorensen *et al.* (1984 a-c) showed histopathological changes in fish in the liver, kidney, heart, and ovaries and also a decrease of rough endoplasmatic reticulum in tissue cells. Changes in mitochondria in nerve and muscle cells were also found (Schultz *et al.* 1980).

The rate of uptake and final concentration of Se accumulation is dependent upon the available ambient concentrations as well as the bioavailability of the element. Se can occur in three oxidation states (II, IV, VI) but when it is dissolved in an oxygenated environment Se (IV) and Se (VI) will dominate, of which Se (IV) is more rapidly accumulated compared with Se (VI). The work of Cutter (1991) in cooling ponds and reservoirs in the U.S. proved that Se(IV) is the predominant species found in ponds associated with power stations, indicating that the oxidation of Se (IV) in Se (VI) is a very slow process. Besides, Se can exist in different organo-Se compounds which are also biological available (Sandholm *et al.* 1973; Niimi & LaHam 1976). Earlier marine mesocosm studies with PFA using the polychaete *Nereis virens* established that equilibrium between uptake and depuration of Se were not reached after 9 weeks (Jenner & Bowmer 1992). In the freshwater experiments with *Unio pictorum* reported here, the field situation was simulated by using sediment from the River Rhine and PFA artificially enriched with Se (IV) and additionally by means of dosing experiments with Se (IV) in similar systems.

The freshwater mussel *Unio pictorum* was chosen for this study because of its elaborate reproductive cycle. The mussel is a sediment dweller and is exposed directly to elements leaching from PFA sediments via interstitial water. The mussel is also exposed indirectly due to its filtering feeding habit. After fertilisation, unionids generally retain their eggs within the outer gills, which act as a mesh basket and form a so-called marsupium. There, the embryos are brooded until they are large enough to be released. They then take up the second phase of their larval development as a parasite attached to the gills of fish. The cycle is completed when the fully grown glochidia release themselves from the gills. The fact that the *U. pictorum* broods its young provides an ideal opportunity to examine them for a wide variety of reproductive effects as a result of contamination.

MATERIALS AND METHODS

The painter's mussel *Unio pictorum* is a common and widely distributed mussel in The Netherlands. This mussel has a slight preference for more solid muddy substrates compared with *Anodonta anatina* and *A. cygnea* which are found in really muddy sediments. *U. pictorum* was collected in a small river with a muddy clay substrate in which normally high densities of mussels up to 100 /m^2 are, usually, found. Exposure of the mussels took place in flow-chambers placed in a laboratory situated next to the Rhine. The flow chambers (l 200 cm; w 20 cm; h 20 cm) were built in two blocks of 4, each with a separate water supply from the Rhine, with an adjustable flow of 1 to 5 l/min. Rhine sediment (15 cm in height) collected next to the laboratory was used as a substrate for the mussels to burrow and crawl. Excess of suspended matter in the incoming water was removed by a lamellae filter system in a settlement chamber. Both controlled and exposed mussels received Rhine water with the existing plankton as a food source. As far as is known, no accidental releases of chemicals which could have interfered with the experiments occurred in this sector of the Rhine during the experimental period

Survey of the experiments

Experiment I.

An accumulation experiment was carried out with Rhine sediments and PFA sediments charged with Se (IV) to study uptake and translocation. Se was added as Na_2SeO_3 and the exposure lasted for 6 weeks. Four types of sediments were used: Rhine sediment (RS); PFA sediment (PFA); the Rhine sediment was charged with 50 µg/l Se (RS + Se) and the PFA was charged with 50 µg/l Se (PFA + Se). Four groups of 15 mussels were used over a period of 6 weeks. Five mussels out of each sediment type were sacrificed after weeks 2 and 6. The length and fresh-weight of each mussel were measured, the flesh was carefully removed from the shell and divided to three fractions: foot, gills and rest fraction.

Experiment II.

A long-term dosing experiment with Se (IV) was carried out with the aim of investigating effects on the reproduction and the histological condition of the mussels. The experiment lasted for 75 days and the sampling of 5 mussels for Se accumulation was carried out on days 0, 50 and 75. Twenty-five individuals were sampled on days 50 and 75 for histological preparation. The mussels (including the 25 control ones) were fixed in neutrally buffered formalin for histological analysis. Two transverse slices were taken perpendicular to the line of the valve opening, through the region of the hepatopancreas. A piece of both the marsupium, outer demibranch and the inner gill was enclosed with each of the visceral slices. The slides (4 µm) were stained with Ehrlichs haematoxylin and eosin (HE). Histopathological screening was done according to the methods of Bowmer *et al.* (1990). The cytological quality of the tissues was assessed on a scale of 1 to 5, of which 5 was considered 'normal'. The screened tissues were: stomach; digestive gland; style-sac; intestine and chlorogogen tissue; gonad sacs, (gametes and blood spaces); muscle tissue of the body wall; nerve tissue and the gills. The methodology followed in this experiment was focused on obtaining an overall picture of the health of the exposed mussels. In addition to the quality analysis of individual tissues, a general index of each individual was also used, based on the extent and severity of cytological abnormalities or lesions in all tissues. This type of index has been used by a number of authors in recent years (Peters & Yevich 1989; Bowmer *et al.* 1990; Bowmer *et al.* 1993). The grading system only summarizes the health of invertebrates in a very general way according to the criteria stated in Table 1.

Table 1. Index of histopathological score for individuals.

SCORE	CRITERIA
5	An individual showing no apparent tissue abnormalities, well nourished.
4.5	Individual showing no obvious tissue abnormalities but where feeding, condition is sub-optimal (winter/post-spawning).
4	An individual showing slight tissue abnormalities in one organ or part of its system, e.g. abnormal vacuolation or slight/local inflammation.
3.5	Individual showing moderate abnormalities / lesions in one organ or part of its system, e.g. inflammation, necrosis, etc.
3	A definite pathological condition is recognizable, i.e. serious abnormalities in one organ, or a more diffuse general condition, e.g. lesions, inflammation, evidence of parasitisation, starvation, etc.
2	An individual showing extensive pathology, e.g. lesions, neoplasia, extensive parasite infestations/infection. Condition apparently critical.
1	A terminal case, substantial wasting of key tissue, little apparent function remains.

Experiment III.

From the results of Experiment II a comparable experiment was set up in which Se was dosed in 2 concentrations, 50 µg/l and 250 µg/l. The 50 µg/l dosing period lasted for 152 days and the 250 mg/l for 75 days. This experiment was set up primarily for screening teratogenic effects on the larvae (glochidia) in the gills of the female mussels. Therefore, every 14 days, mussels were sampled to a maximum of 20 animals, or as many as sufficed to obtain 5 female mussels. The outer-gill was fixed in neutrally buffered formalin and stained with HE. The number of female animals with swollen gills are listed in Table 2. Se dosing started on April 10th. From the stained section the numbers of embryos and larvae were estimated and scored from 1 to 4, corresponding with <5 embryos, 2 with 5 - 50, 3 with 50 - 100, and 5 with >100 respectively. The stages of development of the embryos from the controls and the exposed mussels were divided as follows:

stage 1: early blastula, large eosinophilic cells, jelly coating, no invagination

stage 2: late blastula, undergoing invagination

stage 3: early gastrula, having completed gastrulation, showing the beginning of differentiation in a jelly envelope

stage 4: advanced gastrula: differentiation of tissue under way

stage 5: early glochidia, without shell, showing adductor muscle, intestinal epithelium and primitive mantle

stage 6: advanced glochidia: shelled, round in shape, tissue well differentiated.

Table 2. Sampling dates and the corresponding number of female mussels with brooded marsupiums recovered. The total number of mussels sampled is given in brackets. This number does not necessarily represent the males. After 15/07 no mussels were found with swollen gills for 8 succeeding sampling dates. Concentrations in µg/l.

date	14/05	27/05	03/06	10/06	17/06	24/06	02/07	08/07	15/07
Control	5(10)	5(9)	6(15)	6(15)	4(15)	5(10)	4(26)	-	-
Se 50	5(10)	4(16)	6(17)	6(10)	5(15)	5(15)	2(10)	3(10)	1(14)
Se 250	5(8)	3(15)	5(15)	3(15)	3(15)	3(19)	-	-	-

The tissues of the gills, i.e. the filaments of the gill lamellae and the supporting interlamellar junctions, as well as the embryo and larval content were examined for signs of pathological anomalies and parasite infestations.

The Se concentration in both sediment and different tissues were measured after digestion of the freeze-dried material with HNO_3 - HF - $HClO_4$ in teflon-coated bombs (Berghof) with AAS (with Zeeman correction).

RESULTS

Experiment I

In Experiment I, the mussels were exposed to sediments loaded up with Se (IV) for 6 weeks. The Se concentration was measured weekly in both Rhine sediment and PFA substrate. The results show a fairly constant Se concentration in the PFA substrate but a much higher and less stable concentration in the Rhine sediment, varying from 132 to 77 µg/l (Table 3). These deviations were caused by the organic binding of Se by the river sediment and the imperfection of the sampling procedure. The accumulation of Se in foot, gill and rest fraction of the body showed a rapid increase in 2 weeks after the start of the experiment. Concentrations in the foot, gills and rest fraction were significantly (T-test; $P < 0.05$) higher after 2 weeks and after 6 weeks for both sediment types (with the exception of Rhine sediment and rest fraction), which indicates a direct transport of Se from pore water into the foot. The differences between the samples taken after 2 and 6 weeks are small, which means that the accumulation of Se took place predominantly during the first 2 weeks (Fig. 1). However, Se concentration in the foot fraction was significantly higher after 6 weeks (T - test; $P < 0.05$). Se concentrations in the control group in the Rhine sediment were at detection limit levels, but in the PFA control group concentrations for all 3 tissue fractions were >2 µg/g D.W.

Experiment II

Selenium exposure

In this experiment the mussels were exposed to a nominal concentration of 50 µg/l Se (IV) for 75 days with the aim of finding differences in the development of the eggs and embryos due to prolonged exposure to Se (IV). The Se concentration in the flow chambers is shown in Figure 2. It took about 12 days to reach the premised concentration.

Table 3. Measured Se concentrations in the substrates (µg/g).

time in weeks	Rhine sediment + Se	PFA substrate + Se
week 1	130	42
week 2	132	52
week 3	78	61
week 4	77	47
week 5	116	57
week 6	93	43

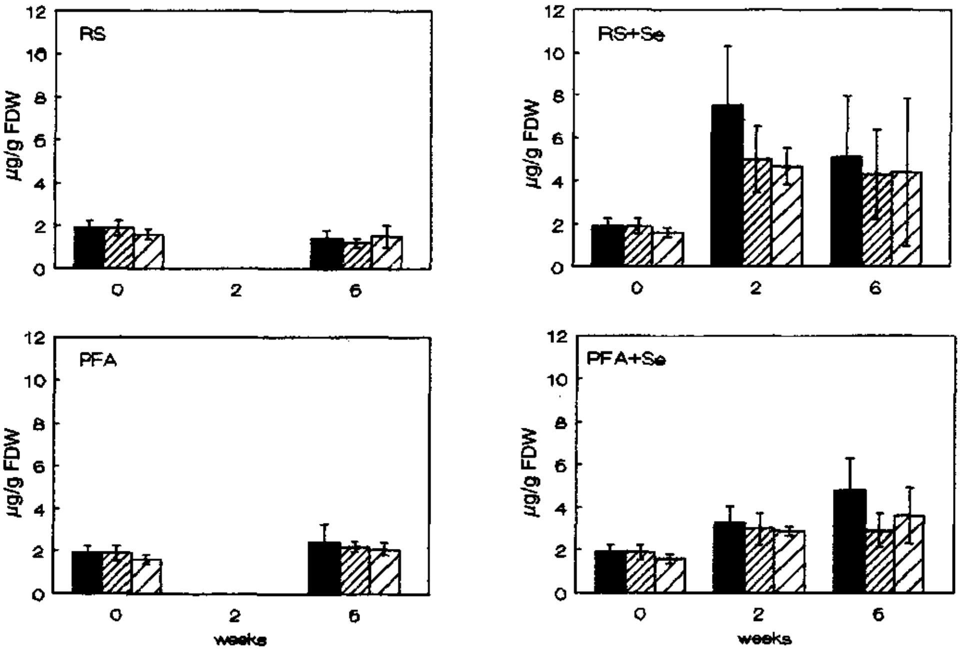

Figure 1. Se concentrations (from left to right) in foot, gill and rest fraction of *Unio pictorum* on day 0, after 2 weeks and after 6 weeks, respectively, exposed to Rhine sediment and PFA substrate with (RS/PFA + Se) and without extra Se (RS/PFA).

Se concentrations in the gills increased by a factor of 2 on day 53 and finally reached a mean of 6.7 µg/g D.W. which proved to be equal to the concentrations found in Experiment III a year later. Se concentrations on days 0, 53, and 74 of gills and whole animals are given in Table 4.

Mussel weight and length

During the exposure period no real change in growth was found regarding length and fresh weight (Table 5). This result was to be expected because the mussels used were of a considerable age (>10 years) and growth is known to be extremely slow. Also, their wet weight showed a stable pattern for both control and Se exposed animals. Some loss in weight had been expected due to a release of gametes, but weight losses of some importance (3 to 4 g per individual) were found only for 8 mussels in the exposed group on day 74. This group consisted of males and females with and without trematode and mite infestations. No specific pathological condition was found to account for the weight loss. In the control group one individual showed a decline in weight.

Table 4. Se concentrations in the gills and entire bodies of *Unio pictorum on* day 0, day 53 and day 74. The data in parenthesis represent the S.D.

Concentration µg/g D.W.	day 0	day 53	day 74
Gills	2.6 ± 0.5	5.4 ± 0.4	6.7 ± 0.7
Whole clam	2.1 ± 0.3	3.2 ± 0.5	4.4 ± 0.7

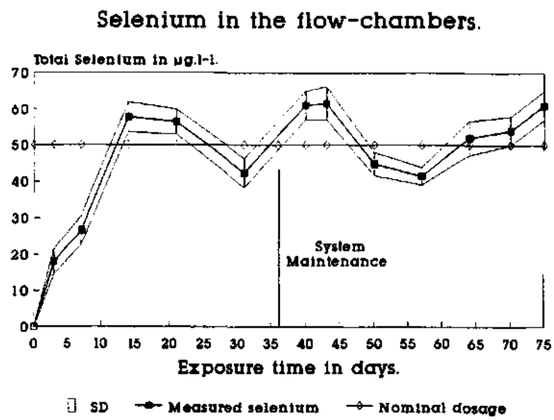

Figure 2. Selenium (IV) concentration in µg/l during of the experiment. The band represents the S.D. of the analyses (3 replicates).

Table 5. Mean shell length and live weight

SAMPLE	Mean shell length		Mean live weight	
	day 0	day 50 - 75	day 0	day 50 - 75
Control 50 d.	88.3 ±7.4	88.2 ± 7.4	51.2 ± 14.2	51.3 ± 14.0
Control 75 d.	87.7 ± 6.4	87.4 ± 6.5	47.6 ± 10.7	47.4 ± 10.9
Selenium 50 d.	92.7 ± 4.9	92.5 ± 4.9	54.6 ± 8.9	54.8 ± 9.2
Selenium 75 d.	87.5 ±.7	87.5 ± 5.5	47.2 ± 9.5	46.1 ± 9.8

Reproduction

All mussels were sexed prior to the analyses of reproductive maturity. The sex ratio was slightly shifted to the females (42 % males and 47 % females, the rest being hermaphrodites). One mussel could not be sexed due to parasitic castration.

From the oocyte diameter of each female *U. pictorum*, a histogram of diameter frequency was created which gave insight into the reproductive maturation of the individual. These histograms provided an insight into the reproductive maturation of *U. pictorum* and provided the data for Figure 3. in which the median oocyte diameter of each female mussel of the 5 exposed groups is presented together with the associated trend line. The median size was used instead of the mean size because the mathematical distribution of the oocyte diameters in the histograms rarely followed a normally distributed pattern. Each point in Figure 3 represents the state of maturity and it is obvious that the start population shows a higher degree of maturity than all the other samples. Furthermore, even the less ripe individuals on the right-hand side are more advanced than some of the other series. The trend for the Se group which was exposed for 75 days lies about 5 - 10 µm lower, suggesting that in a situation of generally declining maturity Se exposed mussels decline much more rapidly than the control group.

An important discovery was the degree of synchronization amongst the embryos and glochidia in the brood chambers in the gills. Apparently, the entire population on the gills of a female mussel originated from one single abrupt spawning. The stage of development of the embryos and glochidia larvae is summarized in Figure 4. The start population, which was ripe with large amounts of spawnable oocytes, showed 6 out of 10 female mussels carrying broods in the outer gills. All these females possessed early developing embryos. By contrast, the majority (9 out of 11) of the Se exposed females were still brooding after 50 days *exposure*, 7 were *found with fully developed glochidia and* only 2 mussels were found with gastrulas. Finally, 4 out of 9 females were carrying gastrulas after 75 days of exposure and no glochidia. Of the control group, after 75 days only one mussel was carrying glochidia, indicating the end of the spawning period.

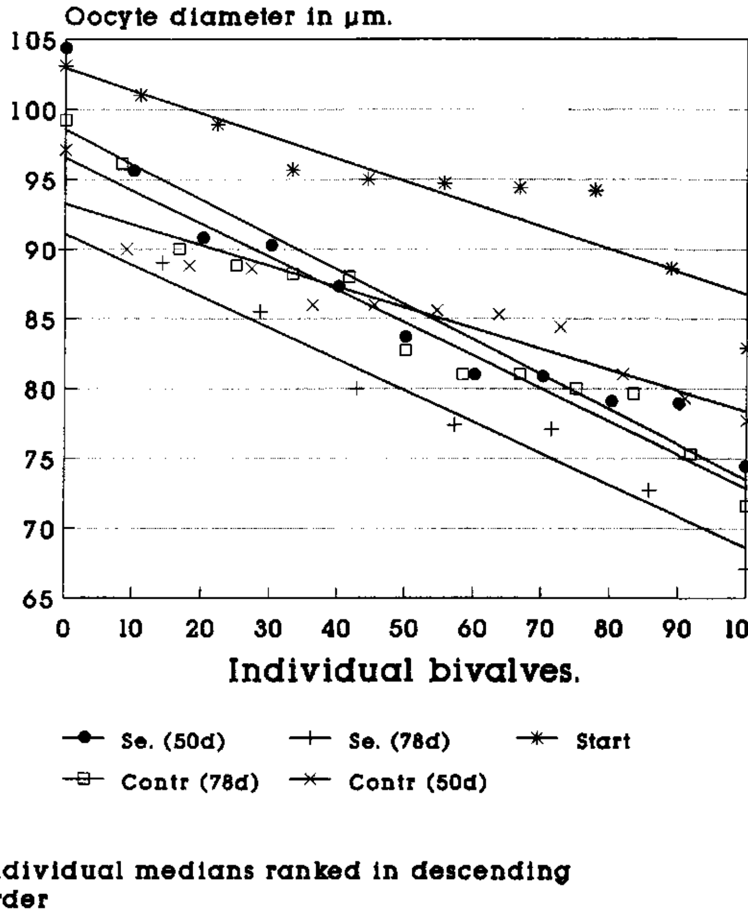

Figure 3. Individual median oocyte diameters (µm) are plotted across an equal distance for each exposed *Unio pictorum* population and for the start group. The trend lines give an indication of the relative state of maturity of each population.

Experiment III

This study is the continuation of experiment I, in which Se (IV) was dosed at 50 μg/l for 22 weeks (152 days) and at 250 μg/l for 11 weeks (75 days). Sampling dates and the number of female mussels with swollen gills prepared for light microscopy are given in Table 2.

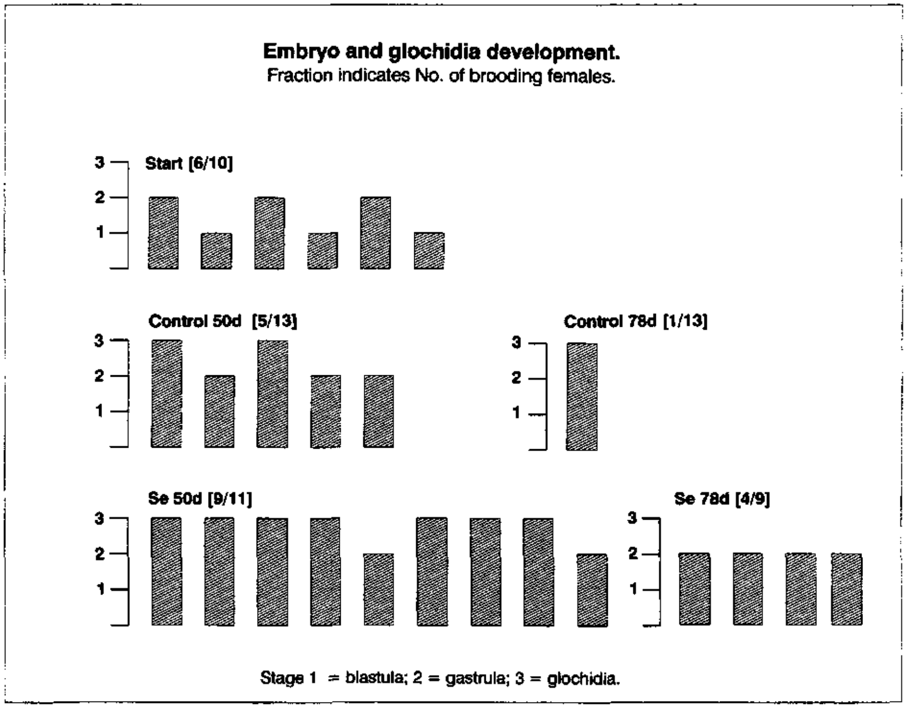

Figure 4. The number of individual female *Unio pictorum* bearing embryos and larvae on their gills of each population is shown. The height of the bar indicates the stage of development.

Selenium exposure

The 50 μg/l dosing took place for 152 days and the 250 μg/l Se was dosed for 8 days. The measured Se concentrations in the flow chambers during the dosing period are shown in Figure 5. Se concentrations in the gills were measured at regular intervals and are presented in Figure 6. For the mussels exposed to 50 μg/l the accumulation level was approx. 5 μg/g (D.W.) and for the 250 μg/l-exposed animals the concentrations were about 15 μg/g (D.W.). For both series a constant level seemed to be reached.

Reproduction

From each microscopical preparation of the gills, which were rather uniform, the numbers of embryos and larvae within the section of the gills were roughly estimated as follows:

<5: score of 1; 5 to 50: a score of 2; 50 to 100: a score of 3; >100: a score of 4.

The stages of development were divided in stages 1 to 6 as explained in the section on material and methods.

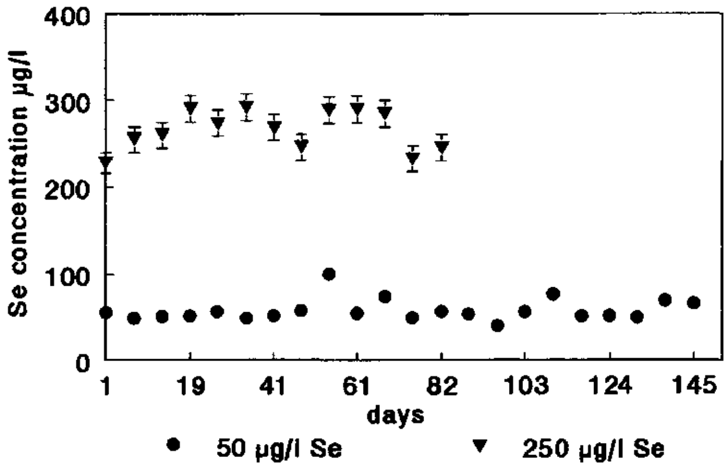

Figure 5. Measured Se (IV) concentrations at the outlet of the flow-chamber for the 50µg/l and the 250 µg/l.

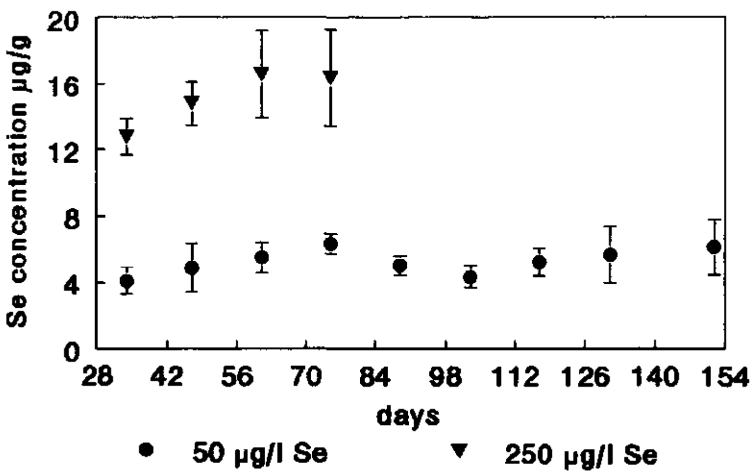

Figure 6. Se concentration µg/g FDW in gills at both dosing concentrations of 50 µg/l and 250 µg/l.

The number of embryos and larvae found per section (*c.* 1 cm^2) generally varied between 50 and 100 or was greater than 100, except in the control sample from the beginning of July, which contained very few, suggesting that the larvae had already been detached from the gills. In Figure 7 the score of embryos/larvae is shown. As can be seen, no drastic reduction in numbers was found.

The stages in the development of the control samples were as follows: from mid-May to mid-June, the embryos showed a general development from early gastrulae to early glochidia. This was apparently followed by a second development cycle, as evidenced by the presence of a large number of early blastula in mid-June and early gastrulae towards the end of the same month. At the beginning of July another development cycle is suggested but not confirmed by the data.

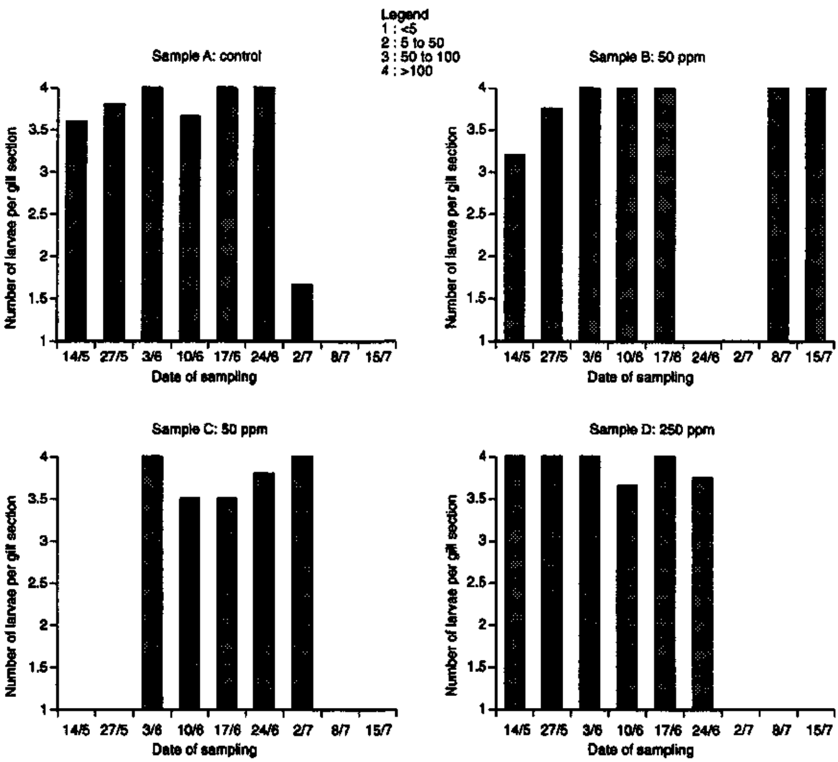

Figure 7. Number of embryos/larvae per section.

With the 50 µg/l exposed animals a similar pattern was found in the development of oocytes and larvae. The sampling on June 17 and 24, and on July 02, again showed a second cycle, suggesting that a development cycle of about two weeks is possible at that time of the year. More irregular development stages were found at the 250 µg/l-exposed animals, but no severe variation or malformations. Frequently, more than one stage was present in a single individual, unlike in to the other two groups where development stages were more or less uniform (Fig. 8).

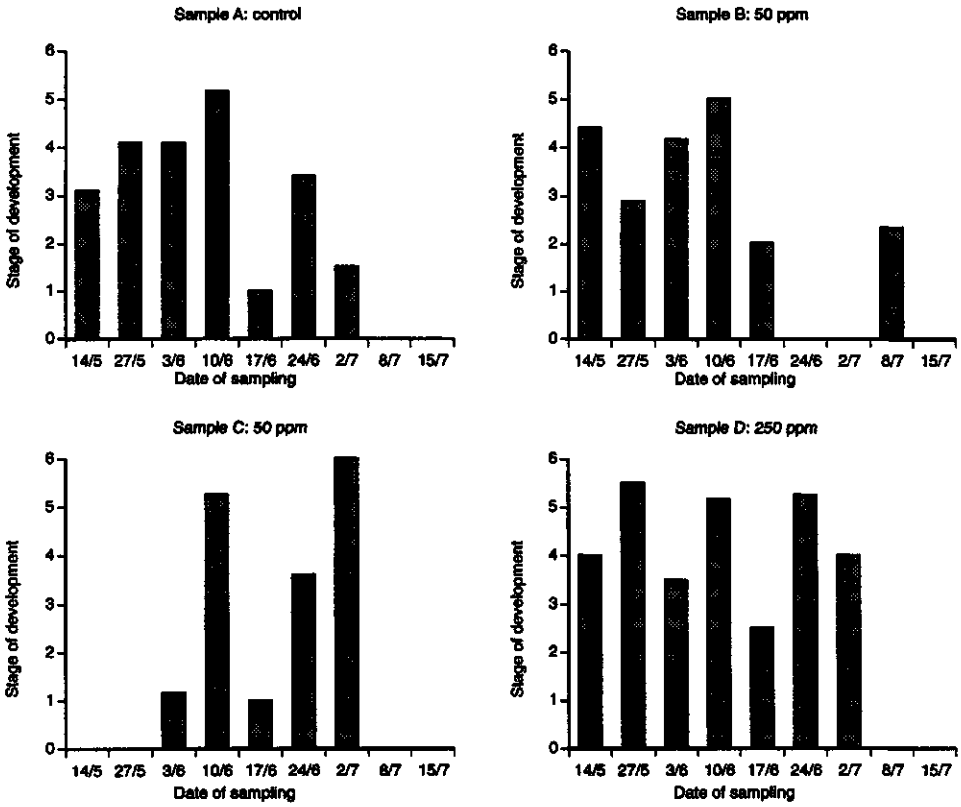

Figure 8. Stage of development of the eggs and embryos: 1= early blastula, 2= late blastula, 3= early gastrula, 4= advanced gastrula, 5= early glochidia, 6= advanced shelled glochidia.

DISCUSSION AND CONCLUSIONS

In the first experiment with Se charged sediments it was shown that Se was expected to be transported directly from pore water to the foot, but the accumulated final concentrations were relatively low and accumulation also took place primarily in the first 2 weeks of exposure. The concentrations in the foot were higher throughout the experiment. Se concentrations in the gills varied between 4 and 5 µg/g FDW, which is in agreement with dosing experiments II and III, in which Se concentrations in the gills varied from about 5 to 6 µg/g (FDW). From the Se concentrations in the gills, foot and the rest of the body it can be concluded that rapid translocation of Se occurs. Hilton *et al.* (1982) showed that Se can be accumulated from water by the gills and is stored perhaps in anorganic form, unlike Se accumulated by digestion. The latter route transforms Se into a methylated (fat soluble) form which is far more transportable and can be eliminated easily by the kidneys. The extensive investigations of Sorensen *et al.* (1982a - b; 1983a - b - c; 1984a - b - c) proved that Se produces histopathological abnormalities in the liver, kidney, hart, ovaria, and erythrocytes. Moreover, it causes mitochondrial changes and a decrease in rough endoplasmatic reticulum. Chronic toxicity data for fish show effects in the concentration range of 10 to 30 µg/l Se(IV) (Hunn *et al.* 1987; Lemly 1985).

Due to biomagnifaction, long term effects (dying out) were found for fish at concentrations as low as 10 µg/l (Barnhart 1958; Gillespie & Bauman 1986; Lemly 1985). Lemly mentions that even concentrations as low as 2 - 3 µg/l may lead to the elimination of a fish population as a result of biomagnification. Hunn *et al.* (1987) found that Se accumulation via food in fish concentrated especially in the ovaria. Also Sager and Cofield (1984), and Lemly (1985) found high Se concentrations in the ovaria of fish exposed to Se; however, Lemly (1985) exposed the fish to Se in water and the others in food. It seems evident in our study that if there were any effects caused by Se they would have to be found in the gills with marsupia filled with developing fertilized eggs and glochidia.

Oocyte development was at its peak in early April during Experiment I, followed by ovulation in both control and Se exposed groups of *U. pictorum*. Oocyte development was synchronized, but ovulation was not epidemic, occurring over a period of weeks in the entire test population. Ovulation occurred in one burst in each individual and embryos on nearly 25 to 30% of a gill were in an almost identical state of development throughout. All the time a substantial amount of viable oocytes was present in the gonad. The decline in oocyte diameter *i.e.* the median oocyte diameter per individual or relative % oocytes >100 µm in diameter, levelled out between 50 and 75 days in the control group from late May to late June, but continued to decline in the Se dosed group. The pattern of embryo occurrence also differed between the control group and the Se exposed group as shown in Figure 4. The control group showed evidence of spawning by late May, whereas the majority of the Se exposed group still possessed developing glochidea. By the end of July, almost all mussels in the control group had released their first brood and were in an apparent rest phase, while part of the selenium group had a fresh brood consisting of phases of blastulas and gastrulas.

On the basis of these results the hypothesis was formulated that,although no signs of severe damage were seen after Se exposure, there was a delay in the development of the glochidia which may have resulted in less viability or malfunction of the brood. Therefore, Experiment III was set up with a relatively low (50 µg/l) and a high (250 µg/l) Se concentration for maximum impact on the oocyte formation, ovulation and embryology after fertilization. However, no drastic reduction in numbers of glochidia was seen at both concentrations. Also, no particular number of deformed glochidia was found in the exposed groups compared with the control group.

This leads to the conclusion that within the chosen experimental layout no definite effects could be demonstrated which could be attributed to Se (IV). Crane *et al.* (1992) found that the hatching of eggs in experimental ponds after long exposure times (32 weeks) of the perch (*Perca fluviatilis*) at 25 µg/l Se (mixture of Se(IV) and Se (VI)) failed completely. With an exposure time of 22 weeks and the levelling off of the Se concentrations found in the gills and other body parts more pronounced effects would be expected. In a study on the determination of Se bioavailability to *Macoma balthica,* Luoma *et al.* (1992) concluded that selenite in solution contributed little to Se bioaccumulation by *M. balthica*. Still, efficient food web transfer via diatoms is possible and also particulate Se - *M. balthica* being a particulate-feeding consumer - can lead to severe biological effects in fish and waterfowl (Johns *et al.* 1988).

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CONCLUDING REMARKS TO PART TWO

In Part two (chapters 7, 8 and 9), the situation as it pertains to the freshwater environment is considerd, with the emphasis on the clam *Unio pictorum* and the accumulation of cadmium and selenium.

In Chapter 7, the substrate acceptance by the clam was tested by measuring growth and the behaviour expressed as activity with the aid of the Musselmonitor®. It can be concluded that PFA is not an ideal substrate when compared with Rhine sediment. Average growth in PFA is lower, which is thought to be caused by the physical structure of PFA. The spherical particles of PFA enable an extremely dense packing of the substrate and also perhaps the so-called 'pozolanic' feature of PFA creates a substrate which is too hard for optimal growth to *U. pictorum*. This lesser acceptance of PFA is supported by the observed activity. In the first weeks, activity in PFA is enhanced compared with the Rhine sediment. Translated into behaviour, this means that the clams crawl longer in time seeking for a resting place. Once, the surface of the PFA substrate was covered with a layer of about 1 cm fine sedimentated suspended matter, the situation improved for the clams.

In Chapter 8, a cadmium dosing experiment is described which was performed with Cd (50 µg/l) inorder to gain more insight into accumulation and elimination patterns of *U. pictorum* in relation with their activity. A large number of Cd studies have been carried out with mussels and clams in controlled, relatively short laboratory tests of a few weeks duration in which the role of food, temperature, substrate and suspended matter was neglected (Nriagu *et al.* 1981; Graney *et al.* 1984). Also, only a few long term elimination studies have been described in literature (Borchardt 1983; 1985).

Our results show that the process of Cd accumulation was rapid and almost irreversible, with concentration plateaus occurring after 3 - 6 weeks depending on temperature and Cd concentration in the surrounding water. Production of metal binding proteins and lysosomal vesicles may be responsible for the plateau in the accumulation pattern found in this study (Hemelraad *et al.* 1990). The kidney proved to be the target organ with a Cd concentration factor of 6000. Elimination from the gills and hepatopancreas and the maintenance of high levels in the kidney are in agreement with the studies of Holwerda *et al.* (1988). Unionidae prooved to be rather resistant to cadmium toxicity. According to the work of Van Urk and Marquenie (1989), the slowly diminishing population of the freshwater mussel *Dreissena polymorpha* in the Rhine, around the years 1980 was caused by the negative energy balance of *D. polymorpha* due to a constant drain of energy and proteins. The rapid accumulation of Cd and the low elimination caused a steady increase in Cd concentrations. The same situation can be expected for the Unionid population in the long term, however the sedimentary existence of the clam could have evolved some resistance to Cd. Activity (opening/closure) of the valves was found to decline as was also reported by Balogh and Salanki (1984) and Herwig (1989). The clam *U. pictorum* performed to be well suited for accumulation studies in both laboratory and field experiments. At least the expected differences in accumulation within the experimental setup in the laboratory and the flow chambers, in respect to the large differences in activity of the clams, were not confirmed.

In Chapter 9, the clams were subjected to Se(IV) spiked sediments and Se(IV) dosing (50 µg/l and 250 µg/l). Disposal of PFA in the direct vicinity of power stations is common practise all over the world. In the Great Lake area of North America some 74 coal fired power stations are situated, which discharge PFA (usually wet-sluiced) to basins created adjacent to the power stations (Hatcher *et al.* 1992). Hatcher *et al.* found that Se was one of the elements found in higher concentrations in oligochaetes and fish. Runoff from these sites smothers the benthic fauna (Guthrie and Cherry 1976; Dvorak *et al.* 1977), alters pH beyond the limits tolerable for local benthic macrofauna (Coutant 1978) and elements are leaching out. Studies by Hilton *et al.* (1980) and Lemly (1985) have indisputably prooven the effect on the reproduction of fish in Se contaminated waters. Se concentrations in the water bodies used in the fish studies were <15 µg/l. Information on the speciation of Se is not considered in most studies. Chronic toxicity data for fish showed teratogenic effects (Lemly 1985; Hunn *et al.* 1987). The extensive studies of Sorensen *et al.* (1982; 1983; 1984) with fish exposed to Se showed quite a few abnormalities in liver, kidney, heart, ovaries and erythrocytes.

In our study at least effects on the oogenesis were expected. Results show that the diameter of the amount of viable oocytes in the gonads compared with the control group continued to decline in the Se dosed group. The spawning in the controls occurred in May but spawning was delayed in the Se exposed group. The unexpected conclusion is that, no signs of severe damage were seen, although a delay in development exists which can lead to a less viable brood and possible effects on population level. Even an increase in concentration up to 250 µg/l Se(IV) showed no drastic increase in histopathological effects.

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PART 3. DUCKWEED AND TERRESTRIAL STUDIES

In Part three (chapters 11, 12 and 13) studies are carried out with the plant *Lemna minor*, the yellow nut sedge *Cyperus esculentus* and the worm *Eisenia fetida*. The study with the duckweed *Lemna minor* was initiated with the purpose of finding an easy to handle and rapid plant monitoring organism for leachates of waste products in general. Duckweed is a plant with a root, stem and one specialised leaf. It grows vegetatively but can flower also, belonging as it does to the angiosperms. According to Nasu and Kugimoto (1981) and Nasu *et al.* (1984) *L. minor* is one of the more sensitive plants for heavy metal and other aquatic pollutants. Compared with other higher water plants *L.minor* has a certain number of advantages: it is geographically widespread and shows a rapid vegetative (clonal) reproduction. Its size is small, it is easy to handle and it can be sterilized, which is necessary for culturing, and has a high reproducibility in experiments (Adema and de Zwart 1984; Wang 1986; Huebert & Shay 1993). The value of testing with plants, which form the major part in biomass in nature, has often been underestimated.

In Chapter 11 and 12 experiments with *L. minor* are described with separate elements, combinations of elements (artificial leachates) and leachates of PFA, bottom ash and coal gasification slag (CGS). A division is made between PFA from conventional burners and so-called low NOx PFA/bottom ash from a new generation of burners developed in order to reduce the NOx emission from power stations. Besides coal residues also sediments, leached equal to the different PFAs were tested in order to get more comparative data on the framework in which the phytotoxicity found could be presented. Therefore, sediment from the Apeldoornsch Kanaal and from the River Rhine was sampled.

In Chapter 13 the effects of CGS were studied next to PFA (as a reference) with *Cyperus esculentus* and the worm *Eisenia fetida*. The ash resulting from coal gasification consists of about 90% bottom ash and 10% fly ash, which is quite the opposite of conventional coal burning. The fly ash from coal gasification is left out of consideration. In an earlier study by Marquenie *et al.* (1988) *C. esculentus* and *E. fetida* were also used in a 'worst case' experiment with PFA and Rhine sediments. A major difference is that in our experiment nutrients were added in order to avoid malnutrition of the plants. *C. esculentus* is a nitrogen fixing plant that can grow in wetland as well as upland conditions and is a well known phytomonitor (van Driel *et al.* 1983). The purpose of using wetland and upland conditions was to create oxic and anoxic conditions which could influence the bio-availability of elements. *E. fetida* is a common indicator organism and is recommended as an indicator organism in the EEC directive for determination of ecotoxicity (EEC 1985). In an extensive study by Van Gestel (1991) the usefulness of using earthworms in ecotoxicology was demonstrated in laboratory experiments and by means of field experiments. Effects on earthworm populations can be predicted on the basis of laboratory studies if the chemical form and the environment is stable and the organism could not avoid the toxicant.

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CHAPTER 11

DUCKWEED *LEMNA MINOR* AS A TOOL FOR TESTING TOXICITY OF COAL RESIDUES AND POLLUTED SEDIMENTS

INTRODUCTION

Duckweed (*Lemna minor)* is used in water quality studies to monitor heavy metals and other aquatic pollutants, because duckweed, like other water plants, may selectively accumulate certain chemicals and may serve as biological monitors (Ray & White 1976; Werff van der & Pruyt 1982; Nasu & Kugimoto 1981; Nasu *et al.* 1984). The possibilities of using water plants as monitoring species were investigated by Roulet (1975), Kenaga and Moolenaar (1979), Rowe *et al.* (1982), Wang (1984; 1986), and Adema and de Zwart (1984). As far as the performance of the test is concerned both static and flowthrough systems are used (Eichorn von & Augsten 1969; Walbridge 1977; Bishop & Perry 1981).

In the present study the duckweed test is applied to coal residues and polluted sediments. With the construction of new coal burning power stations, the amount of coal waste products, *e.g.*, pulverized fuel ash (conventional and low NOx PFA), bottom ash, and coal gasification slag, will increase over the next few years. Low NOx PFA is formed at a lower temperature stage in the boiler due to the application of a new type of burners for reduction of NOx emission. As a whole the coal combustion can lead to surpluses of unused fly-ash and CGS. Questions are raising concerning the arrangements for dumping the coal residues regarding the release of heavy metals and other potentially toxic materials.

In The Netherlands, dumping in aquatic environments is out of the question, but large-scale

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Jenner HA, Janssen-Mommen JPM (1993) Duckweed *Lemna minor* as a tool for testing toxicity of coal residues and polluted sediments. Arch Environ Contam Toxicol 25:3-11.

inland deposition on recoverable sites is under study. An important element of the study is to assess the possible environmental impact in connection to various optional management strategies. So far, research at fly-ash basins has been focused on the accumulation of elements in water, sediment, and biota. It is also considered that the potential of duckweed to accumulate heavy metals could also be used to remove and/or relocate elements in the ecosystem (Guthrie & Cherry 1979a; 1979b; Clark 1981; White *et al.* 1986). This is feasable, because in existing fly-ash basins in the USA duckweed is one of most common plants having been the subject of research (Rodgers 1978). The toxicity of leachates of CGS on *Daphnia*, algae and duckweed was studied by Cushman & Brown (1981) and Klaine (1985). Specific tests for determining the toxicity of PFA leachates were conducted by Epler *et al.* (1980). Others have carried out research for application of duckweed in toxicity tests of industrial effluents and their conclusion was that bioassays with duckweed are both sensitive and comparable with methods using Daphnia or fish (Wang 1986; 1990; Wang & Williams 1990; Taraldsen & Norberg-King 1990).

The main objective of the present study was to obtain insight into the accumulation and toxicity of leached elements. In a previous paper (Jenner & Janssen-Mommen 1989) we examined phytotoxicity in experiments involving PFA leaching with acetic acid, as prescribed in US-EPA (1980), as well as the use of a standard, artificial leachate. The EPA method for leaching resulted in effects attributed to the acetic acid itself rather than to the metals in the leachate. In the study presented here, special attention was paid to 'low NOx' ash and to coal gasification slag (CGS) and a number of methodological aspects between the effect of repeated leaching on element concentrations was investigated. Additional monitoring experiments with sediments from a known polluted canal form a case study, providing evidence to support the establishment of a better system for judging the ecotoxicity of coal residues.

MATERIAL AND METHODS

Lemna minor (duckweed) was obtained from the Department of Plant Physiology of the Wageningen Agricultural University in The Netherlands. Duckweed was disinfected by immersing the fronds in 70% ethanol, followed by 1% NaOCl and rinsing with sterilised water (Bowker 1980). The sterilized (120 °C; 20 min.) growth medium used in the experiments was a medium modified by Rombach (1976) according to Gorham (1950), see Table 1. Instead of 10 µM EDTA as applied originally in the 'Gorham' medium was reduced to a concentration of 2.5 µM, since preliminary experiments demonstrated that 10 µM EDTA caused growth decline. The pH value of the medium is 5.0 ± 0.1. For the culture of *L. minor* 200 ml of the medium is transferred to high petri dishes (ϕ 9.5 cm, height 5 cm). The stock culture was kept in glass petri dishes (sterilized at 150 °C for 2 h). The stock culture was maintained sterile and transferred to fresh medium every 2 wk. All glassware and the disposable high petri dishes, used for the experiments, were cleaned in approx. 0.1 N HNO_3 for 24 h and were rinsed with demineralised water (Milli-Q). The experiments were performed in triplicate, each experiment was inoculated with 10 fronds, and lasted for 2 weeks. All incubations were performed in a laminar flow chamber. For the aseptic culture of *L. minor* a specially designed cabinet was used. In the cabinet the illumination was provided by cool white fluorescent lamps (16 h light: 8 h dark) with a light intensity of 80 µmol/m^2/s at water level. Inside the cabinet the temperature is measured at two locations, recorded continuously, and maintained at 23 ± 1 °C. The cabinet itself is placed in a room climatized at 18 °C.

The conventional PFA originated from KEMA batch A8850 (Amer power station); the low NOx ash was sampled at the Borssele power station. The coal gasification slag (CGS) was 'Drayton slag' produced according to the Shell process at a plant in Houston, USA. The low NOx PFA was divided into a course fraction and a fine fraction by a wind shifter (Heyd Sichter D1,2, type DNC, Germany). The composition of the fine fraction is 90% finer than 24 µm, and the course fraction is 90% finer than 146 µm.

The sediment samples from the Apeldoornsch Kanaal were taken using a 'Van Veen' sediment sampler. Samples were taken from three locations: a control site (outlet of a brook into the canal) called the 'Veldhuizer spreng'; a pigment factory near the city of Apeldoorn; and a electroplating plant in the town of Dieren. One sample was taken from the control site and three were taken from each of the other locations. The samples were dried at 105 °C and heated to establish the organic weight loss (at 600 °C). The organic content of the control was 0.9%; Apeldoorn 20.3%; Dieren 9.1%.

Table 1. Gorham medium (1950) modified by Rombach (1976).

- $Ca(NO_3)_2.4H_2O$	0.5 g
- $MgSO_4.7H_2O$	0.25 g
- KH_2PO_4	0.136 g
- H_3BO_3	2.86 mg
- $MnCl_2.4H_2O$	1.81 mg
- $ZnSO_4.7H_2O$	0.22 mg
- $(NH_4)Mo_7O_{24}.4H_2O$	0.18 mg
- $CuSO_4.5H_2O$	0.07 mg
- $Co(NO_3)_2.6H_2O$	0.08 mg
- NH_4VO_3	0.01 mg
- Fe-EDTA-solution	5 ml
- Milli-Q water to make up to	1000 ml

Fe-EDTA-solution consists of: 934 mg $FeCl_3.H_2O$ and 800 mg Na_2-EDTA (Titriplex III) in 1 litre Milli-Q water. The solution is airated for several hours to acomplish complete oxidation.

The coal residues and sediments were leached, according to the extraction procedure (EP) described by USEPA (1980), but nitric acid instead of acetic acid was used due to the toxic action on *L. minor* of the latter. The conventional PFA was leached three times in succession. The Low NOx and CGS was leached at three different L/S ratios (10, 20 and 40) at pH 5, 7 and 9 in two separate successive leaching experiments.

Of the coal residue/sediment samples 100 g was added to 1,600 ml (16x weight) of demineralized water. The sample was stirred for 24 h at ambient temperature while the pH was kept constant at 5, 7, and 9 with 0.5 N HNO_3 /NaOH. After stirring and checking the pH, demineralized water was added to make up the final volume of the sample to 2,000 ml (L/S 20) in correspondence to the formula:

$$V = 20(W) - 16(W) - A$$

V = volume demineralized water added (ml); W = weight of the solids (g); A = volume added 0.5 N HNO_3/NaOH (ml)

For the purpose of studying the influence of pH and solid-liquid ratio on the susceptibility to leaching of elements in the waste products, the concentrations were converted to leached percentages, i.e. the fraction of the total amount of the given element present in the solid material that had been leached out. The leached percentage was calculated as follows:

$$\frac{(L/S) \times \text{conc. in leachate } (\mu g/l)}{\text{conc. sediment (mg/kg dry matter)}} = \% \text{ leached}$$

For the toxicity tests with *L. minor*, the leachate was diluted with Gorham's medium containing 2.5 µM EDTA, pH 5, with leachate concentrations in the range 0 - 100%. The number of fronds was enumerated twice a week, *viz.* on day 4, 7, 11 and 14. A distinction was made between fully grown (1), near-fully grown (3/4), half-grown (1/2) and newly formed (1/4) fronds. The multiplication rate (MR) of *L. minor* was calculated for various concentrations of metal additions. The multiplication rate is a measure for the rate of increase in the number of fronds (Rombach 1976).

$$MR = 1000(\log n_1 - \log n_0)/t;\ t = t_1 - t_0 \text{ (days)};$$

n_1 = number of fronds on day t_1; n_0 = number of fronds on day t_0.

Growth was also measured and expressed as a percentage of total surface covering of the petri dish (= 100%), using image processing (PC Vision Plus framegrabber) and software package TIM (Difa Measuring Systems BV, Breda, The Netherlands). The surface coverage parameter can be established rapidly and easily, and this parameter has a much greater resolution than can be achieved by the conventional way of counting the number of fronds. In experiments with the leachates the counting of fronds is therefore omitted. The effects on growth are expressed as the percent difference of the leaf area coverage between the exposed culture and the control. At the end of each experiment, fresh weight by fast but carefully blotting, and dry weight (60 °C for 24 h) was determined. Concentrations of elements in the duckweed were determined by standard methods. Dried duckweed was digested in a pressure bomb with a Teflon liner (Berghof, Germany) with 1:1 diluted nitric acid. Depending on concentrations in the samples, elements in PFA, CGS, sediments, growth medium, leachate and duckweed were measured either by means of atomic absorption spectrophotometry (AAS) or by inductively coupled plasma (ICP). The element concentration is exposed in µg/l for the solutions and in µg/g dryweight for sediments and in µg/g fresh weight for duckweed samples. With these data the concentration factor (CF) in the duckweed was calculated (CF = concentration duckweed/concentration 'initial' medium).

RESULTS

Element composition in the coal ashes

The concentrations of most of the analyzed micro-elements in the fine fraction of the low NOx PFA were comparable with those in conventional PFA (Table 2). However, the As, Cr and Ni concentrations in the fine fraction were lower, while that of Pb was higher. In the coarse fraction of the 'low NOx' PFA, all analyzed micro-element concentrations were about twice as low as those in the fine fraction. In the CGS the concentrations of most of the macro-elements were lower than in the conventional fly-ash, but the calcium concentration in particular was appreciably higher. The micro-element concentrations in CGS were in general also lower than in conventional PFA, except for chromium and nickel.

Leaching behaviour of elements

Conventional PFA

When the PFA was subjected to repeated leaching (three times), the element concentrations in the first leachate (I) proved to be the highest, dropping rapidly in the second and third leachates (II and III) (see Table 3). The leaching percentages of the anionic elements are clearly higher than those of the cationic elements. B mainly leached out in the first step. The total cumulative leaching percentages given in Table 3 show that at the final liquid-soil ratio of 60 (3 times 20), 38% of Se was leached out. The percentages for As and Mo amounts to 21 and 22%, respectively. To summarize, the final leached element concentrations after the three steps vary between 10 and 40% for the anionic elements and are less than 1% for the cationic elements.

Low NOx ashes and coal gasification slag

In previous leaching experiments, with conventional PFA, pH was kept at pH 5, although, as is well known, the behaviour of elements in PFA changes at higher pH values. Especially As and Se are leaching in higher concentrations at higher pH values. The pH of the PFA produced in The Netherlands is normally between 9 and 11. The element concentrations in leachates of low NOx PFA and CGS at pHs 5,7,and 9, of two successive leaching procedures were performed, at solid-liquid ratios of 10, 20 and 40. The pH was kept at the above mentioned values during the leaching procedure (Table 4).

With the low NOx PFA, the leached percentages of B calculated were over 100%, which, of course, should not be possible. However, the composition of PFA is not homogeneous, resulting in deviations in the calculations for element distribution in the solid material. The anionic elements in the low NOx PFA leached out very well. After the first leaching, the leached percentages of B were between 50 and 100%, of Se and Mo 30 - 60% and 20 - 50% respectively. The leached percentages of arsenic were lower (2 - 35%). The second leaching resulted in a further 10 - 20% leaching of B and Se, 5 - 15% of Mo, and 5 - 10% of As. The leaching of the cationic elements (Cu, Ni, Pb and Zn) is generally in the order of a few percent. Only when the coarse fraction was leached at pH 5, with liquid-solid ratios of 20 and 40, really high leached percentages for Cu were measured. Such values not being credible, Cu contamination has been suspected. Cr can leach out either as a cation or as an anion, and its leached percentage is comparable with the cationic elements.

A comparison of the leaching of elements from the fine fraction of the low NOx PFA with the leaching of elements from the coarse fraction shows that at a pH of 5, the anionic elements (As, B, Mo and Se) leach out better from the fine fraction. There is little difference in the leaching of Mo from the two fractions at pH 7 and 9. Less of the Se was leached from the fine fraction at pH 7, and less of the B was leached from the fine fraction at pH 7 and pH 9. Differences in the leaching of the cationic elements from the two fractions were limited, the only difference being that Cu and Zn appeared to leach out better from the coarse fraction at pH 5. One must bear in mind, however, that these observations are not credible, especially where Cu is concerned.

The leaching of the elements from the CGS was low compared with the leaching from the low Nox PFA. Leaching of the anionic elements As, Mo and Se was not demonstrable, and leaching of boron was in most cases only $\leq$1%. Similarly, almost no leaching of cationic elements was detectable, except for Ni and Zn, the leaching of which was comparable with their leaching from low NOx PFA.

Toxicity tests with *Lemna minor*

Single elements

An assessment was made of the toxic effects on growth of the cations Cd, Cu, Zn and the anions As and Se to duckweed. The parameter surface coverage showed to be far more sensitive (EC_{50}) by a factor 15 - 20 for Cd and Zn than the parameter MR (Table 5). For the NOEC values no difference can be demonstrated between the two parameters for the cations. With the anions the values differ up to a factor 10, whereby the parameter surface coverage also turns out to be far more sensitive. From this point onwards it was decided only to use surface coverage as a parameter.

The EC_{50} values show a clear difference in toxicity between the cations and the anions (Table 5). On the basis of their toxicity the elements analyzed can be subdivided into three groups of toxicity:

- the cations Cd, Cu, and Zn with EC_{50} <0.5 mg/l
- the anions As (III) and Se (IV) with EC_{50} 0.5 - 3 mg/l
- the anions As (V) and Se (VI) with EC_{50} >5 mg/l

The NOEC values of the cations are also considerably lower than those of the anions, with the exeption of arsenite and selenite.

The data on the accumulation of metals by duckweed and the corresponding concentration factors are also presented in Table 5. It turns out that especially for Cu and Se, low concentrations in the duckweed are toxic (approx. 20 - 50 µg/g), followed by Cd, As (III), and finally by Zn and As (V), with concentrations ranging from 200 to 300 µg/g. As with the EC_{50} and NOEC values, there is a clear difference in concentration factor (CF) between the cations and the anions, with the exception of arsenite. The CF for the cations and the anion arsenite is many times greater than that of the remaining anions (Table 6).

Conventional PFA and low NOx PFA

In the case of the conventional PFA, only the leachate from the first leaching (step I) was demonstrated to have a toxic effect on growth of *L. minor*. The 75% leachate was associated with the EC_{50} (Fig. 2).

The toxic effects of the low NOx PFA on *L. minor* were tested using the leachates obtained at pH 5 with liquid-solid ratios of 10, 20 and 40 respectively (Fig.1). The element concentrations in the leachates obtained at pH 5 were generally higher than those obtained at pH 7 and 9, which implies that the toxicity tests may be regarded as 'worst case' testing. The toxicity of the leachates obtained from the CGS was not tested because of the low element concentrations in the leachates. Only Ni may be expected to have toxic effects.

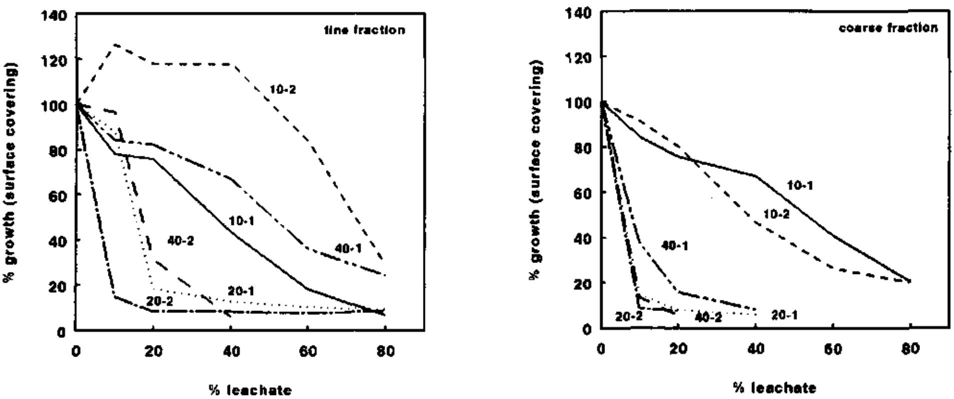

Figure 1. Growth of *L. minor* exposed to leachates of 'low NOx' PFA obtained at pH 5 and various liquid-solid ratios. 10-1 = liquid-solid ratio of 10, first leachate.

An erratic relationship appeared to exist between the toxicity of the leachates of the fine fraction and the liquid-solid ratio (Table 6). The growth reduction of the duckweed was greatest with the leachate obtained using a liquid-solid ratio of 20. The tests using leachates from the coarse fraction did seem to reveal a tendency for the toxicity to be greater at higher liquid-solid ratios, but again the growth reduction was greatest with the leachate obtained at a liquid-solid ratio of 20 (Fig. 1).

The effects of the leachates from both fine and coarse fraction increased as the copper concentrations in the leachates rose. The copper concentrations in the leachates are to be considered as potentially toxic. Only the leachates obtained from the (first) leaching of the fine fraction with L/S 10 displayed a toxicity higher than one would expect from the copper concentrations alone. Since the leachates contain a mixture of a large number of elements, other elements are likely to contribute to the toxicity as well.

Table 2. Element concentrations in coal ashes (dry wt.)
– = not measured

Element	Conventional PFA	"low NOx" PFA Fine	"low NOx" PFA Coarse	Coal gasific. slag
Macro-elements (%)				
Si	25.6	—	—	15.8
Al	15.2	—	—	10.5
Fe	4.8	—	—	7.3
Ca	1.29	—	—	23.2
Mg	0.56	—	—	0.48
Na	0.32	—	—	0.06
K	1.94	—	—	0.10
Ti	0.91	—	—	0.79
P	0.18	—	—	0.23
S	—	—	—	1.30
C	5.1	—	—	0.58
Micro-elements (µg/g)				
As	42	14	<10	21.6
B	170	187	105	143
Cr	150	71	37	216
Cu	156	139	69	65
Mo	25	28.0	9.8	<3
Ni	142	80	35	190
Pb	88	255	62	<3
Se	17	24.8	13.5	5.4
Zn	120	128	26	25

Sediment testing

Low element concentrations were found in the leachates of the Veldhuizer brook sediments (Table 7). At both other sampling points the Fe, Cr, and As concentrations were a factor of 2 - 12 higher, Cd and Ni concentrations were 20 - 30 times higher, Pb (Apeldoorn) was 65 times higher and Zn was even 170 - 370 times higher. The leachate of the brook sediment has no clear toxic effect on duckweed growth. However, at higher concentrations the fronds become smaller and the roots shorter. *L. minor* grew very poorly on the leachates of the samples from Apeldoorn and Dieren. The fronds were fewer in number, smaller and lighter in colour, and displayed a tendency to overlap. At high leachate concentrations, the old fronds were yellow, the roots white and liable to drop off.

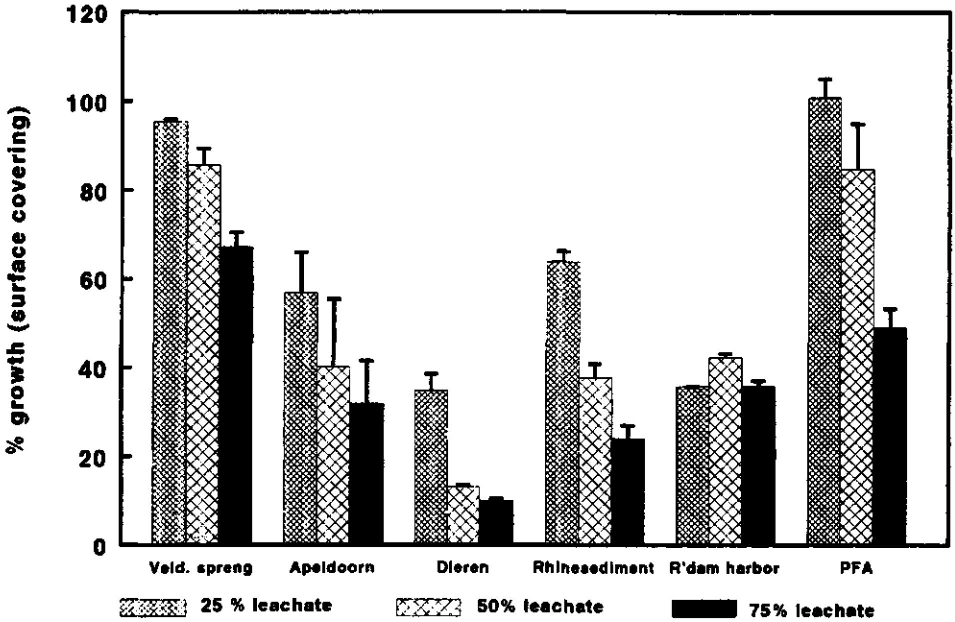

Figure 2. Comparison between the growth percentages of *L. minor* at 25%, 50% and 75% added leachate of different bottom sediments and conventional PFA.

In the experiments using leachates of samples obtained from the brook location, the concentrations of added leachate associated with EC_{50} were over 75%. Leachates of Apeldoorn and Dieren showed an EC50 at concentrations of 35% and 19%, respectively (Fig. 2).

The Zn concentrations in the leachates of the samples from both Apeldoorn and Dieren clearly exceeded the EC_{50} concentration as determined in the experiments with the individual elements. The concentrations in the duckweed associated with the EC_{50} of the Apeldoorn and Dieren leachates were 350 and 450 µg/g, respectively. These concentrations correspond fairly well with the concentrations in the duckweed at EC_{50} concentrations (= 300 µg/g) that were determined in the experiments using the individual elements. The Zn concentrations in the Apeldoorn and Dieren leachates at EC_{50} were, however, clearly higher (1,500 µg/l and

1,900 µg/l respectively), indicating that the availability of Zn in the leachates was less than in the growth medium at single element tests (290 µg/l).

Table 3. Element concentrations in the leachates of conventional PFA after three successive leaching procedures using HNO_3. The last group of columns shows the leached percentage.

	Conc. in leachate (µg/L)			Percentage leached (%)			
Element	I	II	III	I	II	III	Total
As	266	118	52	12.7	5.6	2.5	21
B	834	54	<15	9.8	0.6	<0.2	10.5
Cr	35	3.8	1.3	0.46	0.05	0.02	0.5
Cu	5.4	2.8	2.4	0.07	0.04	0.03	0.1
Fe	3	2.1	<2	<0.01	<0.01	<0.01	<0.01
Mo	181	77	<50	14.5	6.2	<4	22
Ni	70	9.5	5.8	0.99	0.13	0.08	1.2
Sb	31.2	9.9	6	4.8	1.5	0.9	7.2
Se	222	73	31	26.1	8.6	3.7	38

Table 4. Percentages of elements leached from low NOx PFA and CGS at different pH values and L/S ratios in two separated successive leaching experiments. (– = below detection level. All the values obtained for lead were beneath detection limits, so this element has been omitted from the table altogether.).

		Fine fraction						Coarse fraction						CGS					
		L/S 10		L/S 20		L/S 40		L/S 10		L/S 20		L/S 40		L/S 10		L/S 20		L/S 40	
Element*	pH	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
B	5	115	18	106	15	124	12	99	16	108	12	112	10	1.1	1.5	0.6	0.3	1.1	0.8
(1) —	7	73	18	74	12	89	16	87	17	94	11	102	13	0.5	0.4	—	—	0.7	—
(2) <20	9	45	11	45	14	52	11	63	17	66	14	78	13	0.4	0.3	—	—	—	—
Cu	5	0.3	0.7	14	43	5.3	23	2.4	3.6	113	128	90	162	—	—	—	—	—	1.9
(1) —	7	—	—	0.1	0.2	—	—	0.1	0.1	1.2	0.6	0.3	0.1	—	—	—	—	—	—
(2) <2	9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.1	—
As	5	33	6.1	32	4.7	36.3	4.5	—	—	—	—	—	—	—	—	—	—	—	—
(1) <10	7	9.3	—	8.5	5.7	19	6.4	—	—	—	—	—	—	—	—	—	—	—	—
(2) <3	9	1.7	2.9	3.6	5.7	8.7	11	—	—	—	—	—	—	—	—	—	—	—	—
Cr	5	0.1	—	0.1	—	0.2	—	0.1	—	—	—	0.1	0.1	—	—	—	—	—	—
(1) —	7	2.2	0.4	1.5	0.1	2.7	0.4	1.0	0.1	0.2	—	1.2	0.2	—	—	—	—	—	—
(2) 0.7	9	2.0	0.5	2.1	0.6	2.6	0.7	1.0	0.3	1.0	0.3	1.4	0.4	—	—	—	—	—	—
Mo	5	30	10	51	13	47	11	16	2.6	22	3.4	16	4.9	—	—	—	—	—	—
(1) <3	7	43	15	59	11	49	11	40	11	53	8.0	45	8.0	—	—	—	—	—	—
(2) <2.5	9	35	9.3	37	13	37	8.6	38	9.0	38	7.8	42	9.0	—	—	—	—	—	—
Ni	5	2.7	0.7	4.1	1.2	2.5	0.7	2.2	0.5	2.4	1.1	3.1	1.6	2.7	5.0	1.8	1.2	2.9	3.8
(1) —	7	0.6	0.1	0.9	0.3	1.0	0.1	0.9	0.2	1.1	0.2	0.9	—	0.2	0.3	0.3	0.2	0.8	0.6
(2) <3	9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Se	5	57	13	45	16	57	14	27	3.8	—	—	38	9.2	—	—	—	—	—	—
(1) —	7	30	12	33	18	53	15	46	17	58	16	75	20	—	—	—	—	—	—
(2) <4	9	35	13	48	13	58	14	31	13	45	17	53	13	—	—	—	—	7.7	—
Zn	5	1.3	0.9	2.3	2.1	6.4	3.9	4.9	2.3	15	26	15	11	2.4	6.6	1.9	2.7	—	6.2
(1) —	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
(2) <15	9	0.1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

*(1) Detection level solid (µg g^{-1} dry wt)
(2) Detection level liquid (µg L^{-1})

Table 5. EC_{50}- and NOEC-values (expressed as mg/l) of the elements studied in both surface covering and multiplication rate and the accumulated concentration of elements (µg/g fresh weight) at the EC_{50} (surface covering) with the corresponding concentration factor (CF).

Element	Surface coverage		Accumulation	CF	Multiplication rate	
	EC_{50}	NOEC	(µg/g)		EC_{50}	NOEC
Cd	0.1	<0.06	88	816	1.6	<0.06
Cu	0.14	0.06	25	194	0.32	0.06
Zn	0.29	0.16	296	825	5.6	0.16
As (III)	0.63	<0.75	140	±210	1.6	<0.75
As (V)	22.2	<4	214	9	86	37
Se (IV)	1.7	0.08	38	21	3.5	0.8
Se (VI)	±5.3	0.8	±45	±8	±11.5	>2.4
SeO_2	2.9	0.8	46	15	8.6	0.8

Table 6. Leachate percentage associated with 50% growth reduction (EC_{50}) in duckweed, and the associated element concentrations in the Low NOx leachates. These concentrations are compared with the EC_{50} and NOEC figures obtained in tests carried out using the individual elements. (10-1 = L/S 10, first leaching).

Leachate L/S	EC_{50} % leachate	Element concentrations in leachate at EC_{50} (µg L^{-1})									Visual effects
		As	B	Cu	Cr	Mo	Ni	Pb	Se	Zn	
Fine											
10–1	36.0	167	8060	24.9	1.2	370	78	<1	506	92	a
10–2	72.4	62	2580	73.1	<0.5	233	41	<2	239	94	a/b
20–1	15.5	35	1960	168	0.3	201	25	<1	86	65	b
20–2	5.9	2	550	192	<0.1	111	3	<1	12	55	b
40–1	51.5	6.5	3200	103	1.9	52	25	<2	179	130	a/b
40–2	17.2	2.7	510	154	<0.2	101	2.3	<1	15	63	b
Coarse											
10–1	53.2	3.9	5770	95	1.3	134	41	<1	197	92	a
10–2	38.2	<2	950	106	<0.3	75	7	<1	20	54	a/b
20–1	5.9	0.2	800	246	<0.1	106	2.5	<1	0.3	58	b
20–2	5.5	<1	510	260	<0.1	101	1.1	<1	3.3	66	b
40–1	8.0	<1	700	141	<0.1	101	2.2	<1	10	54	b
40–2	5.8	<1	490	179	<0.1	101	0.8	<1	1.8	51	b
EC_{50}	630	8400	140	—	8700	450*	—	1650	290		
NOEC	—	540	65	—	—	45*	—	>80	165		

* = Wang 1986

[a] Fronds smaller and lighter in colour, necrosis (effect presumably caused by B)

[b] Losing of fronds, edges of and eventually whole fronds turn yellowish brown (effect presumably caused by Cu)

DISCUSSION AND CONCLUSIONS

Duckweed, *Lemna minor*, is well suited for testing the toxicity of leachates, and the experiments described here support three conclusions:

1. Toxicity of conventional and Low NOx PFA is only related to the anionic elements and the toxicity of the tested dredged sediments is caused by cations
2. The leaching of elements from CGS is very limited
3. The toxicity of the tested coal residues is far lower than that of sediments from the Apeldoornsch Kanaal.

Roughly, the chemical composition of the PFAs is reasonably alike; CGS showed higher chromium and nickel contents, and significantly lower lead, selenium and molybdenum concentrations. The concentrations of elements leached from conventional PFA (USEPA extraction procedure) can be presented as follows:

	<10 µg/l	:	Cu, Fe
10	- 50 µg/l	:	Cr, Sb,
50	- 100 µg/l	:	Ni
100	- 500 µg/l	:	As, Mo, Se
	>500 µg/l	:	B

Cations as Cu, Fe, and Zn are well bonded in conventional PFA, and appear only in low concentrations in the leachate. The anions As, B, Mo, and Se on the contrary leach well and quickly from the PFA and can appear in relatively high concentrations in the leachate, especially in the short term. Further distinction is possible within the anionic group of elements, which may be categorized on the basis of the rate of leaching and the extent to which they are leached out, as follows: Se > Mo ≥ As > B. An explanation for these elements' different leaching characteristics may lie in the extent to which each is bonded to the surface of the PFA particles (Sloot van der 1990). For it would appear that Se is 80-100% surface bonded, As 50 - 80%, and Mo 50 - 70%. The rate of leaching for 'Low NOx' PFA was in the order of B » Mo ≥ Se > As. Percentual leaching concentrations are higher of low NOx PFA compared with conventional PFA. Element leaching of CGS is neglectable with the exeption of Ni.

Table 7. Element concentrations in the sediments (dry weight) and leachates with the associated leached percentages (mean with SD). A: Veldhuizer brook (control); B: Apeldoorn (pigment factory); C: Dieren (electroplating plant).

Element	Location	Sediment (µg/g)	Leachate (µg/L)	Leached (%)
As	A	6.8 ± 2.3	<5	—
	B	27.2 ± 3.4	10.5 ± 2.7	0.8 ± 0.3
	C	15.9 ± 0.8	37.1 ± 11.5	4.7 ± 1.2
Cd	A	0.1 ± 0.1	0.3 ± 0.1	6.2
	B	8.1 ± 1.6	11.5 ± 2.2	2.9 ± 0.6
	C	24.3 ± 0.4	7.5 ± 1.2	0.6 ± 0.1
Cr	A	7 ± 1	<8	—
	B	748 ± 196	55 ± 24	0.16 ± 0.08
	C	126 ± 6	36 ± 8	0.58 ± 0.11
Fe*	A	10 ± 1	0.1 ± 0.1	0.02
	B	34 ± 6	1.4 ± 0.5	0.09 ± 0.03
	C	12 ± 1	0.9 ± 0.1	0.15 ± 0.01
Hg	A	<0.5	<0.5	—
	B	0.7 ± 0.1	<0.5	—
	C	1.1 ± 0.4	<0.5	—
Ni	A	9 ± 1	<10	—
	B	98 ± 24	242 ± 49	5.0 ± 0.8
	C	71 ± 21	194 ± 39	5.6 ± 0.5
Pb	A	8 ± 4	<3	—
	B	2479 ± 692	197 ± 44	0.17 ± 0.05
	C	189 ± 30	7 ± 2	0.08 ± 0.01
Se	A	<4	<5	—
	B	5 ± 2	<5	—
	C	<4	<5	—
V	A	≤5	<10	—
	B	45 ± 7	<10	—
	C	56 ± 4	14 ± 3	0.50 ± 0.06
Zn	A	36 ± 2	25 ± 4	1.4
	B	1050 ± 251	4247 ± 767	8.2 ± 1.1
	C	1682 ± 139	9200 ± 339	11.0 ± 1.3

* Iron concentration in $mg\ g^{-1}$ and in $mg\ L^{-1}$

The influence of the pH on the susceptibility to leaching was demonstrable for most elements. Leaching of the anionic elements B and As is lowering as pH becomes higher. Leaching of the elements Mo and Se was highest at pH 7, though in the case of Se this was only so with the coarse fraction. Leaching of Cr increased as the pH increased. A clear reduction in the leaching of the cationic elements Cu, Ni and Zn was observed as the pH rose. There was generally no detectable leaching of these elements at pHs 7 and 9.

The influence of the L/S ratio on the elements' susceptibility to leaching was less clear than the pH effect. With the low NOx PFA there was increased leaching of B, As and Se at higher liquid-solid ratios, but in respect of As and Se this was only detectable at pH 7 and pH 9. Leaching of Mo was highest with a liquid-solid ratio of 20. Amongst the cationic elements, leaching of Zn increased at higher liquid-solid ratios. The solubility product of the different elements plays an important role in this context (De Groot *et al.* 1989).

In their free ionic form, the cationic elements are easily taken up by *L. minor* (high CF) and can therefore be toxic in low concentrations, this is also true of As (III). The biological availability of the rest of the anionic elements examined is lower for *L. minor* (low CF), with the result that relatively high concentrations are needed in the leachate before a toxic effect occur.

The results of toxicity tests on the fine and coarse fractions of the low NOx ash using *L. minor* do not reveal the expected relationship between the liquid-solid ratio and growth inhibition. Growth was most strongly inhibited at a liquid-solid ratio of 20. The results are likely to have been dominated by the effects of Cu. Acute effects such as loss of mother/daughter fronds were visually detectable, as were non-acute effects such as fronds being stunted, lighter in colour, or yellowish brown and necrotic.

Toxicity experiments with CGS leachates on *Spirodela oligorhiza* and the algae *Selenastrum capricornutum* showed an EC_{50} of 76% and a NOEC 20% (Klaine 1985). Klaine obtained the CGS from a pilot plant and followed the USEPA (1980) leaching procedure, using acetic acid. A substantial part of the effects found may be attributed to the use of acetic acid. In our experiments the element concentrations in the CGS leachates were low and consequently no toxicity experiments were carried out.

The growth effects on *L. minor* with leachates from the Apeldoornsch Kanaal are probably caused by the high Zn concentrations. Zn has an NOEC of circa 160 µg/l and an EC_{50} of 290 µg/l. Concentrations of Zn in both sediments and leachates were relatively high. Leaching of most other elements was relatively low (less than 1%). Strong growth inhibition was also observed in earlier studies and pilot experiments using sediments from the River Rhine and harbour sludges from Rotterdam treated in the same way as the canal sediments. The effects were probably also caused by Zn. In the harbour and River Rhine sediments Zn concentrations were 1,050 and 650 µg/g (dry wt) respectively. The leached percentage was about 10%. It can be concluded that PFA is less phytotoxic and if there are any effects they are caused by the anions compared to the effects of the tested sediments.

In a review of the accumulation of toxic trace elements by freshwater vascular plants, Outridge and Noller (1991) concluded that the free-floating species may be useful for biomonitoring elements in water. Problematic is the absence of a standardized testing protocol covering leaching tests and effect tests adjusted for the biological availability of elements. The present paper may be a step ahead in the proper dirction.

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CHAPTER 12

PHYTOMONITORING OF PULVERIZED FUEL ASH LEACHATES BY THE DUCK-WEED *LEMNA MINOR*

INTRODUCTION

The forecast for coal combustion in The Netherlands is for about 15 million tons a year in the year 2000, which will result in an annual amount of about 1.5 million tons of pulverized fuel ash (PFA). Compared with 1985, at 0.5 million tons of PFA, the production will triple in 15 years' time (Meij *et al.* 1986). The deposition of PFA in disposal piles arouses concern in The Netherlands about long-term environmental effects due to elevated metal concentrations in PFA and the possibly elevated metal concentrations in leachate or runoff water (Bolt & Snel 1986; Cherry & Cuthrie 1977; Cherry *et al.* 1984). Nriagu (1988) describes the dangers of low-level elevated metal concentrations for higher animals and human populations. He discusses possible links with cardiovascular diseases, and allergies. Sublethal effects can be expected for (aquatic) organisms lower in the food-chain.

Phytotoxicity research is still a minor component of all the tests and bio-assays commonly used in toxicity studies (Wang 1984). The criticism of phytomonitoring with *L. minor* is focussed on lower sensitivity compared with aquatic animals, so that duckweed studies seem to be redunant. Bishop and Perry (1981) also concluded that the ecological significance of the *L. minor* testing was quastionable compared with the testing of daphnias or fish. However, duckweed forms an essential component in shallow stagnant waters. The bio-assay studies of Ray and White (1976), Guthrie (1979), and Nasu and Kugimoto (1981), utilizing higher plants and especially aquatic plants, such as the duckweed *L. minor*, highlighted the advantages of aquatic plants.

This chapter is based on:
Jenner HA, Janssen-Mommen JPM (1989) Phytomonitoring of pulverized fuel ash leachates by the duckweed *Lemna minor*. Hydrobiologia 188/189:361-366.

The importance of metal speciation and related toxic effects is discussed by Wang (1986; 1987), who used duckweed for toxicity tests of Cr, Ba, Cd and Ni in natural water samples. Reproduction of *L. minor* is usually vegetative.

Each mother frond produces two daughter fronds in two separate envelopes. However, flowering also occurs. Duckweed is clone-forming, which means that the starting material can be genetically equal. It can be disinfected and grown in a liquid medium as well as on agar, autotrophically or heterotrophically (Landolt 1957; Hillman 1961). From an ecological point of view it is an important species with a global distribution, eaten by several species of wildfowl, and, for instance, muskrats.

The emphasis in our study is on the toxic effects of PFA leachates prepared artificially and by leaching procedures on growth and accumulation of heavy metals. The artificially prepared leachate mixture is used to avoid natural differences in metal concentrations in coal combustion (Srivastava *et al.* 1986).

MATERIAL AND METHODS

All experiments are performed with a duckweed strain obtained from the Agricultural University of Wageningen. For the aseptic culture a specially designed cabinet was used. In the cabinet the illumination was provided by a cool white fluorescent lamp (16 h light; 8 h dark) with a light intensity of 6000 lux. Temperature was maintained at 22 ± 1.5 °C. High Petri-dishes of glass were used for the stock culture and disposable Petri-dishes, rinsed with approx. 0.1N HNO_3 were used for the experiments. Disinfection of the duckweed was effected by immersing the fronds in 70% ethanol, followed by 1% NaOCl and rinsing with sterile water (Bowker *et al.* 1980). The growth medium used (pH 5) was a Gorham medium (Gorham 1950), modified by Rombach (1976), see Table 1.

The incubation time for all experiments was fourteen days, in which each experiment was inoculated with 4 triplets of *L. minor* on day one. The effects on growth were measured by counting the number of fronds and using image processing techniques to determine the percentage of the water surface covered by duckweed as a measure for biomass. EDTA is necessary (metal complexation) for optimal growth. With the image processing technique it was found that 2.5 µM EDTA resulted in a higher percentage covering than the prescribed 10 µM EDTA according to Gorham. However, the number of fronds was equal at either EDTA concentration, indicating that the fronds in 2.5 µM EDTA were larger. In all other experiments 2.5 µM EDTA was used. All experiments were carried out in triplicate. The effects measured were calculated against the controls within each experiment.

Table 1. Gorham medium (1950) modified by Rombach (1976).

element	mg/L
$Ca(NO_3)_2.4H_2O$	500
$MgSO_4.7H_2O$	250
KH_2PO_4	136
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.22
$(NH_4)Mo_7O_24.4H_2O$	0.18
$CuSO_4.5H_2O$	0.07
$Co(NO3)_2.6H_2O$	0.08
NH_4VO_3	0.01
Fe^{3+} EDTA - solution	5 ml
distilled water to make up to 1 Liter.	

[Fe^{3+} EDTA: 1 L dist. water + 934 mg $FeCl_3.6H_2O$ + 800 mg Na_2EDTA (Titriplex III)]

The leachates of PFA were prepared according to EPA instructions, i.e. solid/liquid of 1:20; stirring for 24 h.; a static pH 5 (acetic acid); filtering at 45 µm (U.S. EPA 1980). This 'natural' type of leachate is used against the 'standard' of an artificially composed leachate, in accordance with (cascade technique) the mean metal concentration values in PFA in the year 1987. The destruction techniques of PFA are in accordance with ASTM D3683 (ANSI/ASTM 1978) and NEN 6465 (NNI 1981). The duckweed was dried for 24 h at 60 °C and destroyed in teflon-coated pressure bombs with 1:1 diluted HNO_3. The results are expressed on a wet weight basis. The metal concentrations in PFA, leachate and duckweed were determined by means of ICP-AES and AAS.

RESULTS

The results of both destruction methods for determining the total metal concentrations in PFA in accordance with NEN and ASTM are listed in Table 2. The differences in micro-element composition clearly show how cautiously a proposed method has to be used. The cation concentrations in the ASTM procedure (Cu, Cr, Ni, Zn) are higher, indicating a better destruction of the matrix. Hence, only part of these cation concentrations can contribute to biological processes or is bio-available to the plant. The results of the leaching experiments in accordance with the EPA standard procedure are presented in Table 3. The differences between the percentage of leaching of cations (Cr, Cu, Ni) and anions (As, Mo, Se) are striking. For Se, however, actual concentrations are low. This metal analysis table has served as the composition list for the artificial 'standard' leachate.

Table 2. Comparison between different methods of PFA element analysis for some micro-elements (μg/g).

element	Dutch stand. NEN 6465	ANSI-ASTM 3683
As	38	45
Cu	100	173
Cr	60	153
Ni	38	136
Pb	50	52
Sb	8	13
Se	15	15
Zn	80	133

The effects in percentage of the addition of 'natural' leachate on the growth of *L. minor* are presented in Figure 1. Noteworthy is the difference in effect due to the acid used to maintain a static pH of 5 during the prescribed 24 hours stirring. Only the leachate prepared with acetic acid seems to cause a dramatic decrease in growth. This phenomenon was studied in a number of experiments with the 'standard' and 'natural' leachates with and without acetic acid (Fig. 1). The decrease in growth is caused by the toxic action of the acetic acid. Effects on growth actually start at a 60% addition. For the 'standard' leachate a more rapid decline is found with clear effects at as little as 20% addition, showing that some components in the 'standard' are missing, or that the 'natural' leachate is less toxic because of some chemical interactions.

Table 3. Element concentration in PFA and leachate (acetic acid) calculated as mean value of coal combustion. Sitution in 1987.

Element	PFA		Leachate	
	%	$\mu g\,g^{-1}$	$mg\,l^{-1}$	%
Al	15.2		0.33	0.004
Ca	1.3		129	20
Fe	4.8		0.012	0.0005
Mg	0.6		9.7	3.5
P	0.18		2.9	32
As		58	0.46	16
B		166	0.9	11
Cr		150	0.02	0.23
Cu		156	0.11	1.4
Mn		667	0.2	0.6
Mo		25	0.36	29
Ni		142	0.09	1.3
Se		17	0.39	46
Zn		150	0.21	2.8

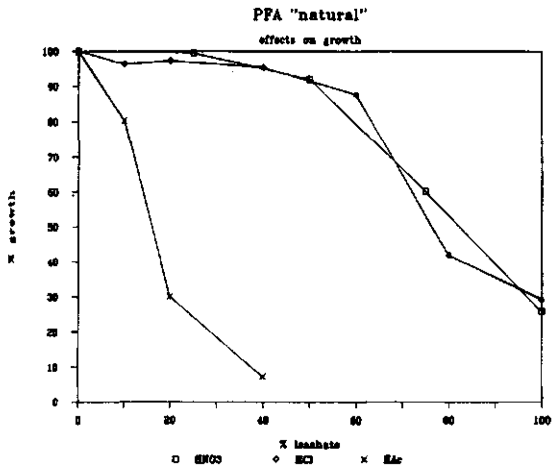
The accumulation of macro- and micro elements by *L. minor* is presented in Table 4. Anions as well as cations are accumulated, but especially cations. The accumulation of micro-elements was studied in other experiments (to be published elsewhere) in which the attention was focussed on the metal speciation of As and Se. In these experiments methods were similar to the PFA leaching study. The EC_{50} values (= concentration resulting in growth reduction of 50%) for the different metals are outlined in Table 5. The concentration factor (CF) is expressed as the ratio of the concentration accumulated in the duckweed and the concentration in the medium after 14 days incubation time. Amongst the elements given in Table 5 Cd, Zn and Cu show a relatively high CF combined with a low EC_{50} value compared with the other elements. The difference in toxicity of As(III) and As(V) was a factor 35.

Table 4. Macro/micro - elements in *Lemna minor* (μg/g ww.) grown on medium with 10% leachate (acetic acid) addition to the medium. CF: Concentration Factor.

Element	Control		10% PFA leachate	
	$\mu g\,g^{-1}$	CF	$\mu g\,g^{-1}$	CF
Al	9.8	-	13.5	409
Ca	790	9.7	764	8.6
Fe	16.7	118	6.4	49
Mg	196	8	239	10.4
P	1275	41	1100	39
As	-	-	2.6	57
B	59	116	58	105
Cu	2.5	139	9.4	347
Mn	192	384	248	527
Ni	1.4	-	3.1	344
Se	-	-	0.7	18
Zn	57	1130	67	1022

Table 5. Element concentration, inducing a growth reduction of 50 % after 14 days. The CF values are calculated from the EC_{50} concentrations.

Element	$mg\,l^{-1}$ (EC_{50})	CF (EC_{50})
Cu	0.13	300
Cd	0.09	825
Zn	0.33	820
Se		
SeO_2	2.75	13
Na_2SeO_3	1.78	20
Na_2SeO_4	>5	8
Ge	6.4	57
B	8.5	18
Mo	64	9
As		
$NaAsO_2$	0.82	210
Na_2HAsO_4	30	12



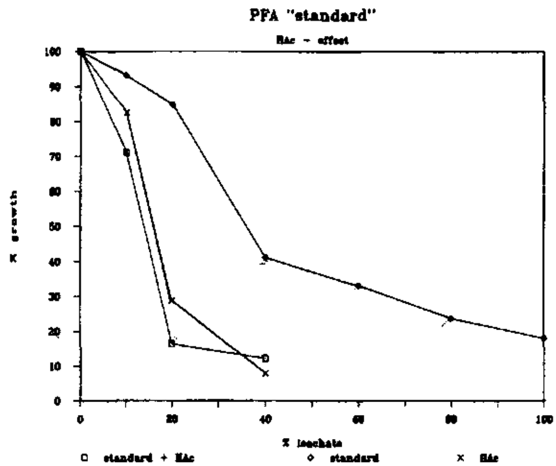


Figure 1. Effects on growth of 'standard'and 'natural' leachates with and without acetic acid.

DISCUSSION

The results in the study of metal accumulation with *Lemna minor* show the ease of applying duckweed as a bioassay. It should be fitted in a larger test scheme complementary to (in-)vertebrate tests. A prerequisite for future applicability of *L. Minor* as phyto monitor for metal pollution is the simultaneous occurrence of high accumulation and relatively strong effect-resistance for metals, see Tables 4 and 5. Effects on growth by the 'natural' leachate occur at addition percentages of 60%; however, at additions of 10% elevated metal concentrations can already be detected. The use of a 'standard' leachate addition as a reference toxicant led to effects at 20% percent addition. This subject needs more attention.

A great deal of research has been focussed on the leaching techniques and the prediction of heavy metal concentrations in leachates (Van der Scoot *et al.* 1982; 1985) with cascade, column and 24-hour stirring tests. Direct use of leachates in bioassays, as demonstrated with PFA leachate prepared in accordance with the EPA standard, has to be used with care. The supposed effects on growth of a combination of heavy metals appear to be attributed to the acetic acid. Acetic acid is a known phytotoxic for barley seedlings (Lynch 1977). Instead of acetic acid, HNO_3 will be a better acid.

Considering the rate of metal leaching of PFA the anions, and especially the Se-anion, show a high washout of about 50% within 24 hours. This is caused by rather poor bonding, due to condensation of the anions (As, Se, Mo) on the PFA particles, compared with the cations, which can also be seen in the data of the two destruction methods for element analysis (Table 2).

As opposed to the low leachability, accumulation of cations by duckweed is high, especially for the elements Cu and Zn (Table 4). The data of EC_{50} values on growth by individual elements show a well-known order. One should be cautious in interpreting the apparently low Se concentration data, because Se accumulates rapidly in the food chain (Lemly 1985) and teratogenic effects on higher animals (fish) are found at concentrations lower than 15 µg/l as Se (Gillespie & Bauman 1986).

Considering the differences in effects by selenite and selenate more research on speciation is required. In the case of arsenite and arsenate, the difference in toxicity is far more pronounced, but arsenite is rapidly oxidized to arsenate (if it exists at all), and besides that As is not accumulated in the food chain and therefore poses no threat in ecotoxicology compared with Se.

The results presented in this paper point to the need for a better understanding of that part of metals that will leach from disposal piles at a certain location and subsequently that part that will be bio-available for the biota. Hence, speciation and bio-assay studies are necessary. A simple determination of total metal concentrations in PFA by means of more or less complete destruction methods, as used in legislation so far, overestimates possible toxicological effects and consequently devaluates PFA potentials for recycling purposes.

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CHAPTER 13

EFFECTS OF COAL GASIFICATION SLAG AS A SUBSTRATE FOR THE PLANT *CYPERUS ESCULENTUS* AND THE WORM *EISENIA FETIDA*

INTRODUCTION

Coal gasification slag (CGS) is one of the residual products produced by the coal gasification process. The amounts produced are comparable with those of pulverized fuel ash (PFA) from the traditional pulverized-coal-fired boilers. The ash resulting from coal gasification consists of about 90% bottom ash (CGS) and only 10% PFA, whereas the ash produced by coal combustion contains 90% PFA. In terms of quality the differences are large both physically (grain size distribution) and chemically (mobility and concentrations of elements). A study of environmental effects requires insight into the mobility and the - closely linked - biological availability of contaminants (Irgolic 1989; Jenne *et al.* 1986). Monitoring the uptake in plant and animal tissue with the relative calculated concentration factors will provide a more accurate picture of the mobility and bioavailability of contaminants (Stafford & Edwards 1985).

The monitoring study was performed on the plant *Cyperus esculentus* and the worm *Eisenia fetida*; effects such as metal accumulation, growth inhibition and mortality of the organisms in the substrates CGS and reference PFA were analyzed. The study is related to previous research by Marquenie *et al.* (1988), who used a worst-case experimental setup to study effects also on *C. esculentus* and in substrates of PFA and Rhine river sediment. In the experiments with *C. esculentus* an N-P-K fertilizer was applied as well to establish the effects of nutrient deficiency. *C. esculentus* (yellow nut

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This Chapter is based on:
Jenner HA, Janssen-Mommen, JPM, Koeman JH (1992) Effects of coal gasification as a substrate for the plant *Cyperus esculentus* and the worm *Eisenia fetida*. Ecotox Environ Safety 24:46-57.

sedge) is a nitrogen fixing plant that can grow under wetland as well as upland conditions and is a well-known used phytomonitor. (Van Driel *et al.* 1983). *C. esculentus* was tested both under relatively dry (upland) conditions and extremely wet conditions (wetland). The purpose was that this would cause aerobic and anaerobic conditions respectively, near the roots which might lead to variations in metal availability.

E. fetida is recommended as an indicator organism in the EEC/OECD procedure for eco-toxicological tests of industrial chemicals (Stafford & Edwards 1985; Adema 1985; Adema *et al.* 1988; Neuhauser *et al.* 1985; EEC 1984). Worms constitute the principal group of soil organisms, about 80% in terms of biomass. Worms improve the soil structure (aeration and drainage) and the nutrient cycle and they contribute to the enrichment of the soil by organic matter. Worms can serve as an indicator of soil pollution because of their metal accumulation behaviour; metal concentrations in the worms may even be toxic to animals in subsequent stages of the food chain (Karnak & Hamelink 1982; Beyer & Cromartie 1987; Marquenie & Simmers 1984). Conversion processes of worms in the soil can cause an increase in the availability of contaminants for plants as a result of the relative increase of the complexable part (Schmidt *et al.* 1986).

MATERIAL AND METHODS

The yellow nut sedge *C. esculentus*, originating from WES (U.S. Army Engineer Waterways Experiment Station) was grown in accordance with the directives of Doty and Sweet (1970). For the upland condition perforated flower pots were used 12 cm in diameter and 9.5 cm in height (volume 0.7 litres). The pots were placed in plant dishes 2.8 cm in height and the water level was kept right up to the edge of the dish, using demineralized water. The plants in the wetland condition were grown in closed flower pots 13 cm in diameter and 12 cm in height, which were filled with substrate up to 2 or 3 cm below the edge (volume 1.2 litres). Here the water level was kept up to the edge of the pot.

The substrates tested are:

- 100% potting soil
- 50% CGS + 50% potting soil
- 75% CGS + 25% potting soil

- 100% CGS
- 100% CGS + nutrients
- 100% PFA + nutrients

The CGS was obtained from a pilot plant working according the Texaco process (Holten) and the PFA was from the Amer power plant (Geertruidenberg). At the start of the experiment nutrients were added in the form of an N-P-K solution ($Ca(NO_3)_2.4H_2O$ - $NaH_2PO_4.H_2O$ - KC1). The concentrations correspond with those of the monitored potting soil. This standard potting soil contains an average of 1.25 g/l PG Mix, of which 14, 16 and 18% in the form of N, P and K respectively.

For each substrate and humidity condition three pots were used (in triplicate), with 4 plants in each pot. Prior to planting, plants grown from tubers were cut at a height of about 10 cm from the roots and washed with demineralized water. Long daylight (16h/8h) conditions of about 8000 lux intensity were used. Average temperatures were 15.9 ± 2.8 °C during the night and 20.1 ± 2.9 °C in the daytime. After 10 weeks exposure time the plants were cut off at the point where they started to grow (10 cm above the roots). Subsequently, the plants were washed with demineralized water and dried with filter paper to be able to determine their fresh weight. Finally, the plants were cut into small pieces (± 3 cm) and dried for 24 hours at 60 °C for the determination of their dry weight.

E. fetida came from the stock culture of the National Institute of Public Health and Environment. *E. fetida* was exposed to the upland situation only, in the same soil mixtures, without N-P-K, as *C. esculentus*. Perspex cylinders of 10 cm in diameter and 20 cm height were used. The cylinders were sealed at the bottom with fine-meshed gauze. The substrate height was 18 cm and the cylinders were placed in plant dishes filled with demineralized water. The temperature during the experiment was 18 °C and the light intensity 300 - 600 Lx.

For each substrate three cylinders were used containing 25 worms each. The exposure time was 7 weeks. At the start as well as at the end of the experiment the total number of worms, including their fresh weight, was determined for each cylinder. To establish their fresh weight, the worms were washed with demineralized water, carefully dried on filter paper and then weighed. At the end of the experiment, the worms were removed from the substrates, weighed and purged for 48 hours on moisted filter paper to clean the guts of the worms. The filter paper was renewed after 24 hours. The worms were frozen and subsequently freeze-dried. The dry samples were homogenised for analysis.

The pH value of all substrates was measured at the beginning and at the end of the experiment. For this purpose 50 ml of demineralized water was added to 20 grams of air-dried soil and mixed for one hour. After the mixture had been set aside for one night to settle, the pH value in the above liquid was measured. The CGS used had an initial pH of 8 and the PFA had a pH of 10. The redox potential (rH in mV) in all substrates was measured each week by means of a combined Ag / $AgCl_2$ electrode. The value measured must be corrected for measuring against a hydrogen electrode (E_H). At a temperature of 20 °C this correction amounts to +211 mV. With the pH value and redox potential measured the rH values can be calculated according to the formula:

$$rH = \frac{2 \,.\, E_H}{U_N} + 2 \,.\, pH$$

U_N = Nernst-constante (58.16 at 20 °C)

The rH value is a measure for the oxidising and reducing properties in a system; see the subdivision below (Nebe 1974):

rH 0- 9, severely reducing properties
rH 9-17, mainly mildly reducing properties
rH 7-25, system with more or less undefinable properties
rH 5-34, mainly mildly oxidising properties
rH 34-42, severely oxidising properties

The pH values and redox potentials were used, in a computer program, to determine for the various elements their theoretical speciation in the water phase by thermo-dynamical equilibrium calculations.

The following elements were determined in substrate, in *C. esculentus* and in *E. fetida*: As, B, Cr, Cu, Mo, Ni, Pb, Sb, Se and Zn. The homogenates were destructed in teflon pressure vessels (Berghof), with HNO_3/HF for the plant material and HNO_3 / HF / $HClO_4$ for the worms. For most elements the method of analysis used was the ZAAS furnace technique (= atomic absorption spectrometry with Zeemann correction), except for B and Zn, which were determined on the basis of ICP (inductively coupled plasma).

Of the substrates only the element concentrations in 100% CGS and 100% PFA were determined. These concentrations were subsequently used to calculate the concentrations for the other substrates, while it was assumed that the concentrations of the above elements in the potting soil were negligible.

RESULTS

The pH value of the potting soil is 6.2 and it increases --from about 6 to approx. 8-- in proportion to the amount of CGS added to the potting soil. The addition of nutrients to the CGS results in a slight drop of its pH value against that of 100% CGS. During both experiments the pH value of the substrate hardly shows any change at all. The differences are below 0.5.

Both with *C. esculentus* and *E. fetida* the redox potential at the outset of the experiment is highest for substrates containing CGS ($\pm$ 600 mV), slightly lower for potting soil (500 - 550 mV) and considerably lower for substrates containing PFA ($\pm$ 300 mV). For *C. esculentus* there is at the start of the experiment no difference in redox between upland and wetland. During the experiment the redox falls sharply with potting soil (both in wetland and upland situation) and with 50% CGS in the wetland condition.

With the substrates containing CGS the redox potential shows a slight decline in all cases, while under wetland conditions it is lower than in the upland situation. This difference between upland and wetland is large indeed with 50% CGS. For PFA-containing substrates no clear change in redox potential was observed. For all substrates tested the rH >17; hence, no reducing circumstances were created.

Cyperus esculentus

Table 1 gives the data on the biomass of *C. esculentus* after 71 days of exposure in various substrates.

Table 1. Biomass of *C. esculentus* after 10 weeks in the various substrates; old/new = newly formed shoots.

Substrate plants		Number (old/new)	Fresh weight (g/pot)	Fresh weight (g/plant)	Dry weight (mg/pot)	Dry weight (mg/plant)	Length (cm/plant)	Moisture (%)
Potting soil	W	12 (0)	5.58 ± 0.71	1.39 ± 0.18	1409 ± 109	352 ± 27	32.3 ± 1.1	74.6 ± 1.4
	U	12 (4)	2.45 ± 0.50	0.48 ± 0.16	680 ± 123	134 ± 48	24.0 ± 3.0	72.1 ± 1.5
CGS								
50%	W	12 (1)	3.22 ± 0.99	0.74 ± 0.16	927 ± 239	212 ± 38	28.0 ± 2.0	71.8 ± 0.7
	U	10 (2)	0.96 ± 0.22	0.24 ± 0.06	304 ± 74	76 ± 19	18.7 ± 1.5	70.9 ± 1.7
75%	W	9 (3)	2.70 ± 0.31	0.67 ± 0.08	786 ± 123	197 ± 31	31.7 ± 1.5	71.0 ± 1.3
	U	9 (4)	1.31 ± 0.25	0.31 ± 0.08	396 ± 54	93 ± 21	23.8 ± 1.3	69.7 ± 1.6
100%	W	5 (3)	0.71 ± 0.23	0.27 ± 0.03	230 ± 67	90 ± 13	25.5 ± 0.9	67.1 ± 3.7
	U	5 (3)	0.34 ± 0.11	0.13 ± 0.04	116 ± 42	44 ± 11	20.2 ± 1.6	66.1 ± 2.9
CGS + NPK	W	6 (2)	3.68 ± 0.96	1.45 ± 0.32	819 ± 246	326 ± 116	40.4 ± 7.5	77.9 ± 3.0
	U	7 (3)	7.34 ± 4.69	2.17 ± 1.41	1563 ± 1066	457 ± 300	37.8 ± 10.2	79.6 ± 2.9
PFA + NPK	W	2 (10)	0.60 ± 0.55	0.16 ± 0.09	130 ± 108	39 ± 11	17.0 ± 1.4	72.1 ± 14.0
	U	1 (6)	0.39 ± 0.21	0.17 ± 0.12	93 ± 75	43 ± 40	28.0	77.8

Note. Old/new = newly formed shoots. W, wetland; U, upland.

It turns out that with an increasing CGS percentage the number of growing plants declines while the number of newly formed shoots increases. In PFA the plants show hardly any growth at all, but the number of newly formed shoots surpasses that of the CGS substrates and potting soil. This could signify that when plant growth is inhibited due to an unfavourable environment, remaining energy will be used to develop new shoots. The average plant length does not show any clear differences between the various substrates. Only with CGS + NPK the longitudinal growth is better as compared with 100% CGS, but then deviation is rather great. For the determination of average length only the plants originally placed are taken, while the newly formed shoots are not taken into account because it is difficult to establish their exact exposure time. With potting soil and the CGS substrates growth is better in the wetland conditions, with the exception of CGS + NPK, which shows equal longitudinal growth in both situations. There is little to be said about longitudinal growth with the PFA substrate on account of the small number of plants growing.

Both per pot and per separate plant fresh- and dry weights (Table 1) decrease with increasing CGS addition. The differences in growth can be demonstrated only in the wetland situation, where growth is clearly better than under upland conditions. The addition of nutrients to 100% CGS results in a significant improvement of growth, especially in the upland situation. The yield of PFA substrate is extremely low: in spite of the addition of nutrients hardly any growth occurs here. When more CGS is added to the potting soil, the plants assume a lighter and more faded colour. This may be indicative of a lack of nutrients, since with CGS + NPK the plants have a much darker and more robust colour.

Eisenia fetida

Table 2 contains the data with respect to the biomass of the worms. It shows that the treatments with CGS and PFA cause a clear weight loss. Only in potting soil is there a noticeable weight increase. With potting soil there is no mortality; with 50, 75 and 100% CGS mortality is 6.7, 2.7 and 9.3 % respectively, while it is 32% with PFA.

Table 3 represents the element concentrations of 100% CGS (Holten, Texaco process) and 100% PFA (Amer power station). These concentrations were taken as a basis for the CGS / potting soil mixtures so as to calculate the concentrations in the substrate. This was based on the, probably incorrect, assumption that the concentrations of heavy metals in potting soil were negligible.

The C content in the used CGS is relatively high which in turn means a relatively low SiO_2 content in CGS. With the micro elements the concentrations of As, Mo, Pb and Se are 2 to 4 times higher in PFA, while the Cu and Zn concentrations are nearly equal and those of B, Cr, Ni and Sb are 1.5 to 3 times higher in CGS.

Table 2. Biomass of *E. fetida* per cylinder, at the start of the experiment and after 7 weeks of exposure in various substrates.

Substrate	Fresh weight (g) Start	Fresh weight (g) End	Fresh weight (g) Purged	Weight increase (end–start) (%)	Mortality (%)
Potting soil	5.67 ± 0.83	6.87 ± 1.00	6.18 ± 0.92	+21.1 ± 0.2	0
CGS					
50%	3.81 ± 0.39	1.85 ± 0.09	1.88 ± 0.23	−51.1 ± 3.1	6.7
75%	5.30 ± 0.08	3.00 ± 0.13	3.04 ± 0.11	−43.3 ± 1.6	2.7
100%	5.32 ± 0.20	2.54 ± 0.33	2.74 ± 0.29	−52.2 ± 4.3	9.3
PFA	3.55 ± 0.25	0.94 ± 0.27	0.94 ± 0.29	−73.6 ± 6.1	32

The metal concentrations in *C. esculentus* are indicated in Table 4. In the CGS-containing substrates the concentrations measured for the elements As, Pb, Sb and Se are nearly all below the detection limit. Low concentrations were measured only in 100% CGS for Sb (wetland) and in CGS+NPK for As (wetland and upland) and for Sb (wetland). For Pb the concentrations in PFA are also below the detection limit, while with these substrates the As and Se concentrations in the plants are slightly higher.

The As and Se concentrations are also higher in the substrate PFA in relation to CGS (Table 3). The uptake of these metals appears to be better in the wetland situation, but the number of observations is too small to demonstrate this conclusively. Only with PFA there is a significant difference in As uptake between the wetland and upland conditions. In *C. esculentus* Cr is accumulated in PFA substrate, however, Cr concentration in PFA is lower than in CGS. In the CGS substrates there is an accumulation of B, with the B concentration gradually decreasing as the CGS percentage rises.

Table 3. Element concentrations in CGS and PFA (dry-weight).

ELEMENT CONCENTRATIONS IN CGS AND PFA (DRY WEIGHT)

Macro (%)			Micro ($\mu g \cdot g^{-1}$)		
Element	CGS	PFA	Element	CGS	PFA
SiO_2	29.4	55.3	As	26	82.3
Al_2O_3	19.5	24.2	B	184	113
Fe_2O_3	8.6	8.7	Cr	280	190
CaO	4.6	3.1	Cu	255	268
MgO	—	1.4	Mo	17	29.3
Na_2O	0.8	0.2	Ni	539	187
K_2O	1.8	2.7	Pb	76	142
TiO_2	0.9	1.2	Sb	24	16.4
SO_3	1.6	—	Se	5.1	18.7
C	30.8	3.8	Zn	228	276

Extremely high B concentrations were measured in *C. esculentus* in CGS and PFA, while the B concentration in PFA is lower than in CGS. Unlike the CGS substrates, the PFA substrates show a higher B uptake in the upland situation.

The Mo concentration in *C. esculentus* increases gradually with the CGS percentage. In all substrates the uptake is higher under wetland conditions, but with 75 and 100% CGS this difference is not significant. High Mo concentrations are found in the plants from PFA. These plants contain concentrations up to 40 or 50 times higher than those in the plants from CGS, while the concentration in the PFA substrate is only twice as high as in CGS.

The concentrations of the cations Cu, Ni, and Zn in *C. esculentus* increase gradually with the CGS concentration. In the plants from PFA the Cu and Zn concentration are of the same order, while the Ni concentration is considerably lower. This corresponds with the metal concentrations in the substrates.

Table 4. Metal element concentrations in *C. esculentus* (µg/g DW) after 71 days of exposure in various substrates. W= Wetland; U= Upland.

Substrate	As	B	Cr	Cu	Mo	Ni	Pb	Sb	Se	Zn
Potting soil										
W	<1.2	13.5 ± 0.34	0.82 ± 0.62	2.6 ± 0.4	3.5 ± 1.0	1.7 ± 0.2	<0.8	<1.5	<1.5	31 ± 4
U	<1.2	14.9 ± 2.2	0.86 ± 0.49	2.8 ± 0.3	4.2 ± 1.1	7.0 ± 7.7	1.0 ± 0.4	<1.5	<1.6	32 ± 5
CGS 50%										
W	<1.2	84.2 ± 10.7	0.56 ± 0.25	3.1 ± 0.8	7.3 ± 3.3	1.7 ± 0.2	<0.8	<1.5	<1.5	24 ± 2
U	<1.3	56.8 ± 10.0	0.21 ± 0.16	3.4 ± 0.5	6.5 ± 1.4	1.3 ± 0.5	<0.9	<1.5	<1.6	27 ± 6
CGS 75%										
W	<1.2	54.2 ± 7.9	0.61 ± 0.41	4.3 ± 0.3	8.6 ± 1.5	2.1 ± 0.2	<0.8	<1.5	<1.5	41 ± 7
U	<1.2	31.0 ± 4.0	0.39 ± 0.29	4.3 ± 0.1	3.8 ± 0.3	2.1 ± 0.4	<0.8	<1.5	<1.5	33 ± 1
CGS 100%										
W	<1.6	46.0 ± 10.5	0.43 ± 0.40	8.3 ± 0.7	15.7 ± 1.9	5.5 ± 0.1	<1.1	2.3 ± 0.7	<2.0	50 ± 2
U	<3.1	36.1 ± 1.2	0.95 ± 0.55	8.5 ± 0.9	10.9 ± 3.3	5.4 ± 0.3	<2.1	<3.1	<3.9	51 ± 3
CGS + NPK										
W	2.7 ± 0.6	68.5 ± 6.2	0.37 ± 0.25	10.2 ± 0.6	10.4 ± 0.4	9.7 ± 4.7	<0.8	2.1 ± 0.1	<1.4	38 ± 1
U	3.6 ± 1.0	29.2 ± 11.7	2.08 ± 0.99	9.0 ± 0.7	9.4 ± 9.3	8.3 ± 0.4	<0.8	<1.5	<1.5	32 ± 4
PFA + NPK										
W	14.9 ± 4.3	268 ± 57	19.53 ± 5.1	7.2 ± 0.7	526 ± 148	1.9 ± 0.2	<2.7	2.7 ± 1.6	8.3 ± 2.8	46 ± 14
U	6.9 ± 4.4	335 ± 40	36.64 ± 30.0	5.7 ± 1.7	388 ± 241	0.8 ± 0.5	<3.2	<4.8	7.0 ± 2.5	48 ± 19

Note. W, wetland; U, upland.

The concentration factor (CF = concentration in *C. esculentus*/concentration in the substrate) is indicated in Table 5.

The CF is influenced by the metal concentration in the substrate. In the substrates with an increasing percentage of CGS this is clearly to be seen for B: the CF declines with a rising percentage of CGS and therefore with an increasing B concentration in the substrate. For the remaining elements the CF is nearly constant at the various CGS percentages.

Table 5 shows that the CF of the cations Cu, Ni, Pb, and Zn is in the same low order for all substrates. For B and Cr the CF is highest, but for the CGS substrates they remain below 1. Only for B and Mo the CF determined is greater than 1 in PFA substrates. The CF for B and Mo in these substrates lies between 1 - 3 and 5 - 18, respectively.

Table 5. Concentration factor of the elements in *C. esculentus* in the various substrates, in which the range is indicated for the CGS substrates. W= Wetland; U= Upland.

	CGS		PFA	
Element	W	U	W	U
As	0.10	0.1	0.2	0.1
B	0.3–0.9	0.2–0.6	2.37	2.97
Cr	<0.01	<0.01	0.10	0.19
Cu	0.02–0.04	0.02–0.04	0.03	0.02
Mo	0.6–0.9	0.30–0.8	18	13
Ni	0.01–0.02	0.01–0.02	0.01	<0.01
Pb	—	—	—	—
Sb	0.09–0.1	—	0.17	—
Se	—	—	0.4	0.4
Zn	0.2	0.1–0.2	0.2	0.2

Note. W, wetland; U, upland.

The element concentrations in *E. fetida* are shown in Table 6.

Table 6. Metal concentrations in (μg/g DW) after 7 weeks of exposure in various substrates.

	Potting soil	50% CGS	75% CGS	100% CGS	PFA
As	3.1 ± 0.1	24.8 ± 2.1	14.2 ± 0.9	9.5 ± 1.4	100 ± 7
B	<10	<10	<10	<10	14.0 ± 5.3
Cr	2.4 ± 2.3	9.6 ± 13.9	1.8 ± 2.1	1.7 ± 0.7	7.2 ± 1.9
Cu	8.0 ± 1.0	8.0 ± 0.7	7.4 ± 1.0	9.9 ± 1.8	16.4 ± 2.5
Mo	2.2 ± 0.3	2.2 ± 1.1	3.0 ± 0.9	1.9 ± 0.9	<2.7
Ni	5.7 ± 1.0	3.1 ± 2.4	1.2 ± 0.4	3.6 ± 0.9	2.1 ± 1.1
Pb	3.8 ± 0.8	2.5 ± 0.1	1.4 ± 0.8	3.6 ± 3.1	3.3 ± 0.3
Sb	<1.6	<1.6	<1.6	<1.6	<3.5
Se	<2.0	<2.1	<2.0	<2.1	9.2 ± 0.2
Zn	117 ± 4	124 ± 2	127 ± 4	140 ± 2	115 ± 2

With the CGS substrates only a reduced concentration of As and, to a lesser extent, Zn can be shown in the worms. The As concentration decreases with the CGS percentage. In PFA the As concentration is about 10 times higher than in 100% CGS.

Table 7 shows the concentration factors of the elements in the various substrates.

With the CGS substrates the CF is highest for the elements As and Zn and it declines with an increasing CGS percentage. In PFA the CF of As is considerably higher than in 100% CGS.

Table 7. *Concentration factors of the elements in E. fetida.*

	CGS			
Element	50%	75%	100%	PFA
As	1.9	0.7	0.4	1.2
B	—	—	—	0.1
Cr	0.07	0.01	0.01	0.04
Cu	0.06	0.04	0.04	0.06
Mo	0.3	0.2	0.1	—
Ni	0.01	0.01	0.01	0.01
Pb	0.07	0.03	0.05	0.02
Sb	—	—	—	—
Se	—	—	—	0.5
Zn	1.1	0.7	0.6	0.4

DISCUSSION

The growth of *C. esculentus* declined with the CGS percentage added. In 100% PFA growth was extremely poor. A remarkable feature was that the poorer the growth, the more new shoots were formed. This is possibly a response of the plant to survive under poor circumstances. It is well known that *C. esculentus* produce tubercles when the days are short, so that new plants can originate when the days become longer (Doty & Sweet 1970).

The addition of nutrients to CGS results in a significant growth improvement: the plants have a darker and robuster colour. The poor growth on CGS is probably caused by a lack of nutrients. Marquenie *et al.* (1988) also found an extremely poor growth and they did not attribute this to toxicity but to insufficient availability of nutrients. The results of this study, however, show that despite the addition of N-P-K growth on PFA remains poor. Hence, this is not caused by a lack of nutrients, but probably by certain physical or chemical properties, such as soil structure, which is highly dense for PFA, and there may also be an effect from excessive B concentrations. B levels in soil above 75 µg/g (dry weight) are considered to be phytotoxic (Chaney 1983). Francis *et al.* (1985) found a maximum B concentration of 61 µg/g (dry weight) in a CGS study with slag from pilot plants with fluidized-, fixed- and entrained bed-type gasifiers.

With *E. fetida* there was a clear effect of the substrate on the weight and mortality of the worms. Unlike potting soil, the CGS and PFA substrates caused a weight loss and mortality occurred as well, particularly in PFA.

In the course of the experiments the pH value of the substrates hardly showed any change at all. The pH value of potting soil was $\pm$ 6; with a rising CGS percentage the pH value increased as well and at 100% CGS it was approx. 8. The PFA used was a basic fly ash with a pH value of 10 - 12. The pH value of the CGS substrates and PFA was not modified at the outset of the experiment, as was the case in the experiments of Marquenie *et al.* (1988) with acetic acid and $CaCO_3$ respectively. The pH value mainly influences metal adsorption on organic matter and therefore also biological availability; e.g. the concentrations of cations turn out to be higher at lower pH values (Van Gestel & van Dis 1988). Hence, modification of the pH value of the CGS substrates and PFA would give a false indication of the metal availability.

The rH values show that no reducing (anaerobic) circumstances have occurred in any of the substrates, even though this was in fact expected. The pH value and the redox potential served as a basis to analyse by means of Pourbaix diagrams in what form the elements can theoretically occur in the (pore) fluid of the substrates; however, mutual interactions and mobility between the elements was not taken into account. Of the anions As appears to occur in all substrates in the form of arsenate (H_2AsO_4 / $HAsO_4^{2-}$). The free arsenate ion shows good biological availability, while biological availability of the fractions bound to Al, Fe and Mo oxides and the Al, Ca and Fe precipitates is poor. The highly mobile element B appears to occur as $B(OH)_3$. Especially with low pH values boron occurs in a form that is weakly bound in the soil, i.e. $B(OH)_3$, and its biological availability is then good. Under alkaline conditions borate $B(OH)_4^-$ is dominant and adsorption should be greater while biological availability should be smaller; nevertheless, this was not found for CGS and PFA with respective pH values of 8 and 10 and initial concentrations of 184 and 113 µg/g. Cr occurs in natural soils as trivalent Cr and its biological availability is poor due to strong fixation in the soil. The hexavalent form is found only in extremely oxygenous conditions. Mo occurs in the substrates as $HMoO_4^-$ / MoO_4^{2-}. Biological availability is good for both forms, but it may decline due to binding on Al and Fe oxides. In alkaline, wet soils the availability of Mo is good. Uptake of Sb in the form of SbO_3^- may be poor and SbO_3^- is mainly bound on Al, Fe, and Mn oxides. Sb shows a rather diverse speciation pattern: with potting soil as Se/$HSeO_3^-$ (low redox), in 50% CGS and PFA as SeO_3^{2-} /SeO_4^{2-} and in the remaining CGS substrates as SeO_4^{2-} (high redox).

In a terrestrial environment the uptake of selenate by organisms is better than that of selenite, since selenite is fixed in the soil better (low mobility). The cations Cu, Ni, Pb, and Zn occur mainly as bivalent ions; only with high pH values can they be found in the form of metal oxides or hydroxides.

In the CGS substrates the uptake of a number of elements is clearly different for *C. esculentus* and *E. fetida*. In *C. esculentus* accumulation of B and Mo occurs, while in *E. fetida* there is hardly any uptake of these elements. On the other hand, in the worm *E. fetida* uptake of As (CF >1) occurs and the Cr, Pb and Zn concentrations exceed those in the plant. The worms can contain high background concentrations of, e.g. Pb and Zn and are therefore poor indicators for these elements (Stafford & Edwards 1985; Beyer & Cromartie 1987).

The comparison between 100% CGS and 100% PFA shows that for the uptake of As, Cr, Cu and--to a slightly lesser extent--Se is higher in PFA. In *C. esculentus* the B, Mo, and Cr concentrations especially are far higher (10 to 50 times) and the As and Se concentrations that are slightly higher with PFA than with CGS. For both kinds of organisms there are clear differences in the uptake of anionic metals, the concentrations of which are generally higher in PFA than in CGS. These differences cannot be accounted for by the metal concentration in the substrate. The As, Mo and Se concentrations are higher in PFA, whereas those of Cr and B are lower. These differences in uptake are caused by differences in biological availability of the metals. In CGS the biological availability of the metals is poorer, since the coal gasification process produces a highly glassified matrix from which the elements are released rather more poorly. During combustion anions such as Se and As condensate on the route of the flue gases on the outer side of the PFA particles and thus mobility is higher.

The results concerning *C. esculentus* are in good agreement with those found by Marquenie *et al.* (1988). The higher As uptake in our experiment is likely to be caused by the higher pH value in the substrates, since arsenate has a high mobility in alkaline soils. Cr generally has a very poor biological availability and is freely mobile in oxygenic circumstances only. Whether these circumstances were more favorable in the experiment is rather difficult to verify, as Marquenie did not check them. For *E. fetida* Marquenie *et al.* (1988) found a higher CF for cations and Se (3 to 10 times) compared with our data.

Research into the availability of cations to worms by Morgan and Morgan (1988), Ireland (1979), Stafford and Edwards (1985) demonstrates that the tissue concentrations of Cu and Zn are regulated and that the CF of most anions is less than 1, except that of Cd. The results of the study show that with *C. esculentus* and *E. fetida* the accumulation of elements from CGS hardly differs from that of potting soil, but that great differences do indeed occur in comparison with PFA.

CONCLUSIONS

Three important conclusions may be drawn from the results presented.

- First, as was anticipated, growth inhibition occurs for *C. esculentus* and *E. fetida* in CGS and PFA substrates. The growth inhibition is caused by shortage of nutrients and not by toxic components.
- Second, accumulation of B and Mo has found only in *C. esculentus*, while in *E. fetida* there only is accumulation of As.
- Third, the availability of the cations turns out to be less than that of the anions, while the anions from PFA show greater availability than those from CGS.

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CONCLUDING REMARKS TO PART THREE

In a toxicity study for testing chemicals by Kenaga and Molenaar (1979) a higher sensitivity of animals was found compared to algae and plants, which lead to the conclusion that standards for animals also would protect plant life. According to Wang (1983) this conclusion is only partly correct because phytotoxicity tests are not well developed. Rowe *et al.* (1982) and Lewis (1995) also indicated the need for further development of plant testing for aquatic toxicity studies for the completion of existing tests with animals. Even flow-through systems have been developed for duckweed testing (Walbridge 1977; Bishop 1981). The conclusion that standards for animals will do the job in protecting nature seems short-sighted. Duckweed *Lemna minor* proved to be a suitable plant for phytomonitoring purposes. Surprisingly the *surface cover parameter by image processing showed to be far more sensitive in comparison* with the Multiplication Rate parameter. Depending on the tested elements it proved to be 15 - 20 times more sensitive.

The leaching procedure for PFA, low NOx PFA and Coal Gasification Slag (CGS) used in the *Lemna* experiments (chapter 11 and 12) was a modified EPA leaching test (U.S. EPA 1980), which has the advantage of needing only 24 hours stirring at a constant pH. Nitric acid was used instead of acetic acid which proved to be highly toxic for *Lemna*. Results of the triplicate leaching tests, final L/S 60, show that 38% of Se, 21% of As and 22% of Mo was leached out. The leaching of cations was less than 1%. It is known that leaching of As and Se increases at a higher pH, which was tested for Low NOx PFA and CGS at L/S 10, 20 and 40. Most anionic elements leach out far better at a higher pH, *e.g.* Se and Mo leach between 30 - 60% at L/S 10, decreasing with higher L/S ratios. In contrast, the highest leaching of As (36%) was found at pH 5 with a L/S of 40.

Acute toxicity is directly related to the concentration of free metal ions. Metal complex ations are less easily adsorbed by organisms. A complexing agent like EDTA is necessary for testing under static laboratory conditions. The growth medium was adjusted to pH 5 and sterilized, including 2,5 μM of Fe-EDTA. The toxicity (EC_{50}) of cations and anions at EDTA concentrations of 2,5 and 10 μM declines with increasing EDTA concentration. For the essential elements (Cu, Zn, and Se) the decrease is caused by a shift in the concentration *between deficiency and toxicity. For the non-essential elements (Cd and As), this is due to* a decrease of the growth inhibition. A 4 times increase of the EDTA concentration (2,5 to 10 μM) also increases the EC_{50} by a factor of 4.

The toxicity of single elements tested with *L.minor* can be divided into three groups:

- the cations Cd, Cu, and Zn with an EC_{50} of <0.5 mg/l
- the anions As(III) and Se(IV) with EC_{50} of 0.5 - 5 mg/l
- the anions AS(V) and Se(VI) with EC_{50} of >5 mg/l

Accumulation of the cations is clearly higher compared to the anions with the exception of As(III), which is accumulated in relatively high concentrations. This result demonstrates the importance of speciation analyses (chapter 11). It is still not evident which species of As occur in PFA. The toxicity of As(III) is about 15 times more toxic (EC_{50}) than As(V) for although, this latter species is accumulated to higher concentrations (factor 1.5). Results of the toxicity experiments of all elements are summarised in Figure 1A and 1B. A clear difference exist in concentration factor (CF) between cationics and anionics with the exception of As (III) with a CF >200. This leads to the conclusion that the cationics and As(III) are accumulated in higher concentrations which is expressed in Figure 1B by the EC_{50} for the latter elements.

For assessing the toxicity of the different PFAs in a broader framework, sediments from the Apeldoornsch Kanaal, the River Rhine and Rotterdam harbour were also tested with *L. minor*. This resulted in an unexpectedly high toxicity of Rhine and Apeldoornsch Kanaal sediment. The effects at the latter sediment leachates can be ascribed to really high Zn concentrations, which were above 4000 μg/l. The accumulated Zn concentration in *L. minor* was more than 350 μg/g and the EC_{50} for accumulated Zn is 300 μg/g which corresponds fairly well.

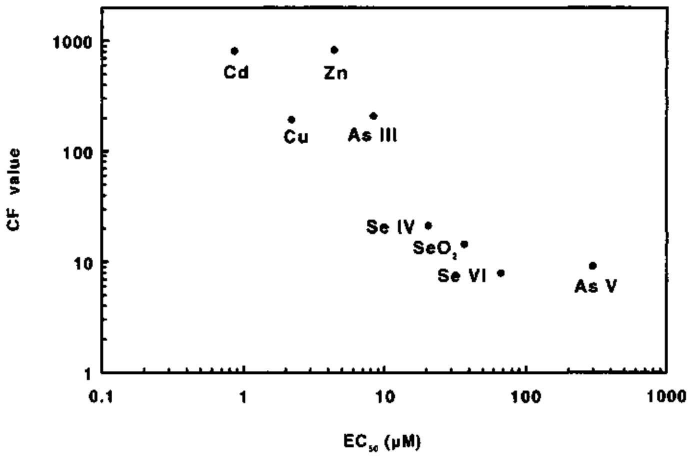

Figure1A. Concentration factor (CF = ratio of element concentration in *L. minor* versus concentration in the growth medium at day 0) expressed against the EC_{50} values (log/log scale).

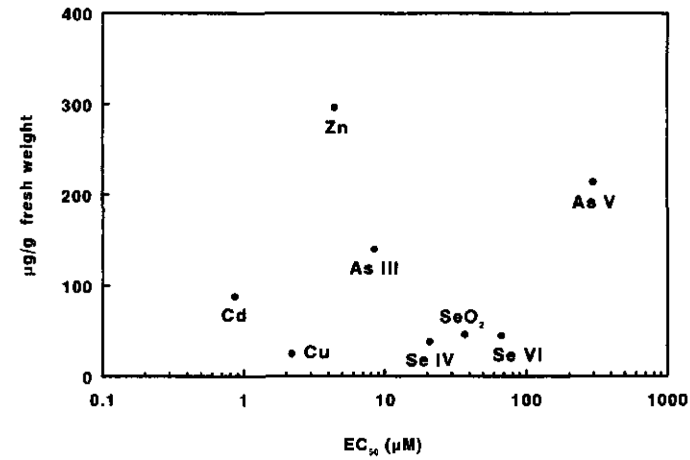

Figure 1B. Accumulated element concentration against the EC_{50} values (single log scale).

In our leaching experiments with CGS (Drayton slag) element concentrations were generally low, only at a L/S of 10 some Ni (5 µg/l) and Zn (<7 µg/l) was found. Toxicity experiments with *L. minor* were omitted due to the low element concentrations in the leachates.

In Chapter 13, laboratory experiments with the plant *Cyperus esculentus* and the worm *Eisenia fetida* are described, which were carried out with conventional PFA (Amer 8 batch) and CGS (Texaco process). PFA was used as a reference substrate and both PFA and CGS were tested in wetland and upland conditions. Both in substrate and in plant tissues concentrations of the cations Cu, Ni, Pb, and Zn and the anions As, B, Cr, Mo, Sb, and Se were determined. The availability of anions for *C. esculentus* and *E. fetida* is greater in PFA than in CGS. The extent and the rate of uptake in the plants is generally higher in the wetland situation. With *C. esculentus* a CF >1 was found for B and Mo and in *E. fetida* a CF >1 was found only for As in PFA. Mortality of *E. fetida* was high in PFA (32%). General conclusion of this chapter is that the used CGS hardly shows any serious potential toxicity.

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SUMMARY AND CONCLUSIONS

This thesis describes the consequences of the disposal of the combustion residues of coal, especially the uptake of elements from such residues and their effects on various organisms. The effects on benthic organisms in fresh and in seawater are considered in the first two parts. The third part looks at the uptake of elements from coal residues and their effect on the growth of plants and worms.

The central theme is the combustion residue known as pulverized fuel ash (PFA), or 'flyash'. Coal is a product of natural origin that was formed from plant remains in the period between 255 and 203 million years ago. During the coal forming process the elements from plants become 100 to 1000 times more concentrated. In all probability, further concentration occurred through airborne deposition and the salts from sea and freshwater. The elements present will be concentrated a further 10 times during combustion in a boiler. This means that following combustion, the unburned elements are re-released to the environment, whether or not they are bound to flyash particles. Some elements are released directly in the gaseous phase, other elements condense on the surfaces of the flyash spheres and another fraction is bound up in the matrix of these particles. The question is whether an adequate estimate can be made, from the ecotoxicological point of view, as to what effect on the environment PFA will have when used in unbound applications.

The elements in PFA can be divided into macro-elements which occur in relatively high concentrations (g/kg) and trace elements which occur at concentrations of less than 1 mg/kg. It is largely these trace elements which reach the environment through leaching from PFA and accumulate in organisms. In The Netherlands, however, discharge of PFA in unbound form (not as cement/concrete 'stabilized' products) is not a concern at the moment. It may possibly be (re)considered as an option in the future, at which point in time, knowledge of possible environmental effects will be essential. At present, nearly all PFA is used by the cement industry. Additionally, a small quantity is used in pellet form as artificial gravel in the concrete industry.

The anion forming elements are especially important from the point of view of environmental protection. In order of leaching potential, these elements are in order of highest to lowest: selenium > molybdenum >> wolfram > vanadium > antimony > arsenic > chrome. Accumulation of these elements by organisms in excessive concentrations can lead to undesirable effects. Such effects can be increased (biomagnification) in organisms which are higher in the food-chain.

Part 1

In the first part of this thesis research in the marine environment is described with organisms which are indigenous to intertidal sedimentary biotopes, i.e. the lugworm *Arenicola marina*, the ragworm *Nereis virens*, the baltic tellin *Macoma balthica* and the cockle *Cerastoderma edule*. The accumulation of such elements is described in the above species and their survival during a 90-days exposure to 100% PFA and a 50% PFA mixture with sand. A sand control and a reference of moderately contaminated harbour dredged sediment from Rotterdam harbour were also used. In addition, the effect of a daily dose of PFA was examined in an effort to simulate regular disposal. At the end of this period, the organisms were sampled and the elements zinc, arsenic, chrome copper, nickel, cadmium antimony and selenium were determined in animal tissue and sediment. In the ragworm experiment, young worms were used to examine the colonisation of disposed PFA. Moreover, the effects of a 230-days exposure to a PFA substrate is described in the cockle. Alteration in the reproductive processes, tissues and organs was assessed in these animals. Parasites were also examined, as organisms living in sub-optimal conditions are often more strongly parasitised.

Results

The concentration of the elements measured in the PFA substrates appeared to have been reduced by 10% during the test period. However, it was clear that the arsenic and selenium concentrations had not been reduced to the extent expected (<5% only), as it is known that both elements are easily leached. The accumulation patterns in the lugworm demonstrate that arsenic in particular is accumulated (a factor of 15 in 50% PFA / 50% sand and a factor of 5 in 100% PFA on basis of tissue concentrations). This is probably caused by the binding of arsenic to sediment particles which are eaten by the lugworm in its sedimentary diet. Accumulation in the cockle was only a factor of 2 above the sedimentary levels. The small accumulation levels in the cockles from the 230-days experiment were unexpected. The accumulation factor for arsenic was a factor of 3 in the baltic tellin. The concentration of zinc in the baltic tellin was remarkably high in animals exposed to all sediment types, including the control, at >550 mg/kg (dry weight). However, these concentrations do not appear to be abnormal and existed at the start of the experiments. From this it can be seen that the 'lifestyle' of the organisms used in bioassays can strongly determine the accumulation of the anionic elements. Where the cationic elements copper, chrome and nickel are concerned, no unexpected accumulation patterns were found; only zinc was accumulated. Several elements were analyzed after 4, 8 and 12 weeks exposure in the ragworm experiment. Here also, the arsenic concentration increased up to week 8 (from 23 to 76 µg/g in 100% PFA, on basis of tissue concentrations), after which a decrease was observed after12 weeks. It is interesting to note that in the ragworm, selenium was slowly but steadily accumulated and no equilibrium was reached after 12 weeks exposure. The copper concentration in ragworms increased by a factor of three. The other cation forming elements zinc, chrome and nickel did not however accumulate.

With the lugworm, a high mortality was found in all substrates, i.e. 96% in the 100% PFA and 55.5% in the control. The lugworm appears sensitive to transplantation and 100% PFA is absolutely unacceptable as a substrate in which to burrow. By contrast, the baltic tellin showed a low mortality (20% in 100% PFA) and a wild population existed in the substrate, originating from the Wadden Sea. Cockle mortality reached 40% in the 100% PFA exposure and 15% in the control. Unexpectedly, only slight microscopic changes in the reproductive tissues were observed. In the experiment with the ragworms young animals with an average weight of 200 mg (ca. 2 cm in length) were used and an appreciable mortality of 50 - 60% was seen in the first weeks of the experiment. This mortality was to be expected as the delicate young animals were placed in the tanks by hand at the start of the experiment.

Conclusions

The general conclusion concerning the leaching of elements from PFA and subsequent uptake in the benthic fauna is that a species dependent differential situation occurs. Arsenic and selenium are the most prominent elements. A second conclusion is that PFA caused a physical effect which was reflected in an altered behaviour of the organisms examined. Even in a substrate of 50% PFA / 50% sand after 60 days none of the burried worms came to the surface, indicating severe stress. The consequences of this are that, a radical change in the species composition of the benthic fauna will take place upon dumping PFA in the marine environment and recovery will take years. A lesser sedimentation through deposition over larger areas will reduce the mortality of species or populations, but effects on species composition will still occur.

Part 2

The experiments described in Part 2 were carried out in flow chambers with the painters mussel *Unio pictorum*. This mussel species was chosen because it crawls freely in the upper layers of sediment unlike other sessile bivalve species such as the Zebra mussel (*Dreissena polymorpha*). The painters mussel moves by sticking out their foot, which then changes shape to form an anchor with which the mussel then pulls itself along. The mussels are rather active in summer and remain permanently open, but during the winter, dig themselves in.

The growth of mussels in PFA substrates was compared with data from Rhine sediment. Additionally, growth was compared with tagged mussels which were followed for several years in the River Linge, a tributary of the Rhine. Behaviour was examined with a laboratory version of the Mussel Monitor®, where activity was measured as the opening and closing actions of the valves. In a simultaneous research project at the University of Utrecht with a similar mussel species in aquaria without any substrate, the mussels remained closed and only opened for short periods. The specific question examined during these experiments was whether the mussels showed an abnormal behavioural pattern in PFA substrates and did this influence the accumulation of metals.

The accumulation of the metal cadmium is examined in a separate study. Although cadmium only occurs in very small amounts in PFA, it was none the less chosen for this accumulation experiment, because much experience had already been gained with cadmium in laboratory experiments at the University of Utrecht. The expectation was that accumulation would be considerably different in the laboratory than in semi-field experiments due to the large difference in opening / closure behaviour.

The accumulation of selenium and the occurrence of effects on the development of eggs in *U. pictorum* was examined. Arsenic and selenium leach readily from PFA, whereby arsenic can be strongly accumulated in contrast to selenium. Selenium (Se) is taken up only very slowly, but bioaccumulation and biomagnification does occur. Selenium is concentrated in proteins and especially in the developing oocytes which form a target organ. The literature indicates that some fish species in selenium rich waters in California die out at concentrations of 15 µg/l Se. An important fact is that Se can occur as different forms or 'species', *e.g.* selenite [Se(IV)], selenate [Se (VI)] and organo-selenium compounds. It is usually the Se (IV) form which occurs in PFA leachates, *i.e.* this species which accumulates more rapidly than Se (VI). The accumulation of Se was examined in three experiments with dosed Se (IV).

Results

The growth of mussels in PFA was somewhat retarded when compared to growth in river sediment (Rhine and Linge). The mussels had initial difficulties in burrowing into the PFA and were only comparable with the Rhine situation after 5 days. The individual mussels nearly always stood open and showed a high level of activity interspersed with short periods when the valves were closed.

In the cadmium experiment, mussels were exposed to four separate dosages of 50 ug/l. The accumulation of cadmium appeared to progress rapidly, and a plateau was reached after 3 weeks with kidney concentrations of *ca.* 400 µg/g (freeze dried weight); which means a bioconcentration factor (BCF) of 8000. No elimination of cadmium was observed during a consecutive depuration period of 29 weeks. The lack of any difference in accumulation in the presence or absence of a substrate was unexpected.

At the selenium experiments the effect of enriching PFA and Rhine sediment with selenium was examined. The highest concentrations were observed in animals exposed to the enriched Rhine sediment. Mussels were continuously exposed for 11 weeks to 50 µg/l of Se (IV) in order to examine the possible effect on the development of oocytes and embryos. Selenium accumulation had not reached a steady state after 11 weeks of exposure. Differences (not significant) were found in the synchronisation of glochidia production (larvae on the gills of female mussels) as well as in the average size of the oocytes at the end of the exposure period. Following exposures of 22 and 11 weeks to concentrations of 50 and 250 µg/l, an accumulation of 5 and 15 µg/g was observed, with BCFs of 100 and 60 respectively (freeze dried weight). The most unexpected result was that no differences were observed in the numbers of glochidia and that no deformations were found. This implies that no demonstrable (significant) effects were found in the chosen experimental setup. Se resembles sulphur in uptake behaviour and is built in proteins (eggs). The element is an essential nurient, but can be toxic at slightly higher concentrations than ambient. On the basis of literature data for the development of eggs in a perch species during a 32 week experiment, the expectation was that effects would indeed be found.

Conclusions

The accumulation of cadmium in *U. pictorum* occurred rapidly. The kidneys formed the the main target organ and a steady state is apparently reached after three weeks of exposure. Elimination of cadmium hardly occurr over a period of 28 weeks whithout dosing. No mortality was found during the exposure and during the elimination period. An important finding is that the presence or absence of substrate, which has a clear effect on behaviour (valve movement), did not influence the uptake of cadmium.

One apparent effect of selenium on oocytes, embryos and larval development is that the timing of the appearance of larvae in the gills of the females is altered. The consequence of this is that the mussels retain the larvae longer on the gills and release them later. Such longer retention might have consequences for the life cycle of this species through a delay in the settlement on fish and the ditribution of the larvae.

Main conclusion from this section is that PFA has a marked influence on the quality as a sediment by physical changes leading to abnormal behaviour and secondly through the leaching of elements.

Part 3

Part 3 is concerned with research carried out with plants and earthworms. Phytomonitoring as illustrated here with duckweed, is still a rather uncommon monitoring technique but admirably suited to the purpose of testing leachates from PFA. Experiments with the duckweed *Lemna minor* are described where the effects of conventional PFA, PFA from low NOx burners, bottom ash and coal gasification ash were examined. In order to be able to compare the observed effects properly, sediments from a canal and a river were sampled and tested also. At the same time different methods of producing leacheates were examined. A distinction is made between natural leacheates and artificially produced leacheates, where element concentrations were in agreement with the 'cascade' leachate technique (successive leaching of 5 times). An image analysis technique was developed to measure the growth of duckweed in a simple and effective manner, in the various experiments. The effects of coal gasification slag (a type of bottom ash) were also examined. In the coal gasification process, the flammable gaseous components are first removed from the powdered coal with the aid of steam under high pressure and reducing conditions. The gas mixture thus produced is burned by conventional means. During this process, only a relatively small fraction of flyash is produced and is removed. The bulk of the residue is formed by coal gasification slag (CG slag) which remains after the gasification process. The growth of yellow nut sedge (*Cyperus esculentus)* and the elements which accumulate in the plant were examined in the laboratory. The effects on the earthworm *Eisenia fetida* were examined in a similar manner.

Results

Duckweed appeared to be very suitable organism to monitor leacheates from PFA. It appears from the experiments that the anion forming elements cause the effects in the conventional PFA and the low NOx leacheates, while the cations cause the effects in the sediments from the Apeldoornsch Kanaal and the Rhine. The toxicity of PFA is considerably lower when compared to that of the Apeldoornsch Kanaal. This difference is mainly caused by zinc (>4000 μg/l) which occurs in the leachate from the Apeldoornsch Kanaal; the 'no effect concentration' for duckweed is about 160 μg/l.

The order of toxicity for elements such as those found in separately executed tests with duckweed was: cadmium >copper >zinc >arsenic (III) >selenium (IV) >boron > molybdenum.

The leaching of elements from coal gasification slag appeared to be minimal. The growth of the yellow nut sedge decreases with increasing concentration of coal gasification slag through lack of nutrients and not as a result of the toxic elements from CG slag. Addition of nutrients resulted in a marked improvement in growth. Growth in PFA was equally retarded, in this case because the fresh PFA contained boron which shows growth effects on plants above 60 µg/g. Accumulation was found to be restricted to boron and molybdenum. The physical effect of compaction of the PFA, through which the substrate becomes so hard as to prevent proper root growth, should also be considered as a serious effect. A clearly negative effect on the growth and mortality of the worm *E. fetida* was found in PFA and CG slag. The mortality was especially high (32%) in PFA; growth was only found in potting soil. Accumulation was only observed for arsenic. The availability of the anion forming elements from PFA was greater that from CG slag.

Conclusions

The contention that PFA in general should be evaluated as a chemical waste needs to be reconsidered in the light of the results presented here. At present, The Government of The Netherlands is considering a revision of this standpoint, through which PFA will be placed in a category with raw materials. The effects of conventional and low NOx PFA are related to the anion forming elements. The leaching of elements from CG slag appears to be very small. The sediments from the Apeldoornsch Kanaal show a far higher toxicity than PFA, which is caused by the high zinc concentrations. The effects of the sediments from the Rhine and the Apeldoornsch Kanaal are associated with the cation forming elements.

Final Conclusions

The main conclusion is that through the disposal of PFA, the greatest changes are caused by physical effects, as appears from the marine and freshwater studies. The acceptability of PFA as a substrate for benthic organisms is far worse that expected. Within the relatively short time period of the tests, accumulation of elements and acute effects appears to be far less important than expected. A reduction in number of oocytes in *U. pictorum* was found prior to spawning. The later appearance of the larvae of *U. pictorum* point to possible effects at the population level. Where accumulation of elements is concerned, the anions arsenic and selenium are the most important elements in leacheates.

Phyto-monitoring of PFA leacheates and sediments from the Apeldoornsch Kanaal and Rhine with the duckweed (*L. minor*) shows that PFA has but a comparatively low toxicity. The leaching of elements from CG slag is minimal and the observed effects can be accounted for by a lack of nutrients. The main thrust of the "Bouwstoffenbesluit" which is in preparation at present and aims at preventing soil pollution, is the leaching of unbound and stabilized residues. For PFA in unbound form, this means that the leaching characteristics must be measured in a column test and the composition determined following Aqua Regia digestion. During digestion, the matrix is broken open and a composition spectrum arises which is far removed from the natural situation as illustrated by the duckweed experiments, for example. For the elements antimony, chrome, fluorine, molybdenum, selenium, vanadium and the sulphate component, the threshold value of the "Bouwstoffenbesluit" will in all probability not be achievable when based on the proposed column test. More knowledge is needed concerning the leaching of PFA in unbound and stabilized form under natural conditions, when considering the biologically available fraction. This means that more information is needed on the speciation of the elements in bioassay studies. In unbound form, the physical effects on the aquatic environment (substrate) and thereby on species and populations is an area where as yet, little is known. The possibilities for stabilized applications of non-usable wastes such as bottom ashes, and flyash from coal- and waste combustion, in 'artificial waste blocks' requires more attention. Applications can certainly be found in the form of artificial reefs, aimed at biotope improvement for sessile organisms and young fish.

In general, toxicological risks of unbound PFA deposition show to be less severe as initially assumed. However, physical effects for benthic organisms will be severe.

SAMENVATTING EN CONCLUSIES

Dit proefschrift beschrijft de gevolgen bij eventuele deponie van kolenreststoffen zonder afdekking. Het gaat hierbij om de opname door organismen van elementen uit reststoffen en de effecten hiervan. In de eerste twee delen wordt ingegaan op de effecten voor bodembewonende organismen in zeewater en zoetwater. In Deel 3 wordt ingegaan op de groei van planten en de opname van elementen uit reststoffen door planten en wormen.

Poederkoolvliegas (PKVA) vormt het centrale thema. Deze reststof wordt gevormd bij de verbranding van steenkool. Steenkool is een produkt van natuurlijke oorsprong dat in een periode tussen 255 - 203 miljoen jaar geleden gevormd is uit plantaardig materiaal. Gedurende het verkolingsproces zijn de elementen uit de planten 100 tot 1000 maal geconcentreerd. Waarschijnlijk is er extra verrijking opgetreden door stof uit de lucht en zouten uit zoet- en zeewater. Bij verbranding in de ketel worden de aanwezige elementen opnieuw ongeveer een factor 10 geconcentreerd. Dit betekent dat na verbranding van de koolstof de niet verbrande elementen, al of niet gebonden aan vliegasdeeltjes, opnieuw in het milieu terecht kunnen komen. Sommige elementen komen direct vrij in de gasfase, andere elementen condenseren op het oppervlak van de vliegasbolletjes en een deel wordt in de matrix van de bolletjes gebonden. De vraag is: Is er een schatting te maken van wat nu het effect op het milieu is van PKVA, uit ecotoxicologisch oogpunt, bij toepassing in ongebonden vorm. Anders gezegd: is er een schatting te maken wat de risico's zijn als PKVA gestort wordt in zee of in zoetwater.

De elementen in de PKVA kunnen worden onderverdeeld in macro-elementen, die in relatief hoge concentraties (g/kg) en in spoorelementen die beneden de 1 mg/kg voorkomen. Het zijn met name deze spoorelementen die door uitloging uit de PKVA in het milieu terecht kunnen komen en vervolgens in organismen. Hoewel in Nederland deponie in ongebonden vorm op dit moment (nog) niet aan de orde is, kan het in de toekomst een optie zijn waarvoor kennis over mogelijke milieu-effecten noodzakelijk zal zijn. Momenteel wordt bijna alle PKVA gebruikt in de cement industrie. Daarnaast wordt een klein deel gebruikt, na pelletering, als kunstgrind in de betonindustrie.

Het zijn vooral de anion-vormende elementen die vanuit milieuhygiënisch oogpunt van belang zijn. In volgorde van uitlooggedrag van hoog naar laag zijn dit: seleen > molybdeen >> wolfraam > vanadium > antimoon > arseen > chroom. Accumulatie van deze elementen door organismen in te hoge concentraties kan leiden tot ongewenste effecten. Deze effecten kunnen zich versterken (biomagnificatie) in hoger in de voedselketen staande dieren.

Deel 1

In het eerste deel van dit proefschrift wordt het onderzoek beschreven dat in het mariene milieu is uitgevoerd. De onderzochte organismen zijn specifiek voor het wad te weten: de zeepier *Arenicola marina*, de zager *Nereis virens*, het nonnetje *Macoma balthica* en de kokkel *Cerastoderma edule*. De overleving en de accumulatie van elementen is onderzocht bij bovengenoemde soorten na een verblijf van 90 dagen in 100% PKVA en een mengsel (50% PKVA / 50% zand). Als controle is alleen zand gebruikt en als referentiesediment verontreinigde baggerspecie uit de Rotterdamse haven. Daarnaast is onderzocht wat het effect is van een dagelijkse dosis PKVA waarmee een regelmatige stort werd gesimuleerd. Aan het eind van de periode zijn de organismen bemonsterd. De elementen zink, arseen, chroom, koper, nikkel, cadmium, antimoon en seleen zijn bepaald in zowel het sediment als in de dieren. In een van de experimenten zijn jonge zagers gebruikt om een kolonisatie van een PKVA-stort te simuleren. Bij de kokkel is naar veranderingen gezocht in (voortplantings)organen na een verblijf van 230 dagen in een bodem van PKVA. Apart hiervan is naar parasitaire aandoeningen gekeken omdat dieren die in niet-optimale omstandigheden leven, vaak sterker door parasieten worden geïnfecteerd.

Resultaten

De concentratie van de onderzochte elementen in de PKVA bodems bleek na de test periode met ongeveer 10% te zijn afgenomen. Opvallend was dat het arseen- en seleengehalte veel minder was gedaald (<5%) dan verwacht. Van beide elementen is bekend dat ze goed uitlogen. De accumulatie bij de zeepier in PKVA sediment laat zien dat vooral arseen wordt geaccumuleerd in vergelijking met de wadbodem (factor 15 in 50% PKVA / 50% zand en een factor 5 in 100% PKVA op basis van concentraties in de weefsels). Dit wordt waarschijnlijk veroorzaakt doordat het arseen uit de PKVA zich bindt aan bodemdeeltjes waarmee de zeepier zich voedt. Bij de kokkel is deze accumulatie van arseen slechts een factor 2. Onverwacht was de geringe accumulatie van elementen bij het 'kokkel experiment' van 230 dagen. Bij het nonnetje is de accumulatie voor arseen een factor 3. Opvallend is dat de zink concentraties in het nonnetje in alle sedimenttypes zeer hoog is > 550 mg/kg (drooggewicht). Deze hoge concentraties zijn evenwel niet abnormaal en bestonden al bij de aanvang van de experimenten. Hieruit blijkt dat de levenswijze van de gebruikte organismen sterk bepalend is voor de accumulatie van de anionvormende elementen. Voor de cationvormende elementen koper, chroom en nikkel zijn geen onverwachte accumulatie patronen gevonden. Alleen zink wordt geaccumuleerd.

Bij het zager experiment zijn na 4, 8 en 12 weken een aantal elementen geanalyseerd. Ook hier nam de arseen concentratie toe tot en met week 8 (van 23 µg/g naar 76 µg/g in 100% PKVA, op basis van weefsel concentraties), waarna een daling werd gevonden na 12 weken. Evenals arseen accumuleert seleen in de zager, zij het dat de accumulatie trager verloopt. Na 12 weken is nog steeds geen evenwichtssituatie bereikt. Ook de koper concentratie in zagers uit PKVA was met een factor 3 toegenomen. De andere cationvormende elementen zink, chroom en nikkel accumuleren echter niet.

Van de onderzochte soorten is de zeepier het gevoeligst voor PKVA als substraat, wat blijkt uit de relatief hoge sterfte. In 100% PKVA was de mortaliteit 96% maar ook in de controle (wadbodem) was de sterfte hoog (55,5%). De zeepier blijkt gevoelig voor overplaatsen en 100% PKVA absoluut niet als substraat te accepteren. Het nonnetje daarentegen vertoonde een lage mortaliteit (20% in 100% PKVA) en er bleek zelfs een 'wilde' populatie te bestaan afkomstig uit het gebruikte waddensediment. Bij de kokkel lag de sterfte op 40% in 100% PKVA en op 15% in de controle. Onverwacht was dat in de kokkel slechts geringe microscopisch aantoonbare veranderingen in het reproduktieve weefsel zijn waargenomen. In het experiment met zagers werden jonge dieren gebruikt van gemiddeld 200 mg (circa 2 cm lengte) en hier werd alleen in de eerste weken een aanzienlijke sterfte gevonden van 50 - 60%. Deze sterfte was te verwachten omdat de tere jonge dieren 'met de hand' in de bakken zijn uitgezet aan het begin van het experiment.

Conclusies

Milieuhygiënisch gezien zijn, op grond van de gevonden accumulatie, de elementen arseen en seleen zeker het belangrijkst, echter de uitloging uit de PKVA was geringer dan verwacht.

PKVA blijkt als substraat zelfs bij 50% PKVA / 50% zand sterk te verschillen met een natuurlijke wadbodem. Dat leidt tot een ander gedrag van de onderzochte organismen. Een uitzondering vormt het nonnetje dat geen enkel probleem met PKVA heeft. Bij deze soort treedt geen sterfte op, hetgeen wel het geval was bij wadpier, kokkel en zager. Men mag derhalve op grond van de resultaten concluderen dat bij stort zeer ingrijpende veranderingen zullen plaatsvinden in de soortsamenstelling van de bodemfauna en herstel van deze fauna jaren zal kunnen vergen.

Deel 2

De experimenten in Deel 2 zijn uitgevoerd in stroomgoten met de schildersmossel *Unio pictorum*. De keuze voor deze soort is gemaakt omdat het een mossel is die vrij rondkruipt in de bovenste laag van de bodem. Dit in tegenstelling tot bijvoorbeeld de Driehoeksmossel *Dreissena polymorpha* die zich vasthecht op hard substraat. In de zomer zijn schildersmosselen behoorlijk actief, waarbij ze bijna voortdurend openstaan en water filtereren. In de winter graven de mosselen zich echter in.

Onderzocht is hoe de groei van de mossel verloopt in een bodem met PKVA in vergelijking met Rijnsediment. Daarnaast is de groei vergeleken met gemerkte mosselen die een paar jaar in hun groei zijn gevolgd in het riviertje de 'Linge' dat met Rijnwater wordt gevoed. Het gedrag is met de laboratoriumversie van de MosselMonitor® bestudeerd. Hierbij is gekeken naar de activiteit, het open- en dichtgaan van de beide schelpen (klepbeweging). In gelijktijdig uitgevoerd onderzoek bij de Universiteit Utrecht met een andere Unio-soort bleek dat in de aquaria zonder substraat de mosselen bijna voortdurend dicht stonden en slechts voor korte perioden open. De actuele vraag was toen: is een ander klepbewegingsgedrag aantoonbaar in PKVA en heeft dit invloed op de opname van elementen?

Voorts is de accumulatie van het element cadmium bestudeerd, hoewel dit slechts in zeer geringe hoeveelheden in PKVA voorkomt. Toch is voor cadmium gekozen in het accumulatie-experiment omdat hiermee al veel ervaring, op laboratoriumschaal, was opgedaan bij de Universiteit Utrecht. De verwachting was dat in de semie-veldomstandigheden de accumulatie duidelijk anders zou verlopen in vergelijking met de laboratorium experimenten, gezien het verschillende klepbewegingsgedrag.

Daarnaast is de accumulatie van seleen en het optreden van effecten op de ontwikkeling van eieren van de schildersmossel *Unio pictorum* bestudeerd. Arseen en seleen logen gemakkelijk uit PKVA. Daarbij kan arseen wel sterk accumuleren maar wordt het niet verder in de voedselketen opgenomen dit in tegenstelling tot seleen. Seleen (Se) accumuleert weliswaar veel langzamer in organismen, maar biomagnificatie treedt wel op. Se concentreert zich onder andere in eiwitten en met name de zich ontwikkelende eieren in organismen vormen dan het doelorgaan. In de literatuur is beschreven dat bepaalde vissoorten in seleenrijke wateren in Californië al uitsterven bij concentraties van 15 µg/l seleen. Een belangrijk gegeven is dat Se in verschillende vormen (speciatie) kan voorkomen zoals: seleniet [Se (IV)], selenaat [Se(VI)] en organische seleenverbindingen. In percolaat van PKVA is het vooral Se(IV) dat voorkomt en juist Se(IV) wordt sneller geaccumuleerd dan Se(VI). In een drietal experimenten met de schildersmossel is de accumulatie van Se(IV) onderzocht in doseringsexperimenten.

Resultaten

De groei van de mossel in PKVA bleef iets achter vergeleken met de groei in riviersediment (Rijn en Linge). De schildersmossel blijkt in het begin moeite te hebben om zich in te graven in PKVA, pas na 5 dagen is het gedrag vergelijkbaar met de Rijnsediment situatie. De oorzaak is waarschijnlijk dat sedimentatie, van zwevend stof in de stroomgoten, het gedrag van de mosselen beïnvloedt. De individuele mosselen staan bijna voortdurend open en vertonen een hoge activiteit die wordt afgewisseld met korte perioden waarbij de schelpen zijn gesloten.

Bij de cadmium proeven werden de mosselen blootgesteld aan 50 µg/l cadmium. De accumulatie van cadmium bleek zeer snel te verlopen en na 3 weken werd in de nieren een plafond bereikt van circa 400 µg/g (vriesdrooggewicht). Bij de kieuwen en hepatopancreas was de concentratie circa 200 µg/g. Onverwacht was dat het wel of niet aanwezig zijn van substraat geen invloed had op de accumulatie van cadmium. Ook was het opvallend was dat eliminatie van cadmium over een aansluitende periode van 29 weken in gewoon Rijnwater nauwelijks optrad.

In het seleen onderzoek is zijn experimenten uitgevoerd waarbij PKVA en het Rijnsediment werden verrijkt met Se om een zogenaamde "worst case" situatie te creëren. De hoogste concentraties in het weefsel werden bij de experimenten met het verrijkte Rijnsediment gevonden. In een doseringsexperiment werden de mosselen gedurende 11 weken aan 50 µg/l Se(IV) blootgesteld om effecten te vinden in de ontwikkeling van de eieren en embryo's. De accumulatie van Se bleek na 11 weken nog geen plafond te hebben bereikt. Verschillen werden gevonden in een tijdsverschuiving van de aanwezigheid van glochidiën (larven op de kieuwen van vrouwelijke mosselen) en in de gemiddelde grootte van de eicellen aan het eind van de doseringsperiode. Deze verschillen waren echter niet significant. Bij een dosering gedurende 22 weken met 50 µg/l Se(IV) en een 11 weken lange dosering met 250 µg/l Se (IV) bleek een plafond bereikt te worden in seleen accumulatie in de kieuwen van respectievelijk 5 µg/g en 15 µg/g (vriesdrooggewicht). Het onverwachte resultaat was dat geen significante verandering werd gevonden in de aantallen glochidiën en ook zijn er geen gedeformeerde glochidiën gevonden. Op grond van literatuurgegevens over de ontwikkeling van eieren bij baarsachtigen bij blootstelling aan Se in een 32 weken durend experiment was de verwachting dat effecten bij *Unio pictorum* gevonden zouden worden.

Conclusies

De accumulatie van cadmium in *U. pictorum* verloopt snel. In de nieren wordt al na 3 weken een "steady state" bereikt maar eliminatie van cadmium, over een periode van 28 weken in gewoon Rijnwater, blijft achterwege zonder dat evenwel sterfte optreedt. Een belangrijk gegeven is dat het wel of niet aanwezig zijn van substraat, hetgeen een duidelijk effect heeft op het gedrag (klepbeweging), niet van invloed is op de opname van cadmium.

Het belangrijkste effect van seleen op eieren, embryo's en larvale ontwikkeling is dat er een verschuiving plaatsvindt in het tijdstip waarop de larven op de kieuwen van de vrouwelijke mosselen verschijnen. Het gevolg hiervan is dat deze mosselen de larven op een later tijdstip op de kieuwen gaan dragen en later vrij komen. De vraag blijft open of er op dat tijdstip de vissen in de buurt zijn die als gastheer moeten dienen voor de verdere verspreiding en groei van de larven.

Vastgesteld kan worden dat PKVA als substraat minder geschikt is voor *U. pictorum* en daarmee waarschijnlijk ook voor andere vrijlevende bodembewonende mosselen.

Deel 3

In Deel 3 is onderzoek uitgevoerd met planten en wormen. De nadruk ligt op de experimenten met het eendekroos Lemna minor. Daarin zijn de effecten op eendekroos onderzocht van elementen in percolaten van conventionele PKVA, PKVA afkomstig van ketels met low NOx branders, bodemas en kolenvergassingsslak. Om een vergelijking te kunnen maken in opgetreden effecten zijn op vergelijkbare wijze sedimenten (waterbodems) uit een kanaal en de Rijn getest. Daarna wordt ingegaan op de verschillende methoden voor het verkrijgen van percolaten. Een onderscheid is gemaakt in 'natuurlijke' percolaten en 'kunstmatig' samengestelde percolaten waarin de elementconcentraties overeenkwamen met de zogenaamde 'cascade' uitloogtechniek. Er werd een beeldverwerkingsmethode ontwikkeld om de groei van het eendekroos in de verschillende experimenten op een snelle en eenvoudige manier te meten. Tevens worden de effecten beschreven van kolenvergassingsslak (type bodemas) op planten en wormen. Bij kolenvergassing worden eerst de brandbare gasvormige bestanddelen uit poederkool verwijderd met behulp van stoom onder hoge druk en reducerende omstandigheden. Het verkregen gasmengsel wordt op conventionele wijze verbrand. Hierbij ontstaat slechts een relatief kleine fractie vliegas die wordt afgevangen. De bulk wordt gevormd door kolenvergassingsslak (KV-slak) die na het vergassingsproces achterblijft. Op laboratorium schaal is onderzocht hoe de groei van de plant *Cyperus esculentus* verloopt en welke elementen accumuleren in de plant. Op soortgelijke wijze is bij de worm *Eisenia fetida* gekeken naar de mortaliteit en accumulatie van elementen.

Resultaten

Eendekroos blijkt zeer geschikt te zijn voor het testen van percolaten van PKVA, kolenvergassingsslak en waterbodems. Uit de experimenten komt naar voren dat de anionvormende elementen de effecten bij PKVA veroorzaken. Het zijn daarentegen juist de cationvormende elementen die de effecten van de waterbodems veroorzaken. De toxiciteit van PKVA ligt vergeleken met de waterbodems aanmerkelijk lager. Dit verschil wordt voornamelijk veroorzaakt door het element zink dat in hoge concentraties (>1000 µg/l) in het percolaat van het kanaal sediment voorkomt, terwijl de 'geen effect concentratie' voor eendekroos bij 160 µg/l ligt. De toxiciteitsvolgorde voor elementen zoals deze is gevonden in afzonderlijk uitgevoerde testen met eendekroos is: cadmium >koper >zink >arseen (III) >seleen (IV) >borium >molybdeen. Een 'natuurlijke' uitlogingsmethode (24 uurs uitloging met een constante pH van 5) geeft pas bij 60% additie effecten op groei, terwijl bij de 'standaard' test reeds effecten optraden bij 20% additie.

De uitloging van elementen uit KV-slak is minimaal gebleken. De groei van knolcyperus neemt bij toenemende concentratie KV-slak af door gebrek aan nutriënten en niet door toxische effecten van elementen uit de KV-slak. De toevoeging van voedingsstoffen resulteerde in een sterke verbetering van de groei. De groei in PKVA bleef evenwel achter, hetgeen verklaard wordt doordat verse PKVA relatief veel borium bevat dat bij concentraties boven de 60 µg/g al groeiremming geeft. Bij *C. esculentus* is alleen accumulatie van borium en molybdeen gevonden. Daarbij speelt eveneens het fysische effect van het inklinken van PKVA waardoor de grond zo dicht wordt dat de wortelgroei ongunstig wordt beïnvloed. Er is een duidelijk negatief effect gevonden voor de groei en mortaliteit van de worm *E. fetida* in PKVA en KV-slak. Met name in PKVA was de mortaliteit hoog (32%). Accumulatie is alleen gevonden voor het element arseen. De beschikbaarheid van de anion-vormende elementen uit PKVA was groter dan van KV-slak.

Conclusies

De uitspraak dat vliegas in zijn algemeenheid als chemisch afval moet worden beoordeeld, komt met de hier gepresenteerde resultaten in een ander daglicht te staan. Momenteel wordt door de overheid gewerkt aan een herziening van dit standpunt waardoor PKVA in de categorie grondstoffen zal kunnen komen. De effecten van conventionele en low NOx PKVA kunnen worden gerelateerd aan de anionvormende elementen. De uitloging van elementen uit KV-slak blijkt zeer gering. De veel hogere toxiciteit van de sedimenten uit de Rijn en het bemonsterde kanaal zijn toe te schrijven aan cationvormende elementen en met name zink.

Eindconclusies

De grootste veranderingen bij deponie van PKVA zal teweeg worden gebracht door fysische effecten zoals is gebleken uit de zeewater en zoetwater studies. De acceptatie van PKVA als substraat voor bodemorganismen is slechter dan werd verwacht. Binnen het relatief lange tijdsbestek van de proeven bleek de accumulatie van elementen en acute effecten bij de gebruikte bodemorganismen veel geringer dan verwacht. Opmerkelijk was dat geen significante verschillen zijn gevonden in histopathologische afwijkingen in het reproduktieve weefsel van *C. edule* en effecten op de larvale stadia bij *U. pictorum*. Wel werd een duidelijke reductie in het aantal oöcyten gevonden bij *U. pictorum* vlak voor de eiafzetting. De latere verschijning van larven op de kieuwen bij *U. pictorum* is moeilijk te interpreteren. Mogelijk kunnen op lange termijn effecten gaan optreden op het niveau van populatieomvang. Wat accumulatie van elementen betreft zijn arseen en seleen, beide anionvormende elementen, de belangrijkste elementen in de percolaten.

Fytomonitoring van percolaten met eendekroos (*L. minor*) van PKVA en waterbodems uit de Rijn en een kanaal laten zien dat PKVA vergelijkenderwijs slechts een geringe toxiciteit vertoont. De uitloging van elementen uit KV-slak is minimaal gebleken en de gevonden effecten zijn toe te schrijven aan gebrek aan nutriënten. Uitgaande van het Bouwstoffenbesluit, dat nu in voorbereiding is, wordt aan uitloging en uitlooggedrag van reststoffen groot belang gehecht. Immers het Bouwstoffenbesluit heeft als doel het voorkomen van bodemverontreiniging. Voor PKVA in ongebonden vorm, betekent dit dat uitloogkarakteristieken bepaald moeten worden volgens de zogenaamde kolomtest en de samenstelling na koningswaterontsluiting. Bij ontsluiting wordt de matrix opengebroken en ontstaat een samenstellingsbeeld, dat ver is verwijderd van de natuurlijke situatie zoals deze is benaderd in het 'kroos' onderzoek. Voor de elementen antimoon, chroom, fluor, molybdeen, seleen, vanadium en de component sulfaat kan hoogst waarschijnlijk niet worden voldaan aan de gestelde grenswaarde die gebaseerd is op de kolomtest. Meer kennis is nodig omtrent de uitloging onder natuurlijke omstandigheden van PKVA in gebonden en ongebonden vorm waar het gaat om de biologische beschikbare fractie. Dit betekent dat onderzoek nodig is naar de speciatie van de elementen in bio-assay studies. In ongebonden vorm zijn de fysische effecten op het aquatisch bodem-milieu (substraat) en daarmee op soorts- en populatieniveau een onderzoeksgebied waar nog weinig over bekend is. Vooral de mogelijkheden van gebonden toepassingen van niet meer te gebruiken reststoffen afkomstig van kolenstoken en vuilverbranding, in zogenaamde 'waste blocks' verdient meer aandacht. Vormen van toepassingen zijn zeker te vinden in kunstmatig aangelegde riffen langs de kust.

Alles overziend, blijkt dat de toxicologische risico's bij deponie van PKVA in ongebonden vorm veel geringer zijn dan aanvankelijk verwacht. Echter de fysische effecten voor bodemorganismen zullen aanzienlijk zijn.

CURRICULUM VITAE

Henk Arnold Jenner werd geboren op 3 april 1948 te Palembang (Indonesië). Na het behalen van het diploma h.b.s.-B te Bussum begon hij in 1968 zijn studie biologie aan de Vrije Universiteit te Amsterdam. In 1969 is eerst de militaire dienstplicht vervuld. Na het afzwaaien als 2e luitenant der artillerie werd de biologiestudie voortgezet. In de doctoraalfase was Histologie het hoofdvak en Ethologie en Plantensystematiek waren de bijvakken. Na het behalen van het diploma in 1976 volgde een korte onderzoeksperiode bij de subfaculteit Biologie van de Vrije Universiteit. In februari 1977 werd de betrekking bij KEMA aanvaard. In de eerste jaren richtte het onderzoek zich op de problematiek rond (mossel)-aangroeibestrijding in koelwatersystemen van elektriciteitscentrales en industriële koelwatergebruikers. Doel was het vinden van alternatieve bestrijdingsmethoden, in plaats van chloordosering, om de ongewenste aangroei tegen te gaan. De thermische bestrijdingsmethode ook wel de 'thermoshock' methode genoemd was onderdeel van deze studie.

In 1985 verschoof het onderzoeks- en werkterrein voor een belangrijk deel naar de ecotoxicologie. Om "erkend" toxicoloog te kunnen worden, werden de vakken pathobiologie en proefdierkunde gevolgd aan de Universiteit Utrecht en de bijbehorende tentamens afgelegd. Het onderzoek in de ecotoxicologie lag in eerste instantie bij de uitloging van (zware)metalen in relatie met reststoffen en de effecten op de biota. Het in deze periode uitgevoerde werk ligt onder andere ten grondslag aan dit proefschrift. Momenteel vormt de problematiek rond gechloreerde organische verbindingen, welke ontstaan tijdens de chlorering van koelwater, een belangrijk aandachtsgebied.

In 1987 volgde de benoeming tot sectiemanager van de sectie biologie welke na de reorganisatie in oktober 1992 de sectie Water, Bodem en Reststoffen werd. Deze functie wordt tot op heden vervuld. De sectie is onderdeel van de "Business Unit Mlieu Services" van KEMA Nederland B.V.

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