Research Articles

A New Sediment Contact Assay to Assess Particle-bound Pollutants Using Zebrafish (*Danio rerio*) Embryos

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Abstract

Goal, Scope and Background. Based on a bioassay battery covering only primary producers and consumers as well as degraders, the potential ecological hazard of sediments to vertebrates cannot be estimated comprehensively. Therefore, there is an urgent need to develop and standardize integrated vertebrate-based test systems for sediment investigation strategies. Whereas vertebrate-based *in vitro* systems have frequently been used for the investigation of aqueous samples, there is a significant lack of whole sediment assays. Thus, the purpose of the present study was: (1) to develop a rapid and reliable, but comprehensive method to investigate native sediments and particulate matters without preceding extraction procedures; (2) to compare the hazard potential of solid phase sediments to the effects of corresponding pore waters and organic extracts in order to characterize the bioavailability of the particle-bound pollutants; and (3) to relatively evaluate the embryotoxic effects of sediments from the catchment areas of the rivers Rhine, Neckar and Danube.

Methods (or Main Features). To investigate the toxicity of sediment samples on vertebrates, the standard embryo toxicity test with the zebrafish (*Danio rerio*; Hamilton-Buchanan 1922) according to DIN 38415-6 was modified with respect to exposure scheme and toxicological endpoints. Sediments from the catchment area of the Neckar River were assessed using pore waters, acetonic extracts and native sediments in order to get inside into the potential bioavailability of particle-bound pollutants. A comprehensive test protocol for the investigation of native sediments in the embryo toxicity test with the zebrafish is presented.

Results and Discussion. The fish embryo assay with *Danio rerio* can be carried out with both aqueous and organic sediment extracts as well as native (whole, solid phase) sediment samples. Elongation of exposure time from 48 to up to 196 h significantly increased the mortality. Using the fish egg assay with native sediments, a broad range of embryotoxic effects could be elucidated, including clear-cut dose-response curves for the embryotoxic effects of contaminated sediments; in contrast, absence of embryotoxic effects could be demonstrated even for the highest test concentrations of unpolluted sediments. With native sediments, embryotoxicity was clearly higher than with corresponding pore waters, thus corroborating the view that – at least for fish eggs – the bioavailability of particle-bound lipophilic substances in native sediments is higher than generally assumed. The relative ranking of sediment toxicity was identical using both native sediments and sediment extracts, EC_{20} values of the latter, however, being eight time lower higher than with the native sediments. A comparison of the embryo toxic effects of samples from the Neckar area with locations along the Rhine and Danube rivers elucidated a broad range of results, thus indicating different levels of contamination.

Conclusions. A modified protocol of the zebrafish embryo test allows the assessment of sediment toxicity in both aqueous extracts and native sediments. The isolated investigation of pore waters may result in a clear-cut underestimation of the bioavailability of lipophilic particle-bound substances (as determined by native sediments).

Recommendations and Perspectives. The zebrafish embryo test with native (whole, solid phase) sediments appears very promising for the evaluation of the bioavailable fraction of lipophilic particle-bound substances and can therefore be recommended for the evaluation of vertebrate toxicity in tiered sediment test strategies and dredging directives such as the HABAB-WSV. Whereas acetone extracts may be tested as a rough estimation of embryotoxicity, native sediment samples will provide a more comprehensive and realistic insight into the bioavailable hazard potential.

Keywords: Acetone extracts; bioavailability; *Danio rerio*; early life stage; embryo toxicity; native samples; pore water; sediment contact test; suspended matter; whole sediment assay

Introduction

Over the last 15 years, contaminated sediments have increasingly not only been recognized as a major sink for persistent toxic substances released into the aquatic environment, but also as a potential source. Thus, numerous concepts using chemical analyses, bioassays or integrated approaches have been developed for the assessment of sediment quality (Ahlf et al. 2002a, b, Burton 1991, 1995, Chapman 2000, Chapman et al. 2002, Heise and Ahlf 2002). Chemical analysis, a highly effective analytical tool, is capable of recording both source substances and their metabolites; however, this method inherently fails to provide data about biological effects to organisms. The number of substances present in aquatic ecosystems requiring chemical analysis is such that a complete chemical screening would be much too labor- and time-consuming as well as costly. Moreover, even the most elaborate chemical analysis would not be able to provide any information about synergistic / antagonistic factors. In con-

trast, ecotoxicological tests do provide data about biological effects, however, without identifying neither the substance nor its potential source. Previous studies have repeatedly documented that a comprehensive ecotoxicological assessment cannot be made on the basis of only one system of testing, rather, a combination of biological test procedures has been recommended (Ahlf and Förstner 2001, Ahlf et al. 2002a, b, Hollert et al. 1999b, 2002b, Wenzel et al. 1997).

In current German sediment investigation strategies, e.g. the HABAB-WSB (BfG 2000) for dredged sediments and an integrated hierarchical approach combining toxicological, chemical and ecological information of sediments presented by Ahlf and colleagues (Ahlf et al. 2002a, b), ecotoxicological investigations are used to evaluate sediment quality. However, in both the HABAB-WSB and the strategy followed by Ahlf and colleagues, only (a) primary producers (algal growth inhibition test – DIN 38412 L33), (b) prokaryotic organisms (bacterial contact assay with whole sediments – DIN 38412 L48 and Microtox assay with *Vibrio fischeri* – DIN 38412 L48), and (3) invertebrates (e.g., *Daphnia magna* – OECD Guideline for Testing of Chemicals 202, Part I adopted 7.6.84) are employed for initial screening. Previous studies elucidated that – based on a bioassay battery based on only primary producers and consumers as well as degraders – ecological hazard potential of sediments to vertebrates could not be estimated (Ahlf et al. 2002a, Hollert 2001, Hollert et al. 2002a, b, Ulrich et al. 2002). Thus, there is an urgent need to develop, standardize and implement integrated vertebrate-based test systems into sediment investigation strategies.

In current biotest approaches, intact organisms or *in vitro* systems are exposed to sediments using different exposure scenarios (Harkey et al. 1994). Maybe, the most important issue in sediment toxicity testing protocols is the question as to which should be used as the test phase. Test phases can be categorized as follows: (a) organically extractable phases (in solvents other than water), (b) elutriate phase (water-extractable), (c) interstitial water phase (pore ater), (d) whole sediment, and (e) *in situ* assays (Burton 1991). There is broad consensus that whole-sediment exposure protocols represent the most realistic scenario to simulate *in situ* exposure conditions in the laboratory. Nevertheless, organic extracts of whole sediments have frequently been used to assess the potential ecotoxicological burden of sediments and in situations that require identification of soluble toxicants *via* toxicity identification evaluation. Elutriate fractions, although originally intended to mimic the open-water disposal of dredged materials and flood events, have also been used to determine the toxicity of contaminated sediments.

Whereas quasi-natural solid phase exposure has frequently been selected for both microbial (Ahlf et al. 2002b, Kwan and Dutka 1995, Liß and Ahlf 1997, Rönnpagel et al. 1995) and invertebrate test systems (Hoss et al. 2002, Nendza 2002, Traunspurger and Drews 1996, Traunspurger et al. 1997a, b, Triebskorn et al. 2001), there is a striking lack of whole sediment fish-based test systems. To date, only very few studies have used fish-based *in vitro* test systems in combination with native sediments as the exposure phase (Ensenbach 1998, Hollert et al. 2000b, Kocan et al. 1996, Ulrich et al. 2002).

Testing the toxicity of sediment to fish has been focused on studies with fathead minnow (*Pimephales promelas*); although other species have been used such as rainbow trout (*Oncorhynchus mykiss*), bluegill bream (*Lepomis macrochirus*), largemouth bass (*Micropterus salmoides*), and goldfish (*Carassius auratus*; Burton 1992). A significant amount of sediment testing with fish has been devoted to bioaccumulation of sediment-associated toxicants. In addition, fish are the principal focus of biomarker-based studies using a wide range of genotoxicity, biochemical, histopathological methods, as well as other endpoints indicative of sublethal exposures to sediment contaminants (Benson and DiGiulio 1992). In addition, preliminary tests have been conducted as acute exposure studies with adult zebrafish (*Danio rerio*), although they proved to be relatively insensitive, if compared to early life-stages (Ensenbach 1998). Rather, early embryonic and larval stages in fish and bird development have frequently been shown to be more sensitive to the effects of toxicants, if compared to juvenile and adult fish (Ensensbach and Nagel 1995, 1997, Fent and Meier 1994, Harris et al. 1994, Murk et al. 1996, Strmac et al. 2002, Wiegand et al. 2000) and have, thus, become essential elements in environmental hazard assessment for chemicals. The use of zebrafish in early life-stage tests provides several advantages. Zebrafish are relatively easy to breed in the laboratory, and eggs are readily available in high numbers throughout the year (Laale 1977). Due to the transparency of both eggs and post-hatch larvae, zebrafish development can continuously be monitored and effects can easily be identified by light microscopy. Finally, embryonic development is relatively rapid and synchronous (Bresch 1991, Nagel 2002, Nagel and Isberner 1998). As a consequence of mainly animals' rights considerations, an embryo test with the zebrafish has recently been suggested as a substitute for the acute fish test and internationally validated (Nagel 2002). Based on promising results from tests with both pure chemicals and waste water, the test design was validated and standardized by a German DIN-working group under DIN 38415-6.

Whereas vertebrate-based *in vitro* systems have frequently been used for the investigation of aqueous samples (Behrens et al. 1998, Brack et al. 2000, 2002, Castano et al. 1994, Ekwall et al. 1998, Giesy et al. 2002, Hilscherova et al. 2000a, b , Hollert et al. 2002a, b, Machala et al. 2001, Nehls et al. 1998, Segner and Segner Braunbeck 1997), there is a significant lack of whole sediment assays (Ensenbach 1998).

Thus, the purpose of the present study was: (1) to develop and present a comprehensive method to investigate native sediments and particulate matters without preceding extraction procedures; (2) to compare the hazard potential of native (whole, solid phase) sediments to the effects of corresponding pore waters and organic extracts in order to characterize the bioavailability of the particle-bound pollutants; and (3) to relatively evaluate the embryotoxic effects of sediments from the catchment areas of the rivers Neckar, Danube and Rhine.

1 Material and Methods

1.1 Sampling locations

In order to evaluate the ecotoxicological contamination of 12 selected aquatic sites in streams within the catchment area of the Neckar river, a small river system in Southern Germany, a sediment quality triad (SQT) approach was used. Results of these SQT studies study have been published elsewhere (Hollert et al. 2002a, b, 2003).

Samples were taken from four separate regions in the Neckar catchment area (**Fig. 1**; Hollert et al. 2002a, b): (1) The site F1 at the small stream Forellenbach was selected due to discharge of treated hospital wastewater. In contrast, site F2 was an uncontaminated reference site located at a small stream with a comparable ecomorphological situation. F3 was located some distance downstream the confluence of the two streams (F1, F2), and the site F4 was located further downstream and characterized by regeneration in earlier studies (Becker 1992). (2) In order to assess the efficiency of a sewage treatment plant at the Elsenz river, sites upstream (El1) and downstream (El2) of the plant, as well as a reference site (Hil) characterized by, at best, agricultural input were investigated. (3) In order to evaluate the ecological and ecotoxicological effects of sediments temporarily resuspended by currents caused by currents from shipping traffic, a fish-spawning site connected to the Neckar River (Eb2) was examined in comparison to a reference site (Eb1) close to the mouth of the spawning site to the Neckar River. (4) In order to assess background levels as well as effects of air-borne pollutants emitted by the highly industrialized Ludwigshafen/Mannheim region located 20 km west of the sampling site, the small stream Mühlbach was investigated close to its spring region (Mb1) and after passing the city of Heidelberg and an adjacent agricultural area (Mb2).

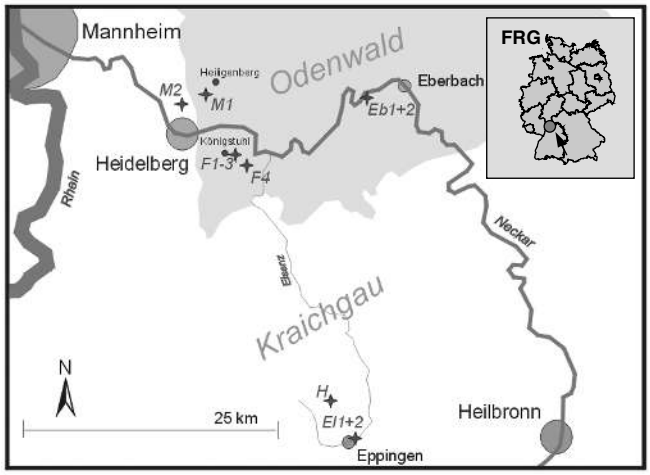

Fig. 1: Locations of the investigated sites at small streams within the catchment area of the Neckar river in Southern Germany. The sample locations are indicated by asterisks. F 1 – F 4: Forellenbach sites, M 1 – M 2: Mühlbach sites, El 1 – El 2 and Hil: sites in the Elsenz area, Eb 1 – Eb 2: Neckar River fish-spawning site Eberbach

1.2 Sample processing and extraction

Near-surface sediment samples (0–5 cm depth) of the small streams were collected by means of a Van-Veenen gripper or a sediment corer. After homogenization, pore water was obtained *via* centrifugation at 3000 x g, and samples were shock-frozen at –30°C and freeze-dried immediately (beta 1–8 K; Christ, Osterode, Germany). Freeze-dried samples were stored in plastic jars (polyethylene, Greiner, Frickehausen, Germany) at 4°C in the dark.

Subsamples of the freeze-dried sediments were used for preparation of organic extracts. In a Soxhlet apparatus, 50 g dried sediment of each site was separately extracted at 6 cycles per hour over 24 h with acetone (Baker, Gross Gerau, Germany). Extracts were reduced in volume using a rotatory evaporator (WB 2001; Heidolph, Kehlheim, Germany; 400 mbar, 36–38°C). Extracts were concentrated close to dryness with N_2, the solvent was changed to dimethylsulfoxide (DMSO), and samples were stored at –30°C until testing. Maximum concentration of DMSO was 0,25% in the test assay. The fish egg assays with organic extracts and pore water were carried out according to Strmac et al. (2002).

1.3 Testing of whole sediments

The whole dry sediment samples were tested at concentrations of 3 g, 2.5 g, 2 g, 1.5 g, 1 g, 0.5 g, 0.25 g and 0.1 g per 5 ml of artificial water (ISO 7346/3) which was produced according to DIN 38415-6 (stock solution: 58.8 mg/L $CaCl_2$ x 2 H_2O, 24.6 mg/L $MgSO_4$ x 7 H_2O, 12.6 mg/L NaHCO3, 5.5 mg/L KCl; stock solutions were diluted 1:5 with double-distilled water; prior to utilization, water was ventilated for 24 h (pH 7.8 ± 0.2); corresponding to concentrations between 600 and 10 mg sediment per ml artificial water). The samples were filled up to a total weight of 3 g with artificial sediment (quartz powder, grain size W4, Quarzwerke, Frechen, Germany) and homogenized in a mortar to avoid sediment or silica dust hot spots. These mixtures were transferred into 6-well microtiter plates. Each well was filled up with 5 ml of artificial water, which had been ventilated to oxygen saturation. As negative controls, 3 g of quartz powder plus 5 ml of artificial water as well as 5 ml artificial water only were used. As positive controls, 3 g of quartz powder filled up with 5 ml of a 3.7 µg/ml 3,4-dichloraniline (3,4-DCA) solution were used. Five replicates (wells) per sediment concentration and controls were tested, each containing 5 eggs (n = 25). At the end of the exposure period, oxygen concentrations were controlled in order to ensure that toxic effects were caused by particle-bound sediments and not by lack of oxygen.

1.4 Fish, maintenance and egg production

To investigate the toxicity of sediment samples on vertebrates, the embryo toxicity test with the zebrafish (*Danio rerio* Hamilton-Buchanan 1922) according to DIN 38415-6 was modified with respect to exposure scheme and toxicological endpoint. *Danio rerio* is a small cyprinid with easily distinguishable sexes. It is relatively insensitive to varying water-conditions and is therefore easy to keep and breed (Laale 1977). Adequate maintenance conditions provided (Strmac et al. 2002), spawning groups of 1 (2) females and 2 (4) males may provide up to 300 eggs at regular intervals of 2–3 days throughout the year. Zebrafish can easily be obtained

from local pet shops and dealers and perfectly suits for microscopic studies due to the transparency and rapid development of its eggs and embryos. Finally, embryonic development is relatively rapid and synchronous (Eaton and Farley 1974, Laale 1977, Nagel 1986, Bresch 1991, Kimmel et al. 1995).

Adult zebrafish were bred at the Department of Zoology, University of Heidelberg, and kept in 160 L tanks under flow-through conditions at a water temperature of 27.0 ± 0.5°C. The quality of the charcoal-filtered well water was maintained at 379 mg/L $CaCO_3$ (21.3°dH hardness), 744 S (conductivity), pH 7.36 ± 0.2.3, and 10.5 ± 0.5 mg/L O_2 (95 ± 5% saturation) throughout the rearing and experimental periods. Ammonia, nitrite and nitrate were kept below detection limits (ammonia: 0–5 mg/L; nitrite: 0.025–1 mg/L; nitrate: 0–140 mg/L). Aquaria had neither decoration nor sediment substrates. The photoperiod was adjusted to 12 h light and 12 h darkness. Fish were fed twice a day with TetraMin dry flakes (Tetra, Melle, Germany) and *Artemia* nauplii; prior to spawning, additional 3 to 4 *Artemia* nauplii rations were provided to stimulate optimal egg production. Faeces and excess food particles were removed twice a day, and aquaria screens were cleaned daily.

For breeding, up to eight 6.5 L plastic tanks were placed inside a large glass tank (water temperature 27.0 ± 0.5°C, pH 7.5 ± 0.25, **Fig. 2**). The bottom of each plastic tank was replaced by a stainless steel mesh through which the fertilized eggs could drop into rectangular glass dishes placed underneath to prevent the brood from being eaten by their parents. Each breeding tank was equipped with a green plastic or glass plant imitate as a breeding substrate. The day before a test was performed, 4 male and 2 female individuals were selected and transferred into one of the breeding tanks shortly before the beginning of the darkness period. If the fish are placed into the breeding tanks during light period they immediately start mating, and eggs cannot be used for testing the next day.

Spawning and fertilization take place within 30 minutes after light is turned on in the morning (Nagel 1977). The glass dishes with the eggs were removed, and eggs and water were transferred into 18 cm petri dishes. A fertilization rate of more than 80% was required to provide adequate test material. Once the 4-cell stage is reached (1 h after fertilization; Kimmel et al. 1995), fertilized eggs can easily be distinguished from non-fertilised opaque eggs. Fertilized eggs were individually selected and directly transferred to the sediment-containing cavity of a 24-well plate using a 2 ml plastic pipette with a widened opening. Since no pre-incubation is possible in the whole-sediment test, this step had to be performed rapidly so that exposure during early developmental stages was guaranteed. The microtiter plates were sealed with adhesive film (Renner, Darmstadt, Germany), and incubated at 27.0 ± 0.1°C with a photoperiod of 12 h light and 12 h dark for 196 h.

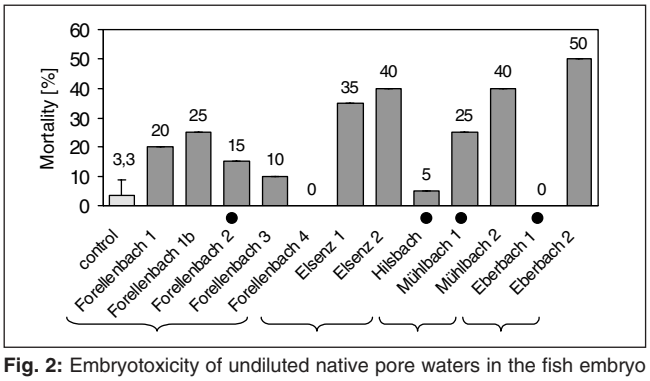

Fig. 2: Embryotoxicity of undiluted native pore waters in the fish embryo assay with zebrafish (*Danio rerio*) using 20 fish eggs for each sample after 48 h of incubation. The mortality of the control is given as median and standard deviation from 3 independent experiments. Locations in the same catchment area are indicated with clamps, and the corresponding reference locations are indicated by black circles

Embryos were inspected after 24, 48 and 196 h after initiation of the exposure using an inverse microscope (CK-2 equipped with a SC-35 camera, Olympus, Hamburg, Germany). For examination, the eggs they were transferred into microtiter plates filled with fresh artificial water (water temperature 27.0 ± 0.1°C). During transfer and retransfer of the eggs, it is essential to keep the amount of transferred water to a minimum and avoid transfer of sediment particles.

The endpoints regarded lethal are Presence of somites, coagulation of embryos, detachment of tail, development of eyes, developmental retardation, heart functioning, blood circulation, pigmentation, formation of edema, spinal deformations and hatching; for the timing of the endpoints (**Table 1**). Mortality was expressed as EC_{50} values, which were calculated using probit analysis.

Table 1: Toxicological endpoints used to determine the mortality of the embryos and larvae in the embryo toxicity test with zebrafish (*Danio rerio*; modified after Ensenbach 1999)

Toxicological endpoints	24 h	48 h	72 h	144 h
Lack of somite formation	†	†	†	†
Coagulation of embryos	†	†	†	†
Non-detachment of tail	†	†	†	†
Non-development of eyes	•	†	†	†
Developmental retardation	•	•	†	†
Lack of heart function		†	†	†
Lack of blood circulation		†	†	†
Lack of pigmentation		•	†	†
Edema formation		•	†	†
Lack of hatch		•	†	†
Spinal deformations			†	†

† = lethal criterion used to determine mortality rate

• = documented, but not evaluated as a lethal criterion

1.5 Quality control and statistics

As a control for the validity of a given test, the results of the positive control (3,7 mg/L 3,4-dichloroaniline) had to be within a range of 20 to 80%. In contrast, negative controls were regarded valid, if the mortality did not exceed 10%.

Since the pT value (potentia toxilogica = negative binary logarithm of the first non-toxic dilution using a dilution factor of two, HABAB-WSV, BfG 2000) is easy to compute by EC_{20} values, not only EC_{50} values were determined. Moreover, according to the German DIN regulation, only 20% effect are regarded as a positive effect, whereas 10% mortality are accepted for a valid control. For these reasons, both EC_{20} and EC_{50} values were computed from the dose-response curves using non-linear regression analysis (Prism 2.01, GraphPad Inc., USA). For a comparison of results obtained for the different catchment areas, data were analyzed with the H-test according to Kruskal and Wallis (ANOVA; SigmaStat 2.03; SPSS-Jandel, Erkrath, Germany). In cases of significant differences between the groups, a post-hoc test according to Dunn was used to identify groups that differed significantly.

2 Results and Discussion

2.1 Embryo toxicity of pore water

Fig. 2 provides a survey of the embryotoxicity of native pore water samples from native sediments, as determined by means of the zebrafish egg assay after an incubation period of 48 hours.

For the Elsenz river sites both upstream (Elsenz 1) and downstream (Elsenz 2) of the sewage treatment plant, high embryotoxicity could be determined. In contrast, the pore water of the reference location Hilsbach, which is characterized by, at best, agricultural input, induced a statistically non-significant mortality of only 5%. Especially for the fish-spawning site connected to the Neckar River (Eberbach 2), a very high mortality could be determined, when compared to the reference location Eberbach 1. This result clearly gives evidence as to why there is a complete lack of juvenile fish at Eberbach 2 (Hollert et al. 1999a), although this site had originally been designed as a fish spawning site. Continuous changes in water levels at Eberbach 2 in conjunction with currents caused by shipping traffic might account for both continuous remobilization of sediments and repeated temporary falling dry of the habitat followed by accumulation of easily exchangeable metal species (Song and Müller 1993). The high embryotoxicity of the pore waters at site Eberbach 2 is of greatest importance for the ecosystem of the spawning site, since several studies identified pore water as a major route of exposure of infaunal and epibenthic organisms to sediment contaminants (Burton 1991, Carr et al. 2001).

Whereas the procedures to collect and assess sediment pore waters as a primary measure of sediment quality are still under discussion (Chapman et al. 2002, Wang 1999), the use of whole sediment assays is unanimously recommended (Chapman et al. 2002, Wenning and Ingersoll 2002).

2.2 Embryo toxicity of native sediments and organic extracts

Fig. 3 provides a survey of the appearance of zebrafish *Danio rerio l*arvae after incubation with native sediments. Most of these alterations proved to be not site-specific; rather, general developmental retardation, underdeveloped eyes and ears, lack of somite formation or yolk detachment as well as edemas of heart and yolk sac are common effect not only typical of exposure to sediments, but also to pure chemicals. However, except for edema formation, which is very frequent, the frequencies of these changes vary with the specific exposure scenario. In the experiments, oxygen concentrations were consistently higher than 2 mg/L. Since in preliminary experiments oxygen levels of 0.5 mg/L had been identified to be sufficient for normal development of zebrafish eggs and embryos (details not shown), toxic effects had to be regarded as a consequence of exposure to particle-bound substances and not of oxygen depletion.

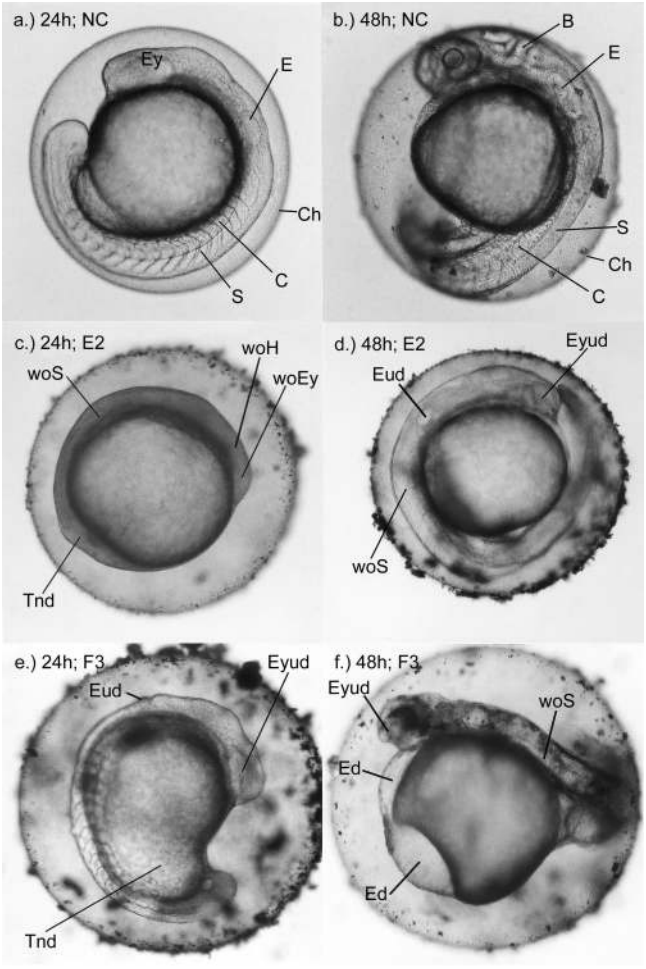

Fig. 3: Embryos of zebrafish (*Danio rerio*) after 24 and 48 h incubation with native sediments (a) 600 mg/ml W4 quartz powder, negative control, 24 h, × 700; (b) W4 control, 600 mg/ml, 48 h, × 700; (c) location Eberbach 2, 143 mg/ml 24 h, × 650 revealed retardations in the development in comparison to the 24 h control; (d) location Eberbach 2, 330 mg/ml, 48 h, × 650 displaying underdeveloped eyes and ears as well as lacking somites, (e) 330 mg/ml, location Forellenbach 3, 24 h showing underdeveloped eyes and ears and the tail is not detached from the yolk; (f) location Forellenbach 3, 230 mg/ml, 48 h showed distinct edemas in the heart and yolk sac regions as well as a lack of somites. B = brain; C = chorda; Ch = chorion; E = ear; Ed = edema; Eud = ear underdeveloped; Ey = eye; Eyud = eye underdeveloped; NC = negative control; Tnd = tail not detached; woEy = without eyes; woH = without head; woS = without somites

Figs. 3a and b show normally developed zebrafish larvae. After 24 h, the tail is detached from the yolk, ears and the eyes can clearly be identified. The somites as well as the chorda are visible, and spontaneous movement has started. After 48 h, eye and skin pigmentation due to the melanophore development are evident, and a well-structured spinal cord can be easily distinguished. At this stage, both heart beat and blood circulation can be easily observed. Following 24 h exposure to, e.g., native sediments from the fish spawning site Eberbach 2 (Fig. 3c), the larvae had only reached the epiboly stage and had, thus, failed to reach the 24 h-typical developmental stage. After 48 h exposure to a sediment concentration of 330 mg/ml from Eberbach 2 (Fig. 3d.), both eyes and ears were underdeveloped., and somites could not be distinguished.

After 24 h exposure to the native sediment from the Forellenbach site F3 (concentration of 231 mg/ml), the tail was not detached from the yolk sac, and the eyes and ears were underdeveloped (Fig. 3e); after 48 h exposure to a concentration of 330 mg F3 sediment per ml, the larvae displayed formation of huge edemas, and somites were not fully developed (Fig. 3f).

Fig. 4 visualizes the embryo toxicity of a native (whole, solid phase) sediment from the fish spawning site Eberbach 2 (Eb2) in the zebrafish egg assay in relation to incubation time. Prolonged exposure increased the mortality of zebrafish embryos significantly.

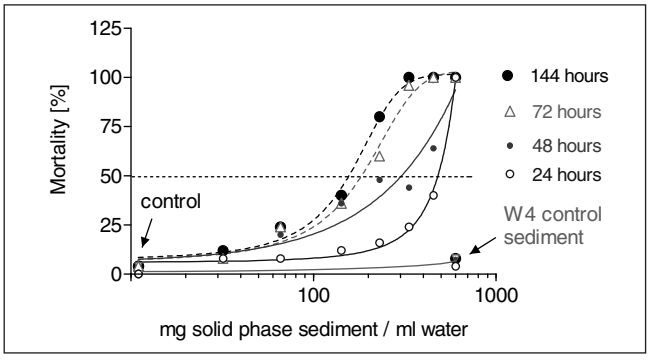

Fig. 4: Embryo toxicity of a native (whole, solid phase) sediment from the fish spawning site Eberbach 2 (Eb2) in the zebrafish (*Danio rerio*) embryo assay in dependence from incubation time. Every concentration was investigated by means of 25 eggs

If compared to the pore waters, the native sediments clearly induced stronger effects. Thus, it seems important to repeat that the bioavailability of lipophilic particle-bound substances to zebrafish embryos exposed to native sediments may be considerably higher than generally assumed. Again, as for the pore water, a very high mortality of the fish-spawning site Eberbach 2 could be determined for the native sediments, when compared to the reference location Eberbach 1 (**Fig. 5**), elucidating the high ecological relevance of the results. Most importantly, the lack of embryotoxic effects in sediments from the site Eberbach 1 documents that the zebrafish embryo contact assay is capable of identifying non-toxic native sediments showing an embryotoxicity comparable to those of (sterilized) W4 quartz powder and pure water controls.

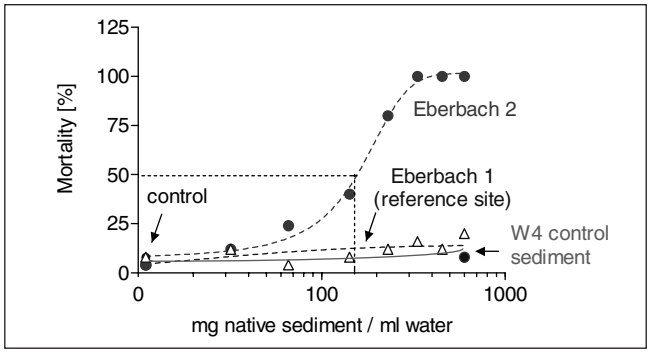

Fig. 5: Embryo toxicity of two native (solid phase) sediments from a fish spawning site in a prolonged zebrafish (*Danio rerio*) embryo assay with an incubation time of 144 h. 3 g SiO powder (grain size W4) as negative controls

The undiluted pore water of the sediment sampled downstream the sewage treatment plant located at the Forellenbach (F1, F1b) induced the strongest embryotoxic effects in the catchment area of the Forellenbach creek with mortality of 20 and 25% (Fig. 2). However, the toxicity of the reference site F2 was only unimportantly lower (15%). In contrast to the minor differences (factor 2) of the pore water samples, the acetonic extracts of F1 and F1b clearly revealed higher embryotoxicity, when compared to the corresponding reference sediment (factor 10 for the EC_{50} values, **Fig. 6**).

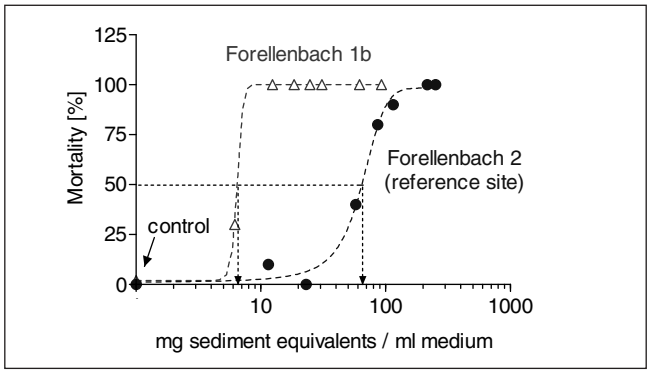

Fig. 6: Embryo toxicity of two acetone sediment extracts in the zebrafish (*Danio rerio*) embryo assay after 48 h incubation. Mortality is calculated from 20 eggs per concentration. Forellenbach 1b is a location heavily contaminated by effluents from a sewage treatment plant, Forellenbach 2 is a reference site. Control: artificial water

Acetonic extracts are commonly believed to assess the total potential toxicity and to, therefore, overestimate the actual bioavailable toxicity of sediments (Ahlf 1995, Burton 1991). As a consequence, a critical re-evaluation of the relevance of results obtained by means of organic extracts has repeatedly been postulated. A typical dose-response curve for the embryotoxicity by native sediments from the Forellenbach is given in **Fig. 7**.

Since for the reference location Forellenbach F 2 (F2) and the regeneration site Forellenbach 4 (F4) only minor or no effects could be determined, the native sediment sampled downstream the sewage treatment plant (F1b) induced a mortality of 60%, and the whole sediment of the location downstream the confluence of the two creeks killed 100%

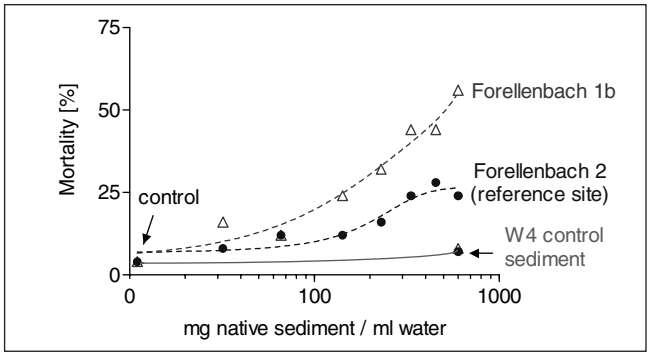

Fig. 7: Embryo toxicity of two native (whole, solid phase) sediments from the Forellenbach creek in a prolonged zebrafish (*Danio rerio*) embryo assay with an incubation time of 144 h. 3 g Quartz powder (grain size W4) was used as a negative control

of the embryos when tested at a concentration of 600 mg sediment per ml. Thus, a much bigger difference between the native samples could be determined than between the corresponding pore water samples. As a conclusion, differentiation of toxicity was more pronounced in native sediment than in pore water samples.

2.3 Comparison of the embryotoxicity in different exposure phases

A comparison of the embryo toxicity caused by different sediments collected from the Forellenbach and tested as native sediment samples and pore waters (**Fig. 8**) revealed that the isolated use of pore water as the only test phase may result in a clear-cut underestimation of the bioavailability of particle-bound lipophilic particle-bound substances as are determined by testing native sediments. The pore water derived from the sediments at location F3, e.g., induced only a mortality of 15%, whereas the native sediment damaged 100% of the embryos. In fact, for all the sediments assessed in the present study, native sediments definitely induced stronger embryotoxicity than the corresponding pore waters (Fig. 8). This result corroborates the view that the bioavailable hazard potential of sediments may not be evaluated by pore water investigations only. Particularly the German directive for handling of dredged material from inland waterways (HABAB-WSV), which dictates the exclusive use of pore waters and aqueous eluates as test phases for the evaluation of sediment toxicity, should be revisited under the light of the present results.

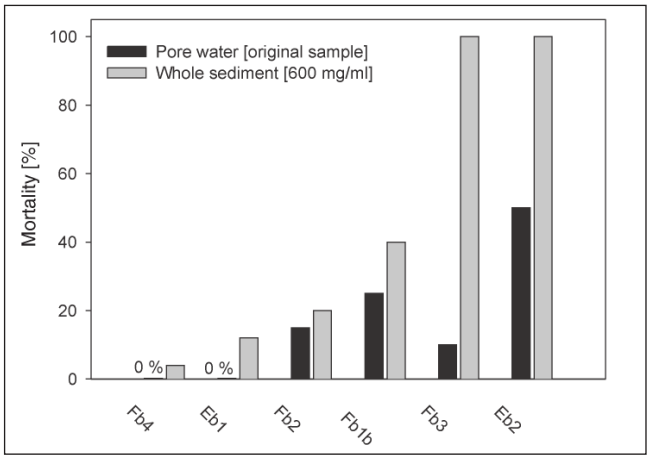

Fig. 8: Comparison of the mortality induced in zebrafish (Danio *rerio*) embryos by exposure to pore water (original undiluted sample) and native sediment (maximum concentration 600 mg/ml) for the locations Eberbach (Eb) and Forellenbach (Fb)

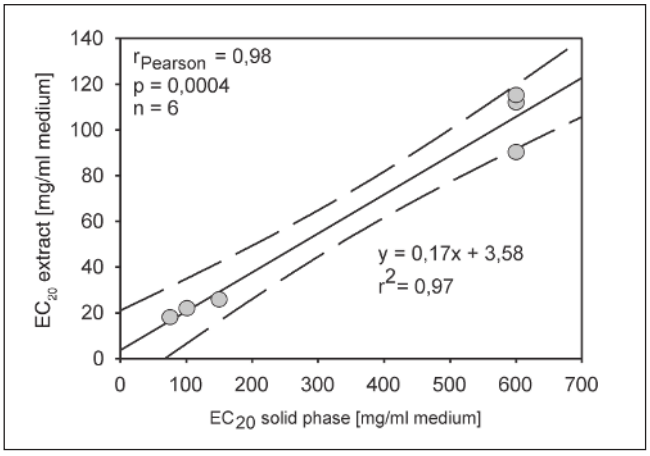

Fig. 9: Correlation of the EC_{20} values derived from zebrafish embryo assays with acetone extracts and native sediments: Although limited in its relevance due to a low number of experiments, there seems to exist a good correlation ($r^2 = 0.97$)

Fig. 9 shows a regression analysis of EC_{20} values obtained from results from tests with native sediments and organic extracts. Although only a total of six direct comparisons could be made, a high correlation coefficient according to Pearson of 0,98 could be determined. Based on this data set, the use of extracts seems to result in the same ranking of sediment toxicity as with native samples. In absolute terms, the EC_{20} values of acetone extracts differ from those obtained with native samples by a factor of 8. As a conclusion, acetone extracts can be recommended as a rough estimation of embryo toxicity, whereas native sediment samples should be tested whenever a more comprehensive and realistic insight into the bioavailable hazard potential is required.

2.4 Hatching rate – A sensitive sublethal endpoint

In the negative control batch with ISO standard water, only 8% of larvae hatched early (i.e. before day 3) and 88% hatched on day 3; none of the larvae hatched later than day 4 (**Fig. 10**). For the quartz powder (the second negative control), a similarly low toxicity and comparable hatching behavior was found. In contrast, all of the native sediment samples tested (600 mg/ml) except for the regeneration site Fb4 clearly modified both hatching rates and time course of hatching (Fig. 10). Since the regeneration site of the Forellenbach FB 4 was definitely comparable to the other locations along the Forellenbach in terms of sediment grain size distributions, the toxic effects were not induced by matrix effects, but by particle-bound substances contained in the native sediment samples. However, this result also documents that the zebrafish embryo assay unambiguously identifies non-contaminated sediment samples such as FB 4, which also proved non-toxic in a whole series of other bioassays

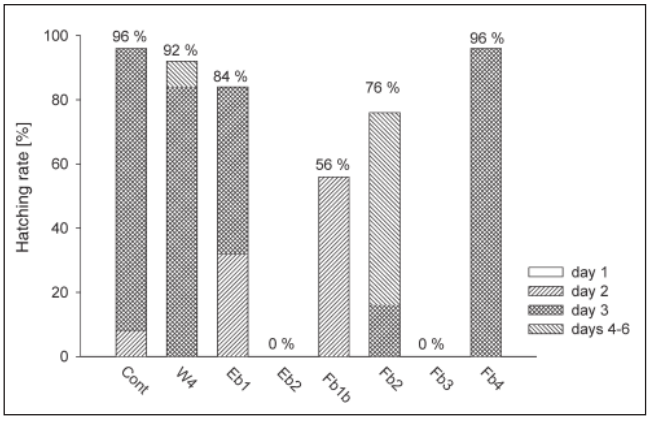

Fig. 10: Daily hatching rates of zebrafish (*Danio rerio*) after exposure to 600 mg/ml native sediment of the triad locations Eberbach (Eb1, 2) and Forellenbach (Fb1 – 4); artificial water (Cont) and quartz powder (mesh size W4) served as negative controls

(Hollert et al. 2002a, b). In more general terms, it seems worth noticing that even in industrialized areas it is possible to identify sediments free of negative effects in bioassays such as the zebrafish embryo test.

In contrast to the regeneration site Fb4, sediments obtained from the reference site Fb2, which also induced no mortality in the fish embryo assay, caused significant changes of the hatching rate. About 60% of the larva hatched late (i.e., between days 4 and 6), when compared to the water control and the W4 quartz powder. In comparison to the other locations investigated within the present sediment quality triad, relatively high PCB concentrations could be determined for site Fb2 (Duerr et al. 2001): 0.055 mg/kg PCB #138 and 0.068 mg/kg PCB #153 classified the sediment sample Fb2 into the worst quality class IV according to the classification strategy of Ahlf and colleagues (Ahlf et al. 2002b). In fact, PCBs are well known to increase mortality and to influence hatching rate in early life stage-tests within different fish species (Andersson et al. 2001, Billsson et al. 1998, Matta et al. 1997, Olsson et al. 1999, Westerlund et al. 2000, Zabel et al. 1995). In another early life-stage test with zebrafish, Strmac et al (2001) recorded a significant delay in hatching after exposure to PCB-contaminated sediment extracts from another small river in Southern Germany. The time-course of hatching thus seems to represent a more sensitive endpoint than pure mortality.

2.5 Comparison of embryo toxicity and acute toxicity in the permanent cell line RTG-2

Toxicity testing in sediment systems with fish has been limited by both technical problems, costs and ethical motivations. Thus, the replacement or reduction of *in vivo* fish tests by *in vitro* cytotoxicity tests (Braunbeck and Strmac 2001, Segner 1998, Strmac and Braunbeck 2000) as well as embryo assays (Ensenbach 1998, Strmac et al. 2002) have repeatedly been recommended. Given the high correlation with data from the fish test, both the cytotoxicity test and the fish embryo assay may provide good estimates of the potential toxicity of sediment-bound contaminants. Cytotoxicity assays are particularly suitable for screening and ranking purposes at lower tiers of testing (according to indication), because they can be performed at small scale with numerous replicates, thus allowing simple and rapid measurement of extracts, pore waters and aqueous elutriates in large sample numbers. Acute cytotoxicity has frequently been investigated with the epithelial-like cell lines RTL-W1 (Lee et al. 1993) and RTG-2 (Wolf and Quimby 1962, Zahn et al. 1996) (both obtained from *Oncorhynchus mykiss*) in combination with the neutral red (Borenfreund and Puerner 1984) and succinic acid dehydrogenase (MTT) (Denizot and Lang 1986) assays, lactatedehydrogenase release into the medium (Weishaar et al. 1975), as well as light microscopic inspection (Hollert et al. 2000a). With these cell lines, acute cytotoxicity tests have sucessfully been used to determine the cytotoxicity of pure substance (Ekwall et al. 1998, Segner 1998, Segner et al. 1994), seewage waters from garbage dumps (Zahn et al. 1995), waste waters (Castano et al. 1994) and sediments (Hollert et al. 2000a, b).

In order to evaluate the correlation between fibroblast-like cell lines and the slightly more work-intensive fish embryo assay, a correlation analysis of EC_{20} values derived from 12 acetone sediment extracts in both assays was made (**Fig. 11**).

The comparison of the sediment extract toxicity revealed a very good correlation ($r_{Pearson}$ = 0.81) between the acute cytotoxicity test with RTG-2 cells and the zebrafish embryo assay. Most importantly, for the 12 sediment samples from the catchment area of the Neckar river, the zebrafish embryo test clearly responded more sensitive. Overall, only two sediment samples gave a significant deviation from the overall correlation: At the site Forellenbach 3 (Fb3), which was characterized by an elevated contamination by heavy metal, both tests showed the same sensitivity, and at location Forellenbach 1 (Fb1) with a striking predominance of lipophilic

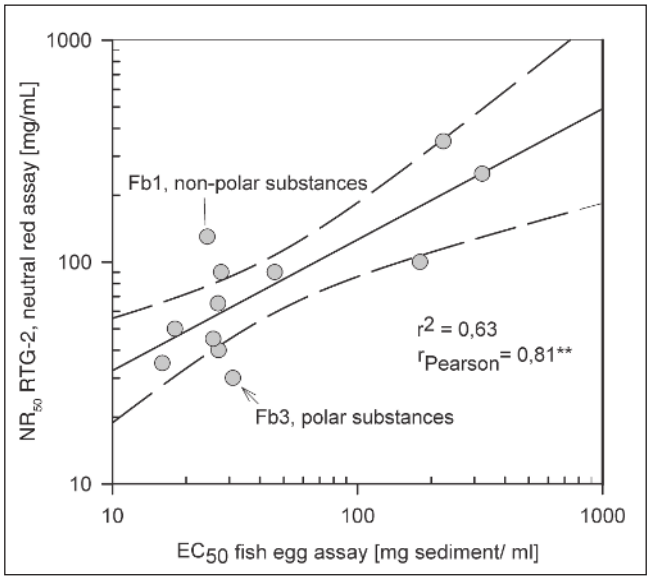

Fig. 11: For the correlation of cytotoxicity of acetone extracts of sediments as determined by means of the neutral red assay with the cell line RTG-2 and by the zebrafish (*Danio rerio*) embryo assay after 48 h exposure, a correlation coefficient of 0,81 (** good correlation according to Pearson) can be calculated. NR_{50} = effective concentration for 50 % cell death in the neutral red test

pollutants the cell test responded less sensitively (independently of S9 supplementation). Thus, based on the results of this study. the zebrafish embryo assay can be strongly recommended for the assessment of sediment toxicity due to its sensitivity.

2.6 Comparison of the embryo toxicity by native (whole, solid phase) sediments from location within different river catchment areas

In order to relatively validate the embryotoxic potential of the whole sediment samples obtained from the Neckar catchment area comprehensively, a comparison with results from tests with sediments collected at other catchment areas was made (**Fig. 12**).

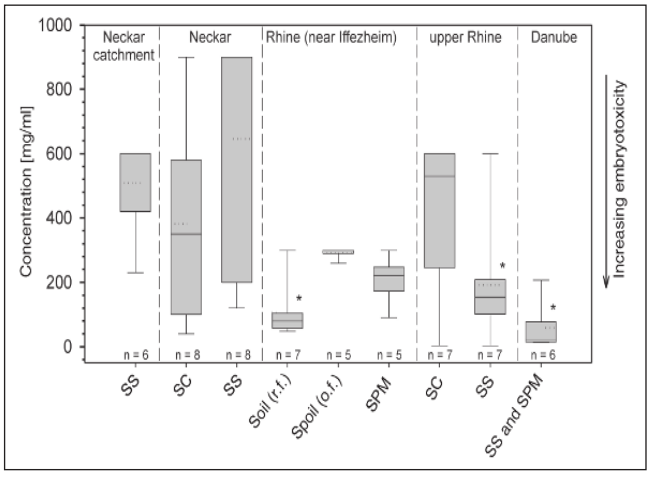

Fig. 12: Comparison of EC_{50} values from native sediment samples collected from the river Rhine close to Iffezheim (Ulrich et al. 2002), the upper Rhine (König et al. 2002), different locations from the river Neckar (Hollert et al. 2000c), the catchment area of the Neckar river near Heidelberg (this paper) and the upper Danube river (Keiter et al. 2003) in the zebrafish (*Danio rerio*) embryo assay after 48 h exposure. Data are represented by box and whisker plots, including 25 and the 75% percentiles (shaded box) as well as standard error (whiskers). Dotted lines represents the means, and solid lines the medians. Stars indicate significant embryotoxic effects (H-test followed by *post-hoc* test according to Dunn, $p < 0.05$), when compared to the group revealing the lowest effects (i.e., Neckar surface sediments). o.f. = often flooded, r.f. = rarely flooded, SC = sediment core, SS = surface sediment, SPM = suspended particulate matter

The whole sediments from the Neckar catchment area showed a comparable embryo toxicity potential as whole sediments from the Neckar river itself (Hollert et al. 2000). In detail, the mean EC_{50} of sediments from the Neckar catchment area is slightly higher than that of sediment cores from the Neckar itself and slightly lower than that of surface sediments sampled from the Neckar river. This results corroborates the conclusion that for the Neckar river and its catchment area older sediments are of particular ecotoxicological importance.

In contrast, surface sediments (König et al. 2002) and suspended particulate matter (SPM; Ulrich et al. 2002) of the upper Rhine river caused significantly higher toxicity in zebrafish embryos than the Neckar catchment area sediments, revealing that surface sediments and remobilized sediments of the Rhine have a very high embryotoxic burden. Likewise, sediments and SPM of the upper Danube river (Keiter et al. 2003) showed a definitely higher embryotoxic potential than the sediments investigated in this study.

3 Conclusions and Recommendations

Toxicity testing in sediment systems with fish has been limited by both technical problems, costs and ethical motivations. Thus, the replacement or reduction of *in vivo* fish tests by *in vitro* cytotoxicity tests (Segner 1998), as well as embryo assays (Ensenbach 1998) are recommended.

The present study clearly documents that the embryo toxicity assay with zebrafish (*Danio rerio*) is capable of detecting lethal and sublethal toxicity of sediments using the test phases whole sediments, pore waters and organic extracts. The findings thus demonstrate the suitability of the zebrafish embryo test for the analysis of complex environmental samples and especially whole sediments in a vertebrate-based test system. The application of the fish egg assay with solid-phase sediments appears to be very promising for the evaluation of the bioavailable portion of particle-bound substances and can, therefore, be recommended for the evaluation of vertebrate-based toxicity in tiered sediment test strategies (Ahlf et al. 2002a, b) and dredging directives such as the HABAB-WSV (BfG 2000). A comparison of the embryotoxicity of sediments assessed by the different exposure phases native sediment and pore water revealed that the isolated use of the pore water as the only test phase may readily lead to an underestimation of the bioavailable portion of particle-bound substances (as determined by native sediments). Whereas organic extracts may be tested in order to roughly estimate embryotoxicity, native sediment samples will provide a more comprehensive and realistic insight into the bioavailable hazard potential.

Although the sensitivity of the assay could be improved significantly using a prolonged exposure time, results documents that an exposure time of 48 h is sufficient to reliably evaluate the embryotoxic effects of particle-bound sediment contaminants. Inclusion of hatching rate and, above all, the time-course of hatching as an additional endpoint significantly adds to the overall sensitivity of the test.

Over the last years, several solid phase assays have been developed for the ecotoxicological assessment of sediment- and suspended matter-bound pollutants. However, so far endpoints such as genotoxicity and dioxin-like activity of sediments in vertebrate-based test systems could only be assessed after transfer of particle-bound substances into the aqueous phase. Processing and extraction of the samples are suspected to not take into account bioavailability. Consequently, the transferability of *in vitro* data to the field situation is critical. Moreover, as national and international water management authorities suggest, there is a need to implement specific effects into regulations, and solid phase assays for the assessment of sublethal effects have to be developed. A promising approach would be to use the sediment contact test with zebrafish in order to develop a combined sediment assay for the examination of embryotoxic, genotoxic and dioxin-like activities of particle-bound substances.

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