ENDOCRINE DISRUPTING, MUTAGENIC, AND TERATOGENIC EFFECTS OF UPPER DANUBE RIVER SEDIMENTS USING EFFECT-DIRECTED ANALYSIS

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Abstract—Effect-directed analysis (EDA) can be useful in identifying and evaluating potential toxic chemicals in matrices. Previous investigations of extracts of sediments from the upper Danube River in Germany revealed acute nonspecific and mechanism-specific toxicity as determined by several bioassays. In the present study, EDA was used to further characterize these sediments and identify groups of potentially toxic chemicals. Four extracts of sediments were subjected to a novel fractionation scheme coupled with identification of chemicals to characterize their ability to disrupt steroidogenesis or cause mutagenic and/or teratogenic effects. All four whole extracts of sediment caused significant alteration of steroidogenesis and were mutagenic as well as teratogenic. The whole extracts of sediments were separated into 18 fractions and these fractions were then subjected to the same bioassays as the whole extracts. Fractions 7 to 15 of all four extracts were consistently more potent in both the Ames fluctuation and H295R assays. Much of this toxicity could be attributed to polycyclic aromatic hydrocarbons, sterols, and in fraction 7-naphthoic acids. Because the fraction containing polychlorinated biphenyls, polychlorodibenzo-p-dioxin/furan, dichlorodiphenyltrichloroethane, and several organophosphates did not cause any observable effects on hormone production or a mutagenic response, or were not detected in any of the samples, these compounds could be eliminated as causative agents for the observed effects. These results demonstrate the value of using EDA, which uses multiple bioassays and new fractionation techniques to assess toxicity. Furthermore, to our knowledge this is the first study using the recently developed H295R assay within EDA strategies. Environ. Toxicol. Chem. 2012;31:1053–1062. © 2012 SETAC

Keywords—Fractionation Steroidogenesis Mutagen Polyaromatic hydrocarbon Polychlorinated biphenyls

INTRODUCTION

According to the U.S. Toxic Substances Control Act, more than 70,000 organic chemicals are currently used in industry. For some of these chemicals, there is a general lack of toxicity data, even though there is the possibility of some of these substances being present at biologically active concentrations in the environment that might pose a risk to organisms [1–4].

Despite extensive stocking efforts, populations of some fish in the upper Danube River, Germany, have been reported to be declining over the last few decades. One concern in the upper Danube River is the toxicity of sediments to fish [5–7]. To date, several studies have assessed toxicity of sediments taken from the upper Danube River and have found several sites where extracts of these sediments caused significant toxicity. Effects include genotoxicity, mutagenicity, teratogenicity, or estrogenicity [1–4]. For example, Keiter et al. [2] reported concentrations of 16 priority U.S. Environmental Protection Agency (U.S. EPA) polycyclic aromatic hydrocarbons (PAHs) to be as great as 26 μg/g dry weight in sediments of the upper Danube River. Extracts of sediments from the same sites elicited significant aryl hydrocarbon receptor-mediated effects in three separate cell lines. In addition to dioxin-like compounds, other chemicals have been reported to occur in these sediments. Specifically, a variety of compounds that were previously shown to modulate the endocrine system such as certain pesticides, plasticizers, sterols, and PAHs have all been measured in sediments [1,2,8,9]. The potential of these sediments to disrupt endocrine functions has been confirmed in a recent study that reported in vitro estrogen agonist potency and modulation of steroidogenesis [9]. However, according to that study only 2 to 6% of the observed estrogenic effect in the sediment studied could be attributed to the routinely monitored estrogenic chemicals estradiol (E2), estrone (E1), ethinylestradiol (EE2), bisphenol A (BPA), or nonylphenol (NP). It has also been reported that exposure to sediments sampled from the same locations but at different times in the Danube River resulted in lesser hatching rates and increased time-to-hatch in zebrafish (Danio rerio) embryos [1].

Chemicals in environmental matrices such as sediments and biota occur as mixtures and can include bioactive compounds and isomers of both natural and anthropogenic origin. Because there may be no a priori knowledge of the chemicals present in
samples, analysis of all of the possible chemicals present in an environmental sample would not only be prohibitively expensive, but because of limits in the available analytical methodologies or authentic standards for some chemicals, not possible. Therefore, approaches have been developed to supplement the identification and quantification of chemicals by instrumental analyses with bioanalytical techniques that make use of the specific properties of certain groups of chemicals to interfere with specific biological processes. This type of analysis has been defined as effect-directed analysis (EDA) and is based on a combination of fractionation procedures, biotesting, and subsequent identification and quantification of individual compounds or classes of compounds [10–15]. In EDA, matrices containing mixtures of chemicals, such as extracts of sediments, are first analyzed using one or a combination of several bioassays that are responsive to different modes of biological action. If a sample has been identified as causing significant effects, it is then subjected to fractionation, which separates the chemicals based on polarity, molecular size, or other physicochemical properties or combinations [10]. These fractions are then retested in the bioassays in which they were previously shown to be active. This procedure is repeated until the activity can be identified and isolated in one or multiple fractions. These fractions are then subjected to instrumental analyses to identify and quantify individual compounds. Because the chemical physical characteristics of chemicals in specific fractions are known, this information can be used to select the most appropriate analytical techniques to identify and quantify specific constituents of that fraction. The information obtained during this process can be used to find the source of the contaminants, such as industry or agriculture, or decide on remedial efforts if needed.

Several assays have been developed to evaluate the biological activities of chemicals. Considering the previously reported mutagenic, teratogenic, and endocrine disrupting potential of Danube River sediments, in the present study we focused on endocrine disruption, mutagenicity, and teratogenicity. One cell line that has been shown to be a useful in vitro model for investigating effects on steroidogenic pathways and processes including the production of sex steroids is the human H295R adrenocarcinoma cell line [16–18]. Under the guidance of the U.S. EPA and the Organization for Economic Co-operation and Development (OECD), an OECD H295R steroidogenesis test guideline was developed to address regulatory needs for the screening of effects of chemicals on steroidogenic pathways [19–21]. Thus, standardized protocols and procedures are available for this assay.

Mutagenic and teratogenic effects can be measured using different types of assays, including the Ames fluctuation assay, to measure mutagenic effects and the D. rerio embryo assay to measure teratogenic effects. Danio rerio embryos have a transparent chorion; therefore, lethal and sublethal effects are easily monitored in the egg [1,22] and have been shown to be sensitive to toxicants. The Ames fluctuation assay has been shown to be a useful tool for assessing mutagenic activity in sediments because it is a rapid and sensitive test that measures the ability of a sample or chemical to cause back mutations in Salmonella bacteria [23,24]. In addition, different types of mutations caused by different chemicals can be determined depending on the type of Salmonella strain used; the TA100 Salmonella strain indicates base pair substitutions and the TA98 Salmonella strain measures frame-shift mutations. Metabolic activation or deactivation of toxicants can also be evaluated by treating the extract with CYP450-enriched liver microsomes (S9) to the assay.

The objective of the present study was to use EDA with a new fractionation technique that uses an online fractionation procedure with multiple coupled high-performance liquid chromatography columns [13]. This fractionation technique was used because, compared to other similar fractionation techniques, it decreases loss of material during fractionation and decreases formation of artifacts. During the first phase of the present study, extracts of sediments from four sites in the upper Danube River were analyzed in the following three assays: the D. rerio embryo assay, the Ames fluctuation assay, and the H295R assay. During the second phase, each extract was fractionated into 18 fractions with different physicochemical properties, and the biological activity of each fraction was characterized in the D. rerio embryo, the Ames fluctuation, and H295R assay. To our knowledge, this is the first study using the recently developed H295R assay within EDA strategies.

MATERIALS AND METHODS

Sampling

In 2006, near-surface samples of bottom sediments were collected at two locations along the upper Danube River (Sigmaringen [Sig2006] and Lake Oepfingen [Opt2006]) as well as at one tributary stream just upstream of its confluence with the Danube River (Lau2006). Samples were collected using a Van Veen grabber or a stainless steel shovel. Furthermore, an archived Lauchert sediment sample collected in 2004 (Lau2004) was included as an uncontaminated reference site based on the results of previous studies [2,25]. All 2006 sampling sites were chosen based on a suspected gradient of sediment contamination at these locations [1,2,25], and because of their exposure to sewage treatment plants effluents (Fig. 1).

Soxhlet-extraction and accelerated membrane-assisted clean-up of sediments

Sediments were pooled, homogenized, lyophilized, and sieved (mesh size 1.25 mm) immediately after returning to the laboratory. Dried and sieved sediments were Soxhlet extracted for 14 h with dichloromethane and acetone (3:1 v/v, 400 ml) according to previously described methods [26]. Extracts were concentrated first by use of rotary evaporation and then by a gentle stream of nitrogen. Elemental sulphur was removed by treatment of the extract with copper. Whole extracts of sediment from each sample were redissolved in 1 ml dimethyl sulfoxide and were used for testing in the D. rerio embryo assay and the H295R assay. Residues were also redissolved in 1 ml hexane (Hx) and acetone (7:3 v/v) for subsequent clean-up procedures.

A new accelerated membrane-assisted clean-up (AMAC) technique was used to purify whole extracts of sediments according to an optimized protocol described previously [27]. Briefly, an aliquot of the whole extract of sediment equivalent to 20 g of sediment was transferred to dialysis membranes (commercially available polyethylene tubes) and diazoyzed using an ASE 200 device (Dionex). The temperature, pressure, number, and duration of cycles were chosen as described previously [13]. After evaporating the extracts to dryness, the residue was redissolved in dimethyl sulfoxide for in vitro testing and in 1 ml Hx:dichloromethane (9:1 v/v) to a final concentration of 25 g sediment equivalents (SEQ)/ml for further fractionation. Furthermore, because of a limited sample volume, in vitro testing was prioritized and AMAC extracts were
only used with the *D. rerio* embryo assay and Ames fluctuation assay.

**Fractionation of extracts of sediments**

Fractionation was performed by using a recently developed automated online fractionation procedure for polycyclic aromatic compounds in extracts of sediment on three coupled, normal-phase high-performance liquid chromatography columns as described in Luebcke-von Varel et al. [13]. All solvents used within the fractionation procedure were Suprasolv or LiChrosolv grade (Merck). Briefly, in the first step, medium polar and polar compounds are trapped on cyanopropyl silica with Hx as mobile phase, while nonpolar substances are flushed to the nitrophenylpropyl silica and porous graphitized carbon stationary phases. To separate PAHs with more than two aromatic rings from the more polar PAHs such as nitro- and keto-PAHs, the cyanopropyl silica-column is switched offline. Flushing of the nitrophenylpropyl silica and porous graphitized carbon phases with Hx continues and the remaining chlorinated diaromatic compounds elute from nitrophenylpropyl silica to the porous graphitized carbon column. Afterwards, a sequential fractionated elution from each of the columns was conducted, starting with the separation of chlorinated diaromatic compounds on porous graphitized carbon in forward and back-flush mode using Hx and toluene as mobile phases. The nitrophenylpropyl silica phase was then eluted with Hx:dichloromethane (95:5). Finally, the cyanopropyl silica column is eluted with Hx, dichloromethane and acetonitrile. After passing through the detector the eluent is collected by the fraction collector into 18 glass bottles [13]. After fractionation, the 18 fractions of each sample were evaporated to dryness and the residue was redissolved in dimethyl sulfoxide for subsequent use in bioassays and chemical analysis.

**Ames fluctuation assay**

All AMAC extracts and fractions were analyzed by using bacteria strains TA98 (measures frameshift mutations) and TA100 (measures base pair substitutions) with or without metabolic enzymes (S9 fraction). Details of the procedure are explained in Reifferscheid et al. [24]. Briefly, in 24-well plates, *Salmonella* were diluted in exposure medium and exposed to either AMAC extracts, fractions, positive control, or a 2% dimethyl sulfoxide solvent control. The plates were incubated for 90 min at 37°C. Different positive controls were used depending on the bacteria strain and if S9 was used, including 4-nitro-0-phenyldiamine (0.05 mg/ml) for TA98 without S9, nitrofurantion (0.06 mg/ml) for TA100 without S9, and 2-aminoanthracene (0.025 mg/ml) for both TA98 and TA100 with S9. After 90 min, a bromocresol purple indicator medium was added to all wells. An aliquot of 800 μl from each well of the 24-well plate was transferred into 16 replicate wells of a 384-well plate in triplicate for a total of 48 wells per sample. The plate was incubated at 37°C for 48 h to allow any bacteria to grow that had been back mutated and able to produce histidine. After 48 h, if a back mutation occurred and the bacteria reproduced, the media turned from purple to yellow. The number of yellow wells per replicate group (16 wells per one replicate) were counted and compared to the solvent control.

Accelerated membrane-assisted clean-up extracts of sediments from Opf2006, Sig2006, Lau2006, or Lau2004 were each tested at six dilutions (12.5–400 mg SEQ/ml) with a solvent control and positive control. All dilutions, solvent controls, and
positive controls were conducted in triplicate. All sediment fractions were investigated at one concentration of 400 mg SEQ/ml in triplicate.

**Danio rerio embryo assay**

Fertilized *D. rerio* eggs were exposed to four or five dilutions of whole extracts of sediments and AMAC extracts of sediments from Opf2006, Sig2006, Lau2006, or Lau2004. For a detailed description of the assay see Holfert et al. [22]. For the fractionated samples, only Opf2006 and Sig2006 extracts were analyzed because no toxicity was observed in any concentration of AMAC extract of either Lau2006 or Lau2004. All fractionated samples were investigated at a concentration of 100 mg SEQ/ml.

In brief, whole extracts of sediments, AMAC extracts of sediments, or fractions were diluted in water containing the following compounds (0.294 g/l CaCl$_2$ $\times$ 2 H$_2$O, 1.23 g/l MgSO$_4$ $\times$ 7 H$_2$O, 0.63 g/l NaHCO$_3$, 0.055 g/l KCl). A total of 10 eggs were added to test tubes containing the diluted extract or fraction. One egg and 100 μl of test solution were removed and placed in one well of a 96-well plate. This was repeated for the other nine eggs in each sample. Whole extracts of sediments and fractions were analyzed in replicate exposures. After seeding and dosing, the 96-well plates were covered and placed in an incubator at 26°C for 48 h. At the end of the incubation period, four endpoints including lack of heartbeat, failure to detach tail, retarded somite formation, and coagulation of the embryo were analyzed.

**The H295R steroidogenesis assay**

Human adrenocortical carcinoma cells, H295R, were obtained from the American Type Culture Collection (ATCC CRL-2128) and cultured and exposed under standardized conditions as described previously [16,17,19–21]. Cells were exposed to extracts or fractions for 48 h in 24-well plates. Dimethyl sulfoxide was used as a carrier solvent and did not exceed 0.1% v/v. For each whole sediment extract, test plates included five concentrations and a solvent control in quadruplicate. Each of the 18 fractions was dosed at a concentration of 20 mg SEQ/ml in triplicate. Cell viability was evaluated and the culture medium was stored in an Eppendorf tube at −80°C prior to analysis for hormones. Cell viability was evaluated with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) bioassay [28]. Hormones in culture medium were extracted and measured by competitive ELISA using the manufacturer’s recommendations (Cayman Chemical Company; Testosterone [Cat # 582701], 17β-Estradiol [Cat # 582251]).

**Gas chromatography/mass spectrometry analysis**

Compounds of interest in the fractions were identified by use of gas chromatography/mass spectrometry (GC/MS; Agilent 7890A GC interfaced to an Agilent 5975C MSD). Helium was used as the carrier gas. The GC program for all analyses consisted of a 4 min hold at 100°C followed by a 4°C/min ramp to 320°C, which was held for 15 min. The injector and GC/MS transfer line were held at 285°C. Fractions were analyzed for polychlorinated biphenyls (PCBs), polychlorodibenzoxin (PCDD), and common pesticides, including dichlorodiphenyltrichloroethane (DDE) group compounds, the hexachloroethanes, heptachlor, aldrin-R, heptachlor-epoxide, endosulfan, $p,p’$-dichlorodiphenyldichloroethylene, diechlorod, endrin, endosulfan, endrin-aldehyde, endosulfan-sulfate, methoxychlor, by using single-ion monitoring (detection limits $= 1$ ng/ml for PCBs and 2 ng/ml for organic pesticides for each 20 g SEQ/ml fraction). Two target analytes were monitored for each compound.

Fractions were also screened by using the GC/MS in scan mode from m/z 50 to 550 to tentatively identify unknown compounds. Sample spectra at maximum peak height were compared to the National Institute of Standards and Technology 2008 database to identify compounds. This database contains spectra of roughly 220,000 compounds. Only a qualitative assessment of the compounds identified in the MS scan was performed and is listed in Table 1.

**Statistics**

All samples were analyzed using the statistical software SPSS Statistics 17.0. Homogeneity of variance was assessed with the Levene’s test. Normality of data distributions was assessed by analysis of a normality plot. A one-way analysis of variance was used to compare results of whole extracts of sediment, AMAC extracts and fractions in the Ames fluctuation assay and H295R assay to the appropriate controls. The parametric post hoc Dunnett’s test was used to compare doses of raw extract to control. Where normality or homogeneity of variance tests failed, the nonparametric Kruskal Wallis test was performed, followed by the Mann–Whitney U post hoc, to analyze differences of all the fractionation samples from controls. Differences were considered significant at $p < 0.05$. Median lethal concentration values for the whole extract of sediment and AMAC extracts determined by the *D. rerio* assay were calculated using the binomial test function of the TOXCALC software (Michigan State University).

**RESULTS**

**Ames fluctuation assay**

Accelerated membrane-assisted clean-up extracts of sediments. With the exception of Lau2004, all AMAC extracts of sediments resulted in significant genotoxic effects in the Ames assay (Fig. 2). However, this significant increase in revertants was observed only with the TA98 strain and after adding bioactivation enzymes S9 (Fig. 2). The most potent extracts of sediments were Sig2006 and Opf2006. For these samples, statistically greater rates of revertants were observed at concentrations as small as 50 or 100 mg SEQ/ml, respectively. Exposure to the Lau2006 extract caused a significant mutagenic effect only at the greatest sediment concentration (400 mg SEQ/ml) tested. The magnitude of the response observed was approximately three- to fourfold less (5–4 revertants) than that of the positive control 2-aminoanthracene (16 revertants). The AMAC extract of Lau2004 sediment did not induce any revertants at the tested concentrations. Furthermore, none of the four AMAC extracts caused a significant increase in revertants in the TA100 bacteria strain either with or without S9 (data not shown).

**Fractionation samples** In general, fractions 8, 10, and 15 produced consistent mutagenic responses among sites. Specifically, significant mutagenic effects were observed in the TA98 strain with S9 when exposed to Sig2006 fractions 9, 10, 11, 15, and 16 (Fig. 3). Fractions 8, 10, and 11 of sediments collected at the same site caused significantly greater numbers of revertants when using the TA100 strain in the presence of S9. In addition, fractions 3 and 15 caused significantly greater numbers of revertants when using the TA98 bacterial strain without S9. No effects occurred in any of the Sig2006 fractions when using the TA100 bacteria strain without S9.
<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Sigmaringen</th>
<th>Optingen</th>
<th>Lauchert</th>
<th>Lauchert Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical measured by GC/MS</td>
<td>Result of assay performed&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Chemical measured by GC/MS</td>
<td>Result of assay performed&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Chemical measured by GC/MS</td>
</tr>
<tr>
<td>F</td>
<td>1 2</td>
<td>1 2</td>
<td>1 2</td>
<td>1 2</td>
</tr>
<tr>
<td>2</td>
<td>Cyclic octaatomic sulfur</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Long-chain alkanes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Naphthoic acid</td>
<td>/+</td>
<td>/+</td>
<td>/-</td>
</tr>
<tr>
<td>5</td>
<td>Flexol plasticizer 3GO; alkylated PAHs</td>
<td>/+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5-ring PAH-like compounds</td>
<td>/+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>6-ring PAH-like compounds</td>
<td>/+</td>
<td>5-ring PAH-like compounds</td>
<td>-/+</td>
</tr>
<tr>
<td>8</td>
<td>Sterol derivatives; 6-ring PAH-like compounds</td>
<td>/+</td>
<td>5- and 6-ring PAH-like compounds</td>
<td>-/+</td>
</tr>
<tr>
<td>9</td>
<td>Fatty acids; sterols or derivatives</td>
<td>/+</td>
<td>Long-chain hydrocarbon with unidentified functionality</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Disooctyl phthalate; sterols or derivatives</td>
<td>/+</td>
<td>Sterols or derivatives; Friedelan-3-one</td>
<td>-/+</td>
</tr>
<tr>
<td>11</td>
<td>Sterols or derivatives; coprostanol</td>
<td>/+</td>
<td>Sterols or derivatives</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Cyclic octaatomic sulfur; Phthalate</td>
<td>/+</td>
<td>Long-chain hydrocarbon with unidentified functionality</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>Naphthoic acid</td>
<td>/+</td>
<td>Flexol plasticizer 3GO; phthalate</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>Sterols or derivatives; coprostanol</td>
<td>/+</td>
<td>Flexol plasticizer 3GO; phthalate</td>
<td>/+</td>
</tr>
<tr>
<td>15</td>
<td>Fatty acids; sterols or derivatives</td>
<td>/+</td>
<td>Fatty acids</td>
<td>-/+</td>
</tr>
<tr>
<td>16</td>
<td>/-</td>
<td>/-</td>
<td>/-</td>
<td>/-</td>
</tr>
</tbody>
</table>

PAH = polycyclic aromatic hydrocarbons.

<sup>a</sup> A summary of results of the Ames fluctuation and H295R assays were also included. <i>Danio rerio</i> embryo assay results are not shown because no significant differences were observed in any fraction compared to control.

<sup>b</sup> Bioassays: 1 = H295R (arrows indicate induction (+) or inhibition (-)) of testosterone and estradiol (T/E2); 2 = significant mutagenicity as measured by the Ames fluctuation assay; F = fraction.
Fraction 10 of the Opf2006 sediment caused significant mutagenicity in both TA98 and TA100 with S9 (Fig. 3). Fraction 13 induced significantly greater numbers of revertants for TA100 with S9. Fractions 15 and 16 induced a significant mutagenic response for TA98 without S9. No effects were observed in any of the Opf2006 fractions with TA100 without S9. Fractions 15 and 16 induced a significant mutagenic response in TA98 (Fig. 3), whereas fractions 8 to 10 were mutagenic in TA100, both with and without S9. Furthermore, Lau2006 fractions 9, 14, and 15 significantly induced a mutagenic effect in TA98 without S9. No effects occurred in any of the Lau2004 fractions of sediments in TA100 without S9 and in TA98 with S9. Significant mutagenic effects were shown in TA100 with S9 when exposed to Lau2004 fractions 4, 8, 9, 10, and 11. In addition, fractions 15 and 17 caused significantly greater numbers of revertants when using TA98 without S9.

**Danio rerio assay**

Whole extracts of sediments from Opf2006, Sig2006, or Lau2006 caused lethality of *D. rerio* embryos in a dose-dependent manner (data not shown) with Opf2006 and Sig2006 being most toxic with median lethal concentrations of 18.9 mg SEQ/ml and 21.5 mg SEQ/ml, respectively, while the Lau2006 extract was less toxic by a factor of 3 (Table 2). After clean-up by AMAC, median lethal concentrations of Opf2006 and Sig2006 were enhanced to 75.6 mg SEQ/ml and 100 mg SEQ/ml, respectively (Table 2), while no toxicity was observed for Lau2006 and Lau2004. None of the fractions of sediments from any of the sites caused a significant increase in mortality compared to the solvent control at the greatest concentration tested (100 mg SEQ/ml).

**The H295R steroidogenesis assay**

Whole extracts of sediments. Cytotoxicity was observed at the greatest concentrations (20 mg SEQ/ml) for all whole extracts of sediments except for Lau2004 (data not shown). Cell viability was significantly less when cells were exposed to 20 mg SEQ/ml from Lau2006 or Sig2006 and at concentrations equal to or greater than 10 mg SEQ/ml at Opf2006. In contrast, the whole extract of sediments from Lau2004 resulted in a significantly greater growth of cells exposed to 5 or 10 mg SEQ/ml, and no significant effects on cell viability were observed at any concentrations tested.

All whole extracts of sediments resulted in significantly greater E2 production by H295R cells. Sediments from Lau2006 and Opf2006 caused significant and dose-dependent greater E2 production at all concentrations tested, whereas sediments collected from Lau2004 and Sig2006 altered E2 production at concentrations greater than or equal to 2.5 mg SEQ/ml (Fig. 4). The greatest effects on E2 production were observed for whole extracts of sediments from Opf2006 and Sig2006 with maximum E2 production being more than fourfold greater than those in the controls. Concentrations of whole extracts of sediments required to induce a maximum response were 2.5 and 5 mg SEQ/ml from Lau2006 or Sig2006 and at concentrations equal to or greater than 10 mg SEQ/ml at Opf2006. In contrast, the H295R steroidogenesis assay could not be determined because no lethality was observed in any of the concentrations.

**Table 2. Median lethal concentration (LC50) values for whole and accelerated membrane-assisted clean-up (AMAC) extracts of sediments in the *Danio rerio* assay**

<table>
<thead>
<tr>
<th>Site ID</th>
<th>LC50 Whole extract of sediment</th>
<th>LC50 AMAC extract of sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opf2006</td>
<td>18.9 mg SEQ/ml</td>
<td>75.6 mg SEQ/ml</td>
</tr>
<tr>
<td>Sig2006</td>
<td>21.5 mg SEQ/ml</td>
<td>100 mg SEQ/ml</td>
</tr>
<tr>
<td>Lau2006</td>
<td>58.6 mg SEQ/ml</td>
<td>CBD (&gt;100 mg SEQ/ml)</td>
</tr>
<tr>
<td>Lau2004</td>
<td>NA</td>
<td>CBD (&gt;100 mg SEQ/ml)</td>
</tr>
</tbody>
</table>

Sig2006 = Sigmaringen; Opf2006 = Oepfingen; Lau2004 = Lauchert collected in 2004, Lau2006 = Lauchert collected in 2006; NA = not available; CBD = cannot be determined because no lethality was observed in any of the concentrations.
Only exposure to Opf2006 and Lau2004 whole extract of sediments produced significant changes in production of testosterone (data not shown). Significant increases in testosterone (T) production were observed for extracts from Opf2006 (1.25, 2.5, and 5 mg SEQ/ml) and Lau2004 (5 and 10 mg SEQ/ml). The greatest induction of T production occurred after exposure to 10 mg SEQ/ml of the extract from Opf2006 (1.5-fold from controls). No significant effects on T production were observed for Lau2006 and Sig2006 at any concentration and for Opf2006 at the greatest concentration tested.

**Fractionation samples.** In general, fractions 7, 10, 14, and 15 were the four most potent fractions that significantly changed E2 or T in at least three of the four sites studied. For example, E2 production was significantly greater in fraction 15 at all four sites and resulted in threefold greater production of E2, the most of any fraction at any of the sites. In sample Lau2004, production of E2 was significantly greater when cells were exposed to fractions 10, 14, and 15. Furthermore, only fraction 15 and the 1 to 18 combined samples resulted in significantly greater T production. Fraction 7 of Lau2006 resulted in significantly less T, but the magnitude of the effect was relatively small. In contrast, fractions 10, 11, 13, 14, 15, and 16 increased E2 production by up to threefold. Fraction 7 of Opf2006 resulted in less production of T, but more production of E2 compared to controls (Fig. 5). Exposure to Opf2006 fraction 18 resulted in a significantly greater production of T. Estradiol production was greater than that of the control when cells were exposed to fractions 14, 15, and 17. Similar to Opf2006, fraction 7 of Sig2006 resulted in significantly less T production, but greater E2 production compared to that of the control cells (Fig. 6). Exposure to Sig2006 fraction 10 resulted in significantly greater T production compared to controls. Production of E2 was significantly greater in cells exposed to fractions 9, 10, 13, and 15 compared to controls.

**Chemical residues**

Results of the MS run in scan mode for compounds identification are given in Table 1. Briefly, various PAH-like compounds, sterols, and naphthoic acids were the predominate chemicals detected among all samples. Naphthoic acids were observed in fraction 7 in all samples except Lau2004. Fractions 8 to 13 contained mostly PAHs, which occurred to some extent in all samples. Sterols or their derivatives were detected primarily in fractions 13 to 16 of all samples except Lau2004. Of the compounds quantified, no PCB, PCDD, or DDT group com-
pounds were detected in any of the fractions or samples. Other chemicals that were quantified and not detected in any fraction include the hexachlorohexanes, heptachlor, aldrin-R, heptachlor-epoxide, endosulfan, \( p,p' \)-dichlorodiphenyldichloroethylene, dieldrin, endrin, endosulfan, endrin-aldehyde, endosulfan-sulfate, and methoxychlor.

**DISCUSSION**

**Whole and AMAC extracts of sediments**

The battery of tests performed in the present study detected significant mutagenicity and endocrine effects of sediment extracts. The patterns of effects observed in bioassays in the present study were similar to those observed in other studies that have shown that sediments from the Sigmaringen and Lake Oepfingen locations were generally more potent. For example, Keiter et al. [2] measured ethoxyresorufin O-deethylase activity in extracts of 10 upper Danube River sediments and found that the Opf sediment extract caused the greatest induction of ethoxyresorufin O-deethylase activity among all the sediment extracts studied, and Lau sediments were among the least potent. The same pattern was observed by Seitz et al. [25] and Böttcher et al. [5] who measured genotoxicity by use of the comet assay and the micronucleus test. In the present study, whole extracts of sediments and AMAC extracts of Opf2006 and Sig2006 exhibited significant potencies in the *D. rerio* embryo survival assay, the Ames fluctuation mutagenicity assay, and the H295R endocrine modulation assay.

The studies by Seitz et al. [25] and Keiter et al. [1] investigated sediments that were collected in 2004. In 2006, new samples were collected from the same sites. When bio-TEQs derived from RTL-W1 cells were compared between sites and years, it was found that the Lau and Sig sediments collected in 2004 were significantly less potent than were extracts of sediments collected in 2006 [29]. In the present study, a similar trend toward greater potencies in 2006 was observed in the Ames fluctuation and H295R assays when the results of the Lau2004 and Lau2006 extracts were compared in both the AMAC and whole sediment extracts, respectively. It is unclear why greater toxic potency was observed after these two years, but it is possible that flood events either deposited new sediments or eroded the original sediment exposing more contaminated sediment underneath.

Several rationales can be used to explain the lesser potency of the AMAC extracts compared to the whole extracts to cause effects in the *D. rerio* assay. The AMAC procedure removes larger lipids and lipid-like matrix components that can interfere with chemical analysis of environmental samples that is not removed in the whole extracts of sediments [27]. Furthermore, the AMAC procedure was shown to have good recoveries of 10 spiked compounds, so it was unlikely that the lesser toxicity of the AMAC extracts was due to poor extraction efficiency [27]. However, extraction efficiencies were not determined for all chemicals. Thus, it cannot be excluded that the lesser toxicity of AMAC extracts might have been because of removal of some of the biologically active compounds.

There are two possible explanations for the fact that further separation of Opf2006 and Sig2006 extracts into 18 fractions completely eliminated the toxicity of all samples in the *D. rerio* assay. One reason for this might be the removal of some active compounds during the fractionation procedure. Alternatively, it might be because of the removal of additive or synergistic interactions between chemicals that were present in the different fractions after separation. Additive and synergistic effects have been reported for several chemicals. For example, a synergistic effect with PCBs was observed when exposed to H4IIE cells in a mixture compared to each PCB separately [30]. In contrast to the results of the *D. rerio* assay, AMAC extracts were less potent than the fractions when analyzed in the Ames fluctuation assay. This is frequently observed in EDA of mutagens and is probably caused by removal of masking compounds during fractionation [14].

**Ames fluctuation assay and fractionation**

In general, fractions 8, 10, and 15 produced a consistent mutagenic response among sites. Specifically, fraction 10 for all of the sites exhibited greater effects to both TA98 and TA100 with S9 activation, but no mutagenic activity was observed without S9, which is typical for PAHs, because they need to be bioactivated to cause an effect [31]. Furthermore, 5-ringed PAHs were identified in fraction 10 in three of the four sites (Table 1). According to Keiter et al. [2], between 55 and 88% of AhR-mediated responses observed in upper Danube River sediments from the same sites was not accounted for by PAHs, PCBs, and polychlorodibenzoxodianifuran (PCDD/Fs). Of the AhR-mediated responses that could be accounted for, PAHs accounted for between 10 and 42% of the total toxicological effects; in all cases, less than 8% of the response was attributed to PCBs and PCDD/Fs [2]. These results are similar to the present study, in that we observed no effect in the fractions that contain PCBs and PCDD/Fs, but saw an effect in fraction 10 that contains PAHs.

Additionally, in the present study we determined that the more semipolar and polar compounds were active in all assays run; these compounds might account for at least some of the unknown toxicity that was observed by Keiter et al. [2]. For example, fraction 15, which contains more polar compounds, produced significant mutagenic effects at all sites in the TA98 bacteria strain without S9. Typical contaminants in polar fractions of sediment extracts are PAHs including nitro-PAHs and dinitro-PAHs, which have been implicated as possible toxicants in sediments and would be eluted in fraction 15 [32,33]. Although these compounds were not detected in the GC-MS scan analysis it is possible that they can exhibit strong mutagenic effects at concentrations below the limits of detection of the GC-MS analysis, used in the present study [32]. Furthermore, sterols and their derivatives were measured in fraction 15. However, these are unlikely to have caused the observed effects because other research that has shown plant sterols to be antimutagenic and in fact decrease the mutagenic capabilities of many chemicals [34,35].

**The H295R assay and fractionation**

Fractionation of the whole extracts of sediments allowed differentiation of trends in the E2 and T profiles of the fractions among sites studied, and to determine possible groups of chemicals that caused the effects observed. For example, many different types of chemicals, including EE2, estrone, and plant sterols, possibly found in fraction 15, could account for greater E2 production by the H295R cells. However, only sterols or their derivatives were measured in this fraction in three of the four sites. Furthermore, results from Grund et al. [9] found that E2, E1, EE2, BPA, and NP only account for between 2 to 6% of the estrogen receptor-mediated activities of nine upper Danube River sediments, including sediments sampled from Opf, Lau, and Sig. In fact, more than 94% of the estrogen receptor-mediated activities were attributed to unknown components [9]. In the present study, fractionation and chemical
analysis of the sediments found that sterols could account for part of the greater E2 production compared to control in the H295R assay, and it is unlikely that EE2, E1, E2, BPA, and NP is an issue in these sediments. Plant sterols, in particular β-sitosterol, have been shown to modulate E2 production in both fish and mammals [36,37]. One study exposed polecats to β-sitosterol and observed greater plasma E2 and thyroid hormone concentrations compared to control polecats [37]. Furthermore, exposure of extracted wood sterols in environmentally relevant concentrations to zebrafish caused a significant change in the sex ratio of zebrafish toward females in the F2 generation [38].

Research has revealed some PAHs to have endocrine disrupting properties. For example, studies have shown decreased androstenedione, T, and E2 in flounder ovarian tissue associated with the exposure to PAHs [8]. Also, it has been reported that PAHs can act as antiestrogens in breast cancer cell lines [39,40]. Polyaromatic hydrocarbon-like compounds with five aromatic rings (i.e., benzo[a]pyrene and benzo[a]fluoranthene) were detected in fraction 10 at all locations except Opf2006 (Table 1). Consequently, Opf2006 fraction 10 was the only location that did not elicit an effect in the H295R assay. More research on the effect of five-ring PAHs within the H295R assay would be needed to verify that they can cause the observed results.

Fraction 7 was of particular interest because the hormone profile differed from that observed for any of the other fractions. Significant decreases in T compared to control were observed in three of the four sites. Compounds such as naphthoic acid-like compounds were measured in fraction 7 from all three sites at which changes in E2 or T were measured. These results correspond with studies that were performed on oil sands process water. It was found that oil sands process water induced E2 concentrations and simultaneously inhibited testosterone production in H295R cells, which is consistent with our results [41]. Future work would be needed to assess the effect of naphthoic acid on the H295R assay and to demonstrate if the observed effect was caused by the naphthoic acid alone.

Given that exposure to fractions 1 to 6 did not result in any changes in E2 or T production at any of the sites studied indicates that chemicals such as many PCBs, polychlorinated naphthalenes, 2,3,7,8-tetrachlorodibenzo-p-dioxin, and 2,3,7,8-tetrachlorodibenzofuran that co-elute with these fractions, can be ruled out as a cause of the observed effects. Other chemicals that can be found in fractions 1 to 6 could be alkanes or DDT and its metabolites; therefore, these compounds are also unlikely to have caused the observed changes in E2 and testosterone. This was verified with the parallel conducted chemical analyses during which no PCBs, PCDDs, or DDT group compounds could be detected in any of the fractions or samples. In general, the addition of the response of the fractions corresponded well with what was measured in the unfraccionated whole extracts of sediments. The one exception was sample Lau2006 where a less than additive effect of the fractions was observed. This was possibly because of an interaction between some of the chemicals within the fractions. Furthermore, most of the greater E2 production observed in the unfraccionated Sig2006 whole extracts of sediments was accounted for by fractions 7, 9, 10, 13, and 15 and its toxicity was driven mostly by plant sterols, PAHs, and naphthoic acid. However, greater E2 production observed in Opf2006 appeared to be driven primarily by chemicals present in fractions 7, 14, 15, and 17 (mostly naphthoic acid and sterols). For the less potent extracts of sediments from Lau2006, most of the greater E2 production was associated with fractions 10, 14, and 15.

CONCLUSIONS

The present study demonstrated EDA is a useful tool for the identification of candidate chemicals or chemical groups in complex environmental matrices such as sediments. Fractions 7 to 15 were consistently more toxic in both the Ames fluctuation and H295R assays among the four extracts and much of this toxicity was associated with PAHs, nitro-PAHs, dinitro-PAHs, sterols, and naphthoic acids. Furthermore, chemicals such as PCBs, 2,3,7,8-tetrachlorodibenzo-p-dioxin, polychlorinated naphthalenes, DDTs, and several OPs could be excluded as causative agents for the observed toxicity because the fractions containing these compounds did not cause any effects for any of the samples and were not measured in any fraction. Taken as a whole, these results show the value of using EDA, which uses multiple bioassays and fractionation techniques to assess the toxicity of sediments that covers a variety of different biological endpoints that are caused by unknown chemicals. Furthermore, to our knowledge this is the first study successfully using the recently developed H295R assay within EDA strategies.

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