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Contaminated sediments and bioassay responses of three macroinvertebrates, the midge larva *Chironomus riparius*, the water louse *Asellus aquaticus* and the mayfly nymph *Ephoron virgo*

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Abstract

Bioassays are widely used to estimate ecological risks of contaminated sediments. We compared the results of three whole sediment bioassays, using the midge larva *Chironomus riparius*, the water louse *Asellus aquaticus*, and the mayfly nymph *Ephoron virgo*. We used sediments from sixteen locations in the Dutch Rhine-Meuse Delta that differed in level of contamination. Previously developed protocols for each bioassay were followed, which differed in sediment pretreatment, replication, and food availability. The *Chironomus* bioassay was conducted in situ, whereas the other two were conducted in the laboratory. The measured endpoints, survival and growth, were related to contaminant levels in the sediment and to food quantity in water and sediment.

Only the response of *A. aquaticus* in the bioassay was correlated with sediment contamination. Food availability in overlying water was much more important for *C. riparius* and *E. virgo*, thereby masking potential sediment contaminant effects. We conclude that growth of *A. aquaticus* was depressed by sediment contamination, whereas growth of *E. virgo* and *C. riparius* was stimulated by seston food quantity. We discuss that the trophic state of the ecosystem largely affects the ecological risks of contaminated sediments.

Keywords: Food; Sediment contamination; Survival; Growth; Whole sediment bioassay

1. Introduction

Sediment contamination is a serious problem in river sedimentation areas, such as floodplain lakes, delta areas and estuaries. Sediments in the Rhine-Meuse Delta in the Netherlands are moderately to heavily polluted with

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trace metals and organic contaminants like polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Den Besten et al., 1995; Reinhold-Dudok van Heel and Den Besten, 1999).

The relationship between sediment toxicity and effects on biota are studied through various approaches. These include studies into the effects of sediment contamination on in situ macroinvertebrate communities (e.g. Schlekat et al., 1994; Pinel-Alloul et al., 1996; Van Griethuysen et al., 2004), and effects on single species in situ and in laboratory bioassays (e.g. Chappie and Burton, 1997; EPA, 2000; OECD, 2001; Den Besten et al., 2003; Burton et al., 2005a). Biotic responses are usually related to total contaminant concentrations, which give good estimates of the degree of pollution, but may not be fully available to biota. Several extraction techniques, such as extraction with Tenax® beads for organic contaminants (Cornelissen et al., 2001) and the AVS/SEM method for trace metals (Di Toro et al., 1990), have been applied to estimate the pollution that is available for organisms. These concentrations are usually termed bioavailable concentrations.

Many invertebrate species are used worldwide in risk assessment procedures, and their use is described in standardized protocols (e.g. EPA, 2000; OECD, 2001). The outcome of risk assessment procedures will depend on the choice of species used in a bioassay. Several studies have shown that different species can have different responses to the same sediment contamination (e.g. Lyytikäinen et al., 2001; Ingersoll et al., 2002; Milani et al., 2003). Several criteria should be considered when selecting a species. First, the ecology of the test animal is of importance. The test animal should be a sediment dweller or feeder and should be exposed to contaminants in sediment and/or in pore water in nature (Wang et al., 2004). Second, the uptake route of the contaminant is of importance. Animals can obtain contaminants from sediments, from food, and from overlying water. The relative importance of these three routes of exposure depends on the contaminant and on the ecology of the test animal (Wang et al., 2004). Recent studies suggest that species commonly used for sediment toxicity testing do not meet the above mentioned criteria (e.g. Warren et al., 1998; Hare et al., 2003; Wang et al., 2004).

The present study compares the effect of sediment contamination on survival and growth of the midge *Chironomus riparius*, the mayfly *Ephoron virgo* and the water louse *Asellus aquaticus*.

Chironomid larvae are widely used test organisms in acute and chronic sediment toxicity tests (e.g. EPA, 2000; OECD, 2001). Larvae of *C. riparius* are opportunistic tube-dwelling deposit feeders, feeding mainly on detritus and organic matter present in the sediment (Armitage et al., 1995). *C. riparius* larvae are sensitive to food deficiency and addition of (usually artificial) food in bioassays is often necessary to exclude reduction in survival or growth due to food deficiency. However, this may mask toxic effects, especially when advantages of organic enrichment prevail against the potential adverse effects of the toxicants (e.g. Ristola et al., 1999; De Haas et al., 2002). A recent study showed that *C. riparius* selectively feeds on added food, and is therefore capable of avoiding contaminated sediments in standard bioassays (Åkerblom and Goedkoop, 2003). Studies into the different uptake routes of contaminants revealed that for *Chironomus staegeri* the main uptake route of Cd was the overlying water with associated particles (Warren et al., 1998; Hare et al., 2001).

The mayfly *E. virgo* has been used recently in (sediment) toxicity tests (Van der Geest et al., 2000; De Haas et al., 2002). The first-instar nymphs live freely on and in the sediment, feeding on fine particulate organic matter. Later instars burrow U-shaped tubes in the sediment, filtering food from the water, such as detritus and algae (Kureck and Fontes, 1996). Results from bioassays with seven different floodplain lake sediments showed that *E. virgo* seemed to be an appropriate test organism for sediment toxicity bioassays since it responded to the toxicant levels in the sediment rather than to nutritional value (De Haas et al., 2002). No information is available on the different uptake routes of contaminants for *E. virgo*.

The water louse *A. aquaticus* is proposed as a suitable species for use in sediment toxicity tests, since it is in continuous contact with the sediment (McCahon and Pascoe, 1988). It is a shredder that feeds on detritus with associated fungi, bacteria and periphyton (Marcus et al., 1978; Graça et al., 1993a,b). *A. aquaticus* is not routinely used as a test species for field sediments, but it has been used in a large number of laboratory toxicity studies (e.g. Migliore and De Nicola Giudici, 1990; Peeters et al., 2000). Comparative studies indicate that *A. aquaticus* has an intermediate sensitivity to contaminants (Van Hattum, 1995). Studies into the different uptake routes of contaminants show that both water, sediment and food are important. *A. aquaticus* could easily accumulate Cd from water and food, however that study was conducted in the absence of sediment (Van Hattum et al., 1989). Another study using stable isotope tracers showed that *A. racovitzai* accumulated Cd primarily from water (Eimers et al., 2001, 2002). In contrast, PAHs were accumulated mostly from sediments in *A. aquaticus* (Peeters et al., 2000).

These three species were used in bioassays following internal protocols developed at our laboratories. We used sediments from sixteen locations in the Dutch Rhine-Meuse Delta (Fig. 1), as part of a larger study described elsewhere (De Lange et al., 2004). The measured endpoints in the bioassays were survival and growth. These endpoints were related to contaminant levels in the sediment and to food quality parameters in water and sediment.

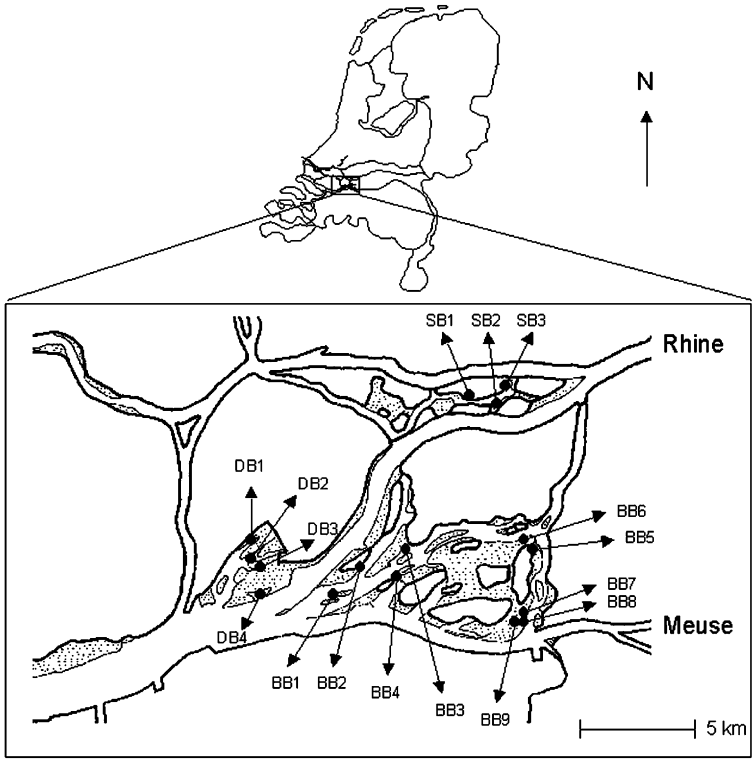

Fig. 1. Sampling locations in the Sliedrechtse Biesbosch (abbr. SB), Dordtsche Biesbosch (abbr. DB) and Brabantse Biesbosch (abbr. BB).

The aims of this study were (1) to measure survival and growth on field sediment in relation to measured sediment contamination and sediment and water food characteristics and (2) to compare the results of the three bioassays and discuss their usefulness for risk assessment.

2. Methods

2.1. Locations and sampling

Sixteen locations were selected in three different parts of the Biesbosch, influenced by the river Rhine only (Sliedrechtse Biesbosch (SB) [$n = 3$] and Dordtsche Biesbosch (DB) [$n = 4$]) or the rivers Rhine and Meuse combined (Brabantse Biesbosch (BB) [$n = 9$]) (Fig. 1). Locations are described in De Lange et al. (2004), and were selected to have different contamination levels based on previous research (Den Besten et al., 1995; Reinhold-Dudok van Heel and Den Besten, 1999). In April 2001, the upper 5 cm of the sediment was sampled using an adjusted Ekman grab; three grabs were taken, pooled and immediately distributed over different storage pots for sediment characteristics, contaminant analyses and bioassays. Care was taken that the storage containers for acid volatile sulfide (AVS) analysis and *A. aquaticus* bioassay were filled without headspace, to ensure minimal disturbance of redox conditions. The sediment for *C. riparius* bioassay was directly used, all other samples were stored frozen (−20 °C). Surface water samples (20 l) were taken back to the laboratory for measurements of characteristics and for use in the *E. virgo* bioassay.

2.2. Surface water and sediment analyses

Surface water characteristics, sediment characteristics and sediment contaminants were analysed according to De Lange et al. (2004). Sediment contamination (5 trace metals, 13 PAHs and 19 PCBs) was estimated both as total concentration and as bioavailable concentration. For trace metals, the AVS/SEM model was used to estimate the bioavailability of trace metals (Di Toro et al., 1990). Acid volatile sulfides (AVS) and simultaneously extracted metals (SEM = sum of Cd-SEM, Ni-SEM, Pb-SEM, Cu-SEM and Zn-SEM) were analysed according to Van Griethuysen et al. (2002), (SEM–AVS)/fOC, the difference between extracted metals and sulfides, normalized on the fraction organic carbon is proposed

as a proxy for trace metal bioavailability (DiToro et al., unpublished). For PAHs and PCBs, the bioavailable concentration was estimated as the rapidly desorbing fraction as extracted by Tenax® beads in 6 h (Cornelissen et al., 2001).

An average contamination rank was calculated to order the locations from least polluted to most polluted (described in De Lange et al., 2004). In brief: locations were ranked separately for total PAHs, total PCBs, and trace metals. These 3 ranks for each location were averaged, resulting in an overall contamination rank from least contaminated (rank 1) to most contaminated (rank 16).

2.3. C. riparius field bioassay

Survival and growth of *C. riparius* was determined in a 1-month field experiment (method described in Den Besten et al., 2003). In brief: 1 day prior to the start of the experiment, second instar *C. riparius* larvae, obtained from a laboratory culture, were counted in sets of 70. At the start of the experiment, sediment was collected on site and sieved through a 500-μm mesh. On each site, three cages (volume 1100 ml) were prepared and filled with 400 ml sieved sediment and 70 larvae. Openings covered with 150-μm mesh size allowed the exchange of water. This inner cage was placed in a protective outer cage. All cages were connected to a pole and positioned just above the sediment. Larvae were thus exposed to the field conditions, i.e. sediment quality, water quality, temperature and light. After 1 month larvae were collected from the cages, counted, and total weight per replicate cage was determined.

2.4. E. virgo bioassay

Survival and growth of the mayfly nymph *E. virgo* on field sediments was determined in a 10-d experiment (method described in De Haas et al. (2002)). Sediment was frozen until the bioassay was performed, within 5 months after sampling. In brief: first instar nymphs (<48 h old) were obtained from field-collected eggs. Sediments were thawed 4 days prior to the start of the experiment. One day before the start of the experiment three replicate glass jars (150 ml) with 25 ml wet homogenized sediment and 100 ml filtered site water (passed twice through 30 μm filter) were prepared and aerated overnight. Control treatments contained 25 ml quartz sand and 100 ml Elendt-M7 medium (OECD, 2001), and were fed according to De Haas et al. (2002). Twenty first instar nymphs were randomly transferred into each test vessel. In addition, the body length of twenty nymphs was measured individually. The experiments were conducted in a temperature controlled room with a temperature of 20 ± 1 °C, moderate light (10 μmol m^{-2} s^{-1}) at a 16:7 L:D regime with 30 min of twilight

Table 1
Overview of environmental variables used in this study: with SD = standard deviation, COV = coefficient of variation, AFDW = ash free dry weight, DW = dry weight, fOC = fraction organic C, C/N ratio as molar ratio, SEM = sum of Cd-SEM, Ni-SEM, Pb-SEM, Cu-SEM and Zn-SEM, PAHs = sum of 13 PAHs, and PCBs = sum of 19 PCBs

Variable	Unit	Average	Minimum	Maximum	SD	COV
Seston						
Chlorophyll *a*	μg l^{-1}	36.2	9.1	142.4	43.1	119.1
AFDW	mg l^{-1}	10.1	2.0	23.5	7.6	75.6
Sediment						
Silt	vol.%	47.5	11.1	88.2	28.0	58.9
N	% DW	0.2	0.0	0.4	0.1	67.0
P	% DW	0.2	0.0	0.9	0.2	95.3
Organic C	% DW	3.2	0.4	7.5	2.0	62.6
C/N ratio	–	39.3	21.7	82.0	17.9	45.6
Sediment-bound contaminants						
Total Cd	mg kg^{-1} DW	5.5	1.3	15.2	4.3	77.6
Total Ni	mg kg^{-1} DW	26.2	6.8	82.0	17.8	68.0
Total Pb	mg kg^{-1} DW	109.1	28.7	297.6	73.0	66.9
Total Cu	mg kg^{-1} DW	51.6	10.8	175.8	41.5	80.4
Total Zn	mg kg^{-1} DW	647.8	175.0	1652.4	396.3	61.2
SEM	μmol g^{-1} DW	8.4	2.3	24.1	5.7	67.4
SEM–AVS/fOC	μmol g^{-1} OC	−316	−1257	292	422	−134
PAHs	mg kg^{-1} DW	7.8	0.8	21.3	5.7	72.9
PCBs	mg kg^{-1} DW	0.3	0.0	1.6	0.4	148.0
Tenax-PAHs	mg kg^{-1} DW	0.3	0.0	1.4	0.4	109.4
Tenax-PCBs	mg kg^{-1} DW	0.1	0.0	1.0	0.2	344.8

before and after each light period. During the experiments, the test systems were constantly aerated.

Nymphs were collected from the sediment at the end of the test, counted and body length was measured with a Leica Image Analyser using the computer program Research Assistant 3 (RVC, Hilversum, The Netherlands). Growth was calculated by subtracting the average initial length from the individual final length.

2.5. A. aquaticus bioassay

Survival and growth of the water louse *A. aquaticus* on field sediments was determined in a 28-d experiment (method modified from Peeters et al. (2000)). Bioassays were performed within 3 months after sampling. Asellids in the size class 4–5 mm were obtained from a culture that had been kept for several months in the laboratory. The origin of this culture was a non-polluted ditch in Wageningen, the Netherlands. Sediments were thawed at 4 °C 4 days before the start of the experiment. Samples were placed in a glovebag under nitrogen, to ensure anaerobic conditions. Thirty millilitre of sediment was placed in a 100-ml jar, with 10 replicate jars for each treatment. Immediately after removal from the glovebag, 40 ml oxygen-free (nitrogen purged) Dutch Standard Water (DSW; Netherlands Normalisation Institute, 1980) was added. The samples were then exposed to air to allow passive reaeration of water and sediment in each jar. Control treatment contained 30 ml quartz sand mixed with 10% organic material (decomposing beech leaves) and 40 ml oxygen-free DSW. After 7 days, when oxygen levels were above 70% saturation, 1 *A. aquaticus* was added in each jar. The jars were placed in a temperature controlled room with a temperature of 17 ± 0.5 °C in dim light (6 μmol m^{-2} s^{-1}) at 16:8 L:D rhythm.

Initial and final length of each individual was measured with a Leica Image Analyser. Growth was calculated by subtracting the individual initial length from the individual final length.

2.6. Statistical analyses

Correlations between survival, growth, and environmental variables were determined with Spearman's ρ correlation coefficient, using SPSS® 10.1 for Windows (SPSS, Chicago, IL, USA). Rank correlations were chosen to reduce the influence of extreme values on results of the correlation analyses.

3. Results

The sixteen locations show a range in seston characteristics, sediment characteristics and contaminants (Table 1). Locations in the SB were the most contaminated, and locations in the BB had the largest range of contaminant concentrations. The least contaminated location was BB1, and the most contaminated was DB1.

Survival in the *C. riparius* bioassay ranged from 2% to 60% (Fig. 2), and did not correlate significantly to any of the measured variables (Table 2). Growth in the treatments varied from 10 to 200 μg ind^{-1}, and was positively correlated with seston chlorophyll *a* concentration (Table 2).

E. virgo survival varied from 0% to 80% (Fig. 2); survival in the control was 90%. Survival was positively correlated with seston AFDW and chlorophyll-*a*, and sediment Cu, PAHs and contamination rank (Table 2). Survival was <10% when seston AFDW concentration

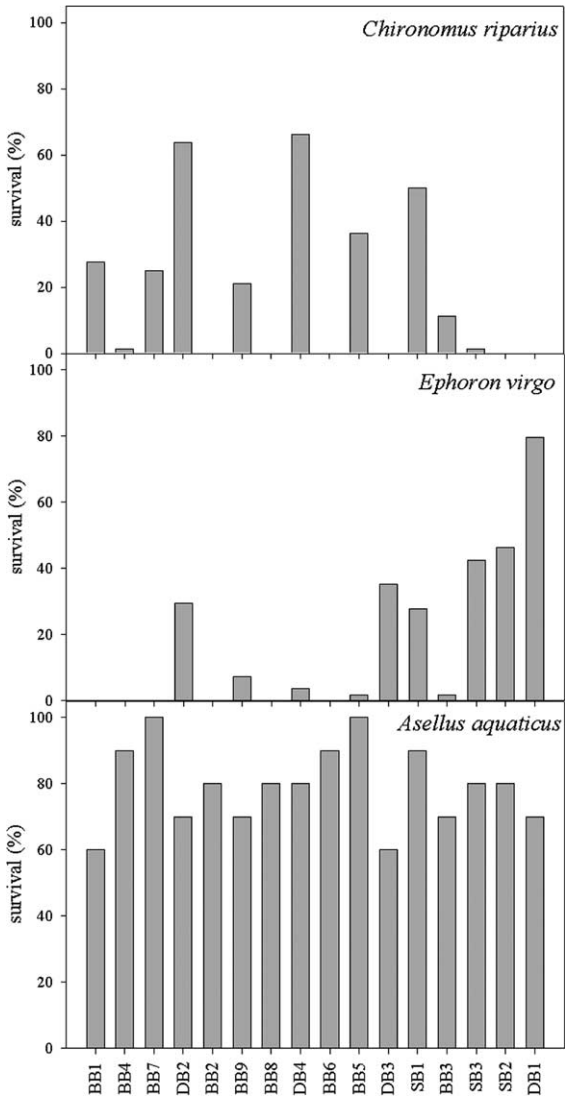

Fig. 2. Survival (%) of *C. riparius* (top panel), *E. virgo* (middle panel), and *A. aquaticus* (lower panel). Locations are ranked from least polluted (left) to most polluted (right).

was below 10 mg l^{-1}, survival was above 20% when seston AFDW concentration was above 10 mg l^{-1} (Fig. 4). Surviving *E. virgo* nymphs had quite high growth rates in the treatments (Fig. 3), control growth during the experiment was 271 μm ind^{-1}. Nymph growth in the treatments showed a positive pattern with seston food abundance, reaching growth similar to that in the controls at concentrations above 10 mg AFDW l^{-1} (Fig. 4). The calculated Spearman correlation coefficient ρ was 0.47, but due to the limited number of values ($n = 9$) this coefficient was not significant (Table 2).

A. aquaticus survival ranged from 60% to 100% (Fig. 2), with 100% survival in the controls. Survival in the treatments was negatively correlated with (SEM–AVS)/fOC (Table 2, Fig. 5). Control growth during the experiment was 50 mm ind^{-1}. Growth in the treatments was negatively correlated with sediment OC, total concentrations of trace metals (Cd, Ni, Pb, Cu, Zn) and SEM (Table 2, Fig. 5).

4. Discussion

Sediment contamination in our study area was moderate to severe (Table 1). However, none of the 16 locations tested resulted in complete mortality for all three bioassays simultaneously. Previous bioassays performed in the same area resulted in a weak response (Reinhold-Dudok van Heel and Den Besten, 1999). We may conclude that despite the high total concentrations of contaminants, the effect on organisms in bioassays is limited, indicating that a large fraction of the contaminants is not available for organisms. This is corroborated by our sediment analysis (see Table 1): bioavailable concentrations of PCBs ranged from 0.4% to 28% of total concentrations, bioavailable PAHs ranged from 0.8% to 10% of total concentrations.

The three bioassays in this study show different correlations with contaminant and environmental variables. Overall performance (survival and growth) of *A. aquaticus* was negatively correlated with SEM and AVS properties of the sediment. This supports the notion that AVS and SEM properties are better related to biological effects than total trace metals (e.g. Ankley et al., 1996; Van Griethuysen et al., 2004). This was proven in a recent field study where total Zn concentrations in freshwater sediments were manipulated; the AVS–SEM model could predict chronic toxic effects on the benthic community, whereas no relation was observed between total Zn concentration and benthic effects (Burton et al., 2005b).

Table 2
Spearman's ρ correlation coefficients between growth and survival in the different bioassays and seston food, sediment characteristics, and sediment-bound contaminants

	Chironomus riparius		*Ephoron virgo*		*Asellus aquaticus*	
	Survival $n = 16$	Growth $n = 16$	Survival $n = 16$	Growth $n = 9$	Survival $n = 16$	Growth $n = 16$
Seston						
Chl-*a*	0.28	**0.55**	**0.55**	0.32		
AFDW	0.39	0.21	***0.69***	0.47		
Sediment						
Silt	0.05	0.00	0.38	0.38	0.24	−0.44
N	−0.11	0.12	0.37	0.28	0.07	−0.45
P	0.19	−0.06	0.42	−0.10	−0.47	0.18
OC	−0.24	0.08	0.36	0.03	0.29	**−0.51**
C/N ratio	0.11	0.01	−0.18	−0.47	−0.12	0.31
Sediment-bound contaminants						
Total Cd	−0.20	−0.02	0.41	−0.08	0.22	**−0.51**
Total Ni	0.04	−0.17	0.20	0.32	0.42	**−0.66**
Total Pb	−0.20	0.12	0.43	0.02	0.19	**−0.59**
Total Cu	−0.03	0.05	**0.59**	0.30	0.11	**−0.54**
Total Zn	−0.27	0.21	0.45	−0.12	0.09	**−0.57**
SEM	−0.23	0.06	0.44	0.08	0.15	**−0.53**
(SEM–AVS)/fOC	0.14	0.19	−0.10	−0.03	**−0.54**	0.19
PCBs	0.17	−0.09	0.66	0.38	−0.08	−0.32
PAHs	0.02	−0.13	**0.62**	−0.03	−0.03	−0.39
Tenax-PCBs	−0.47	0.39	0.44	−0.40	−0.18	−0.20
Tenax-PAHs	−0.24	0.05	0.36	0.17	0.28	−0.41
Contamination rank	0.05	−0.04	***0.73***	0.17	−0.06	−0.40

Underlined values indicate $p < 0.10$; bold values indicate $p < 0.05$, bold and italic values indicate $p < 0.01$. Since the *Asellus* bioassay was conducted with artificial medium, no correlations with seston variables were calculated.

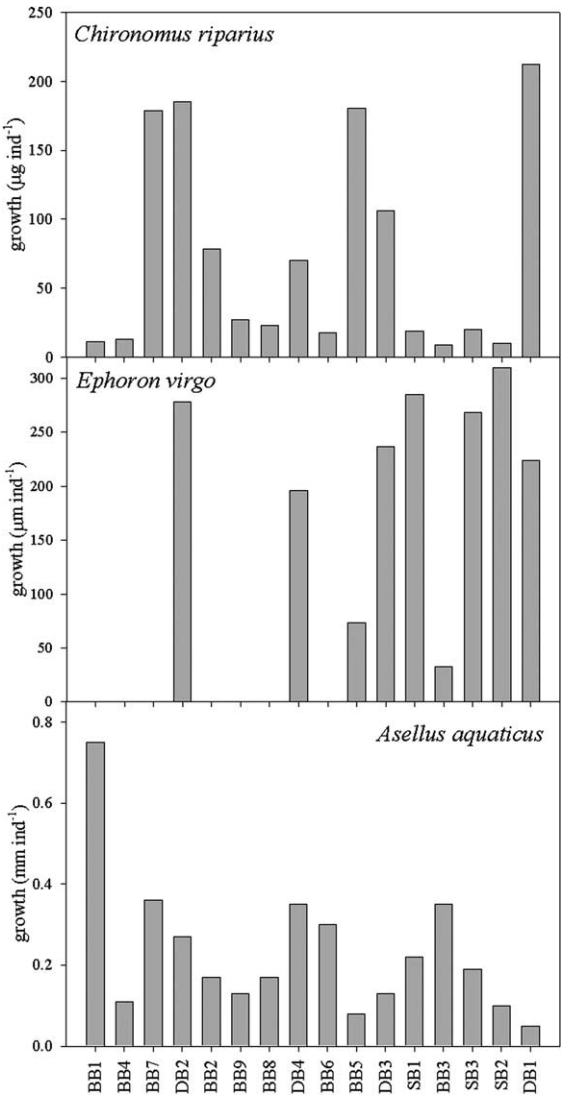

Fig. 3. Individual growth of *C. riparius* (top panel), *E. virgo* (middle panel), and *A. aquaticus* (lower panel). Locations are ranked from least polluted (left) to most polluted (right). Note the different units on the *y*-axis.

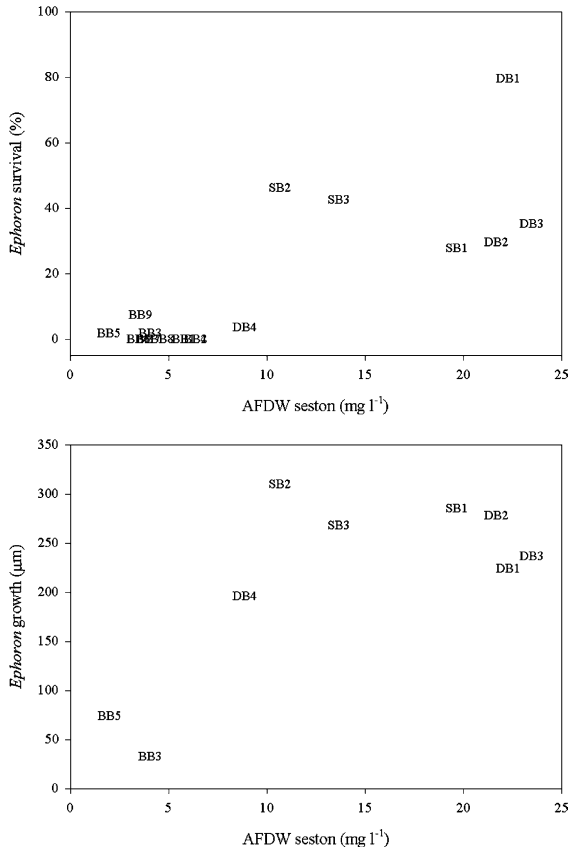

Fig. 4. Results *E. virgo* bioassay: survival (upper panel) and growth (lower panel) versus ash free dry weight (AFDW) seston.

Performance of *E. virgo* and *C. riparius* was stimulated by seston food abundance, masking potential sediment toxicity effects. Our results confirm that *C. riparius* is highly dependent on food availability in the water phase (e.g. Ristola et al., 1999) because growth of *C. riparius* correlated positively with food available in the water phase. More surprisingly, performance of *E. virgo* was also better at higher seston food concentrations. Since seston food availability varied largely amongst locations, interpretation of the results for sediment risk assessment became difficult.

De Haas et al. (2002) proposed that *E. virgo* is an appropriate test organism for sediment toxicity. Our results indicate that it may not be that appropriate. In our study, food quantity in the sediment was lower than in the study by De Haas et al. (2002), and concentrations of sediment-bound contaminants were higher. Our results suggest that this sediment food limitation acted stronger than the concentrations of sediment-bound contaminants on *E. virgo* survival and growth, since food from the water phase could enhance survival and growth, irrespective of sediment contamination.

Differences in life history and feeding habits may explain the different sensitivities to sediment contamination. All three species are sediment dwelling, but only *A. aquaticus* is restricted to the sediment for feeding, whereas *E. virgo* and *C. riparius* both can utilize food from the water phase. Previous studies also have shown that different organisms can have different sensitivities for contaminants (Lyytikäinen et al., 2001; Peeters et al., 2001; Verrhiest et al., 2001; Ingersoll et al., 2002; Milani et al., 2003). This emphasizes that the species choice is an important step in sediment risk assessment procedures.

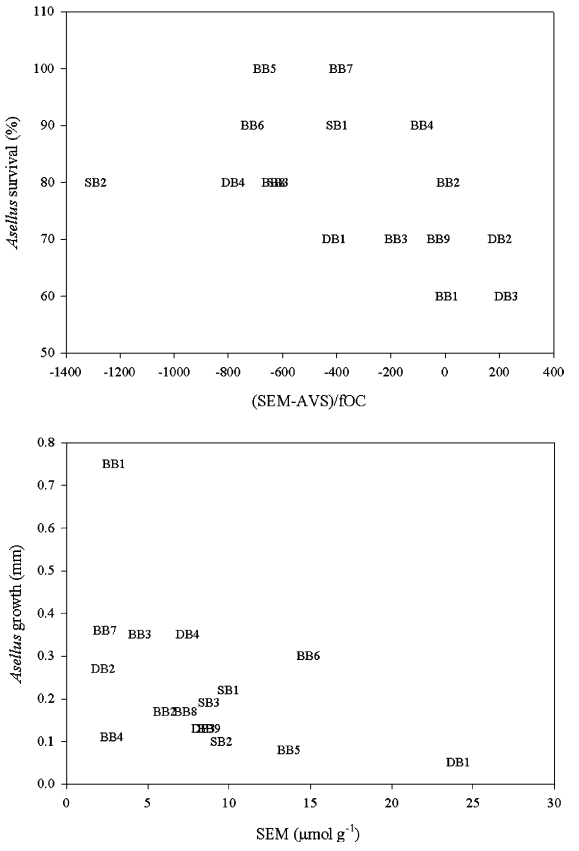

Fig. 5. Results *A. aquaticus* bioassay: survival versus (SEM–AVS)/fOC (upper panel) and growth versus SEM (lower panel).

We did not study the specific uptake routes of contaminants into the test species. The experimental setup of the laboratory bioassays aimed to reflect the natural situation of contaminated sediment with non-contaminated overlying water. In our laboratory experiments, equilibration processes will have resulted in contaminants to be present in pore water and overlying water, similar to nature. Test animals will have been exposed by the different uptake routes to contaminants originating from the sediment.

The differences in sediment treatment may have resulted in the observed differences in bioassay response. Sieving (*C. riparius* bioassay) and homogenizing (*E. virgo* bioassay) of the sediment may result in enhanced toxicity as result of change in redox conditions (Lasorsa and Casas, 1996). Not freezing the sediment (*C. riparius* bioassay) may have left indigenous predacious fauna alive. To avoid this in our experiment with *C. riparius*, sediments were visually checked after sieving for organisms before being used in the bioassay. Freezing the sediment may alter physico-chemical characteristics of the sediment, however prolonged storage without freezing may alter toxicity of the sediment. One study recommends fresh storage (4 °C) for no longer than a few days, and frozen storage (−20 °C) for prolonged storage (Beiras et al., 1998). We chose to freeze the sediments for the laboratory bioassays to kill indigenous fauna, and because we needed to store the sediments for a longer period due to logistic limitations. Handling of the sediment for the *Asellus* bioassay was minimized to maintain redox conditions as much as possible. Results of this bioassay correlated well with AVS and SEM properties of the sediment, indicating that maintaining redox conditions is an important step in pretreatment of the sediment.

Peeters et al. (2001) concluded that results of bioassays give poor predictions of risks of contaminated sediments for in situ macroinvertebrates. This study showed that results of a bioassay are influenced by species selection and sediment pretreatment, offering an explanation of the poor prediction of bioassays for in situ effects on biota. Our results support the notion that the usefulness of bioassays to predict effects on in situ biota is limited.

In conclusion, our results clearly indicate that food availability in overlying water can be an important confounding factor for species that are not restricted to sediment for feeding. The trophic state of an ecosystem will affect the ecological risks of sediment contamination. Furthermore, pretreatment of the sediment seems to be important, especially maintaining redox conditions. Therefore, we recommend that pretreatment of the sediment before used in a bioassay should be kept to a minimum, to maintain redox condition as much as possible.

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