7. Ecotoxicity Characterization of Seepage Waters from a Theisenschlamm Pond Discharging into a Riverine System

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Abstract

An ecotoxicological analysis of a scrubber dust slurry known as Theisenschlamm was performed. Seepage water from a Theisenschlamm pond and water samples from the receiving riverine system Böse Sieben were investigated. The toxic potential of the samples was assessed by using a bacterial luminescence test, the pollen tube growth assay, and in vitro determinations of cytotoxicity and 7-ethoxyresorufin-O-deethylase as a marker of the PAH-inducible cytochrome P4501A system. With all test systems, the seepage water showed massive toxicity. As indicated from EDTA complexation experiments, this effect was at least partly caused by the high Zn contents of the seepage water. In the water samples of the Böse Sieben, only moderate levels of acute toxicity were observed. However, all samples clearly induced the activity of 7-ethoxyresorufin-O-deethylase. This result points to the presence of substantial levels of bioavailable PAHs in the seepage water and the receiving riverine system.

Introduction

Theisenschlamm slurry is a complex contaminated scrubber dust which is generated as a byproduct of copper smelting (LORENZ, 1994). It contains high amounts of heavy metals, particularly Zn, as well as numerous organic contaminants, particularly poly aromatic hydrocarbons (PAHs) (WEISS et al., 1997). In the Mansfelder Land in Saxony-Anhalt, which is an area traditionally characterized by intensive mining activities, large quantities of Theisenschlamm slurry have been deposited. Seepage waters from these slurries represent a potential toxicological hazard for ground- and freshwaters in the Mansfeld area.

The objective of the present work was to provide initial data for an ecotoxicity assessment of seepage waters from Theisenschlamm deposits. The area which has been selected for the investigation is located close to the village of Eisleben in the Mansfeld area. Hydrogeological analyses have shown that the volume of seepage water from this Theisenschlamm deposit accounts for approximately 295 m^3 per day. This volume is at least partly released into the riverine system of the Böse Sieben.

We have analysed the toxic potential of the seepage water from the Theisenschlamm deposit at the former copper state smelter in Helbra, Saxony-Anhalt. In order to learn about the propagation of seepage water toxicity in the Böse Sieben, we have additionally investigated samples of creek water at various distances from the contamination source.

For toxicity characterization, a battery of biotests was utilized. The test battery approach is considered to improve predictability of adverse pollutant effects in ecosystems from toxicity data obtained in the laboratory. Conventionally, a tiered hazard assessment strategy is applied, using test organisms which represent different trophic levels of an ecosystem. In the present study, we have adopted an alternative approach incorporating assays and testing systems that assess phylogenetically conserved mechanisms of toxicity, such as depression of cellular energy metabolism, cytotoxicity, and induction of xenobiotic metabolism (cf. TWERDOK et al., 1997). Metabolic inhibition was measured in luminescent bacteria, cytotoxicity was determined in both plant (pollen tube) and animal (fish) cells, and xenobiotic metabolism was estimated from the activity of the cytochrome P4501A-dependent 7-ethoxyresorufin-O-deethylase (EROD) enzyme activity which catalyzes the monooxygenation of dioxin- and PAH-like substances.

Material and Methods

Sampling sites (see Chapter 6 and Figure 1 of Chapter 1):

Samples were taken

(1) directly from the seepage water of the Theisenschlamm deposit above the well at Stadtborn; the pH of the sample was 6.4;

(2) from creek water of the Böse Sieben at the village of Hergisdorf (approximately 0.5 km distance to Stadtborn); pH 7.7;

(3) from creek water of the Böse Sieben at the city of Eisleben (approximately 4 km distance to Stadtborn); pH 7.6

(4) from creek water of the Böse Sieben at Wormsleben, 1 km west of the entrance into the Süßer See (approximately 13 km distance to Stadtborn); pH 7.8;

(5) from lake water at the outlet of the Süßer See (approximately 18 km distance to Stadtborn; total lake area approximately 246 ha, 8.6 x 10^6 m^3 water); pH 8.1.

Sampling was done during January 1996. The samples were stored in dark flasks at 4°C and were tested immediately or the day after arrival in the laboratory. Adjustment of the pH values of the samples for testing was not performed.

Biotests

Inhibition of bacterial luminescence: This assay measures the toxicant-induced inhibition of energy-dependent luminescence of the bacterium *Vibrio fischeri*. The test was carried out according to DIN 38412, Part 34. An acute exposure time of 30 minutes at a temperature of 15°C was used for effect determination. For conducting the bioluminescence test, commercially available kits containing lyophilized bacteria were purchased from Dr. Lange GmbH, Düsseldorf. Bacterial luminescence was read in a luminometer (Lumistox, Dr. Lange GmbH, Düsseldorf). The bacteria were rehydrated prior to testing and mixed in a 1:1 ratio with a dilution series of the water sample. In order to meet the osmotic requirements of *Vibrio fischeri*, 2 % NaCl had to be added to the samples. Controls were treated with NaCl only. The inhibition of bioluminescence by the samples was calculated in relation to the alterations of lumenscence in the control incubations (BACKHAUS et al., 1997).

Pollen tube growth test: This assay determines inhibitory effects of samples on the growth of pollen tubes from the tobacco species *Nicotiana sylvestris* (KRISTEN and KAPPLER, 1995). The suitability of the pollen tube growth test for toxicity analysis of complex environmental samples has been recently demonstrated by JUNG et al. (1997). For testing, tobacco pollen were suspended in mixtures of growth medium and environmental sample. After an 18 h-incubation period, growth of pollen was quantified by staining with Alcian Blue and subsequent measurement of optical density in a photometer. Sample-induced inhibition of pollen tube growth was calculated in relation to controls maintained in pure growth medium.

Cytotoxicity assay with fish cell lines: Lethal effects of samples on cells can be measured using in vitro cytotoxicity tests (SEGNER and LENZ, 1993). The cell line used in the present study was the continuous cell line, RTG-2, originally derived from gonadal tissue of rainbow trout, *Oncorhynchus mykiss* (WOLF and QUIMBY, 1962). For the test, individual wells of a 96-well tissue culture microplate were inoculated with 30 000 - 40 000 cells in 0.2 ml of cell culture medium (MEM, Minimum Essential Medium). The cells were allowed for 4 hours to attach firmly to the culture substrate. Afterwards, the medium was removed, and 0.2 ml of pure medium (controls) or medium amended with different concentrations of the test sample were added to each well. The wells were tightly covered with sterile plastic foil and the cells were incubated for another 20 h at 19°C. Then the decrease of cell viability was measured using the neutral red uptake inhibition assay (BORENFREUND and PUERNER, 1984). For this, the exposure medium was removed from the wells, and 0.2 ml of culture medium supplemented with 50 µg neutral red/ml was added. The microplate was re-incubated for another 3 h to allow for the uptake of the vital dye neutral red into the lysosomes of viable, uninjured cells. Thereafter, the medium was removed and the cells were fixed for 1 min with a 1 % formaldehyde/Ca solution. Finally, 0.2 ml of 1 % acetic acid/ 50 % ethanol was added for 30 min to extract the dye accumulated by

viable cells. The plate was transferred to a microplate reader equiped with 540/690 nm filters to measure absorbance of the extracted neutral red. Toxicant effects were quantified as percent decrease of neutral red staining in relation to the controls.

Induction of 7-ethoxyresorufin-O-deethylase (EROD): The cytochrome P450-dependent EROD enzyme is induced by dioxin-like chemicals via the aryl-hydrocarbon receptor (cf. SEGNER and BRAUNBECK, 1998). The measurement of EROD induction is an established biomarker for exposure to halogenated hydrocarbons and polyaromatic hydrocarbons (SAFE, 1990). In the present study, EROD induction by the test samples was measured using an in vitro assay with the fish cell line RTL-W1 (LEE et al., 1993). Approximately 60 000 cells in LEIBOVITZ-15 medium were seeded into individual wells of 48 well tissue culture plates and were grown at 19°C for 3 days. Then, the medium was removed and the cells were exposed for another 24 h to culture medium amended with various concentrations of the samples. At the end of the exposure period, EROD activity was measured using a live cell assay (CLEMONS et al., 1996, BEHRENS et al., 1997). Briefly, the exposure medium was removed, the cells were washed with phosphate buffered saline, and DULBECCO´S medium containing 8 µm 7-ethoyresorufin was added. Production of resorufin by the intact cells was followed in a fluorescence plate reader over 20 minutes at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Afterwards, cell protein was determined by using the fluorescent dye fluorescamine.

Ecotoxicological assessment

Seepage water of Theisenschlamm led to a 100% inhibition of energy-dependent bacterial luminescence (Figure1). In contrast, the water samples taken from the creek Böse Sieben and from the Süßer See resulted in only minor effects on bacterial energy metabolism (less than 20 % inhibition of luminescence; Figure1).

The high toxic potential of the seepage water from the Theisenschlamm, as indicated from the results of the bacterial bioassay, is confirmed by the findings of the RTG-2 cytotoxicity test (Figure1). Almost 100% cytotoxic activity was observed for this sample, whereas water samples of the Böse Sieben and the Süßer See showed no cytotoxicity. The results obtained with RTG-2 cells were confirmed by cytotoxicity measurents using a different fish cell line (Figure 5), the RTL-W1 cell line originating from rainbow trout liver (LEE et al., 1993).

Chemicals with prooxidant activity can be a major source of toxicity in environmental samples. In order to estimate the contribution of prooxidants to the observed toxic responses of the bioassays, we performed a cytotoxicity test using glutathione-depleted RTG-2 cells. The tripeptide glutathione acts as intracellular antioxidant and protects against the toxic action of oxidative stress. Intracellular glutathione levels of

RTG-2 cells can be decreased to 20% or less of the normal cell concentration by culturing the cells in the presence of the glutathione synthesis- inhibitor buthionine sulfoxime (BSO) (MARACINE and SEGNER, 1998). When performing the cytotoxicity assay with glutathione-depleted cells, only the sample from Eisleben showed enhanced toxicity compared to untreated cells (Figure 1). This indicates that prooxidants are not critical toxicity factor in the investigated water samples.

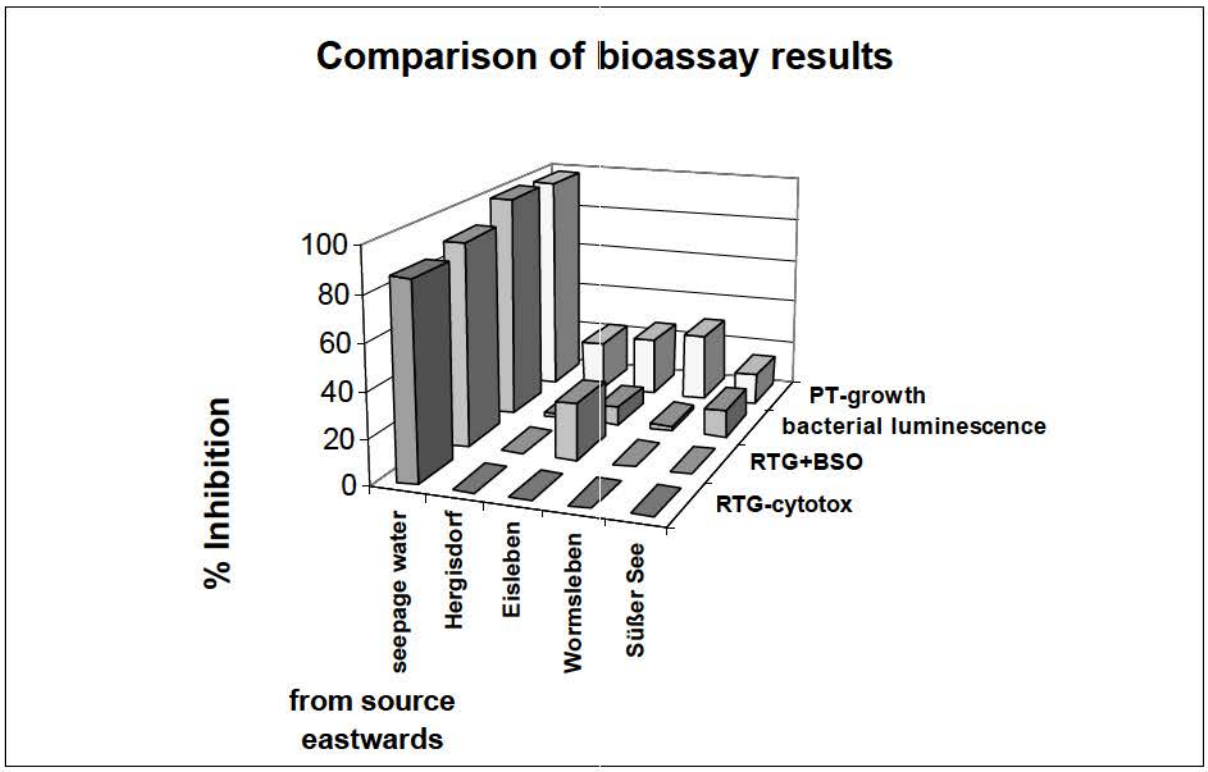

Figure 1: *Toxicity test results as obtained for the 1:2 diluted samples in the various bioassays: Neutral red assay with RTG-2 cells for measurement of acute cytotoxicity („RTG-Cytotox"), neutral red cytotoxicity assay with glutathione-depleted RTG cells for detection of prooxidant toxicity („RTG+BSO"), analysis of toxicant-induced inhibition of bacterial luminescence with Vibrio fischeri („bacterial luminescence"), and toxicant-induced inhibition of the growth of tobacco pollen tubes („PT growth").*

The findings of the pollen tube test confirmed the findings of the bacterial luminescence or cytotoxicity assays. The seepage water from the Theisenschlamm showed 100 % toxicity, whereas for the water samples of the Böse Sieben and the Süsser See only minor or moderate toxic potentials were observed.

Likely candidates possibly responsible for the high acute toxicity of the seepage water sample are metal contaminants, particularly Zn. The seepage water contains massive amounts of Zn, going up to concentrations of more than 50 mM. These concentrations are far above the EC50 values of Zn in the biotests used in this study. EC50 values for Zn were determined to be 0.35 mM in the bacterial luminescence assay and 0.44 mM in the RTG-2 neutral red cytotoxicity assay (unpublished data).

In order to estimate the contribution of Zn to overall toxicity of the seepage water, an EDTA complexation assay was performed (Figs. 2 and 3; HOCKETT and MOUNT, 1996). This approach is based on the assumption that the „free" metal ion is the toxic species whereas complexed metals are not toxic. Through complexation of the free metal ions in the sample by addition of EDTA concentrations which are at least equimolar to the metal levels in the sample, the toxic metal ions will become non-toxic for the biological target system (Figure 2).

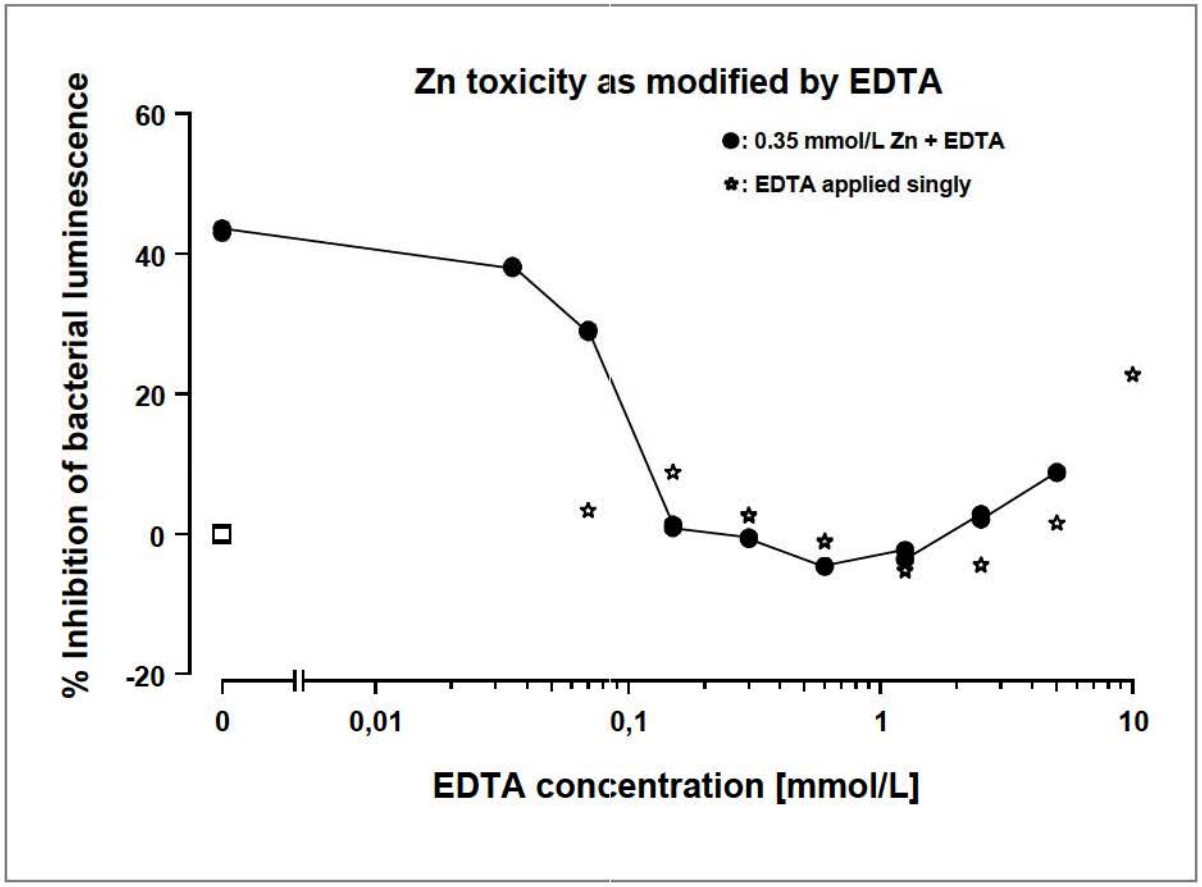

Figure 2: *Principle of EDTA testing: Through EDTA complexation, metals can be removed from a solution. By comparing the toxicity before and after EDTA treatment, the contribution of metals to the overall toxicity of the sample can be estimated. For this purpose, a series of dilutions of an EDTA stock solution were added to the sample (here: pure Zn solution), incubated under constant stirring overnight, and toxicity to luminescent bacteria was recorded the next day, in comparison to pure EDTA dilutions and the pure seepage water. When approaching equimolar concentrations to Zn, EDTA effectively removed Zn toxicity from the sample.*

The results of the EDTA complexation experiment with RTG-2 cells are shown in Figure 3: EDTA was added up to concentrations of 50 mM; at concentrations higher than 25 mM, EDTA itself became cytotoxic. Cytotoxicity of the seepage water from Theisenschlamm was partly reduced by EDTA, with the maximum effect at the highest non-toxic EDTA concentration (25 mM). Complete removal of toxicity could

be not achieved since Zn concentrations in the sample (52 mM) were higher than the highest non-toxic EDTA concentrations (25 mM).

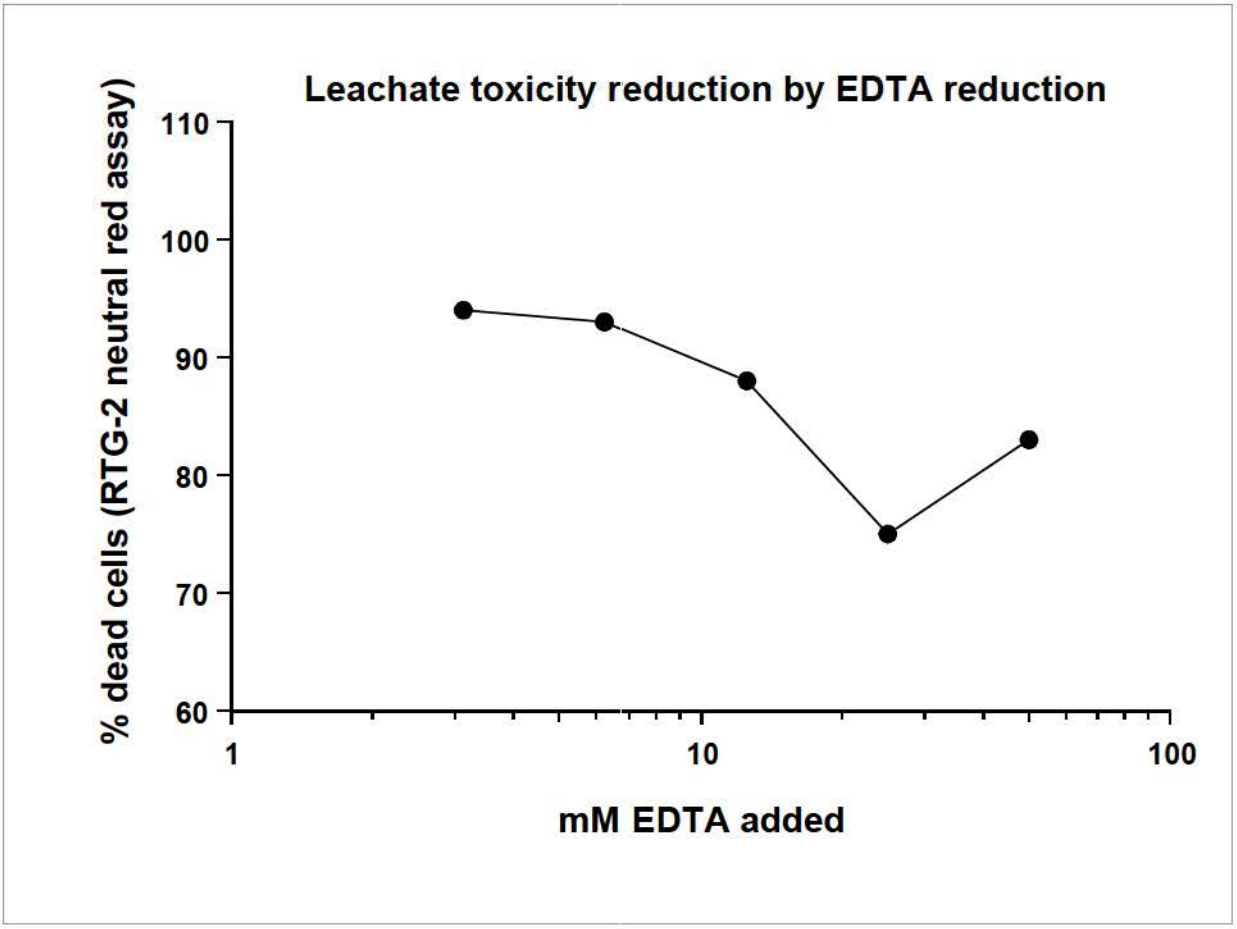

Figure 3: *EDTA complexation of the seepage water from Theisenschlamm (method as described for Figure2).*

Apart from heavy metals, Theisenschlamm slurry contains high concentrations of polycyclic aromatic hydrocarbons. As shown by chemical analyses, the PAHs are leached from the slurry and are released into the water. However, due to their hydrophobicity, the PAHs may attach to DOM (dissolved organic matter) and/or may be transferred into the sediment , leading to their removal from the water phase. To detect the presence of bioavailable PAHs in the water samples, we used the EROD induction bioassay with RTL-W 1 cells (CLEMONS et al. 1996). This assay indicates the presence of dioxin-like substances, including dioxins, furanes, PCBs and PAHs, in a sample (BRACK et al., 2000). The results, as shown in Figure 4, demonstrate a significant induction of EROD in all samples analysed, except for the seepage water itself. Importantly, there is no significant decrease of EROD activity with increasing distance to the entry site of the seepage water into the riverine system. This indicates that substantial levels of bioavailable PAHs stay in the water phase of the Böse Sieben and are not removed into the sediments.

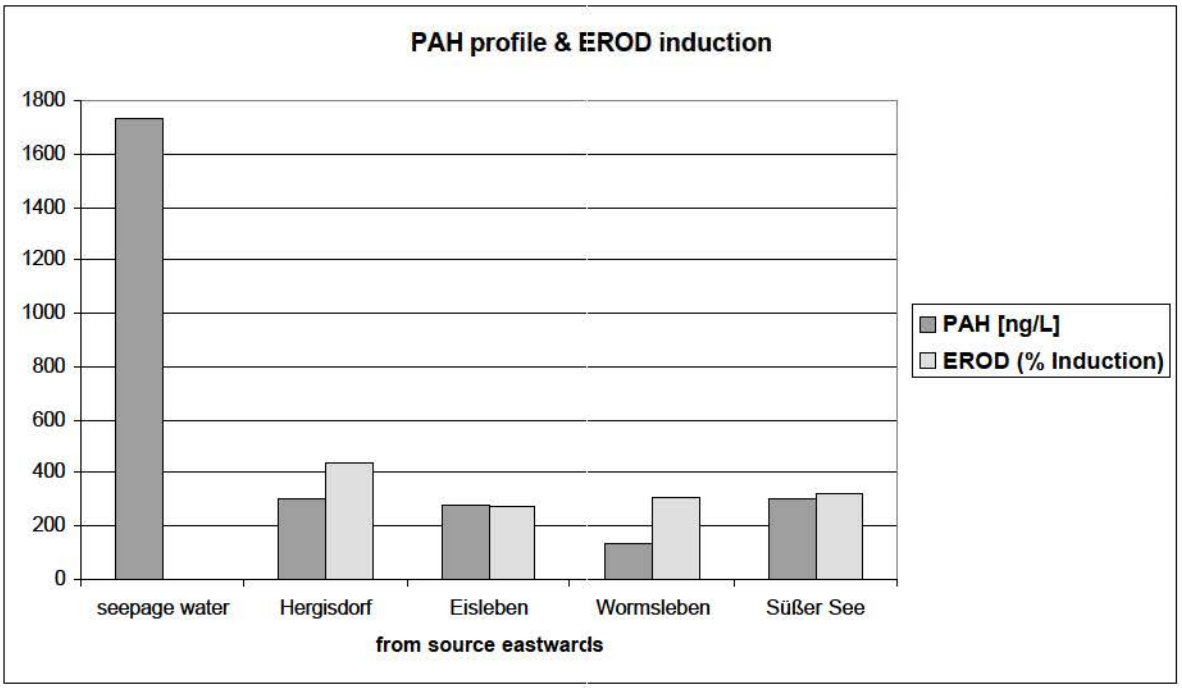

Figure 4: *Total PAH levels in the samples and the corresponding EROD response. The measurement was done in RTL-W1 cells which were exposed for 24 h to 1:2 diluted samples of the seepage water and from the Böse Sieben.*

The lack of EROD induction by the seepage water from Theisenschlamm (Figure 4) is explainable by the extreme acute cytotoxicity of this sample, which hides any sublethal effect such as the EROD response. When diluting the seepage water, an increasing EROD induction with decreasing cytotoxicity is evident (Figure 5).

The bioassay findings on EROD induction are well in line with the findings from chemical analyses on total PAH levels in the water samples (Figure 4). The bulk constituents of the PAH fraction are compounds such naphtalene, fluoranthene, phenanthrene or pyrene (SCHRECK et al., 1998, WEISS et al., 1997) These compounds, however, are non-inducing PAHs, i.e. they lack the ability to induce the cytochrome P450-dependent EROD activity (BEHRENS et al., 2001). The fact that we observe clear-cut EROD induction in the assay with RTL cells indicates that the seepage water from Theisenschlamm, in addition to the quantitatively dominating non-inducing PAH congeners, contains toxicologically relevant amounts of inducing PAHs. This response is of particular relevance since cytochrome P4501A-mediated metabolism of PAHs leads to the generation of mutagenic and, ultimately, carcinogenic metabolites.

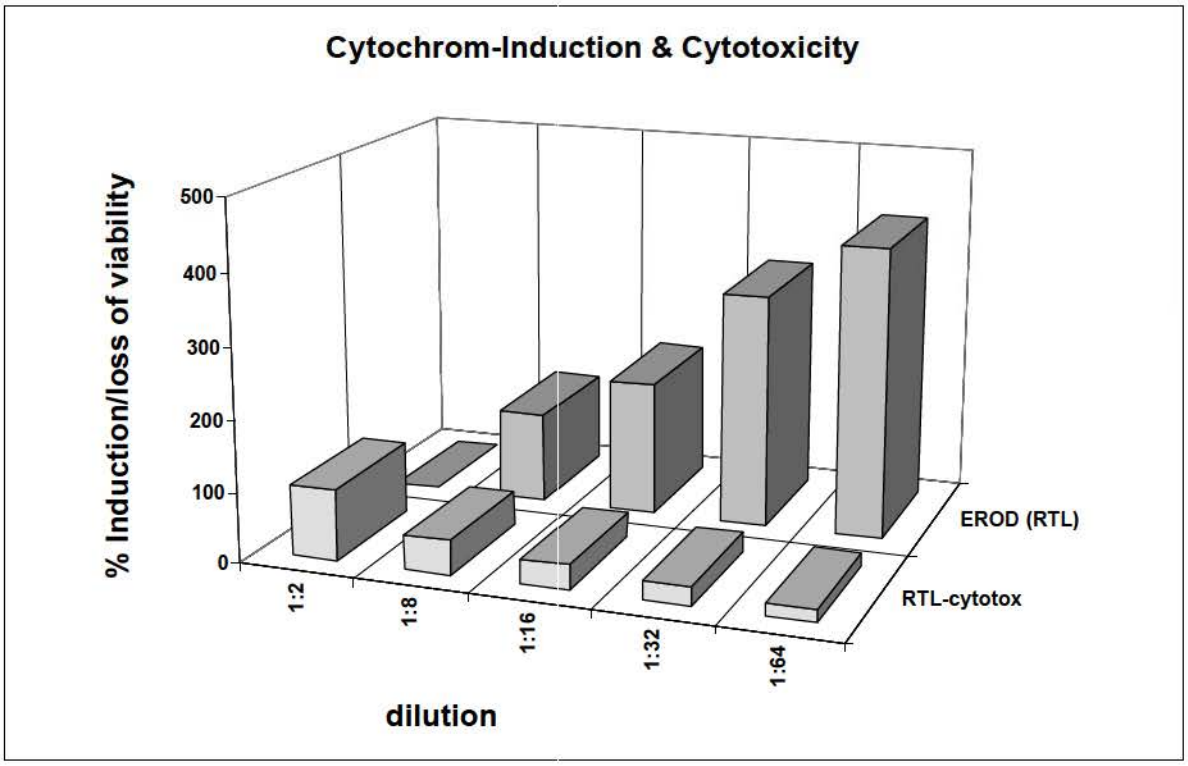

Figure 5: *The 1:2 diluted seepage water from Theisenschlamm is 100% cytotoxic to RTL-W1 cells and, therefore, does not allow the detection of EROD activity. Enzyme induction represents a sublethal response to toxicants and requires the functioning of differentiated cellular functions. Only in sepage water samples diluted 1:8 or more, when cytotoxicity is lower than 50%, EROD induction could be detected.*

Conclusions

The seepage water from Theisenschlamm shows a drastic acute toxicity which appears to be mainly caused by its very high Zn levels. Removal of Zn from this sample lead to a (partial) reduction of the acute toxicity. The high, metal-associated acute toxicity of the seepage water is not detectable in the water samples from the riverine system of the Böse Sieben and the Süsser See. Obviously, Zn ions are sedimented within a short distance after the entry site of the seepage water, and the toxic ions are no longer bioavailable. However, high levels of Zn could be expected in the river sediments close to the seepage water entry site.

Contrary to Zn, PAHs obviously are not quantitatively trapped into sediments but can be found at all sample stations analysed. Accordingly, all investigated stations show a clear-cut induction of EROD activity which is responsive to PAH exposure. The PAHs do not give rise to acute toxicity, however, via EROD-mediated metabolism they may cause mutagenic and carcinogenic effects. This toxic potential must not be overlooked in the hazard assessment of the riverine system of the Böse Sieben. Future studies should address in more detail the presence of genotoxic potentials,

the distribution of chemical toxicity between water and sediment, and their spatial distribution along the river system.

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Fine-grained residues from copper smelting and their environmental impacts

A case study from the Mansfeld District, Germany

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