Ecotoxicology and Environmental Safety **44,** 241–252 (1999)
Environmental Research, Section B
Article ID eesa.1999.1827, available online at http://www.idealibrary.com on IDEAL®

Use of Crayfish in Biomonitoring Studies of Environmental Pollution of the River Meuse

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Received July 29, 1998

The river Meuse, located in western Europe, is contaminated by different pollutants, of both organic and inorganic nature. The predominant sources of Meuse contamination in The Netherlands are agricultural activities and pollution derived from urban areas. Crayfish, water, and sediment samples were collected at four different locations of the river Meuse, in order to cover a large part of the catchment area of this river in The Netherlands. Crayfish may be very useful in biomonitoring studies, since they can integrate body load by pollutants over time in an area-bound manner. In these crayfish, levels of aromatic DNA adducts, heavy metal residues, polychlorinated biphenyls (PCBs), and organochlorine pesticides were determined in hepatopancreatic tissue. Also analyzed were water and sediment samples derived from the same locations, for polycyclic aromatic hydrocarbons (PAHs), heavy metals, and organochlorine compounds. In sediments from the four different sampling sites, no clear differences were observed in PCB levels. Organochlorine pesticide concentrations were highest at location A, the most upstream sampling site, whereas a general decrease was observed following the river Meuse downstream. A similar pattern was observed for the metal compounds. For PAH sediment levels no consistent tendency could be observed. Highest values were detected at site B, followed by, respectively, locations A, D, and C. In water samples, a different pattern was observed. The highest metal concentration was observed at location D, whereas the total organochlorine level was higher at sites B and D, compared to the two other sampling sites. Differences in pollution levels in crayfish between sampling sites were evident. Site D, the most downstream-situated site examined, appeared to be the most polluted site with respect to PCBs, DDT, DDE, and Cu in crayfish. Moreover, DNA adduct levels, which may serve as a dosimeter for the internal dose of aromatic compounds such as PAHs and PCBs, were also significantly higher in hepatopancreatic tissue of crayfish captured at site D, compared to the three other sampling sites. Moreover, significant correlations were observed between DNA adduct levels and the lower chlorinated PCB congeners (PCB 28–PCB 101). By correlating the different pollutants in water and/or sediment with xenobiotic levels in crayfish, no consistency could be observed, indicating that monitoring aquatic species may provide specific information on the presence of surface water pollutants. These results indicate that crayfish can be used as biological indicators of exposure to both organic and inorganic pollution in aquatic systems. © 1999 Academic Press

Key Words: **crayfish; environmental pollution; metals; PCBs; PAHs; DNA adducts; water; sediment.**

INTRODUCTION

The river Meuse is located in western Europe and flows through three countries: France (506 km). Belgium (182 km), and The Netherlands (183 km) (Philippart *et al*., 1988). The river Meuse is a rain-fed river, its natural slope being disturbed by the presence of locks and dams, which have a strong impact on the discharge of the river (Philippart *et al*., 1988; De Ruyter van Stevenick *et al*., 1990). The river Meuse with its high ecological value is used for transport, recreation, fisheries, power generation, and water supply for industry and agriculture (Kraak *et al*., 1991). Moreover, the river Meuse serves as a source for drinking water production for more than five million inhabitants in Belgium and The Netherlands (Philippart *et al*., 1988; De Ruyter van Stevenick *et al*., 1990). Consequently, high demands are made on the water quality but unfortunately, the intensive use of this river has led to hydrographic changes and high levels of toxic compounds in the water, which has resulted in severe degradation of the river's environmental quality (De Ruyter van Stevenick *et al*., 1990). Over the last decades, a tendency toward improvement in concentrations of heavy metals, ammonia, and nitrites has been observed, which is probably caused by the closing of old mines and factories, and by the economical recession (Philippart *et al*., 1988).

By contrast, organic contaminants such as hydrocarbons, PCBs, and chlorinated pesticides still are ubiquitous contaminants of the aquatic environment as a result of

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uncontrolled spillages, surface runoff, river transport, and atmospheric deposition (Porte and Albaiges, 1993; Colombo *et al*., 1990). Their fate and distribution among different biotic and abiotic compartments are a function of their physicochemical properties, i.e., octanol–water partition coefficient (K_{ow}), lipohilicity, and vapor pressure (Colombo *et al*., 1990; Porte and Albaiges, 1993). Because of their strong hydrophobic character, chlorinated pesticides and polychlorinated biphenyls are found in the river system mainly in association with colloids, suspended particulate matter, and sediments (Colomo *et al*., 1990). Moreover, these compounds may bioaccumulate in the fatty tissue of organisms (Colombo *et al*., 1990).

Trace metals, released from both natural sources and anthropogenic activity, may also accumulate in aquatic environments (Rincon-Leon *et al*., 1988), because unlike organic compounds, metals are not biodegradable (Linde *et al*., 1996). Metals are, for the most part, adsorbed to suspended matter and therefore accumulate in sediments (Admiraal *et al*., 1993). Both heavy metals and organic pollutants have been accumulating in the sediment of the river Meuse for decades, and despite relatively lower water concentrations, large deposits are now present (Admiraal *et al*., 1993). Like organic pollutants, these metals tend to accumulate in aquatic organisms.

Aquatic biota, sediments, and water samples may be used for pollution-monitoring purposes (Porte and Albaiges, 1993). The relevance of sediment measurements is limited, since contaminants bound to the substrate by adsorption may not be bioavailable for immediate uptake by aquatic organisms. In addition, varying concentrations on the sediment may reflect changes in the binding properties of the sediments rather than variations in the pollution levels of the sediments (Muncaster *et al*., 1990). The relevance of direct analysis of water is also limited in the case of hydrophobic contaminants, whereas contaminant levels are usually below detection limits (Muncaster *et al*., 1990). Therefore, living organisms should also be used to monitor environmental contamination, since they may reflect the contamination history of a particular location because they have been exposed during their entire lifetime and are capable of accumulating these toxic substances in their lipid tissue fractions (Muncaster *et al*., 1990; Kraak *et al*., 1991).

Crayfish are often used as general pollution indicators in biomonitoring studies. In several studies crayfish have been used as indicators of aquatic metal pollution, since these crustacea tend to accumulate metals in their tissues (Stinson and Eaton, 1983; Anderson and Brower, 1978; Evans, 1980; Diaz-Mayans *et al*., 1986; Madigosky *et al*., 1991). Moreover, in view of their ability to accumulate hydrophobic compounds from water and food, crayfish may be useful indicators of organochlorine contamination and PAH burden. In The Netherlands, the crayfish *Orconectus limosus* has been recorded since 1970, and it is a fairly common species in the river Meuse (Van den Brink *et al*., 1988).

Detection of PAH in organisms is difficult since these compounds are easily metabolized into more polar, readily excretable metabolites. For biomonitoring purposes, measurement of specific DNA adducts may therefore provide a more suitable biological marker of environmental exposure than measurement of the parent compounds. For example, in eels living in PAH-polluted water no high-molecular-weight PAHs (e.g., PAHs containing four rings or more) could be detected but high levels of liver-DNA adducts were observed (Van der Oost *et al*., 1994). Determination of PAH-DNA adducts in aquatic organisms has been successfully applied by means of ^{32}P-postlabeling in environmentally exposed fish and mussels (Dunn *et al*., 1987; Kurelec *et al*., 1989; Varanasi *et al*., 1989; Kurelec *et al*., 1988). Interaction products with DNA may therefore be used as indicators for the biologically available dose (Beach and Gupta, 1996; Schut and Shiverick, 1992). Also, PCBs are assumed to induce their toxic effects after metabolic activation, as has been demonstrated for polycyclic aromatic hydrocarbons and numerous other compounds. Several reports have found that *in vitro* and *in vivo* activation of PCBs resulted in formation of metabolites capable of forming DNA adducts (Oakley *et al*., 1996; Al-Sabti, 1985; Morales and Matthews, 1979; Amaro *et al*., 1996; McLean *et al*., 1996).

This study was conducted to determine the levels of DNA adducts, heavy metal residues (Cd, Pb, Cu, and Zn), chlorinated pesticides (hexachlorobenzene (HCB), dichloro-diphenyl-tri-chloroethane (DDT), and dichloro-diphenyl-di-chloroethylene (DDE)) and the seven indicator congeners of polychlorinated biphenyls (IUPAC Nos. 28, 52, 101, 118, 138, 153, 180) in hepatopancreatic tissue of crayfish caught from four different locations of the river Meuse in The Netherlands. Sediment and water samples were taken at the same locations and analyzed for chlorinated pesticides, PCBs, and heavy metals. In this way, a comprehensive assessment was made of the contamination levels, the sources, and the fate of these pollutants on four different locations of the river Meuse in The Netherlands. Moreover, the relevance of crayfish dosimetry as a biological indicator of water pollution in relation to analysis of water and sediment was assessed.

MATERIALS AND METHODS

Materials

Proteinase K (sp. act. 20 units/mg lyophilisate), RNase A (sp. act. 50 units/mg dry powder), and RNase T1 (sp. act. 100,000 units/ml) were purchased from Boehringer Mannheim (Germany). Alkaline phosphatase (Type VII-N; sp. act. 10,000 units/ml), micrococcal nuclease (100–200 μmolar U/mg protein), potato apyrase (3–10 U/mg protein), and

nuclease P1 (sp. act. 200 units/mg protein) were obtained from Sigma (St. Louis, MO). Spleen phosphodiesterase (2 U/mg) was obtained from Cooper Biomedical Corp., T4 polynucleotide kinase (sp. act. 30 U/μl) and γ-^{32}P from Amersham (Buckinghanshire, UK), and polyethyleneimine-cellulose TLC sheets from Machery–Nagel (Düren, Germany). Methanol was HPLC grade and was obtained from Rathburn (Walkerburn, UK). Tl-(thalium)-standard was derived from Merck (Amsterdam, The Netherlands). Analytical-grade chemicals were used at all other instances. Water was purified by means of a Milli-Q water purification system.

Selection of Crayfish

Crayfish (*Orconectus limosus*), water, and sediment samples were collected from four different locations of the river Meuse (Fig. 1). The first sampling site was in Borgharen (location A): 16 km from the Dutch–Belgian border. The other three sampling sites were Linne (location B), Belfeld (location C), and Boxmeer (location D), which are situated further downstream, 69, 100, and 148 km, respectively, from the Dutch–Belgian border. The four locations were chosen to cover a large part of the catchment area of the river Meuse in The Netherlands. Sediment samples (top 10 cm) were collected by a Van Veen grab. From each location eight subsamples were taken and homogenized. The homogenized samples were stored in polyethylene jars (2 L). The surface water samples were collected in polyethylene bottles which were previously rinsed with diluted nitric acid (5%), and before sampling, the jars were rinsed with surface water. Water samples were centrifuged for 15 min at 2500 rpm and stored at 4°C for further analysis. A subsample was acidified with concentrated nitric acid pH < 2, for metal analysis. Crayfish were collected by means of fike nets. After collection, total length and weight of each crayfish was recorded. From each sampling site 10 crayfish (except for location C, where only 8 animals could be caught) were selected based on weight, length, and sex. To avoid seasonal fluctuations in pollution burden of crayfish, the animals were collected within one season. Crayfish were sedated in ice-cold water containing 1% chloroform for 20 min and sacrificed by exsanguination of the hemolymph; hepatopancreatic tissue was subsequently dissected and stored at -20°C.

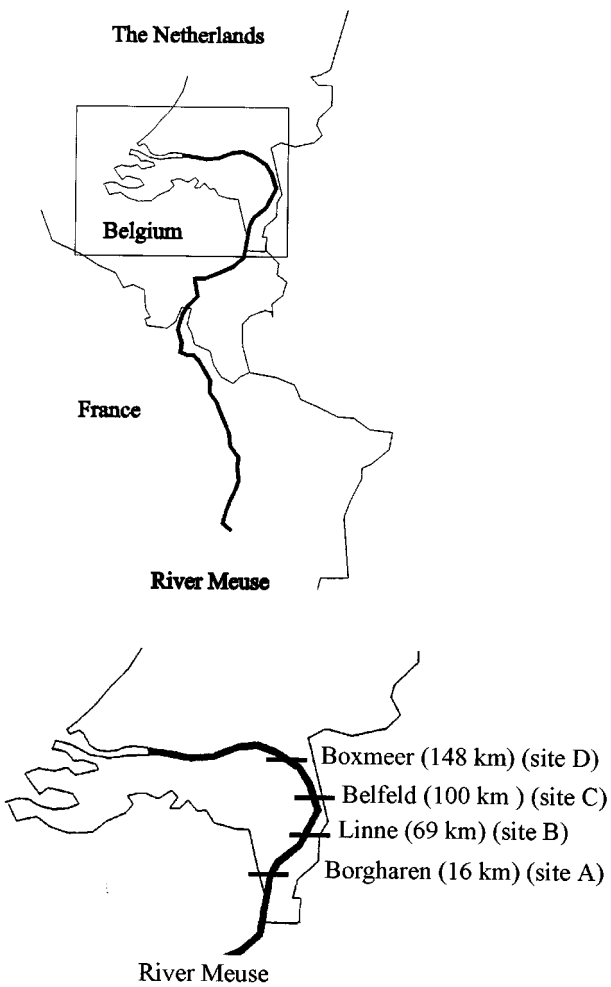

FIG. 1. The course of the river Meuse in The Netherlands, Belgium, and France. The location of the four sampling sites is given in detail.

Atomic Absorption Spectrometry of Metals

Sample Preparation

Preweight samples of hepatopancreas (10–50 mg) were ashed at 450°C for 16 h. Each sample was treated with 2 ml of 1 : 1 mixture of concentrated HNO_3 and water and subsequently boiled for 10 min on a hot plate. The remaining solutions were filled up to 10 ml with water.

Samples of sediments were dried at 90°C to a constant weight and subsequently ground to powder using an agate mortar. Samples (0.5 g) were subsequently boiled under reflux for 2 h using Aqua Regia. After filtration the filtrate was diluted with 0.1% HNO_3. Water samples were acidified with concentrated nitric acid to pH < 2.

Concentrations of Zn were measured by flame AAS with deuterium background correction and concentrations of Pb, Cd, and Cu were measured by graphite furnace AAS with Zeeman background correction. Zn, Cu, Pb, and Cu were carried out at 213.9, 327.4, 283.3, and 228.8 nm, respectively. The ashing temperature was 800°C for Cu, 500 °C for Pb, and 400 °C for Cd; the samples were atomized at 2300, 2300, and 1800°C, respectively. Cu-, Pb-, Cd-, and Zn-nitrate solutions in 0.1% HNO_3 were used for calibration (external standard line). In all instances, paladium-nitrate was used as a modifier. The detection limit was 1 ng/ml for Cu, 2 ng/ml for Pb, 0.05 ng/ml for Cd, and 0.040 μg/ml for Zn.

All glassware was rinsed with 1% HNO_3 to avoid contamination.

Gas Chromatography–Electron Capture Detection of PCBs

Sample Preparation

a. Hepatopancreatic tissue. The sample clean-up procedure was based on Mössner *et al.* (1994). In short, preweight samples (10–60 mg) were mixed with 0.5–1.0 g anhydrous sodium sulfate (heated at 550 °C for 16 h) and subsequently homogenated until a free-flowing powder was obtained. This mixture was placed in a glass column containing a glassfiber filter. After addition of 50 µl internal standard (PCB 143, 100 ng/ml) the mixture was eluted with 10 ml petroleum ether. Part (2 ml) of the eluate was used for lipid determination. The remaining fraction was concentrated under nitrogen to a sample volume of approximately 500 µl.

b. Water samples. Water samples (500 ml) were put in a conical flask with ground stopper. After 50 µl internal standard (PCB 143, 100 ng/ml) was added, water samples were treated with 3 ml sodium sulfite solution to remove remaining sulfur. Samples were extracted twice with 25 ml petroleum ether (40–60°C) and the combined extracts were concentrated under nitrogen to approximately 500 µl and dried on a sodium sulfate-packed column (heated for 16 h at 550°C). Samples were subsequently eluted with 1 ml petroleum ether and concentrated to a sample volume of approximately 500 µl.

c. Sediment samples. Sediments (10 g wet weight) were put in a conical flask with ground stopper. After addition of 50 µl internal standard (PCB 143, 100 ng/ml) samples were treated with 50 ml acetone. To remove sulfur 3 ml sodium sulfite was added. Samples were subsequently shaken with 100 ml hexane. Hexane fractions were evaporated under nitrogen to a sample volume of approximately 500 µl and dried over a sodium sulfate column as described above.

Clean-up Procedure with Silica Gel

For hepatopancreatic, water, and sediment samples a clean-up procedure was applied using silicagel chromatography. The silicagel (Silicagel 60, 63–200 µm) was heated for 16 h at 550°C, deactivated by adding 2% water, and left to equilibrate for at least 24 h. A glass column (diameter, 5 mm) equipped with a glassfiber filter was slurry-packed in hexane with 0.5 g of the deactivated silicagel. After elution of the samples with 5 ml hexane, the remaining fraction was evaporated to dryness under nitrogen, dissolved in a fixed volume isooctane, and analyzed by GC-ECD. All solvents used were suprasolve grade.

Chromatographic Conditions

Chromatographic conditions were as follows: GC, 8560 Mega 2 series with 63-Ni-ECD (Fisons Instr., Milano, Italy); column, 30 m × 0.25 mm i.d., 0.25 µm stationaire phase (CPSil 8 CB low bleed), and a retention gap of 2.5 m × 0.32 mm i.d. (Chrompack International BV, The Netherlands); carrier gas, helium; column head pressure, 80 kPa; make-up gas, nitrogen; injection, cold on column; detector temperature, 300°C; temperature program, 80°C for 3 min, then 4°C/min to 270°C and kept at 270°C for 10 min.

Quantitation of the compounds was performed by measuring peak heights in combination with the internal standard method. Blanks were also carried out but were below detection limits for all compounds analyzed.

Data on seven indicator congeners of PCBs are given separately and are also presented as the total PCB burden (ΣPCB). The PCB load of each organism was estimated on the basis of seven congeners selected from the GC-ECD profile (IUPAC Nos. 28, 52, 101, 118, 138, 153, 180).

HPLC Fluorescence Detection of PAHs

The PAHs analyzed were the 16 PAHs identified as priority pollutants by the U.S. Environmental Protection Agency: naphthalene, acenaphthene, fluorene, phenanthene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, benzo[*g*,*h*,*i*] perylene, dibenzo[*a*,*h*]anthracene, and indeno[1,2,3-*c*,*d*]pyrene, except for acenaphthylene, which cannot be measured by fluorescence detection.

Sample Preparation

Sediment samples (10 g wet weight) were ground with 10 g anhydrous sodiumsulfate and subsequently Soxhlet extracted with 120 ml petroleum ether (40–60°C) for 4 h, after which the sample volume was reduced by rotary evaporation. The extract was dried under nitrogen to a small residue (0.5 ml). Water samples (500 ml) were extracted three times with petroleum ether (40–60°C). The combined extracts were concentrated under nitrogen to a volume of 0.5 ml.

Clean-up Procedure of Water and Sediment Extracts with Silicagel

The silicagel was heated for 16 h at 550°C, deactivated with 6% water, and left to equilibrate for at least 24 h. A glass column equipped with a glassfiber filter was slurry-packed with 0.5 g of the deactivated silicagel and hexane. After elution of the samples with 10 ml hexane the remaining fraction was evaporated under nitrogen to a volume

of 2.0 ml. Subsequently, 1 ml acetonitril was added and the upper hexane layer was evaporated.

HPLC Analysis

Samples were analyzed at 30°C by reversed-phase HPLC with fluorescence detection (Perkin Elmer LS30) using a 5-μm Vydac ODS column (250 × 4.6 mm). The mobile phase consisted of water and acetonitrile. Gradient elution started with 75% of mixture B (40% acetonitrile, 60% water; v/v) and 25% mixture A (100% acetonitrile). A linear gradient was used from 5 to 25 min, resulting in 10% B and 90% A. The excitation wavelength was set at 250 nm while a cut-off filter of 350 nm was installed. The concentrations of individual PAHs were determined by comparing peak heights with those of known standards.

DNA Extraction Procedure

DNA from hepatopancreatic tissue was isolated according to a standard procedure (Gupta, 1984). In short, tissue samples were cut into tiny pieces and homogenized in 1% SDS/1 mM EDTA. The tissue homogenate was incubated with proteinase K and successively extracted with 1 volume of phenol, 1 volume of phenol/chloroform/isoamyl alcohol (25/24/1), and 1 volume of chloroform/isoamyl alcohol (24/1). After DNA precipitation using 0.1 volume NaAc and 2 volumes of ice-cold ethanol, DNA was dissolved in 5 mM Tris–HCl (pH 7.4) containing 1 mM EDTA. RNA was destroyed by addition of RNase T1 and RNase A. After extraction of the digest using 1 volume of chloroform/isoamyl alcohol (24/1), DNA was precipitated and solubilized as described above. DNA concentration was assayed spectrophotometrically.

Determination of Aromatic DNA Adducts by ^{32}P-Postlabeling

DNA samples were analyzed essentially as described by Reddy and Randerath (1986). Five micrograms of DNA were digested for 3.5 h at 37°C using 200 mU micrococcal nuclease and 2.8 mU spleen phosphodiesterase in a total volume of 5 μl containing 20 mM sodium succinate, and 10 mM $CaCl_2$, pH 6. The modified nucleotides were labeled with [γ-^{32}P]ATP (50 μCi) using 5 units of T4 polynucleotide kinase. [γ-^{32}P]ATP was synthesized in the laboratory using carrier free gamma-^{32}Phosphate (DuPont Brussels). The specific activity was determined as described and generally was 3000–5000 Ci/mmol. Purification and resolution of ^{32}P-labeled adducts was carried out on polyethyleneimine-cellulose TLC sheets. The following solvent systems were used: D1, 1.0 M sodium phosphate, pH 6.0; D2, 3.5 M lithium formate, 8.5 M urea, pH 3.5; D3, 0.8 M LiCl, 0.5 M Tris–HCl, 8.5 M urea, pH 8.0; D4, 1.7 M sodium phosphate, pH 6.0.

Quantification was performed by using a storage phosphor imaging technique (Molecular Dynamics, Sunnyvale, CA). Adduct levels were calculated from the amounts of radioactivity on the chromatograms, and the DNA amount, and normalized against the [^{3}H]benzo[*a*]pyrene-diol-epoxide (BPDE)-DNA standards with known modification levels. In each experiment BPDE-DNA standards with a modification level of 1 adduct in 10^7, 10^8 resp. 10^9 nucleotides were run in parallel.

Statistics

The linear association between two continuous variables can be investigated by simple regression analysis. The closeness of this association can be measured by the correlation coefficient *r*. Simple regression analysis was based on log-transformed data to normalize variance among groups. Statistical evaluation of differences between the several groups with respect to adduct level, heavy metal content, and organochlorine burden was performed using Scheffe *F* test (analysis of variance: ANOVA). *P* values < 0.05 were considered significant. Since per site single samples were taken from water and sediments, no statistical tests were performed on these data.

RESULTS

Table 1 presents the concentrations of Cd, Zn, Cu, and Pb in water and sediments at the four different sampling sites. By comparing the metal burden of sediments of the four different sampling locations a general decrease was observed from upstream to downstream sites. Metal concentrations in water samples, however, demonstrated a completely different pattern: highest concentrations of these metals were observed at the upmost downstream location, site D, while water metal content was clearly lower in site A, the most upstream location, and lowest

TABLE 1
Data on Heavy Metals in Sediment and Water Samples of the Four Upstream-to-Downstream Locations of the River Meuse

	Location A	Location B	Location C	Location D
Metals in sediment (mg/kg dry weight)				
Cd	9.38	6.47	8.29	3.20
Zn	1654	985	808	385
Cu	116	78	114	59
Pb	213	121	122	70
Metals in water (μg/L)				
Cd	0.25	0.31	0.22	0.43
Zn	28	43	22	44
Cu	9.8	7.1	6.8	16.6
Pb	3.7	6.1	2.2	6.1

concentrations were observed in site C. Table 2 presents the concentrations of PAHs in sediments that are known to yield DNA adducts through the formation of reactive diol-epoxides (fluoranthene, benzo[*a*]anthracene, chrysene, benzo[*b*]- and benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenzo[*a*]anthracene, and indeno[1,2,3-*c*,*d*]pyrene), the sum of the 16 EPA PAHS, and data on PCBs, HCB, DDT, and DDE in both water and sediment samples of the four different sampling sites. The sum of the PAHs known to produce DNA adducts was highest in location B followed by locations A, D, and C. Comparable values were found for the total of 16 EPA PAHs. The concentrations of PAHs in water samples were below the detection limit. The total PCB burden in sediments of the four different areas was comparable in the four different sites, varying from 167 to 198 ng/g dry weight. With regard to HCB, DDT, and DDE pollution of sediments, a clear decrease was observed following the Meuse downstream. Highest concentrations of the organochloric pesticides were detected in water samples derived from site A whereas lowest values were observed in site D, the most downstream-situated location. On the contrary, in water samples the river appeared to be less polluted with PCBs, HCB, DDT, and DDE in location A, compared to location D, the most downstream sampling site. However, highest values with regard to both HCB and PCBs in water samples were observed in location B.

TABLE 2
Data on Organic Micropollutants (PCBs, HCB, DDT, and DDE) in Water and Sediment Samples of the Four Different Locations of the River Meuse

	Location A	Location B	Location C	Location D
	Water (ng/l)			
PCB 28	0.22	3.87	0.48	0.55
PCB 52	0.38	nd	0.47	0.31
PCB 101	0.05	0.25	0.44	0.63
PCB 118	0.08	0.07	nd	0.64
PCB 138	0.04	0.06	nd	0.48
PCB 153	0.12	0.22	0.27	0.12
PCB 180	0.07	nd	0.39	0.61
Σ PCB	0.96	4.47	2.05	3.34
HCB	0.12	1.64	0.50	0.64
DDT	0.04	0.08	nd	0.50
DDE	0.13	0.23	nd	0.58
Σ DDT + DDE	0.17	0.31	nd	1.08
	Sediment (ng/g dry weight)			
PCB 28	3.48	8.05	7.02	4.93
PCB 52	3.61	5.16	9.07	4.41
PCB 101	23.25	21.36	22.36	17.91
PCB 118	7.72	5.46	17.21	18.64
PCB 138	24.74	28.43	23.45	37.00
PCB 153	62.46	46.96	44.21	58.42
PCB 180	70.60	53.89	41.81	56.56
Σ PCB	195.86	167.19	165.15	198.31
HCB	11.59	8.05	7.02	4.93
DDT	3.32	2.49	2.13	nd
DDE	1.94	2.58	3.02	1.6
Σ DDT + DDE	5.26	5.07	5.15	1.60
	Sediment (μg/kg)			
Adduct-yielding PAHs				
Fluoranthene	684.1	922.5	369.2	602.3
Benz[*a*]anthracene	448.3	577.0	253.4	365.0
Chrysene	396.7	510.9	230.0	332.9
Benzo[*b*]fluoranthene	500.7	495.2	286.0	392.2
Benzo[*k*]fluoranthene	222.1	251.0	132.6	182.1
Benzo[*a*]pyrene	451.5	528.2	259.7	368.4
Dibenzo[*a*]anthracene	557.9	624.6	299.8	412.7
indeno[1,2,3-*c*,*d*]pyrene	555.4	688.9	394.2	397.1
Sum	3817	4599	2225	3053
Sum 16 EPA PAHS	5135	6037	2954	4087

Note. Abbreviations: nd, not detectable; EPA, U.S. Environmental Protection Agency.

Table 3 presents the concentrations of PCBs, HCB, DDT, DDE, and metals: Cd, Zn, Pb, and Cu in the hepatopancreas of crayfish caught at the four different sampling sites. No significant differences were found between the sexes; the data of the two sexes were therefore pooled. Differences in crayfish levels between sampling sites were evident. Crayfish from site D, the most downstream-situated location examined, had the highest level of PCBs: highly significant differences in total amount of PCBs as well as in most individual congeners (PCB 101–PCB 180) were found by comparing site D with the three upstream-located sites ($P < 0.05$). In crayfish from location C the lower chlorinated PCBs 28 and 52 were also significantly enhanced by comparison with locations A and B. HCB concentrations in crayfish from location C were statistically enhanced, compared to locations A, B, and/or D ($P < 0.0001$). Crayfish from location D accumulated statistically higher amounts of both DDT and DDE in hepatopancreas, compared to the three other sampling locations A, B, and C ($P < 0.001$).

The accumulation of Zn in crayfish hepatopancreas in the four different locations was similar. Statistically higher levels of Cu were found in crayfish from location D, compared to the three other sites ($P < 0.001$). Pb concentrations in crayfish hepatopancreas were below detection limit at all locations. For Cd statistically enhanced values were observed in crayfish from locations B and C, compared to location A ($P < 0.05$). The level of Cu (μg/g) in hepatopancreatic tissue was significantly correlated with Cd levels ($P < 0.002$; $r = 0.48$). Furthermore, the hepatopancreatic levels of Cd and Zn were significantly interrelated ($P < 0.05$; $r = 0.34$).

The autoradiograms of the ^{32}P-labeled DNA digest indicated diagonal radioactive zone (DRZ) (Fig. 2). These DRZs are typical for DNA adducts containing aromatic or

TABLE 3

Data on PCBs, HCB, DDT, and DDE (ng/Extractable Lipids), DNA Adducts (DRZ per 10^8 Nucleotides), and Heavy Metals (μg/g wet weight) in the Hepatopancreas of Crayfish Caught at the Four Upstream-to-Downstream Locations of the River Meuse in The Netherlands

	Location A ($n = 10$)	Location B ($n = 10$)	Location C ($n = 8$)	Location D ($n = 10$)
PCB 28	0.102 ± 0.043	0.097 ± 0.036	0.330 ± 0.089[ab]	0.255 ± 0.055[ab]
PCB 52	0.200 ± 0.082	0.268 ± 0.158	0.481 ± 0.145[ab]	0.624 ± 0.146[ab]
PCB 101	1.355 ± 0.586	1.459 ± 0.634	1.874 ± 0.937	3.103 ± 0.821[abc]
PCB 118	0.750 ± 0.335	0.780 ± 0.456	0.694 ± 0.175	1.307 ± 0.378[abc]
PCB 138	2.232 ± 0.828	3.025 ± 1.676	3.030 ± 0.860	4.589 ± 1.052[ab]
PCB 153	3.379 ± 1.286	3.486 ± 1.939	2.810 ± 0.753	5.875 ± 1.250[abc]
PCB 180	2.367 ± 0.912	2.106 ± 1.088	2.041 ± 0.786	4.962 ± 1.273[abc]
Σ PCB	10.385	11.221	11.260	20.715[abc]
HCB	0.020 ± 0.009	0.037 ± 0.018	0.168 ± 0.067[abd]	0.037 ± 0.020
DDT	1.175 ± 0.650	0.503 ± 0.434	0.197 ± 0.045	3.505 ± 1.877[abc]
DDE	0.270 ± 0.100	0.401 ± 0.489	0.356 ± 0.159	0.773 ± 0.651[ab]
Σ DDT + DDE	1.445[c]	0.904	0.553	4.278[abc]
DNA adducts	82.3 ± 57.6	48.0 ± 38.5	88.1 ± 38.6	160.4 ± 89.8[abc]
Cd (μg/g)	0.89 ± 0.51	2.41 ± 1.37[a]	1.48 ± 0.75[a]	1.31 ± 1.00
Zn (μg/g)	36.7 ± 13.5	42.0 ± 11.8	39.0 ± 11.2	36.3 ± 11.6
Cu (μg/g)	411.5 ± 319.0	321.2 ± 148.3	267.4 ± 161.1	721.8 ± 311.4[abc]
Pb (μg/g)	nd	nd	nd	nd

Note. $P < 0.05$ is considered significant; nd, not detectable; abcd, significantly enhanced as compared to, respectively, location A, B, C, D.

bulky hydrophobic moieties and suggest the presence of multiple chemically related adducts. The mean levels of aromatic DNA adducts in crayfish hepatopancreas per area are listed in Table 3. No clear differences in total DNA adduct levels, as reflected by the DRZs, could be observed between crayfish from locations A, B, and C. However, in crayfish sampled at location D, the most upstream sampling site, total DNA adduct levels were significantly enhanced, compared to adduct levels observed in three other locations ($P < 0.05$).

Regression analysis indicated a positive correlation between, respectively, PCB 28 ($P < 0.001$; $r = 0.54$) (Fig. 3), PCB 52 ($P < 0.005$; $r = 0.43$), PCB 101 ($P < 0.05$; $r = 0.42$), and DNA adducts in hepatopancreatic tissue. For the higher chlorinated PCBs, no such correlation was found.

By comparing PCB patterns in the different matrices striking differences were observed. PCB patterns in hepatopancreatic tissue revealed the dominance of penta- to heptachlorobiphenyls. The percentages of total PCBs represented by each chlorobiphenyl (CB) group were as follows: tri-CB, 1.52 ± 1.05; tetra-CB, 2.84 ± 1.24; penta-CB, 20.95 ± 3.18; hexa-CB, 53.71 ± 3.71; and hepta-CB, 20.97 ± 3.11. The dominant hexa-CBs account for 54% of the total PCB burden. The percentages increase from tri- to hexa-CBs and decrease for more chlorinated congeners. In sediments hexa- and hepta-CBs were predominantly found (44.7, respectively, 30.5%) followed by penta-CBs (18.6%). In water tri- and tetra-CBs account for 55.9% of the total PCB burden. Crayfish accumulate high levels of PCBs which are not correlated to the content of PCBs in water and/or sediment compartments.

The general pattern between the metals in the hepatopancreas of crayfish at the four different locations was Pb < Cd < Zn < Cu. This pattern does not reflect the observed pattern found in the environment where Cd < Pb or Cu < Zn were in sediments and Cd < Pb < Cu < Zn in waters.

In summary, highest pollution levels in sediment were observed at locations A and B. In water samples from the different locations the highest metal pollution was observed in location D, whereas the total organochlorine burden was highest in both B and D. However, by comparing the integrated pollution burden of crayfish from the four different sites of the river Meuse, location D appeared to be the most polluted site.

DISCUSSION

The river Meuse is of great importance for The Netherlands especially since one of its major function is the supply

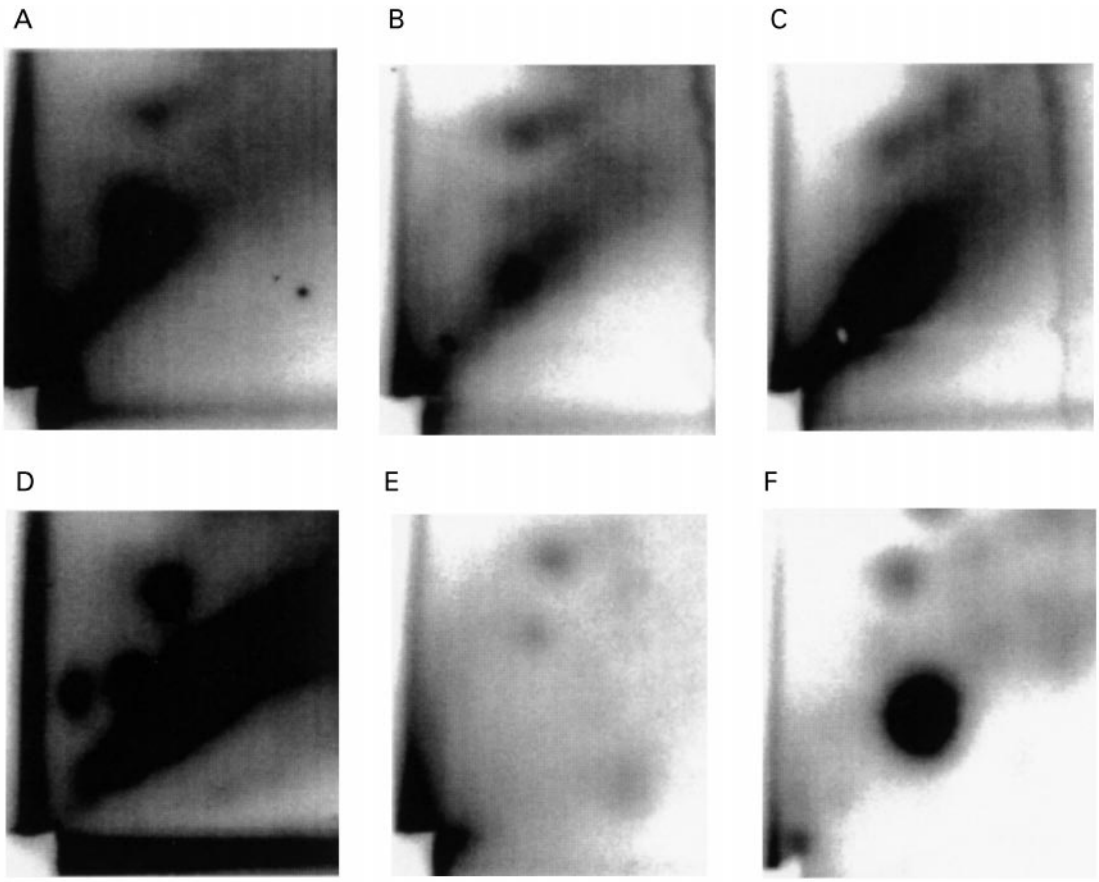

FIG. 2. Representative DNA adduct profiles of ^{32}P-postlabeling DNA digests from hepatopancreas of crayfish derived from (A) Borgharen; (B) Linne; (C) Belfeld; (D) Boxmeer; (E) blank. (F) (+)-anti-benzo[*a*]pyrene-diol-epoxide (BPDE)-DNA standard (level of modification $1:10^7$). The origin (bottom left-hand corner of the chromatograms) was excised after chromatography. Adduct profiles on the chromatograms were visualized and quantitated by using phosphor image analysis (Molecular Image; Model GS-250, Molecular Dynamics, Sunnyvale, CA). For comparison, (E) a blank sample (no DNA), and (F) the adduct resulting from ^{32}P-postlabeling of calf thymus DNA reacted *in vitro* with BPDE, which is the main reactive metabolite of benzo[*a*]pyrene. In each experiment [^{3}H]BPDE-DNA standards with known modification levels (1 adduct per 10^7–10^9 nucleotides) were run parallel for quantitation purposes.

of drinking water for approximately five million people (De Ruyter van Stevenick *et al*., 1990). However, recent measurements have indicated that the water quality of the river Meuse is far above European standards of drinking water, especially with regard to concentrations of trace metals and organic micropollutants, in both the sediments and the water phase (Ploco, 1995). The main pollution in the Dutch part of the river Meuse orginates from diffuse sources. In many cases, the predominant contamination sources are agricultural activities. In addition to agricultural pollution, a significant amount of the diffuse pollution is derived from urban areas (Ploco, 1995). Moreover, many chemical industries located along the river discharge predominantly metals and organic micropollutants into the Meuse (Ploco, 1995).

Water quality with respect to both organic and inorganic pollution can be assessed on the basis of chemical measurements, but this often yields limited information about the bioavailability of these substances (Kraak *et al*., 1991). In biomonitoring studies it may, therefore, be more reliable to analyze indigenous organisms such as crayfish, since they have been exposed for their entire lifetime and may therefore give a reflection of the contamination history of a particular location (Kraak *et al*., 1991).

Anthropogenic emissions of metals into the environment have increased enormously since the industrial revolution (Merian, 1991). For Cd and Zn, nonferrous smelters and secondary production plants are the main sources (Waalkes *et al*., 1992; Merian, 1991), whereas Pb is predominantly derived from automobile emissions (Hoffmann and Wynder, 1977; Merian, 1991). Crayfish have often been considered (biological) indicator species of the heavy metal pollution present in the aquatic environment (Stinson and Eaton, 1983; Anderson and Brower, 1978; Evans, 1980; Diaz-Mayans *et al*., 1986; Madigosky *et al*., 1991). In several

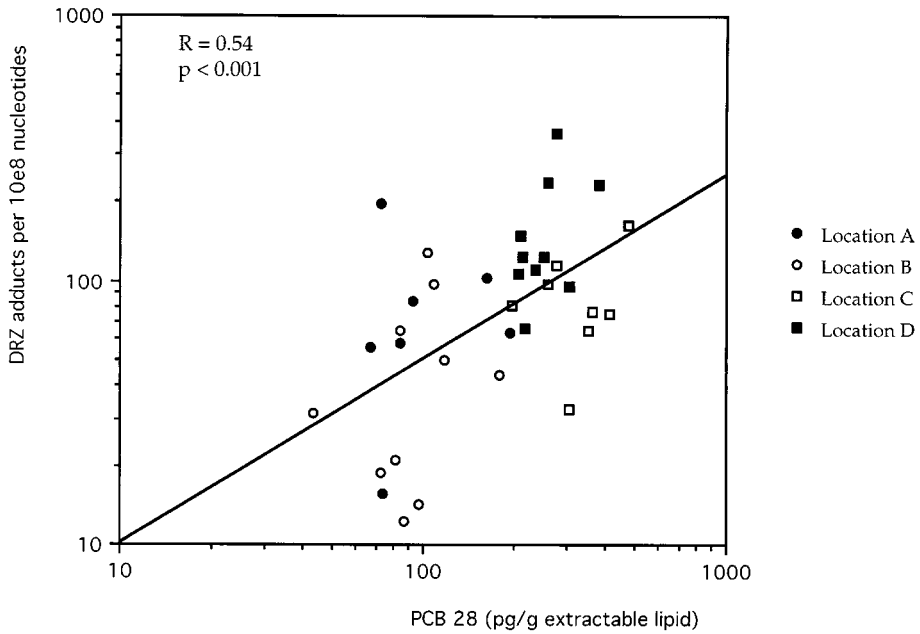

FIG. 3. Correlation between DNA adducts (per 10^8 nucleotides) and PCB 28 concentrations (ng/g extractable lipid) in hepatopancreas of crayfish from locations A, B, C, and D.

studies on heavy metal pollution concentrations of metals in various tissues of crayfish were analyzed. In most studies the highest concentrations of each metal were observed inside the hepatopancreatic tissue (Torreblanca *et al*., 1991; Bagatto and Alikhan, 1987; Madigosky *et al*., 1991). In crustacea species, the hepatopancreas is the main regulatory organ and as such it would be the prime site for metal accumulation and/or detoxification in these animals (Bagatto and Alikhan, 1987).

For all sites along the river Meuse, the concentration of Cu in the hepatopancreas was higher than that of Zn, the concentration of Zn was higher than Cd, and Pb was not detectable in the hepatopancreas of crayfish. In previous studies, relatively high concentrations of Cu in the hepatopancreas of crayfish were observed, reflecting the storage capacity of this organ for Cu (Stinson and Eaton, 1983; Bagatto and Alikhan, 1987). The ability of crayfish to accumulate Cu was apparent from the fact that tissue concentrations were several times higher than water and/or sediment concentrations of Cu. Moreover, the concentrations of Cu were several orders of magnitude higher than those of Cd or Zn and Pb. Regulation of Cu concentrations might be related to the essential biochemical role of this metal in the production of the respiratory protein hemocyanin (Bagatto and Alikhan, 1987). Highest Cu concentrations were observed in crayfish derived from location D followed by locations A, B, and C. In water samples a similar pattern was observed. In sediment samples, on the contrary, lowest Cu concentrations were observed in location D. However, the limits within which Cu is regulated are closely related to the average concentration of all copper sources available in a particular habitat, explaining the relatively high Cu concentrations found in the hepatopancreas of crayfish from location D. In the crayfish *Orconectus virilis* derived from Ramsey lake and Joe lake (Ontario), both heavily contaminated sites, Cu concentrations were comparable to the observed values in location D, while in Wizard lake (Ontario), an uncontaminated site, the Cu concentrations of crayfish fit within the range observed in locations A, B, and C (Bagatto and Alikhan, 1987).

Zn is an essential trace element and similar concentrations in hepatopancreatic tissue of crayfish of all four sampling sites were observed. It has been previously reported that the hepatopancreas of crayfish is capable of absorbing Zn and regulating the body Zn content, maintaining it at a consistent level (Anderson and Brower, 1978). Zn levels in the current study fit well within the range reported for crayfish caught in the Fox river (Illinois) (Anderson and Brower, 1978). The Zn levels in hepatopancreatic tissue did not correlate with Zn concentrations in water and/or sediment. The concentrations of Zn observed in sediments from these areas were several orders of magnitude higher than those found in crayfish tissue (10–40 times), underscoring the capability of crayfish to regulate Zn body content.

Unlike Cu and Zn, Cd and Pb are biologically nonessential metals that enter the animal following the same biochemical pathways as those of essential elements with chemical similarity (Bagatto and Alikhan, 1987). Cd has

been found to be assimilated by the same route as Cu (Bagatto and Alikhan, 1987). Metals such as Pb and Cd are cumulative toxins in both aquatic and terrestrial ecosystems. Continuous exposure to even low concentrations may result in bioaccumulation and resulting health consequences (Madigosky *et al*., 1991).

Cd concentrations in hepatopancreas of crayfish from the four different locations exhibited significantly enhanced Cd levels at both location B and C compared to location A ($P < 0.05$). The observed values fit within the range observed in the crayfish *Procambarus clarkii* collected from contaminated roadside ditches in Louisiana (Madigosky *et al*., 1991).

In sediment samples, on the contrary highest Cd concentrations were observed in location A compared to the three other sampling sites, whereas in water samples highest Cd levels were observed in site D. The Cd concentrations in sediment were several times higher (2–10 times) than those observed in crayfish hepatopancreatic tissue, which might be due to the relatively low bioavailability of Cd from sediments. Pb concentrations in crayfish were below the detection limit (< 5 ng/mg hepatopancreatic tissue).

The discrepancy in Cd concentrations in crayfish and sediments might also be due to metal interactions. In combined exposure, metal compounds may interact with each other, by influencing each other's absorption, distribution in the organism biotransformation, and/or excretion rate (Telisman, 1995, Beyersmann, 1994). The composition of the chemical microenvironment of a metal has, therefore, a major impact on the bioavailability of a certain metal (Beyersmann, 1994). In this study, the concentrations of Cd and Zn in the hepatopancreas of crayfish were significantly interrelated. Moreover, a significant correlation was observed between Cu and Cd levels in hepatopancreatic tissue.

Apart from inorganic pollution by metal compounds, contamination of the aquatic environment by organic xenobiotics such as PCBs seriously affects several functions of the river. When comparing the different locations with each other, it becomes obvious that crayfish from location D are much more contaminated with organochlorine compounds than animals from the three other locations. The total organochlorine burden of crayfish from location D was, on average, 20.7 ng/g extractable lipids in contrast to, on average, 11.0 ng PCBs/g extractable lipids in the three other locations. Detected PCB values fall within the range of those reported in crab and mussel derived from the Catalan Mediterranean coast, whereas in fish derived from this site, much higher values were observed (Porte and Albaiges, 1993).

All the crayfish exhibited a similar pattern of PCB congener distribution with hexachlorobiphenyl being the most abundant congener. The PCBs of lower chlorine contents (tri- and tetra-CBs) constitute a much larger fraction of total PCB burden in water, whereas the concentrations of higher chlorine content (hexa- and hepta-CBs) are predominantly present in sediment. Organisms reveal higher contributions of hexa-CBs and lower percentages of hepta-CBs than sediments which may reflect the differences in bioaccumulation rates of the congeners, increasing from tri- to hexa-CBs followed by a substantial reduction for more chlorinated CBs, which have an unfavorable stereochemistry. This finding is in agreement with the PCB congener distribution in fish derived from the river Seine (Chevreuil *et al*., 1995). The predominance of highly chlorinated PCBs in crayfish also reflects the low rate of metabolism of these highly substituted congeners.

Both PAHs and several relevant PCB congeners are capable of forming DNA adducts after metabolic activation which can be detected by the ^{32}P-postlabeling technique (Morales and Matthews, 1979; Oakley *et al*., 1996; Amaro *et al*., 1996; Shimada, 1978; Kurelec *et al*., 1988; Schut and Shiverick, 1992; Van der Oost *et al*., 1994). Since the postlabeling assay is capable of detecting complex mixtures of aromatic compounds, it appears difficult to separate PCB-DNA adducts from PAH-DNA adducts (Liu *et al*., 1991). It is, therefore, extremely difficult to establish a causal relationship between DNA adducts and one of the components encountered in the environment, which becomes even more difficult since interactions between different pollutants may occur, resulting in synergistic and/or antagonistic effects. It has been suggested that the extent of binding of carcinogenic xenobiotics to DNA in organisms may serve as a dosimeter at the target site level, although the nature of these adducts remains unknown. In the present study, relatively high DNA adduct levels in crayfish hepatopancreatic tissue were observed. In line with these results, other ^{32}P-postlabeling DNA adduct analyses have found similar DRZs in fish obtained from polluted sites (Dunn *et al*., 1987; Varanasi *et al*., 1989; Liu *et al*., 1991). In the current study, a diffuse diagonal zone of aromatic DNA adducts was found, indicating a broad spectrum of adducts (Fig. 2). DRZ adduct levels of crayfish derived from location D were significantly enhanced, compared to the DNA adduct levels of the other locations ($P < 0.05$). PCB concentrations were also significantly enhanced in crayfish from location D, compared to the three other sampling sites. Of particular interest is, therefore, the observed significant correlation between DNA adducts and PCB content in the hepatopancreas of crayfish: by correlating the individual PCB congeners with DNA adducts levels significant correlations were observed for the lower chlorinated congeners (PCB 28–PCB 101), whereas for the higher chlorinated PCBs no such correlations were found, which is in agreement with the low metabolism of the latter. Lower chlorinated PCBs have been found to be metabolized into reactive metabolites (quinones and semiquinones) capable of forming PCB-DNA adducts (Oakley *et al*., 1996; Shimada, 1978; Morales and Matthews, 1979). Although the exact nature of the DNA

adducts is unknown, part of the DNA adducts might be derived from PCBs with low chlorine content. No correlation between PAH concentrations in sediment and DNA adduct levels could be observed.

By comparing the xenobiotic levels in crayfish from various locations of the river Meuse, location D appeared to be the most polluted site, compared to the three upstream situated locations, as is also reflected by hepatopanceatic DNA adduct levels.

Exposure of crayfish to persistent pollutants is mainly via the foodchain. Crayfish, however, also live in close contact with sediment. The sediment–organism interaction could, therefore, contribute to the contamination of crayfish. In general, sediment concentrations of pollutants are higher than levels in the river water due to the hydrophobic characteristics of these organic pollutants. Previous studies have found that pollutants bound to sediment are far less bioavailable than those in solution (Muncaster *et al*., 1990). In this study, it was not possible to correlate pollution burden in crayfish to sediment and/or water values. This discrepancy between pollutants levels in water, sediment, and crayfish, therefore, indicates that for chemical quality assessment in river systems an integrated approach is necessary, analyzing levels of relevant pollutants in water and sediments as well as biotic compartments.

CONCLUSION

From these first results, it appears that crayfish can be used for the biomonitoring of exposure to both inorganic and organic pollutants of river systems. The most striking findings are the regional differences of Cu and PCB pollution, as reflected in the body load of the crayfish. Moreover, the contribution from aromatic organic compounds can be determined on the basis of DNA adduct formation in hepatopancreatic tissue which also resulted in marked regional differences, as has been demonstrated in this study. It is therefore suggested that for chemical quality assessment in river systems both biotic and abiotic compartments should be analyzed for levels of relevant pollutants. Crayfish can be used for this purpose, as has been presented in this study.

ACKNOWLEDGMENT

The authors thank Dr. GECM Strolenberg for technical assistance and helpful suggestions on preparing the crayfish.

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