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Environmental Contamination and Toxicology

Ecotoxicological Examination of Sediment Extracts of Huaihe River, China by *In Vitro* Bioassays

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Extraordinary economic growth, industrialization, urbanization have resulted in widespread water pollution in China. Huaihe River is an important natural water source for central China and is one of the mostly polluted rivers in China. This river is over 1000 kilometers long and flows through four provinces of China, namely Henan, Shandong, Anhui and Jiangsu. Severe pollution incidents have been reported in this area in the past years (Ch. EPA 1996). High concentrations of chemical oxygen demand (COD) and biological oxygen demand (BOD), consisting of many kinds of organic pollutants, were found in the river and caused serious damages of fishery, industry, and agriculture. The contamination of the water has been seriously affected the living standard of the people along the river.

Sediments serve both as a sink and a source for a number of environmental pollutants and exchange chemicals with water phase, especially hydrophobic organic contaminants (Jaffe 1991). Some hydrophobic organic contaminants have slow rates of degradation and can persist in the environment for long periods of time and tend to bioaccumulate and biomagnify in the food chain (Duursma et al. 1986; Fortner and Sick 1985). In rivers, during certain times of year due to floods or human activities, residues associated with sediments can be resuspended and become bioavailable.

The risk assessment of pollutants in aquatic environments is often based on the concentrations detected by analytical chemistry and data on the toxicity of single compounds. Chemical analyses provide little information on the biological effects of complex mixtures, and they do not account for possible interactions between or among individual chemicals (Hilscherova et al. 2001). They provide only part of the knowledge necessary to evaluate and assess their toxic potential for wildlife and humans. Moreover classical chemical analysis of complex mixtures of organic residues present in environmental samples can be both resource and time intensive. In vitro bioassays can be used as a specific chemical detector for complex mixtures. They serve as simple, rapid and sensitive screening systems and represent an alternative to comprehensive chemical analysis. They are practical and reliable for monitoring the toxicity of complex mixture of environmental samples and predicting the potential ecotoxicological effects to environment.

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The bioluminescence inhibition assay with *Vibrio fischeri* is not only described as rapid, sensible and easy to perform, but also as a low cost bioassay which can be recommended as a valuable test method in water pollution control (Nohava and Vogel 1995). The sensitivity of the test correlates well with other bioassays yet requires only 15 or 30 min to obtain a complete reportable assay.

The critical mechanisms of toxicity for some hydrophobic organic contaminants is their dioxin-like activity (Hilscherova et al. 2001). These chemicals bind to the arly hydrocarbon receptor (AhR) present in the cytosol; their binding affinity has been related to the incidence and intensity of toxic effects (Lucier et al. 1993; Hankinson 1995). Binding of a ligand to the AhR initiates a cascade of actions leading to enhanced transcription of AhR-regulated genes and increases in activities of AhR responsive enzymes (Zacharewski et al. 1995). Cytochrome P4501A (CYP1A) is a member of the AhR-responsive enzymes. Organic compounds known to bind to the AhR include PCBs, PAHs, chlorinated dibenzodioxinx, and chlorinated dibenzofurans (Hansen and Addisson 1990; Elskus et al. 1992) which are often found together in environmental matrices. Extensive studies have suggested that the catalytic activity of CYP1A, measured as ethoxy resorufin-O-deethylase (EROD) activity in liver can serve as a biomarker of exposure to PAH and PCB types of pollutants (Narbonne et al. 1991; Goksoyr and Förlin 1992; Stegeman and Hahn 1994) and for assessing the risks of environmental samples in vitro (Pesonen and Andersson 1992).

In the present study, sediment samples were collected along Huaihe River at four sites named as XinYang, FuYang, HuaiNan and BengPu. The EROD induction with a micro-EROD assay in vitro using H4-IIE rat hepatoma cell cultures and bioluminescence test available commercially in Germany through Dr. Bruno Lange GmbH, known as the Lumistox test were used to assess the potential ecotoxicological effects of the sediment extracts from Huaihe River, China to know the pollution extent of the river. Our objective was to evaluate the contamination of Huanhe River by *in vitro* bioassays to investigate the possibility of biomonitoring aquatic pollution.

MATERIALS AND METHODS

Four surface sediments were collected along Huaihe River, named as (1) XinYang (XY), upper river; (2) FuYang (FY), main tributary of Huaihe River, (3) HuaiNan (HN), midway along the river, and (4) BengPu (BP), downstream of HuaiNan as shown in Figure 1. Triplicate sediment samples (0-10 cm depth) were collected from each site by stainless steel grab. The sediment samples from each site were mixed and sub-sampled to ensure the representation. The samples were frozen dried, then homogenized and ground to pass a 2-mm sieve. Ten grams of sediment sample were mixed with anhydrous sodium sulfate and extracted with 60 ml methylene chloride for 24 hours in a Soxhlet apparatus. The extracts were then cleaned up with a column consisted of silica gel (60-80 mess, deactivated, 4 cm long) and anhydrous sodium sulfate (4 cm long), treated with copper wool to remove sulfur and evaporated to dryness with a stream of high purity nitrogen

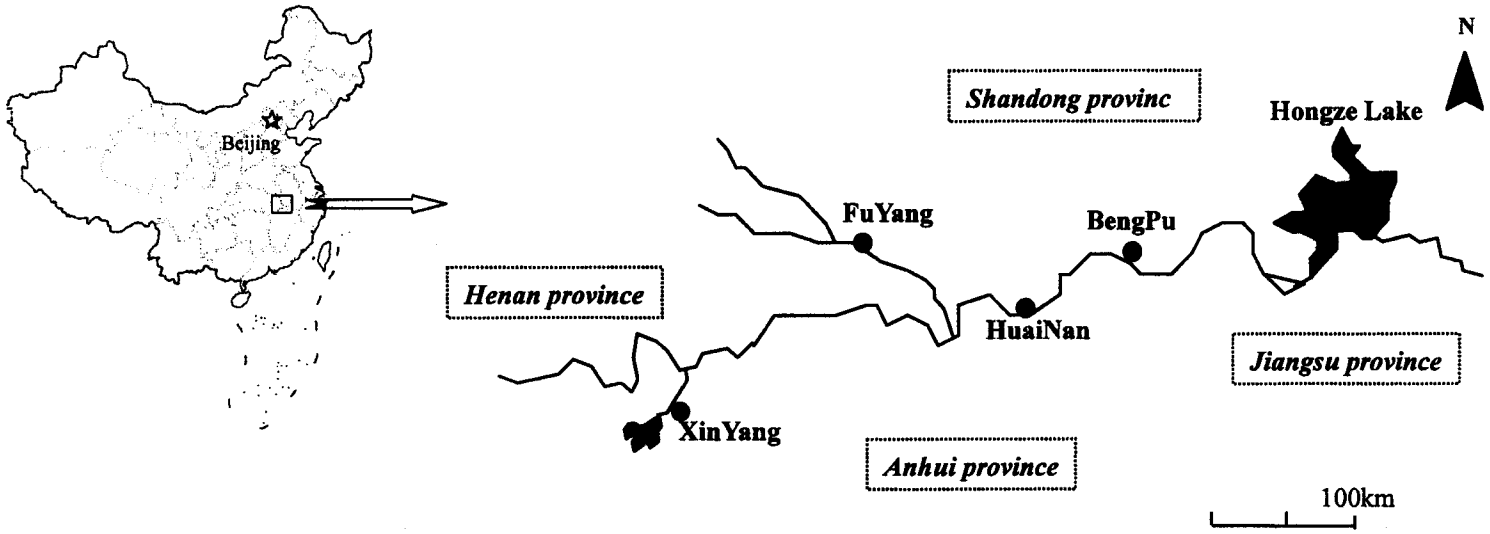

Figure 1. Map of Huaihe River showing the sampling locations

blowing and redissolved in DMSO.

The Lumistox test was conducted following the manufacture's procedures (Dr. Lange GmbH, Düsseldorf, Germany) as described previously (Wang et al. 2002). *Vibrio fischeri* (strain NRRL-B-11177) and other reagents used for the test were purchased from Dr. Lange GmbH, (Düsseldorf, Germany). The 15 min standard bioluminescence inhibition assay was carried out according to ISO and DIN 38412 L34/L341. Bioluminescence was measured on a luminometer (LUMISTox 300, Dr. Lange GmbH). For the purpose of comparison, the bioluminescence inhibition (light loss) after 15-min exposure was calculated by comparing the light with control measured under the same condition and determined as Asami et al. (1996). Concentration of the carrier solvent (DMSO) in the test medium, including control was 0.5% which was a nontoxic dose to *Vibrio fischeri* estimated in preliminary tests.

The H4-IIE rat hepatoma cell line was obtained from the American Type Culture Collection. Dulbecco's MEM (DMEM, containing 1.0 g/l D-Glucose, 3.7 g/l $NaHCO_3$ and 1.0289 g/l N-Acetyl-L-alanyl-L-glutamine, Berlin, Germany) was supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100μg/ml streptomycin and used as cell culture medium. The cell culture and EROD assay were based on Donato's method (Donato et al. 1992). Briefly, 2.5×10^3 cells were seeded into individual wells of a 96-well microtitre tissue culture plate (Coulter counter was used to count the cell numbers). Cells were grown for 24 hours to about 60-70% confluence and exposed with samples for another 24 or 72 hours. 8-well lane was used for replication. The plate was incubated at 37°C, 10% CO_2 incubator (Heraeus instrument).

The sediment extracts dissolved in DMSO were added directly to medium. The final concentration of DMSO was less than 0.5% which showed no toxic effect on the cells. However, to eliminate artifactual effects due to DMSO, the same amount of the organic solvent was added to control cultures.

Determination of EROD enzyme activity was determined directly in intact rat hepatoma cell cultured on 96-well plates. Monolayers were washed twice with PBS (phosphate-buffered saline 20 mM, pH 7.2, containing 0.9% NaCl) to remove detached cells. Assay was started by addition of 200 μl/well of culture medium containing 8 μM 7-ethoxyresorufin as substrate and 20 μM dicumarol to prevent further metabolism of the resorufin formed by the cytosolic enzyme diaphorase. After 30 min incubation at 37°C, a 100-μl aliquot of cell medium was withdraw from each well and transferred to another 96-well plate. 200 μl of ethanol was added to each well to precipate the protein in the medium. Fluorescence of the product resorufin was recorded directly on Microplate Reader (Labsystems Fluoroskan II, Finland) at 550 nm excitation and 585 nm emission wavelength, which could be regarded as relative EROD induction value (Hilscherova et al. 2001) since the wells had appoximate the same number of cells with the same protein concentration. Otherwise, a standard curve of resorufin could be prepared in culture medium and then the EROD activity could be

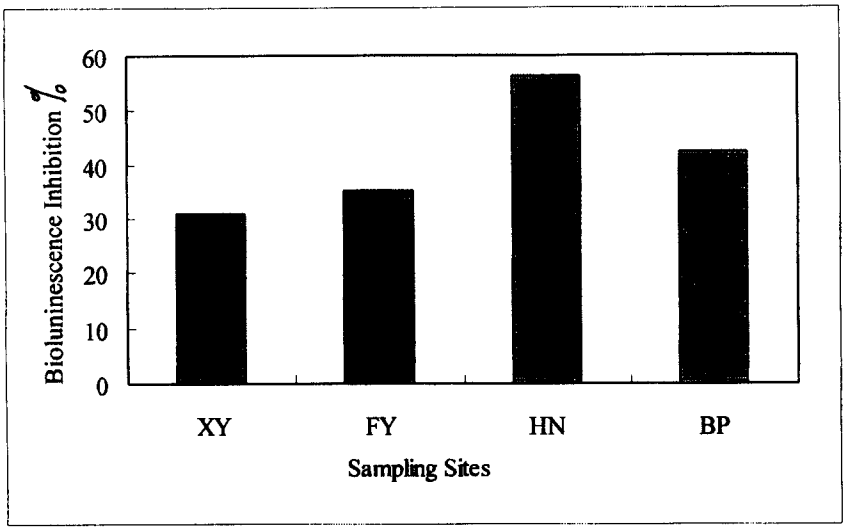

Figure 2. Luminescence inhibition of sediments extracts collected from Huaihe River to *Vibrio fischeri.*

calculated. In this study, the fluorescence value was used directly to express the relative EROD induction level. 7-ethoxyresorufin and dicumarol were purchased from Sigma (St. Louis, MO). All other reagents used were of analytical grade.

RESULTS AND DISCUSSION

The acute toxicity data of the 4 sediment extracts from Huaihe River by a standard bioluminescence test procedure is shown in Figure 2. The acute toxicity for 4 sediments varied by sampling site, the luminescence inhibition to *Vibrio fischeri* from BengPu, HuaiNan was greater than that from FuYang and XinYang.

EROD is generally regarded as being early warning signals for the Ah-receptor-related toxic effects of PCBs, PAHs and related compounds (Brink et al. 2000). Figure 3 is the EROD induction of the sediment samples of Huaihe River as the function of the sample dosage. It could be seen that all 4 sediment extracts contained AhR-active residues which could induce the activity of EROD during the 24 hours incubation with dose-response relationships. Higher concentration of the samples has shown greater induction of EROD. The longer exposure time (72 hr) was chosen as well for potential metabolization of labile compounds. Generally most organic chemicals could be metabolized after 72 hours incubation (Payne et al. 1987), then the level of induced EROD activity decreased, except in the presence of the persistent dioxin-like compounds. The EROD induction values have been reported to be greater at time periods shorter than 72 hr with the activity least variable at 72 h (Hahn et al. 1996). EROD

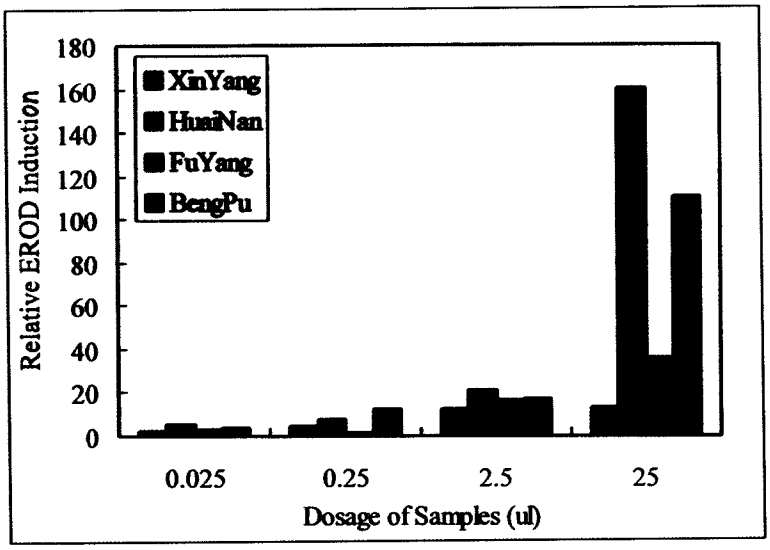

Figure 3. EROD induction as a function of the dosage of the extracts of sediment samples collected in Huaihe River

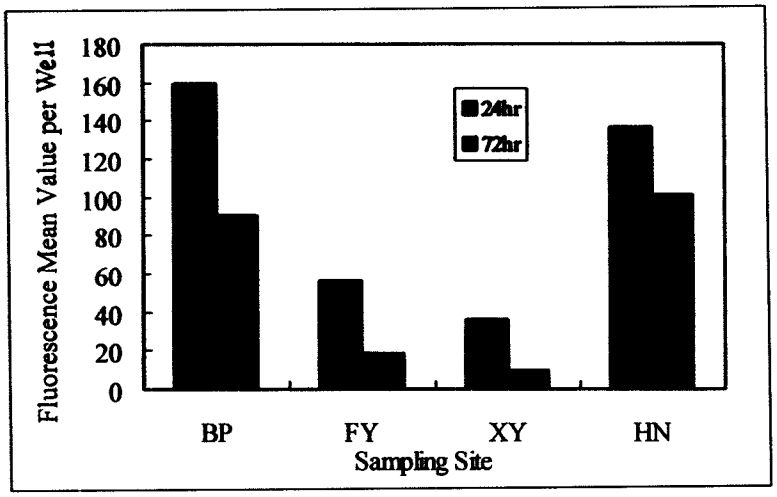

Figure 4. EROD induction after 24 and 72 hours exposure to sediment extract samples from Huaihe River

induction of the cells exposured to 4 sediment extracts after 24 and 72 hours incubation was illustrated in Figure 4. It is obvious that after 72 hours incubation, the high EROD activity still remained. This suggests the presence of the persistent organic contaminants in all samples, such as PCBs and PAHs, and supports the previous chemical analysis results in our lab (Wang et al. 2000; Wang et al. 2001). It demonstrated the pollution extent of the 4 sites of Huaihe River was XinYang < FuYang < HuaiNan < BengPu, according the flow way from upstream to downstream.

Environmental samples are complex mixtures with possible antagonistic or synergistic effect on the EROD induction. Since EROD induction is considered as a biomarker of exposure to PAHs, PCBs and dioxin-like compounds (Stegeman and Hahn 1994), our results suggest the presence of these pollutants in all the samples collected in the river. The results exhibited in figures 2 and 4 show the comparability of the two bioassays for the sediment extracts. Neverthless, more chemical analysis data are necessary in order to show a common cause to the EROD induction and the acute toxicity to *Vibrio fischeri*. Further exploration of the dioxin-like compounds in the river is necessiate because of their persistence and harmful effects on organisms. The results demonstrated that in-vitro bioassays could be developed as a promising technique for monitoring the micropollutants contamination of the river. The in vitro bioassays approach could be a cost-effective alternative to the more resource-intensive and time-consuming instrumental analyses in initial screening of river sediment extracts.

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