Toxicity and Mutagenicity of Upper Danube River Sediments Determined by Chemical Fractionation, the *Danio rerio* Embryo Assay, the Ames Fluctuation Test and the H295R assay.

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By

Eric Bertram Higley

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Toxicology Centre
University of Saskatchewan
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Abstract

Declines in some fish populations in the upper Danube River, Germany, have been reported during the past decades despite extensive stocking efforts. Many theories exist for why such declines have occurred including habitat change, dams, invasive species, disease and pollution. One of the factors of concern in the Upper Danube River is pollution because a number of studies have shown that sediments collected from this area were acutely and/or chronically toxic to fish. Although it can be difficult to link bioassay results to direct effects on the population level, bioassays can give us insight into the potential of exposure of wildlife including fish to sediment. In combination with other researchers a large battery of sediment testing on the Upper Danube River is being performed. Testing includes sediment testing of estrogen receptor mediated processes, dioxin-like responses and genotoxic effects. In this study, four sediment extracts from the Upper Danube River in Germany were used with a novel fractionation technique to characterize the sediment extracts and fractions for their ability to disrupt steroidogenesis, for their mutagenic activities and their teratogenic effects. Fractionation of each of the four sediment samples was performed by separating compounds according to their polarity, planarity, and the size of the aromatic ring system in an on-line fractionation procedure on coupled high performance liquid chromatography columns.

Mutagenic activity was measured in the raw sediment extracts and all 18 fractions using the Ames fluctuation assay and the *Danio rerio* embryo assay was used to assess lethal endpoints. Furthermore, disruptions of steroidogenesis were assessed by first establishing methods and a proof of concept of the H295R assay by exposing H295R cells to 7 model chemicals and measuring changes from a control in estradiol,

testosterone and aromatase activity. Once methods were established all sediments and their fractions were analyzed using the Assay.

Specifically, in the *Danio rerio* assay, two raw sediment extracts killed 100% of *Danio rerio* embryos at a concentration of 33.3 mg sediment equivalents (SEQ)/ml, but none of the 18 fractions of these samples produced any measured toxicity at a concentration of 100 mg SEQ/ml. In the Ames fluctuation assay, significant mutagenic activity was measured in raw sediment extracts and in the fractions. Fraction 10 produced a significant mutagenic response in all sediment samples measured only in S9 bio-activated samples. Furthermore, fraction 15 produced a significant mutagenic response in all sediment samples measured only in non bio-activated samples.

All raw extracts tested in the H295R assay caused an increase in estradiol production up to 4-fold from controls. Testosterone production increased slightly from controls in only two of the raw extract samples. Of the 18 fractions, fractions 7, 10 and 15 increased estradiol in at least three of the samples studied (Sigmaringen2006, Opfingen2006, Lauchert2006 and Lauchert2004). Furthermore, fraction 7 significantly decreased testosterone production compared to controls in three of the four sediment samples.

Taken as a whole, these results show the value of using multiple bioassays and fractionation to characterize sediments that covers a variety of different biological endpoints. This study also demonstrates the usefulness of the H295R assay when combined with a new fraction technique to assess endocrine disrupting chemicals in sediment samples.

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List of Abbreviations

AMAC	Accelerated Membrane-Assisted Clean-up
AMG	Aminoglutethimide
BpA	Bisphenol A
CN	Cyanopropyl silica
d	Day
DCM	Dichloromethane
DEPH	Bis(2-ethylhexyl)phthalate
DMSO	Dimethyl sulfoxide
dw	Dry weight
E2	17β-Estradiol
EDA	Effect-directed analysis
EDC	Endocrine Disrupting Chemical
EE2	17α-Ethinylestradiol
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen Receptor
FOR	Forskolin
GC-MS	Gas chromatography- mass spectrometry
h	Hour
Hx	Hexane
KET	Ketoconazole
Lau2006	Lauchert sediment sampled in 2006
Lau2004	Lauchert sediment sampled in 2004
LET	Letrozole
mo	Month
NO	Nitrohenylpropyl silica
NP	Nonylphenol
OECD	Organization for Economic Cooperation and Development
Opf2006	Oepfingen sediment sampled in 2006
PAH	Polyaromatic hydrocarbon
PCB	Polychlorinated biphenyl

PCN	Polychlorinated naphthalene
PCDD/F	Polychlorinated dibenzodioxin/ furan
PGC	Porous graphitized carbon
PRO	Prochloraz
PrCo	Procedural Control
PC	Positive Control
SC	Solvent Control
SEQ	Sediment Equivalents
Sig2006	Sigmaringen sediment sampled in 2006
T	Testosterone
ww	Wet weight
YES	Yeast Estrogen Screen
YAS	Yeast Androgen Screen

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CHAPTER 1
1.0 GENERAL INTRODUCTION

1.1 Introduction

Severely contaminated sediments from many rivers and lakes have been shown to be acutely and chronically toxic to fish and benthic invertebrate species (Swartz et al., 1994; Keiter et al., 2006; Anderson et al., 2007; Kosmehl et al., 2007). Under certain conditions, sediment toxicity has been shown to contribute to decreases in reproductive success, recruitment, and alterations in community structure, potentially causing population declines. For example, Keiter et al. (2006) reported that zebrafish embryos exposed to sediments from the Danube River showed impaired development and decreased hatching rates compared to control fish. Other sediments have been classified as genotoxic, mutagenic, endocrine disrupting or were recognized for their dioxin-like effects (Keiter et al., 2006; Kosmehl et al., 2007; Keiter et al., 2008; Grund et al., submitted 2009).

Due to the often complex nature of sediment contamination that is the result of the simultaneous presence of a number of different types of chemicals, approaches are needed that allow distinguishing between these different types of pollutants and the associated toxicities. One common approach that is used in this context is the so called weight-of-evidence approach. In this approach, a combination of different biological assays and analytical techniques are utilized to screen for different types of sediment toxicity (Chapman and Hollert, 2006). Depending on the specific exposure scenario sediment toxicity can be assessed by measuring any combination of the following and/or other effects: metal toxicity, endocrine disruption, genotoxicity, mutagenicity, teratogenicity and dioxin-like effects.

A number of *in vivo* and *in vitro* assays have been established and validated to measure the above toxicological effects. However, each assay has its own advantages and disadvantages. For example, *in vitro* assays are less expensive to perform, more replicates per experiment can be tested, and often there is less variability between replicates. However, *in vitro* tests are mostly artificial systems and do not necessarily reflect what happens in a whole organism, and thus, are characterized by relatively great uncertainties regarding the biological relevance of an effect. For example, a chemical being studied might be metabolized by the liver before entering the bloodstream and not cause the effects seen in *in vitro* testing. *In vivo* assays, on the other hand, are more representative of effects of chemicals on whole organisms and populations. However, *in vivo* assays are more expensive and difficult to perform, are typically characterized by relatively great variability, and are subject to extensive rules and regulations because animals are being used.

Some commonly used *in vitro* and *in vivo* assays currently being used in toxicology research include assays to detect endocrine disrupting chemicals, chemicals that cause DNA damage (mutagenic and genotoxic effects) and teratogenic effects. Assays to test for endocrine disrupting potentials of environmental samples mostly include nuclear receptor binding assays (e.g. MVLN, YES, YAS). However, endocrine disrupting chemicals incorporate a wide variety of biological processes, including non-receptor mediated responses involved in hormone production and metabolism. The only currently available and validated *in vitro* test to screen for such effects is the H295R steroidogenesis assay that uses a human adrenocarcinoma cell line, and that has been thoroughly characterized as a screen for endocrine disrupting chemicals. The main

endpoints for the H295R steroidogenesis assay are estradiol and testosterone production, but because this cell line produces all the steroid hormones the capacity to measure any hormone of interest or associated enzymes and their expression also exists. Many other assays evaluate different endpoints, for example genotoxicity, teratogenicity and mutagenicity. The Ames fluctuation assay is one assay used to evaluate the mutagenicity of sediments by measuring back mutations in bacteria (Eisentraeger et al., 2007). The *Danio rerio* (Zebrafish) embryo assay is one of many assays that assess teratogenic activity or genotoxic effects of sediment samples (Kosmehl et al., 2006). Teratogenic activity is measured by monitoring hatching success and mortality of the *Danio rerio* embryo (Keiter et al., 2006). Genotoxic effects can be measured by evaluating DNA strand breaks using the Comet assay. Finally, cell lines (i.e. H4IIE-*luc*, RLT-W1) can be used to assess the dioxin-like properties of sediments (Keiter et al., 2008). Other possible endpoints to evaluate toxicological effects include heat shock protein, Cytochrome P450 enzyme, metallothionein, glutathione and acetylcholinesterase expression values (Allen et al., 2006).

Toxicity assays allow for characterizing the type of toxicity but they do not establish cause (i.e. which chemical caused the toxicity measured). However, because of the sheer number of chemicals possibly present in sediment samples from locations characterized by complex exposure scenarios it would be too resource, labor and time intensive to conduct chemical analyses to identify all potential contaminants in such samples. In these situations, bioassays can serve as important tools in helping to narrow the exposure down to certain chemical groups or types. For example, if EROD induction was measured and shown to increase using the H4IIE bioassay after exposure to sediment

extract, one might suspect the sample to contain chemicals with structures similar to those of the potent EROD inducers called dioxins. To further define what chemical or group of chemicals in the sediment sample caused the toxicity an effect directed analysis (EDA) could be performed. EDA is a procedure that utilizes a combination of bioassays, fractionation techniques and chemical analysis to analyze environmental samples. When a sediment sample has been found to have adverse effects (i.e. an increase in toxicity compared to control) the sediment sample can be fractioned into different chemical groups by size, polarity and reactivity. The different fractions of the sediment are then re-tested in the toxicity assay to identify the fraction with which the toxicity is associated. Manipulation of the sediment sample to inhibit the activity of certain groups of chemicals is also an effective way to evaluate the toxicity of sediment samples (US-EPA 2007). Furthermore, fractionation can help to determine the source from where the toxic chemical or chemicals are coming from (i.e. industry, waste water, farming, etc) by ruling out the toxicity of whole groups of chemicals within the sediment. Once the fraction that caused the toxicity is isolated, that fraction can be analyzed by analytical instruments to determine the chemical or chemicals of interest. Ultimately, this information can then be used in support of remedial actions implemented to remove the toxicity or to reduce its discharge into the system at its source.

1.2 Contaminants and the Danube River

The Danube River is the second largest river in Europe it is characterized by a drainage basin of 817,000 km^2 in 19 different countries (Maljevic and Balac, 2007). Many species of wildlife live within the Danube river basin and are affected by how

humans use the river. The Danube River serves as habitat for over 300 bird and more than 45 fish species. Nonetheless, fish catches in the Upper Danube River have been decreasing since 1990, particularly grayling and sturgeon (Hensel and Holcik, 1997; Keiter et al., 2006). Various theories have been suggested to explain the population declines of fish species on the Danube River, including pollution, habitat destruction, construction of dams and over-exploitation (Keiter et al., 2006).

While overall pollution in the Upper Danube River has decreased over the last decade, several studies have shown certain types of pollution still persists in certain areas including pollution that can cause endocrine disrupting, mutagenic and genotoxic effects (Keiter et al., 2006; Grund et al, unpublished). The recently implemented EU Water Framework Directive identified 33 chemicals as hazardous, 29 of which were used within the Danube Basin. DDT, for example, is still of major concern, and even though it has been banned almost 30 years, 71% of water samples taken on the Danube River contain DDT above threshold levels set by the EU Water Framework Directive (Joint Danube Survey 2). Furthermore, *p,p*'-DDE, the breakdown product of the organochlorine pesticide DDT, has been found in sediments samples of the Danube River at concentrations as high as 10.4 ng/g dry weight (dw) (Skrbic et al., 2007). Moreover, one study found a positive correlation between concentrations of residues in sediment and those in fresh water snails at the same site. Concentrations measured in the sediment reached 50 ng/g dw *p,p*'-DDE and concentrations of 363.6 ng/g dw in the fresh water snails at the same site (Nhan et al., 2001). *In vivo* studies with Japanese Medaka (*Oryzias latipes*) have found *p,p*'-DDE concentrations as little as 0.001µg/ml can cause an increase in ERα gene expression and concentrations of 0.1µg/ml can cause intersex in

Medaka testes (Zhang and Hu, 2008). Furthermore, two *in vitro* studies exposed *p,p*'-DDE to porcine ovaries and measured greater estradiol and aromatase activity and lesser testosterone production compared to control at *p,p*'-DDE concentrations similar to what is found in sediment and biota samples (Nhan et al., 2001; Wojtowicza et al., 2007; Gregoraszczuk et al., 2008).

Another chemical group of concern is petroleum hydrocarbons, and according to Maljevic et al. (2007), concentrations for these compounds ranged from 98-1340 mg/kg dw sediment within the lower Danube River. Finally, other potential endocrine disrupting chemicals (EDCs) nonylphenol, bisphenol A, and estrone were detected in many sediment samples from the Upper Danube River (Grund et al., submitted 2009). These findings have been supported by a wealth of information obtained during analyses of sediments using a range of different bioassays. For example, the same sediment samples reported to be characterized by the presence of EDCs were also shown to induce expression of the CYP11B2 and CYP19 genes in H295R cells (Grund et al., unpublished). Furthermore, one study found significantly elevated aryl hydrocarbon receptor mediated toxicities in 3 cell lines (H4IIE-luc, GPC.2D-luc and RTL-W1) when exposed to Upper Danube River sediment extracts (Keiter et al., 2008). Keiter et al. (2008) also measured 16 PAHs in the sediment samples and found their concentrations to have a maximum concentration of ∑26 ug PAHs/g dw sediment in the most polluted sites, and which could be related to some of the effects seen in 3 cell lines (H4IIE-luc, GPC.2D-luc and RTL-W1). It was also shown that Upper Danube River sediment samples produced genotoxic effects in RTL-W1 cells. In addition, micronucleus formation measured in RTL-W1 cells exposed to the sediments corresponded well with

micronucleus formation in the European Barbel (*Barbus sclateri*) caught in the field (Boettcher et al. (unpublished results)). In conclusion, pollution has been shown to have an adverse affect on the health of the wildlife and many concerns still persist about the general health of the Danube River system (Swartz et al., 1994; Keiter et al., 2006; Kosmehl et al., 2007).

1.3 Objectives

Much work has been performed to characterize the toxicity of sediments in the Upper Danube River. Observed whole sediment toxicities include changes in steroidogenesis, estrogenic effects, genotoxicity, mutagenicity and teratrogenicity. To date, however, there is still uncertainty regarding the specific chemicals that may contribute to the observed effects. Therefore, a weight of evidence approach, in parallel with other researchers, was designed and applied to aid in identifying the group(s) of chemicals that may contribute to the previously observed toxicities. This was done by investigating several fractions of 4 sediment samples previously collected from the Danube River known regarding their potential to elicit certain toxicities.

In a parallel study (Ph.D. thesis of Stefanie Grund, University of Heidelberg, Germany) the above described sediment samples from the Danube River (Grund et al., submitted 2009) have been fractionated into 18 separate fractions. The main objective of this study is to analyze the different fractions of these sediment samples regarding their mutagenic effects, teratogenic effects and their endocrine disrupting potencies, to identify the active fractions.

1.3.1 Objective #1 – Proof of concept for the H295R Assay

In response to concerns about chemical substances that may alter the function of endocrine systems and result in adverse effects on human and ecosystem health, a number of *in vitro* tests have been developed to identify and assess the endocrine disrupting potential of chemicals and environmental samples. Most of these assays measure the potency of direct-acting hormone mimics. However, some compounds can modulate the endocrine system by indirect effects including steroidogenesis, steroid metabolism and hormone transport. One assay that has been recently validated for the assessment of the potential of chemicals to affect hormone synthesis is the H295R Steroidogenesis Assay. The objective here was to characterize the differential effects of seven model compounds on aromatase activity, and E2 and T production using H295R cells to assess if this cell line is a good candidate for screening of steroidogenic effects in sediment extracts.

Testable Hypothesis #1

All of the seven model chemicals will produce a hormone (estradiol and testosterone) and direct, indirect and combined aromatase activity profile in the H295R cells that is consistent with each chemicals mode of action.

1.3.2 Objective #2 – Endocrine Disrupting Activities

Previous analyses of sediments from certain locations in the Upper Danube River revealed endocrine disrupting activities as measured by the H295R Steroidogenesis Assay and the YES assay. Specifically, the objective of this portion of my thesis was to

identify fractions of sediment samples previously reported to elicit endocrine activity for interactions with the production of estradiol (E2) and testosterone (T) using the H295R Steroidogenesis Assay as described by Hecker et al. (2006) to aid in the identification of the causative agents. Several potential endocrine disrupting chemicals (EDCs) have been previously measured in the same sediment samples. Results obtained with the H295R assay was compared with these values to determine whether the observed effects can be associated with these chemicals.

Testable Hypothesis #2

Exposure to sediment extracts will significantly affect estradiol (E2) and testosterone (T) in H295R cells compared to control. Furthermore, effects on E2 and T will be associated with the more non-polar fractions of the sediment samples.

1.3.3 Objective #3 – Teratogenic Activities

The objective of this specific aspect of my research was to perform the *Danio rerio* embryo assay to identify specific fractions of sediment samples that may have teratogenic activity. The data received from this experiment will help narrowing the causative agents of the sediment toxicity down to a smaller group of chemicals. This information can then be used in later analysis for single chemicals to determine the specific chemical(s) responsible for this toxicity type.

Testable Hypothesis #3

Exposure to sediment extracts will result in statistically significant teratogenic effects in *D. rerio* embryos compared to control. Furthermore, teratogenic effects will be associated with the more non-polar fractions of the sediment samples.

1.3.4 Objective #4 – Mutagenic Activities

In addition to endocrine and teratogenic potentials, Keiter et al. (2006) described mutagenic properties of Upper Danube River sediments and extracted suspended particulate matter. All samples analyzed using the Ames test had increased mutagenic properties compared to the controls. The specific objective of my investigations was to follow up the Keiter et al. (2006) work by analyzing fractions of sediment samples collected in January 2006 using the Ames Fluctuation Assay with the aim to pin-point the mutagenic properties to a specific fraction to aid in the identification of causative agents.

Testable Hypothesis #4

Exposure to sediment extracts will result in statistically significant mutagenic effects in all *Salmonella* bacteria strains compared to control. Furthermore, mutagenic effects will be associated with the more non-polar fractions of the sediment samples.

1.4 Methods

1.4.1 Sampling

Sediments were sampled (top 5cm) at seven locations and two tributary streams along the Upper Danube River using a Van Veenen grabber or a stainless steel spoon in January 2006 (Figure 1.1). Four sediment samples that were shown to be toxic were then extracted and fractionated into different chemical groups according to their polarity, planarity and the size of their aromatic system (Lübcke-von Varel et al., 2008).

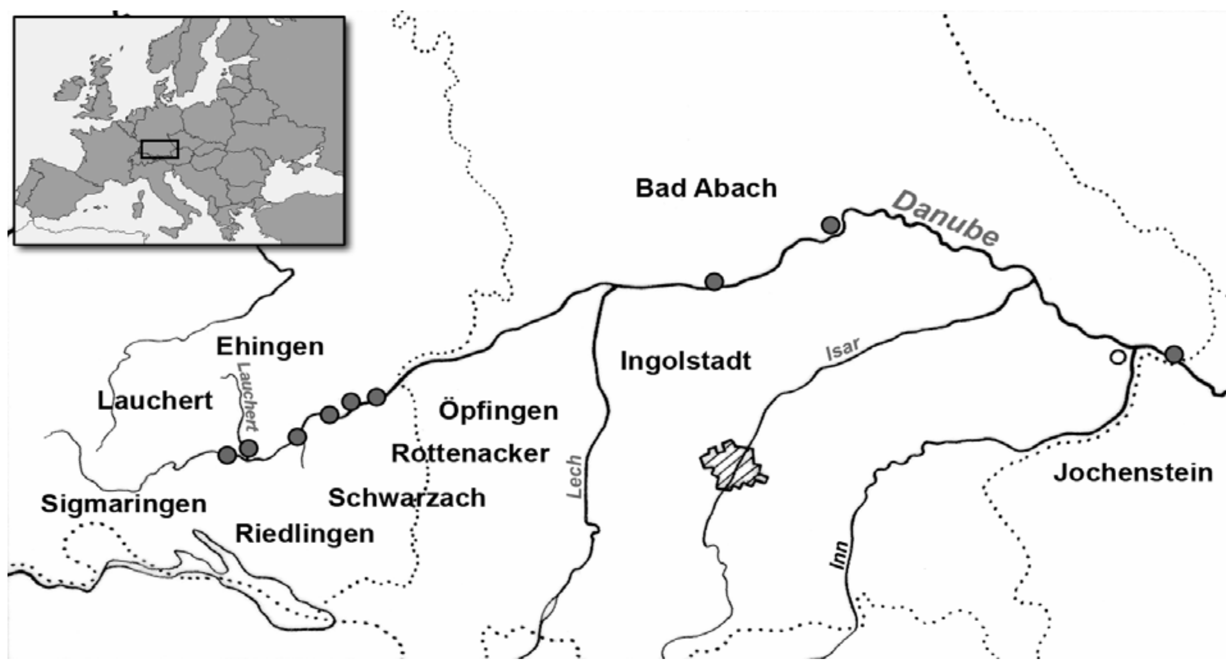


Figure 1.1 Study area and sampling locations.

1.4.2 H295R Assay

The H295R cell line is a human adrenal carcinoma cell line with the ability to produce all the steroid hormones and enzymes of the steroidogenic pathway including cortico- and sex-steroid hormones. These properties render it a valuable tool for identifying and assessing chemicals in the environment that have the potential to interfere with steroidogenic pathways. The potential and advantages of the H295R assay have been recognized by various groups which lead to its validation as part of Tier I of the US-EPA

Endocrine Disruptor Screening Program and an Organization for Economic Cooperation and Development (OECD) test method validation program.

Conduct of the Assay

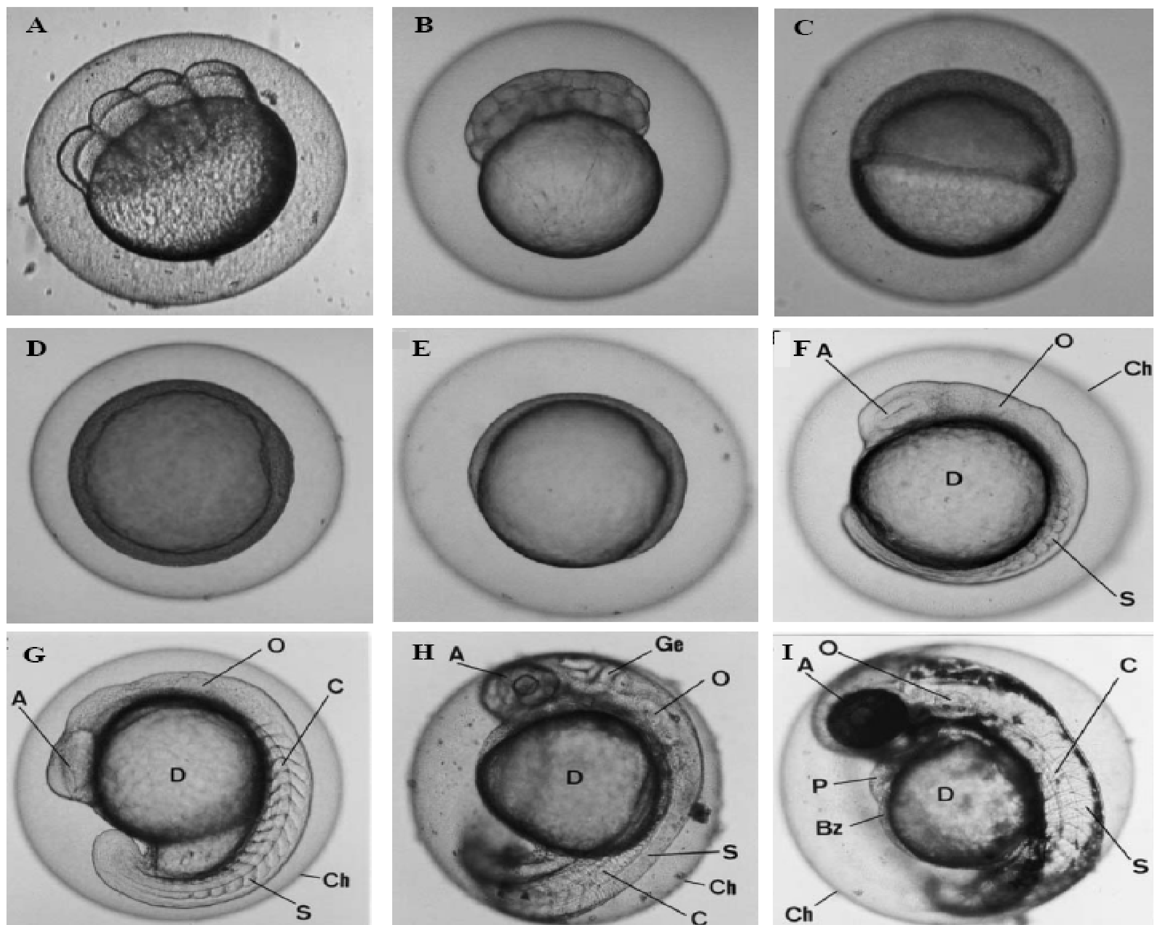
Cells are seeded into 24-well plates at 300,000 cells per ml and left to settle for 24 h. After 24 h the media is replaced and the cells are exposed to the chemical or sample in question through a solvent carrier for 48 h. After 48 h the media is removed, cell viability is assessed with the MTT Assay, the hormones ether extracted and testosterone and estradiol are measured by ELISA. Forskolin and prochloraz was run in the same exposure as positive controls; Forskolin as a general steroid inducer and prochloraz as an inhibitor (Hecker et al., 2006). See Section 2.3 Materials and Methods for more details on the H295R assay.

1.4.3 *Danio rerio* embryo assay

Danio rerio (Zebrafish) is a small, easily cultured freshwater fish that is native to the warm waters of Burma and Sumatra. They reach sexual maturity in ~ 3 mo and produce between 50 and 200 eggs every 2-3 d. The embryos develop rapidly (approximately 96 h) and the eggs have a transparent chorion so their growth can be monitored (Figure 1.2). Because they are easily obtained and cultured, and are rather inexpensive, *Danio rerio* are good candidates for acute and chronic toxicity tests. Furthermore, their small size allows for exposures to be performed in 24, 48 or 96 well culture plates and, therefore, more replicates and doses can be run per experiment.

For the experiments, viable *Danio rerio* embryos 1-2 hours post fertilization were exposed to diluted sediment extracts in 96 well plates in 100µL ISO water (20mM

$CaCl_2 2H_2O$; 5mM $MgSO_4 7H_2O$; 7.5mM $NaHCO_3$; 0.037 mM KCl in Nanopure water). Embryos were covered with an oxygen permeable cover and incubated at 27 °C for 48h. The embryos were analyzed for mortality by monitoring lack of somite formation, coagulation of embryo, non-detachment of tails and eyes, lack of heart function and lack of blood circulation.



Photographs: Marc Rudolf (Rudolf 2000)

A 8-cell stage (1.25 h) **B** 32-cell stage (1.75 h) **C, D** 50 % epiboly stage (6 h)
E 75 % epiboly stage (8 h) **F** Embryo at an age of 16 h **G** Embryo at an age of 18 h
H Embryo at an age of 24 h **I** Embryo at an age of 48 h

A: eye anlage/eye; Bz: blood cells; C: chorda; Ch: chorion; D: yolk sac; Ge: brain anlage; O: ear bud; P: pericardium; S: somites

Figure 1.2 Figure used from Franziska Forster 2008 Masters Thesis.

1.4.4 Ames Fluctuation Assay

TA 98 *Salmonella typhimurium* bacteria strain (measures frameshift mutations) and TA 100 *Salmonella typhimurium* bacteria strain (measures base pair substitutions), neither of which can produce histidine, was cultured in 10ml growth medium and 50µg/ml ampicillin. Specifically, growth medium, ampicillin and either TA98 or TA100 bacteria strain was added to a 100ml flask and placed into a water bath at 37°C. The flask was shaken overnight at 160 rpm for 10 hours to allow bacteria to grow. The *Salmonella* bacteria was diluted in an exposure medium, in 24 well plates, and exposed to either AMAC sediment extracts, sediment fractions, Positive control (PC) or a solvent control (SC) (one sample or dilution per well) and incubated for 90 minutes at 37°C. After 90 minutes, a purple indicator medium that does not contain histidine was added to all wells. 800 µl from each well of the 24-well plate was transferred into 16 wells of a 384-well plate in triplicate for a total of 48 wells per sample. The 384-well plate was incubated at 37°C for 48 hours to allow any bacteria that had been back mutated and able to produce histidine to grow. After 48 hours, if a back mutation occurred and the bacteria reproduced then the media turned from purple to yellow. The number of yellow wells were counted per 16 wells and compared to the solvent control.

All AMAC extracts and fractions were run using both bacteria strains TA98 and TA100 and with and without metabolic enzymes (S9) for each bacteria strain. Opf2006, Sig2006, Lau2006 and Lau2004 sediment AMAC extracts were run in six dilutions (12.5 - 400 mg/ml) with a 2% DMSO solvent control (SC) and a positive control (PC). All dilutions, SC and PC were run in triplicate. All sediment fractions were run at 400mg/ml

sediment equivalents in triplicate. Different PCs were used depending on bacteria strain and if S9 mix was used.

<u>Positive controls</u>

1. 4-nitro-o-phenyldiamine (0.05mg/ml) was used with TA98 bacteria strain without S9

2. Nitrofurantoin (0.06mg/ml) was used with TA100 bacteria strain without S9

3. 2-aminoanthracene (0.025mg/ml) was used with TA98 and TA100 bacteria strains with S9

All PCs were chosen because of there ability to cause a specific mutagenic effect. 4-nitro-o-phenyldiamine was used for its ability to cause frameshift mutations without bioactivation by S9. Nitrofurantoin was used because it can cause base pair subsitutions without bioactivation by S9. Finally, 2-aminoanthracene was used with TA98 and TA bacteria strains and the addition of S9 because of its ability to cause frameshift and base pair mutations but only if bioactivated by S9.

CHAPTER 2

2.0 DIFFERENTIAL ASSESSMENT OF CHEMICAL EFFECTS ON AROMATASE ACTIVITY, AND E2 AND TESTOSTERONE PRODUCTION USING THE H295R CELL LINE

2.1 Abstract

In response to concerns about chemical substances that can alter the function of endocrine systems and may result in adverse effects on human and ecosystem health, a number of *in vitro* tests have been developed to identify and assess the endocrine disrupting potential of chemicals and environmental samples. Most of these assays measure the potency of direct-acting hormone mimics. However, some compounds can modulate endocrine functions through non-receptor mediated effects including steroidogenesis, steroid metabolism and hormone transport. One endpoint that is frequently used in *in vitro* models for the assessment of chemical effects on the endocrine system is the alteration of aromatase activity (AA), the enzyme converting androgens to estrogens. Here we characterize the effect of seven model chemicals on direct and indirect measurements of AA, and compare the predictability of the different types of AA measurement to E2 and T production *in vitro*.

H295R cells were exposed to forskolin, atrazine, letrozole, prochloraz, ketoconazole, aminoglutethimide and prometon for 48 hours. Direct, indirect and combined effects on aromatase activity were measured using a tritiated water release assay. Direct effects on aromatase activity were assessed by exposing cells only during the conduct of the tritium release assay. Indirect effects were measured after exposing cells for 48 h to test chemicals, and then measuring AA without further chemical addition. Combined AA was

measured by exposing cells prior and during the conduct of the tritium release assay. Estradiol and testosterone were measured by ELISA.

Exposure to the aromatase inhibitors letrozole, prochloraz, ketoconazole, and aminoglutethimide resulted in greater indirect aromatase activity after a 48 h exposure due to presumed compensatory mechanisms involved in aromatase activity regulation. Forskolin and atrazine had similar hormone and enzyme profiles and both chemicals resulted in a dose dependent increase in E2, T and indirect AA. Neither of these two chemicals directly affected AA.

Differential modulation of AA and hormone production was observed in H295R cells after exposure to seven model chemicals illustrating the importance of measuring multiple endpoints when describing mechanisms of action *in vitro*. It was found that direct AA measurements were not reliable predictors of effects on E2 for general inducing chemicals. Furthermore, indirect AA measurements were not reliable predictors of effects on E2 of inhibiting chemicals. Furthermore, for most of the chemicals, E2, T, and direct and combined AA were good predictors of the mechanism of action of the chemical, with indirect aromatase activity being a less precise predictor of effects at the hormone level because of presumed feedback loops made it difficult to predict the chemicals true effects.

Future work with the H295R it is recommended that a combination of direct and indirect aromatase measurements is used because it was best in predicting the effects of a chemical on E2 production. Further, it was shown that indirect AA measurements which are a common way to measure AA must be used with caution.

2.2 Introduction

Exposure to natural and man-made substances in the environment has been linked to alterations in endocrine and reproductive systems in wildlife (Ankley et al. 1998; Sumpter and Johnson 2005; Jobling et al. 2006). Some chemicals are receptor agonists and act directly as hormone mimics. One group of chemicals that has received increased attention during the past two decades are environmental (xeno)estrogens such as 17β-estradiol (E2), ethinylestradiol and other estrogen receptor agonists such bisphenol A and some alkylphenolics. Other chemicals can modulate the endocrine system by acting through non-receptor mediated mechanisms. For instance, substances such as some imidazole-like fungicides and phyto-flavonoids have been shown to modulate hormone production by affecting activities of the steroidogenic enzymes aromatase (CYP 19) and 17β-hydroxysteroid-degydrogenase (HSD), respectively (Sanderson et al. 2001; Brooks and Thompson 2005).

One non-receptor mediated pathway of endocrine disrupting chemicals (EDCs) of concern is the interference with sex steroid synthesis, specifically the production of 17β-estradiol, by the enzyme aromatase. Aromatase is a member of the cytochrome P450 family, and catalyzes the conversion of testosterone to estradiol in various tissues of vertebrates. Disruption of aromatase can lead to significant alterations in the endocrine homeostasis in organisms. For example, male and female aromatase knock-out mice had decreased production of estradiol and elevated concentrations of testosterone. Furthermore, disruption of aromatase in these mice also impaired spermatogenesis and sexual behavior in male rats, and resulted in severely under-developed uteri in female rats (Simpson et al. 2002). While the formation of estrogens via aromatase is of great

importance in context with the development and reproductive physiology of vertebrates, it is also discussed in context with the role of estrogens as promoters of carcinogenesis (Ryan 1982).

The aromatase enzyme can be the target of some environmentally relevant chemicals and can affect production of E2 and testosterone (T) (Sanderson et al. 2001; Hecker et al. 2006). Chemicals can affect aromatase activity by reacting directly with the enzyme or through other indirect mechanisms. Direct interactions of a chemical with the aromatase enzyme can include competition of the EDC with the endogenous ligand or by interfering with important chemical processes in the conversion of T to E2. In addition to direct effects of chemicals on aromatase activity, that are typically of an inhibiting nature, indirect effects can result in either decreased or increased aromatase activity. Indirect affects can include induction or inhibition of CYP19 gene expression through cAMP mediated processes (Naville et al. 1999). Furthermore, EDCs can act through feedback mechanisms that can result in up- or down-regulation in aromatase activity but do not necessarily interact directly with the enzyme. For example, if an EDC disrupts the metabolism of estradiol that results in changes in estradiol levels, aromatase activity might also be affected because of an organism's attempt to maintain E2 homeostasis by regulating its production (Ung and Nager 2009).

Assays have been developed to evaluate the potential effects of chemicals on aromatase. Most of these assays, however, only measure a specific endpoint such as aromatase gene expression or aromatase enzyme activity, and it is unclear whether the observed changes are truly predictive of effects at the hormone level. One cell line that has been shown to be a useful *in vitro* model for steroidogenic pathways and processes

including production of sex steroids and the aromatase enzyme is the human H295R adrenocarcinoma cell line (Sanderson et al. 2001; Hilscherova et al. 2004; Hecker et al. 2006; Hecker et al. 2007). Interest in this assay as a screening tool is based on its unique ability to express all the steroidogenic hormones and enzymes. This cell line has been shown to be useful in screening for effects on gene expression of steroidogenic enzymes, steroidogenic enzyme activity and production of steroid hormones (Gazdar et al. 1990; Rainey et al. 1993; Staels et al. 1993; Hilscherova et al. 2004; Gracia et al. 2006; Hecker et al. 2006; Sanderson 2006). Furthermore, under guidance of the US EPA and OECD a H295R Steroidogenesis Assay has been developed to address regulatory needs for screening of the potential effects of chemicals on steroidogenesis pathways (Hecker and Giesy 2008).

The objective of the current study was to investigate the differential effects of selected model chemicals on different aromatase activity endpoints, namely direct, indirect and combined aromatase activity measurements by exposing H295R cells to seven model chemicals with know interactions with the aromatase enzyme. This study further aimed to assess these differential aromatase activity measurement endpoints as predictors of changes of T and E2. Furthermore, multiple endpoints including direct and indirect effects on aromatase activity and production of sex steroids were used to develop a predictive classification scheme for these chemicals. The responses of the H295R cells to seven model EDCs were studied: Letrozole, a specific aromatase inhibitor used in breast cancer treatment; prochloraz and ketoconazole, imidazole fungicides that have been shown to be aromatase inhibitors; forskolin, a cAMP inducer; atrazine and prometon, triazine herbicides and suspected endocrine disruptors that have previously

been shown to induce aromatase activity and E2 production *in vitro*; aminoglutethimide, blocks aromatase and p450 side chain cleavage.

2.3 Materials and Methods

2.3.1 Test Chemicals

Forskolin (FOR), ketoconazole (KET), and aminoglutethimide (AMG) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Prochloraz (PRO) was purchased from Aldrich (St. Louis, MO, USA). Letrozole (LET) was provided by Cstchem (Zhejiang) Co., Ltd. Prometon (PRM) (technical grade, 98.7% purity, Lot 0310070) was obtained from Platte Chemical (Greenville, MS, USA), and Atrazine (ATZ) (CAS number 1912-24-9; purity 97.1%) was obtained from Syngenta Crop Protection Inc. (Greensboro, NC, USA).

2.3.2 Cell culture

The H295R human adrenocortical carcinoma cell line was purchased from the American Type Culture Collection (ATCC CRL-2128; ATCC, Manassas, VA, USA) and grown as described previously (Hilscherova et al., 2004). Cells were cultured in 100 mm2 Petri Dishes with 12.5 ml of supplemented medium at 37 °C with a 5% CO_2 atmosphere. Briefly, the cells were grown in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient mixture (DMEM/F12) (Sigma D 2906; Sigma, St. Louis, MO, USA) supplemented with 1.2 g/L Na_2CO_3, 10 mL/L of ITS+ Premix (BD Bioscience; 354352), and 25 mL/L of BD Nu-Serum (BD Bioscience; 355100) unless specified differently.

2.3.3 Experimental design

All experiments were conducted in 24-well cell culture plates (COSTAR, Bucks, UK) with a cell concentration of 300,000 cells/mL. One mL of cell suspension was added to each well and the cells were allowed to attach for 24 h. After the attachment period, the medium was changed and the experiment was initiated. Cells were exposed to test chemicals for 48 hours. DMSO was used as carrier solvent and did not exceed 0.1% v/v. Test plates included six chemical concentrations and a solvent control (SC), in duplicate or triplicate. At the end of each experiment, the culture medium was transferred to an Eppendorf tube and stored at -80 ºC prior to analysis for hormones and the live cells were subjected to the tritiated water-release assay for determination of aromatase activity.

To identify whether test chemicals could directly interact with the aromatase enzyme activity, a second series of experiments was conducted. For these experiments a subset of three chemical concentrations was selected based on their activity observed during the above-described experiments, reflecting low, medium and high responses of aromatase activity or changes in hormone production (whichever applicable). Each chemical exposure experiment was conducted three different ways: 1) Indirect Aromatase Activity-Cells were exposed in duplicate to each concentration of a chemical or solvent control (SC) for 48 hours and all chemical was rinsed and removed with PBS and aromatase activity was measured as described below, 2) Combined Aromatase Activity-Cells were exposed as described in the indirect assay and the same chemical concentrations were added again during the conduct of the tritium-release assay to evaluate combined effects of pre-exposure and direct interaction with catalytic enzyme activity; and 3) Direct Aromatase Activity- Chemicals were added to untreated cells

during the conduct of the tritium-release assay at the same concentrations as described in the combined aromatase assay to assess their direct interaction with the enzyme.

Prior to exposure, cell viability was evaluated in the SC and the three greatest exposure test chemical concentrations of each chemical with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) bioassay (Mosman, 1983). Cytotoxic chemical concentrations were not included in the data evaluation.

2.3.4 Aromatase activity measurements

Aromatase enzyme activity was measured using a tritiated water release assay as described by Lephart and Simpson (1991) with minor modification (Sanderson et al. 2001). After the H295R cells were exposed for 48 h they were washed twice with 500 μL PBS and then 0.25 ml of supplemented medium containing 54 nM 1β-3[H]-androstenedione (Perkin Elmer, Boston, MA) was added to each well. It is important to note that while most of the chemical was removed during the washing process some of the chemical might still be present within the cells. For the experiments in which direct effects of chemicals on catalytic enzyme activity were measured, the chemical of interest was added at the appropriate concentration to the medium containing 1β-3[H]-androstenedione. DMSO was used as carrier solvent and did not exceed 0.1% v/v. The cells were then placed in an incubator at 37 °C and 5% CO_2 for 1.5 h. After 1.5 h cells were placed on ice to stop the reaction. A 200 μl aliquot of the medium was removed and added to chloroform and Dextran coated charcoal to remove all remaining 1β-3H-androstenedione. Aromatase activity was determined by the rate of conversion of 1β-3H-androstenedione to estrone by aromatase. The quantity of 3H in extracts of medium was

determined by liquid scintillation counting. Aromatase activity was expressed as pmoles of androstenedione converted per h per 100,000 cells. The specificity of the reaction for the substrate was determined by use of a competitive test with non-labeled 1β-androstenedione, and the use of the specific aromatase inhibitor fadrozole (Hecker et al., 2005). Addition of large amounts of 1β-androstenedione reduced tritiated water formation to the concentrations found in the blanks. Furthermore, addition of fadrozole during the tritium-release assay reduced aromatase enzyme activity in a dose-dependent manner with concentrations of 0.3 μM and greater resulting in complete inhibition of enzyme activity to the levels measured in the blanks.

2.3.5 Quantification of Hormones

Frozen medium from exposures was thawed on ice, and hormones were extracted twice with diethyl ether (5 mL) in glass tubes, and phase separation was achieved by centrifugation at 2,000 x g for 10 min. The solvent was evaporated under a stream of nitrogen, and the residue was dissolved in ELISA assay buffer and was either immediately measured or frozen at -80 °C for later analysis. Hormones in culture medium were measured by competitive ELISA using the manufacturers recommendations (Cayman Chemical Company, Ann Arbor, MI; Testosterone [Cat # 582701], 17ß-Estradiol [Cat # 582251]). Extracts of culture medium were diluted 1:2, 1:5, 1:10, 1:50 or 1:100 for estradiol, and 1:50, 1:100, 1:150, 1:250, 1:500, 1:1000 or 1:2000 for testosterone prior to use in the ELISA.

2.3.6 Statistical analyses

Statistical analyses of hormone data were conducted using SYSTAT 11 (SYSTAT Software Inc., Point Richmond, CA). Homogeneity of variance was accessed by the Lavene test and normality was assessed by box plots. All dose-response data were analyzed for significant differences using Kruskal-Wallis One-Way Analysis of Variance. The Mann-Whitney U test was then performed to analyze differences of single doses from controls. The Pearson correlation test with the Bonferroni test was used to evaluate the associations between E2, T and aromatase activity. The Pearson correlation test was also used to test for correlations between all endpoints when the data for all seven chemicals were combined. Differences with $p < 0.05$ were considered to be statistically significant.

2.4 Results

2.4.1 General inducers

Atrazine

Atrazine significantly increased E2 production in a dose-dependant manner at concentrations $\geq$ 1 μM. E2 concentrations were approximately 7.2-fold greater than those of the unexposed (SC) cells for the greatest dose tested (Fig. 2.1A). Testosterone was significantly increased at the two greatest concentrations of 10 and 100 μM but the magnitude of the change was less than observed for E2 (maximum fold change of E2 = 7.2; maximum fold change of T = 1.4). Direct aromatase activity was not statistically different from control levels at any concentration tested. Aromatase activity measured in the indirect and combined aromatase assays was approximately 2.0-fold greater than

control levels. However, the increase in the combined assay was less pronounced than that observed in the indirect assay and was significantly different compared to the solvent control starting at 10µM. Statistically significant, positive correlations were observed between E2 and T ($r = 0.944; p=0.004$), and between the hormones and indirect aromatase activity (T: $r = 0.882$; $p=<0.026$; E2: $r = 0.952$; $p<0.003$).

Forskolin

Forskolin caused a statistically significant dose-dependent increase in production of E2 and T (Figure 2.1B). E2 production increased by 36-fold while T increased by 2.7-fold relative to solvent controls. Indirect aromatase activity was also increased in dose-dependent manner up to 16-fold relative the solvent controls. Direct aromatase activity showed no change when forskolin was added directly to the tritium-release assay for cells that had not been pre-exposed. In the combined aromatase assay, aromatase activity was 9.4-fold greater than that of unexposed cells. This increase was less than that measured in the indirect aromatase activity assay. Statistically significant, positive correlations were observed between E2 and T ($r = 0.899$; $p<0.001$), and the hormones and indirect aromatase activity (T: $r = 0.899$; $p<0.001$; E2: $r = 0.976$; $p<0.001$).

Prometon

No effect on the production of T was observed at any prometon concentration (Figure 2.1C). Relative to controls, E2 production and aromatase activity were significantly greater in cells exposed to prometon at ≥ 3 µM. In the direct aromatase assay, activity

was significantly less than that in the SCs at the two greatest doses tested (10 μM and 100μM). Correlation between concentrations of E2 and indirect aromatase activity levels were statistically significant and positive ($r=0.853$; $p<0.001$). No statistically significant correlation was observed between E2 and T ($r=0.025$; $p=1.00$).

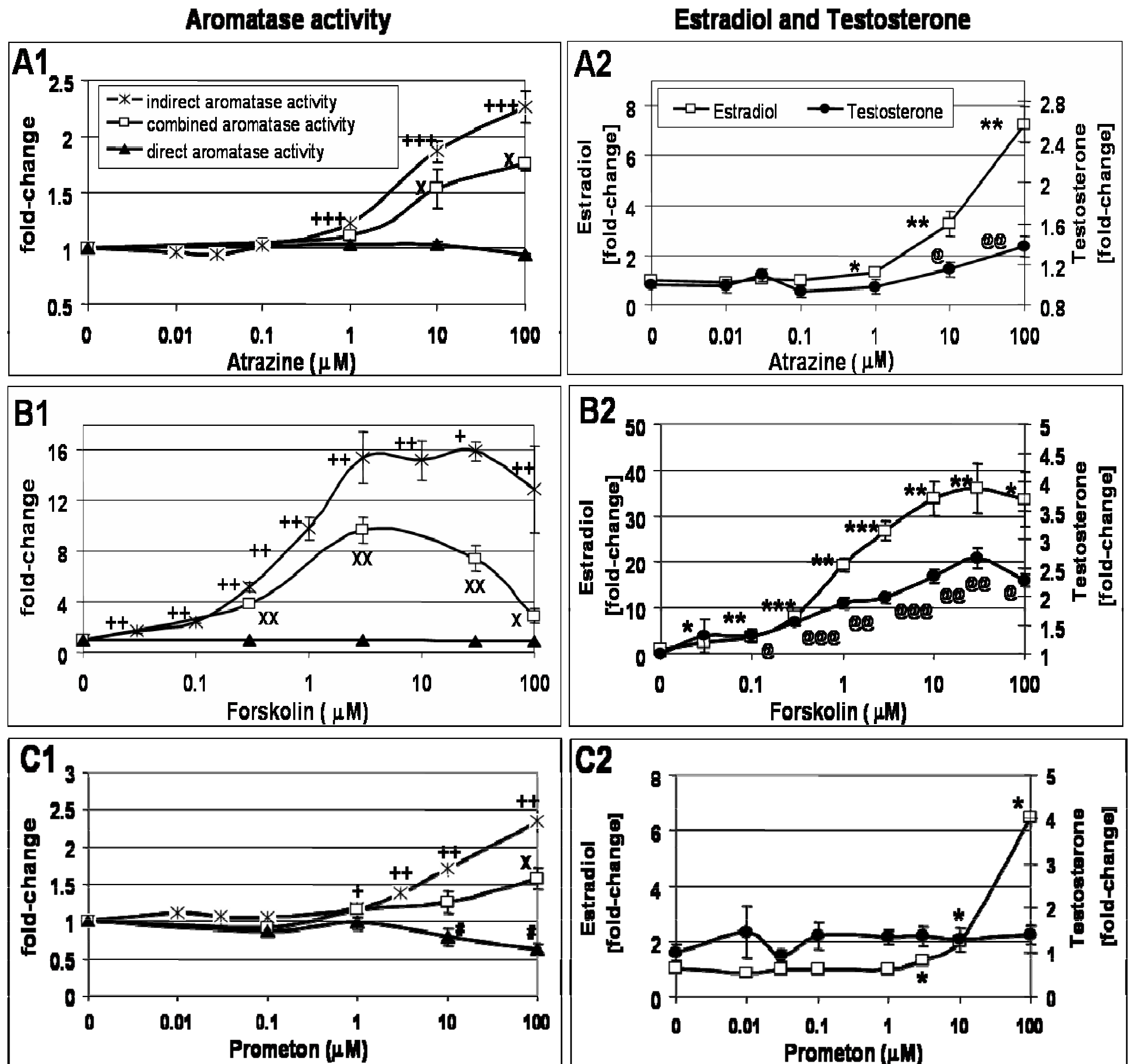


Figure 2.1 General Inducers: Effects of the exposure of H295R cells with forskolin, atrazine and prometon on testosterone, estradiol and direct and indirect aromatase activity. Cells were treated for 48 h with the indicated concentrations of forskolin, atrazine and prometon. Hormone data and aromatase activity is expressed as fold changes compared to solvent controls (SC = 1). Values represent the mean ± sem. Significant differences for estradiol (*), testosterone (@) and indirect (+) and direct (#) aromatase activity are reported relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol = $p < 0.05$; 2 symbols = $p<0.01$; three symbols = $p < 0.001$.

2.4.2 Potent inhibitors

Letrozole

Exposure to letrozole resulted in statistically significant, dose-dependent reduction in E2 and T production as well as a reduction in direct and combined aromatase activities (Figure 2.2A). In the indirect aromatase assay, enzyme activity was significantly greater than in the controls at concentrations between 0.001 and 0.1 μM, while a decrease was observed at concentrations greater than 1 μM. Concentrations of E2 were significantly less than SC levels at concentrations ≥ 0.001 μM letrozole. A statistically significant decrease in T production was measured in the 100 μM letrozole exposure.

Prochloraz

Exposure to prochloraz resulted in a dose-dependent decrease in the production of both E2 and T at all tested concentrations (Figure 2.2B). Direct and combined aromatase assay activities were reduced from control levels with significant reductions being observed in cells exposed to ≥ 0.3 μM prochloraz. The response in the indirect aromatase activity experiment was not monotonic in that activity increased in dose-dependent manner up to 0.3 μM and then decreased at greater concentrations. At 0.3 μM, aromatase activity was approximately 4-fold greater than that in the controls but was significantly less in cells treated with 10 μM prochloraz. A statistically significant, positive correlation was observed between E2 and T ($r=0.0.949$; $p=0.003$) but no statistically significant correlation was observed between either of the hormones and indirect aromatase activity.

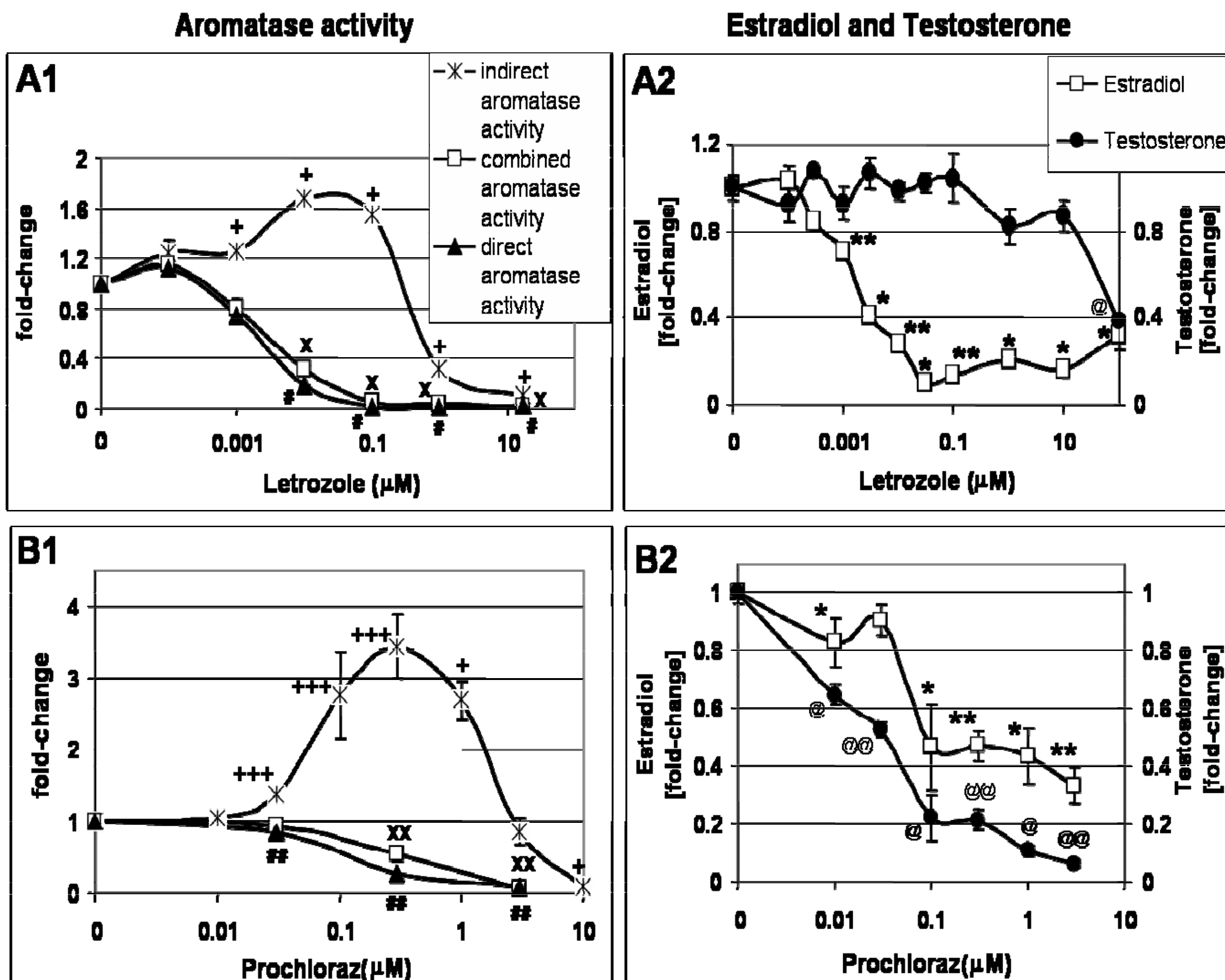


Figure 2.2 Potent Aromatase Inhibitors: Effect of letrozole and prochloraz on testosterone, estradiol and direct and indirect aromatase activity by H295R cells. Cells were treated for 48 h with the indicated concentrations of letrozole and prochloraz. Hormone data is expressed as fold changes compared to solvent controls (SC = 1). Values represent the mean ± sem. Significant differences for estradiol (*), testosterone (@) and indirect (+) and direct (#) aromatase activity are reported relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol = $p < 0.05$; 2 symbols = $p<0.01$; three symbols = $p < 0.001$.

2.4.3 Weak aromatase inhibitors

Aminoglutethimide

Aminoglutethimide significantly reduced production of both E2 and T relative to controls (Figure 2.3A). Testosterone levels were significantly reduced from controls when exposed to ≥1.0 μM aminoglutethimide. While aminoglutethimide affected E2 in a manner similar to T at the greatest concentration tested, a 3-fold greater dose was required (3 μM) to elicit a significant reduction from control levels. Aromatase activity in both the direct and combined aromatase assays were significantly less than that of the SC at concentrations ≥ 1 μM and maximum decreases in activity were 9.6- and 3.5-fold for the direct and combined assays, respectively. In contrast, activity in the indirect aromatase assay increased up to 9-fold in a dose-dependent manner after exposure to aminoglutethimide. This increase was significant at exposure concentrations ≥ 0.3 μM ($p<0.05$). A statistically significant, positive correlation was observed between E2 and T ($r=0.965$; $p<0.001$) while both E2 and T changes were negatively correlated with indirect aromatase activity (E2: $r=-0.939$; $p<0.001$; T: $r=-0.956$; $p<0.001$).

Ketoconazole

Exposure to ketoconazole resulted in dose-dependent reductions in E2, T and direct aromatase endpoints when compared to SC (Figure 2.3B). Statistically significant reductions in E2 and T relative to solvent controls were observed at ketoconazole concentrations ≥1 μM and ≥0.03μM, respectively. In the indirect aromatase assay, a dose-dependent increase in activity occurred in cells exposed to ≥1 μM ketoconazole. The maximum effect was an approximately 13-fold increase relative to the SCs in cells

expose to 10 μM. In the combined aromatase assay, activity was also greater than in the SCs with statistically significant differences observed at concentrations ≥1 μM, and the maximum effect of 4-fold occurring at an exposure concentration of 10μM. In the direct aromatase assay, changes in aromatase activity were bimodal with significantly greater activities (1.5-fold relative to SC) observed at 0.1 and 1 μM, and lesser activities than those of the SCs being observed at 10μM. No statistically significant correlations were observed between aromatase and concentrations of E2 or T. However, a statistically significant, positive correlation was observed between concentrations of E2 and T ($r = 0.875$; $p = 0.013$).

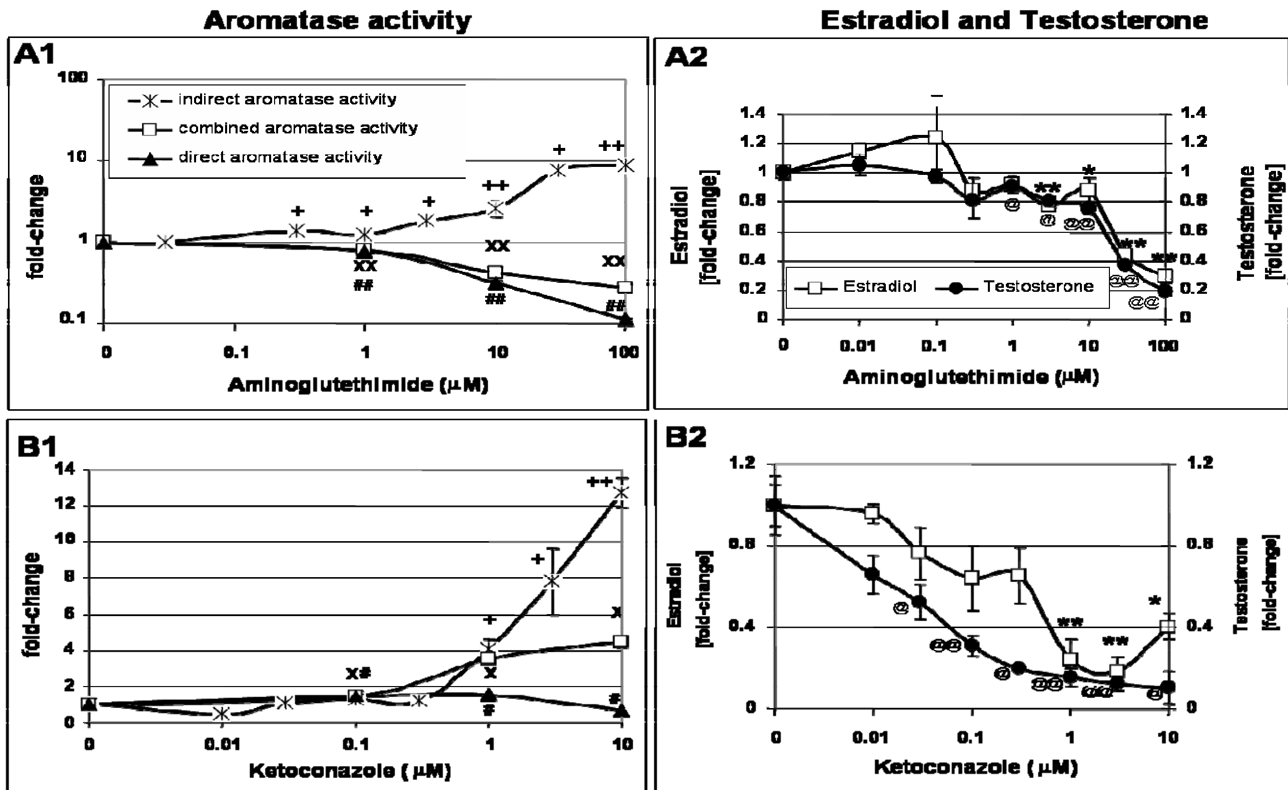


Figure 2.3 Weak Aromatase Inhibitors: Effect of ketoconazole and aminoglutethimide on testosterone, estradiol and direct, combined and indirect aromatase activity by H295R cells. Cells were treated for 48 h with the indicated concentrations of ketoconazole, aminoglutethimide. Hormone data is expressed as fold changes compared to solvent controls (SC = 1). Values represent the mean ± sem. Significant differences for estradiol (*), testosterone (@) and indirect (+), combined (X) and direct (#) aromatase activity are reported relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol = $p < 0.05$; 2 symbols = $p<0.01$; three symbols = $p < 0.001$.

Correlation analysis

The correlation between each set of endpoints was analyzed using the Pearson correlation test (Table 2.1). The strongest correlations were observed between E2 and the combined aromatase activity ($r = 0.66$; $p<0.001$). This relationship was significantly improved when ketoconazole was removed from the data set ($r = 0.84$; $p<0.001$). The combined aromatase activity correlated best with the direct and indirect aromatase activity

measurements ($r = 0.83$; $p<0.001$ and 0.71; $p<0.001$, respectively). T on the other hand only correlated with E2 ($r = 0.64$; $p<0.001$).

Atrazine and forskolin were classified as general inducers because of their ability to stimulate production of E2 and T and aromatase activity in the indirect, but not the direct aromatase assay. Prometon was also classified as a general inducer because of the similarity of E2 and indirect aromatase activity profiles to those observed for forskolin and atrazine. Letrozole, prochloraz, aminoglutethimide and ketoconazole were classified as inhibitors based of their ability to inhibit aromatase activity in the direct aromatase assay and by reducing production of E2 and T. This group of inhibitors was further broken down into two sub-groups, potent and weak inhibitors. Letrozole and prochloraz were grouped together as potent inhibitors because both chemicals caused effects at concentrations less than 0.03 μM. Aminoglutethimide and ketoconazole were grouped together as weak inhibitors because no effects were observed in any endpoint until greater than 0.1 μM.

Table 2.1 Pearson coefficients. Analysis of the correlations between estradiol, testosterone and indirect, combined and direct aromatase activity when the data of all seven chemicals is combined. Numbers in parenthesis is the Pearson coefficients without the chemical ketoconazole included. * $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$

Pearson Coefficient	Estradiol	Testosterone	Indirect aromatase	Combined aromatase
Testosterone	0.64 (0.61) ***	----	----	----
Indirect aromatase	0.52 (0.67) ***	0.079 (0.28)	----	----
Combined aromatase	0.66 (0.84) ***	0.30 (0.60)**	0.71 (0.72)***	----
Direct aromatase	0.53 (0.65) ***	0.23 (0.45)*	0.32 (0.35)**	0.83 (0.84)***

2.5 Discussion

2.5.1 General inducers

Forskolin and atrazine

Forskolin is an extract from the plant Coleus forskohlii and has been shown to induce hormone- responsive adenylate cyclase and increase intracellular cyclic AMP (Seamon et al. 1981). Some genes involved in steroidogenesis, including the CYP19 gene expressed in H295R cells, have a cAMP response-element where cAMP can bind and up-regulate gene expression (Watanabe and Nakajin 2004). The results of this study agree with this mechanism-of-action in that exposure to forskolin resulted in increased indirect and combined aromatase activity, estradiol and T concentrations. Furthermore, since forskolin did not directly affect aromatase activity, the greater aromatase activity in the 48 h exposure was most likely not due to direct interactions with the aromatase protein but rather through an increase in intracellular cyclic AMP.

Exposure of H295R cells to atrazine resulted in a similar hormone and aromatase activity dose-response profile as forskolin. Atrazine has been shown to increase aromatase activity in H295R cells after a 24 h exposure (Sanderson et al. 2001). Furthermore, experimental evidence has suggested that the increase in aromatase activity was due to an increase in cAMP (Sanderson et al. 2002). The observed results in the present study are consistent with cAMP as the mechanism-of-action for atrazine because atrazine did not directly affect aromatase activity, but after a 48 hour exposure, E2, T and indirect and combined aromatase activity all increased.

In conclusion, the hormone and aromatase activity profiles for forskolin and atrazine were comparable and suggest similar mechanisms of action even though exposure to

forskolin resulted in greater concentrations of E2, T and greater aromatase activity at much lesser concentrations than atrazine. Both chemicals increased estradiol levels to a much larger extent than T. These results point out the need to measure more than one endpoint when measuring aromatase activity. For example, if we had only measured the effects of the chemicals directly interacting with the aromatase protein we would have wrongly concluded that forskolin and atrazine does not affect aromatase activity.

2.5.2 Potent inhibitors

Letrozole and prochloraz

Letrozole is a selective aromatase inhibitor and is used in breast cancer treatments to reduce circulating levels of E2 that promote growth of the cancer. Consistent with its pharmacological mode of action, letrozole directly inhibited aromatase activity and resulted in lesser E2 production in H295R cells in our study. Indirect aromatase activity in the cells exhibited a biphasic response, which is due to a presumed positive feedback mechanism caused by the decrease in E2 production with the cells increasing the amount of aromatase enzyme to make more E2. At greater concentrations of letrozole there was sufficient letrozole to completely block aromatase activity while lesser concentrations of letrozole would promote the production of the aromatase protein. This type of biphasic response has been shown in other studies. Villeneuve et al. (2006) found that CYP19A gene expression in fathead minnow ovaries increased after being exposed to fadrozole, an aromatase inhibitor.

In the context of screening chemicals to determine their mechanism-of-action, care has to be taken when interpreting results if indirect aromatase activity was the only

endpoint measured. Furthermore, E2 concentrations were lesser than controls starting at 0.001 µM letrozole which is what is expected from a potent aromatase inhibitor. Interestingly, we observed no change in T concentrations at lesser letrozole concentrations and a slight decrease in T at the greatest concentration, which is opposite to what would be expected for a specific aromatase inhibitor. Instead we would expect T to increase, which was found in several studies with humans and rats, letrozole exposure resulted in an increase in serum T levels. (Kumru et al. 2007; Loves et al. 2008). The increase in T levels observed in these studies may have been due to letrozole blocking the production of estrogens and estrogens effects on luteinizing hormone. Estrogens can suppress the secretion of luteinizing hormone in the pituitary gland. Therefore, when estrogen formation is blocked by letrozole, luteinizing hormone can subsequently increase. Greater luteinizing hormone can lead to greater T production *in vivo* (Loves et al. 2008).

One possible reason why we did not observe an increase in T after the exposure to letrozole in the H295R cells is because the cells do not have the same signaling pathways and interaction between organs that is seen in *in vivo* tests. The H295R cells cannot produce luteinizing hormone and also cannot receive the luteinizing hormone signal from the pituitary gland. Without this signaling pathway, the increase in secretion of luteinizing hormone (caused by the lesser estrogen concentrations) never happens and never signals the cells to produce more testosterone. This interaction between organs signaling pathways is a good example of the limitations of *in vitro* testing. Often *in vitro* testing only evaluates one type of cell or organ and the interactions with other types of cells are missed including signaling among the hypothalamus-pituitary-gonadal axis.

However, the inhibiting effects of letrozole on E2 production would have been correctly predicted based on the in vitro data presented here.

Prochloraz is a widely used fungicide that has been shown to disrupt the action of several enzymes involved in steroidogenesis, including aromatase and those involved in the metabolism of steroid hormones (Vinggaard et al. 2005; Laignelet et al. 1989). In our study, prochloraz directly inhibited aromatase activity and resulted in less E2 production compared to control. As was observed for letrozole, indirect aromatase activity showed a biphasic response when H295R cells were exposed for 48 h. In contrast to letrozole, inhibition of T was the most sensitive response to prochloraz. Existing studies of rats exposed to prochloraz supported our results and found that serum testosterone levels and CYP17 enzyme activity were reduced (Blystone et al. 2007; Laier et al. 2006; Vinggaard et al. 2005). This suggests that the lesser production of T observed in the H295R cells were caused mainly by the reduced CYP17 enzyme activity, and this would account for the large decrease of T.

In conclusion, letrozole and prochloraz resulted in similar enzyme and estradiol profiles. Both chemicals were potent aromatase inhibitors that demonstrated a biphasic dose response when exposed for 48 h. Furthermore, the testosterone profiles between letrozole and prochloraz were slightly different. One theory for this is that prochloraz affects multiple enzymes within the steroidogenic pathway including aromatase and CYP17 whereas letrozole is a highly specific inhibitor of aromatase. However, to date there are no studies that investigated the effects of letrozole on other steroidogenic enzymes such as CYP17, and further research would be required to definitely answer this question.

2.5.3 Other effectors

Ketoconazole

Ketoconazole is a fungicide that is used mostly in pharmaceutical applications including over-the-counter dandruff shampoo. Ketoconazole has been found to decrease both 17, 20-lyase and aromatase activity *in vitro* (Weber et al. 1991) but did not affect 3β-HSD or 17β-HSD (Ayub and Stitch 1986). In our study, exposure to ketoconazole resulted in a decrease of E2, T and directly inhibited aromatase activity, which is consistent with ketoconazole's previously reported mechanism-of-action as an aromatase inhibitor. When the cells were exposed for 48 hours, greater levels of indirect and combined aromatase activity were observed. As with prochloraz and letrozole, it is hypothesized that ketoconazole most likely increases aromatase activity due to presumed feedback mechanisms triggered by E2 concentrations in response to direct interaction with the aromatase protein. Unlike letrozole and prochloraz, aromatase activity after a 48 h exposure to ketoconazole did not exhibit a biphasic response and greater aromatase activity was observed even at the greatest concentrations of ketoconazole. Ketoconazole has the ability to affect the aromatase protein directly, as shown in this study, but most likely does not have the same binding affinity as letrozole and prochloraz and can not shut off the aromatase activity as well at greater concentrations. Furthermore, the lesser concentration of T can be explained by effects on enzymes more upstream in steroidogenesis such as 17, 20-lyase. A decrease in T levels is consistent with *in vivo* studies in humans that ingested ketoconazole. For instance, in a study where boys were dosed with ketoconazole for eight years, significant decreases in serum T production were observed (Almeida et al. 2008).

Aminoglutethimide

Aminoglutethimide is a "generation I" aromatase inhibitor that has also been reported to act as a potent inhibitor of P450 side-chain cleavage. Due to its direct inhibition of P450 side-chain cleavage and the aromatase enzymes, it was used to treat Cushings syndrome (a disease that causes an increase in cortisol) and breast cancer, respectively (Fassnacht et al. 1998; Foster et al. 1983). This mechanism is consistent with the results of our study in the H295R cells, which is characterized by a general decrease in direct and combined aromatase activity, and subsequently E2 production. Also we observed a decrease in T which is what was expected due to inhibition of P450 side-chain cleavage. The 10-fold increase in indirect aromatase activity caused by aminoglutethimide is most likely due to presumed feedback mechanisms that were similarly observed for letrozole, prochloraz, and ketoconazole.

Prometon

Prometon is a widely used nonselective triazine herbicide and has been shown to induce E2 *in vitro* (in H295R cells) and testosterone *in vivo* (fathead minnow reproduction test), respectively (Villeneuve et al. 2007; Villeneuve et al. 2006). While the specific mechanism of action of prometon has never been identified, Villeneuve et al. (2006) found that prometon did not affect the estrogen receptor (ER) in MVLN cells or androgen receptor (AR)-mediated responses of MDA-kb2 cells. In our study, an increase in E2 concentrations was observed when H295R cells were exposed to greater than 3 μM concentration of prometon but no change in T was observed. Indirect and combined

aromatase activity also increased in the 48 hour exposure which would explain the increase in E2. Interestingly, prometon also reacted directly with the aromatase protein by decreasing enzyme activity. These data clearly distinguishes prometon from the other chemicals analyzed in this study because no other chemical both directly inhibited aromatase activity and increased E2 production. A potential mode of action for the observed increase in estradiol caused by prometon might be an interaction with estradiol metabolizing enzymes (i.e. sulfotransferase). However, further experiments are needed to confirm the potential effects of prometon on such metabolizing pathways.

2.5.4 Predictability of aromatase activity for E2 and T

Correlations between E2, T and indirect, combined and direct aromatase activity revealed that E2 correlated strongest with the combined aromatase activity ($r = 0.66$). A correlation between direct aromatase activity and E2 and between indirect aromatase activity was also observed but not to the same degree ($r = 0.53$ and 0.52, respectively). This was expected because the combined aromatase activity integrates both indirect and direct effects on the aromatase enzyme. For example, in ketoconazole a large increase in indirect aromatase activity was observed but in contrast a slight decrease in direct aromatase activity was observed. The combined aromatase activity measurement in this case was more intermediate and a smaller increase was observed than in the indirect aromatase activity. In terms of predicting effects on E2 from aromatase activity the combined aromatase activity was the best predictor of changes in E2 concentrations. For six out of the seven chemicals measured, combined aromatase activity and E2 followed the same pattern (i.e. when E2 increased combined aromatase activity also increased).

Indirect aromatase activity was the least accurate predictor of changes to E2, and for four out of the seven chemicals indirect aromatase activity showed different trends than E2. According to the results presented here the most relevant aromatase activity measurement, and in our opinion the best approach for future studies, is the combined aromatase activity measurement. The combined aromatase activity measurement was better correlated (i.e. greater r value) with E2 and T than any of the other aromatase measurements.

Furthermore, it was observed that the general inducers, forskolin and atrazine, did not directly affect aromatase activity but acted through indirect mechanisms, as oppose to the general inhibitors in which all chemicals acted directly on the aromatase enzyme. Therefore, direct aromatase did not correctly predict what would happen to E2 hormone production in the general inducers and indirect aromatase activity did not correctly predict what would happen to E2 hormone production in the inhibitors.

2.5.5 Conclusion

In conclusion, the results show that measuring only one or two endpoints can be misleading for the determination of the mechanism of action of a chemical. For example, indirect aromatase activity increased in all chemicals that were direct aromatase inhibitors. Therefore, by measuring only indirect aromatase activity it would be easy to falsely conclude that the chemical increases aromatase activity even though one might be measuring a feedback mechanism offsetting direct effects on the enzyme's activity. Additionally, to determine a chemical's mechanism of action it would be more advantageous to measure the effect of a chemical on direct aromatase activity than

measuring indirect aromatase activity because this assay does not involve any feedback loops. In the case of letrozole, comparison of the H295R *in vitro* assay to *in vivo* work in humans and rats demonstrated the limitations of *in vitro* testing in predicting effects in whole animals. Even though the H295R cells exhibit all steroidogeneic enzymes, other signals that are not expressed in this cell line (i.e. luteinizing hormone) can make predicting effects in whole animals difficult. Nonetheless, for most of the chemicals E2, T, and combined aromatase activity were the best predictors of the mechanism of action of the chemical. The only chemical for which combined aromatase activity did not correctly predict effects on E2 production was ketoconazole. In this case E2, T and direct aromatase activity were the best predictors of effect. Based on the findings of this study it is recommended to include all endpoints measured, namely E2, T and direct, indirect and combined aromatase activity to be able to correctly predict the mechanism of action for all chemicals.

CHAPTER 3

3.0 EFFECTS OF UPPER DANUBE RIVER SEDIMENTS ON STEROIDOGENESIS USING CHEMICAL FRACTIONATION AND THE H295R ASSAY.

3.1 Abstract

Despite extensive stocking efforts, some fish populations in the upper Danube River, Germany, have been declining over several decades. Of concern in the upper Danube River is the toxicity of sediments to fish. One class of chemicals of concern that had been identified as part of previous sampling efforts are the endocrine disrupting chemicals (EDC). In an effort to identify the causative agents, in this study, sediment extracts were used with a novel fractionation technique to characterize 4 sediment extracts from the Upper Danube River in Germany for their ability to disrupt steroidogenesis pathways. Sediments were first screened for cytotoxicity and effects on the production of T and E2 *in vitro* by use of H295R cells. To assess which group of chemicals within the sediment sample caused the observed toxicity, sediments that were toxic were fractionated into 18 fractions based on polarity, planarity, and the size of the aromatic ring system. All raw extracts increased estradiol production up to 4-fold relative to controls with a maximum response seen at 5 mg sediment equivalents (SEQ)/mL for all extracts. Fractions 14 and 15 from at least three of the sediments significantly increased estradiol production. These fractions contained steroids, sterols and phthalates. Furthermore, fraction 7 of sediments from three of the four sites significantly decreased production of T compared to controls and increased E2 at two of the sites. This fraction contained PAHs with three aromatic rings. Fraction 10 contained the greatest concentrations of five-ring PAHs and three of the four sediments increased

E2 production. These results demonstrate that sediments from the Danube River have the potential to disrupt steroidogenesis in the H295R cell line.

3.2 Introduction

Despite extensive stocking efforts, decreases in some populations of fishes in the upper Danube River, Germany, have been reported during the past two decades (Wurm, 2001). There are many theories as to why such declines in fish numbers have occurred. These include habitat change, dams, invasive species, disease and pollution. One factor of concern in the upper Danube River is pollution because sediment-borne contamination can cause toxicity in sediment assays (Keiter et al., 2006; Maljevic and Balac, 2007; Otte et al., 2008; Keiter et al., 2008). For example, Keiter et al. (2008) found concentrations of the sum of 16 priority EPA Polyaromatic Hydrocarbons (PAHs) to be as great as 26 μg/g dry weight (dw) sediment in sediments of the upper Danube River and elicit significant AHr-mediated effects in three separate cell lines.

Some chemicals that were reported in the past to act as endocrine disrupting chemicals (EDCs) have been found in Danube River sediments. EDCs such as pesticides, plasticizers, plant sterols, PAHs, etc. have all been measured in sediments and have shown to disrupt the endocrine system in *in vitro* and *in vivo* assays (Tremblay and Van der Kraak, 1998; Arcaro et al., 1999; Higley et al., submitted 2009). These chemicals can pose a threat to wildlife because they can disrupt vital developmental and growth pathways. For example, known estrogen receptor agonists such as 17α-ethinylestradiol (EE2), 17β-estradiol and bisphenol A, have been measured in sediments in several ecosystems (Rempel et al., 2008; Vigano et al., 2008; Robinson et al., 2009).

Sediment extracts from the Upper Danube River produced estrogenic like responses in the YES assay (Keiter et al., 2006). It has also been reported that the same sediments caused embryo toxicity, disruptions in hatching rates and time-to-hatch in *Danio rerio* embryos (Keiter et al., 2006). Other endocrine effects that were caused by sediment-associated contaminants were changes in the expression of key genes involved in steroidogenesis (Blaha et al., 2006), and alteration in the production of the sex steroids testosterone (T) and estradiol (E2) (Grund et al., submitted 2009) using the H295R cell line.

Several assays have been developed to evaluate the potential effects of chemicals on steroidogenesis. One cell line that has been shown to be a useful *in vitro* model for investigating effects on steroidogenic pathways and processes including production of sex steroids is the human H295R adrenocarcinoma cell line (Gazdar et al., 1990; Rainey et al., 1993; Staels et al., 1993; Hilscherova et al., 2004; Gracia et al., 2006; Hecker et al., 2006; Sanderson, 2006; Higley et al., submitted 2009). Interest in this assay as a screening tool is based on its unique ability to express all the hormones and enzymes involved in the adrenal and gonadal steroidogenic synthesis pathways. In addition, most other assays only measure receptor mediated responses and will miss any responses caused by non receptor mediated responses. Using H295R cells allows the determination of receptor-mediated and non-receptor-mediated responses. Furthermore, under guidance of the US EPA and the OECD, a H295R Steroidogenesis Assay has been developed to address regulatory needs for screening of the potential effects of chemicals on steroidogenesis pathways (Hecker and Giesy 2008). The H295R Steroidogenesis Assay has also been shown to be a useful tool not only for screening single chemicals but also

for environmental samples including sewage effluents and sediment extracts (Blaha et al., 2006; Gracia et al., 2008; Grund et al., submitted 2009).

While *in vitro* assays like the H295R Steroidogenesis Assay allow for characterizing the type of toxicity in sediments, they do not permit establishing direct cause-effect-relationships (i.e. which chemical caused the toxicity measured). Because of the presence of multiple chemicals in sediment samples from locations characterized by complex exposure scenarios, it would be extremely resource, labor and time intensive to conduct chemical analyses to identify all potential contaminants in such a sample. Other means to assess which toxic chemicals are found in sediments and if they are found in relevant concentrations are needed.

Effect-directed analysis (EDA) is a method that can evaluate toxicity of single chemicals in complex matrixes (i.e. sediments) (Brack, 2003). This procedure utilizes a combination of bioassays, fractionation techniques and chemical analysis. After sediment has been shown to generate a particular effect in a bioassay the source of that toxicity is still unknown. Fractionation of sediment samples where effects were observed can help narrow down which groups of chemicals caused the observed toxicity in the original whole sediment samples. For example, fractionation of sediment has been used to examine possible estrogen responses in the YES assay and found that estrone, E2 and EE2 resulted in some of the response but other unknown fractions with unknown chemicals resulted in estrogen responses also (Peck et al., 2004). Other examples of the utilization of EDA in sediments include a study by Schlenk et al. (2005) who failed to find any changes in vitellogenin in Halibut (*Paralichthys californicus*) exposed to

sediment fractions that contained estrone or E2 but did observe responses in fractions that contained only unknown chemicals.

In addition, fractionation can help determine the source from where the toxic chemical or chemicals are coming from (i.e. industry, waste water, farming, etc) by ruling out the toxicity of whole groups of chemicals within the sediment. Once the fraction that caused the toxicity is isolated, that fraction can be analyzed by a more focused chemical analysis to determine the chemical or chemicals of interest. Ultimately, this information can then be used in support of remedial actions implemented to remove the toxicity or to reduce its discharge into the system at its source.

The aim of this particular study was to evaluate sediment extracts from the Upper Danube River for their potential to disrupt 17β-estradiol and testosterone production in H295R cells. In addition, the sediments were separated into 18 separate fractions based on polarity, planarity and molecular volume by use of a new fractionation technique by Lübcke-von Varel (2008). Individual fractions were then analyzed using the H295R cells to aid in the identification of possible candidate compounds that are associated with certain fractions.

3.3 Materials and Methods

3.3.1 Sampling

In 2006, near-surface bottom sediment samples were collected at two locations along the Upper Danube River (Sigmaringen (Sig2006) and Lake Oepfingen (Opf2006)) as well as at one tributary just upstream of its confluence with the Danube River (Lauchert (Lau2006)), by means of a van-Veen-gripper or a stainless steel shovel.

Furthermore, a sediment sample collected in 2004 at the Lauchert site (Lau2004) was included as a reference location because based on the results of previous studies it was found to be less contaminated (Keiter et al. 2008, Seitz et al. 2008). All 2006 sampling sites were chosen in accordance with a suspected gradient of sediment contamination at these locations (Keiter et al. 2006; Keiter et al. 2008; Seitz et al. 2008) and because of their exposure to sewage treatment plant effluents (Fig 3.1).

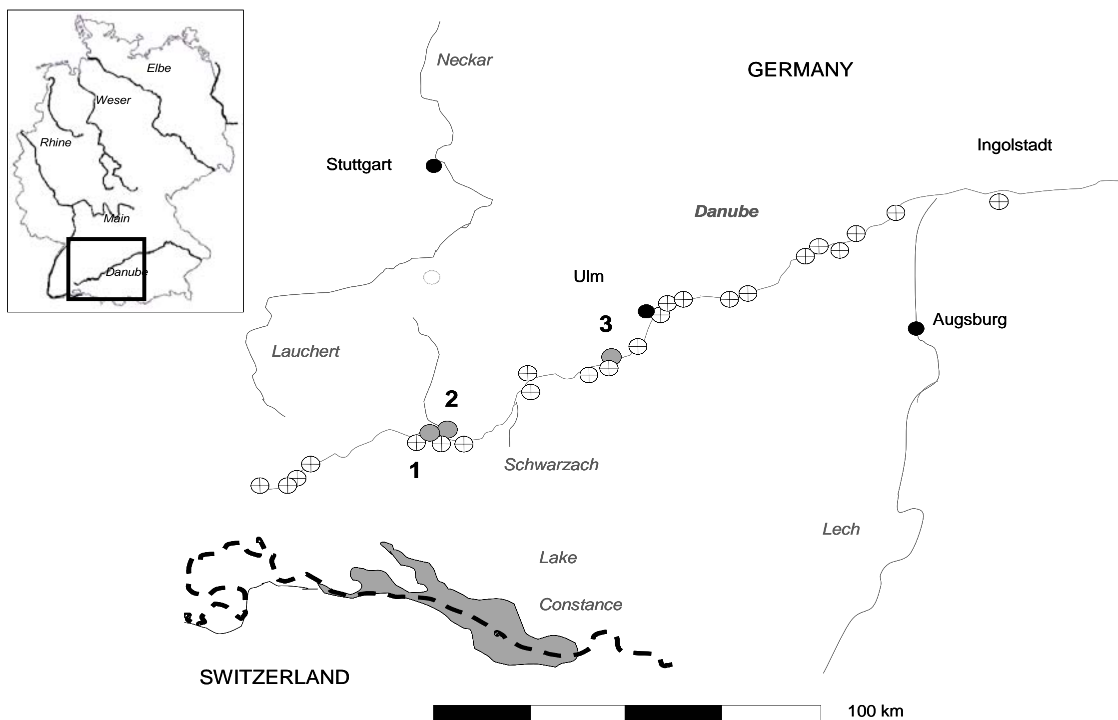


Figure 3.1 Sampling sites along the Upper Danube River. 1 = Sigmaringen, 2 = Lauchert (tributary), 3 = Oepfingen. ⊕ = Sewage treatment plants (> 10,000 residents according to LFW (2005).

3.3.2 Soxhlet-extraction of sediment samples

Multiple samples of sediment from the same location were pooled, homogenized, freeze-dried and sieved (mesh size 1.25 mm) immediately after return to the laboratory. Dried

and sieved sediments were Soxhlet extracted for 14 h with dichloromethane (DCM; p.a.; Merck, Darmstadt, Germany) and acetone (p.a.; AppliChem, Darmstadt, Germany) (3:1 v/v, 400 mL) according to a method described previously (Hollert et al. 2005). After extraction, the extracts were concentrated first by using a rotary evaporator and then by a gentle stream of nitrogen. Elemental sulphur was removed by copper treatment. Residues from each sample were re-dissolved in 1 mL dimethyl sulfoxide (DMSO; Fluka, Buchs, Switzerland) for direct use in the H295R assay or in 1mL hexane (Hx; p.a.; Merck) and acetone toluene (Fluka) (7:3 v/v) for subsequent clean up procedures. To investigate possible biological and chemical interference due to the solvents, all experiments included a procedural control (PrCo) that was extracted in the same manner than the sediment samples.

3.3.3 Accelerated membrane-assisted Clean-up of sediment extracts

A new accelerated membrane-assisted clean-up (AMAC) technique was used to purify the complex matrix of the sediment extracts according to an optimized protocol described previously (Streck et al. 2008). The solvents acetone, Hx, toluene and methanol and DCM (Suprasolv or LiChrosolv grade) were obtained from Merck. Briefly, an aliquot of the raw extract equivalent to 20 g of sediment (20 g sediment equivalents, SEQ) was transferred to dialysis membranes (commercially available polyethylene tubes) and dialyzed using an ASE 200 device (Dionex, Sunnyvale, CA). The temperature, pressure, number and duration of cycles were chosen as described previously (Lübcke-von Varel et al. 2008). Dialysis extracts were collected in glass ASE vials and capped with a PTFE-coated screw cap. After evaporating the extracts to dryness, the residue was weighted

and re-dissolved in DMSO for the H295R assays and in 1mL Hx:DCM (9:1 v/v) to a final concentration of 25 g SEQ mL^{-1} for fractionation procedure.

3.3.4 Fractionation

Fractionation was performed using a recently developed automated on-line fractionation procedure for polycyclic aromatic compounds in sediment extracts on three coupled normal-phase high-performance liquid chromatography columns (Table 3.1; Lübcke-von Varel, 2008). 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 (CN) with hexane (Hx) as mobile phase, while non-polar substances are flushed to the nitrophenylpropyl silica (NO) and porous graphitized carbon (PGC) stationary phases. To separate PAHs with more than two aromatic rings from the more polar PACs such as nitro- and keto-PACs, the CN-column is switched off-line. Flushing of the NO and PGC phases with Hx continues and the remaining chlorinated diaromatic compounds elute from NO to the PGC column. Afterwards a sequential fractionated elution from each of the columns was conducted, starting with the separation of chlorinated diaromatic compounds on PGC in forward and back-flush mode using Hx and toluene as mobile phases. The NO phase was then eluted with Hx:DCM (95:5). Finally, the CN column is eluted with Hx, DCM and acetonitrile. After passing through the detector the eluent is collected by the fraction collector into 18 glass bottles. After fractionation, the fractions of each sample were evaporated to dryness and the residue was re-dissolved in DMSO for the H295R assay and in hexane for GC–MS analyses.

Table 3.1. Solvents, columns used to separate specified compounds into each fraction. Flow rates are 10 ml min^{-1} for fractions 1 to 5 and 20 ml min^{-1} for fractions 6 to 18, respectively. Columns connected in series are hyphenated. Hx = hexane. DCM = dichloromethane. ACN = acetonitrile. CN = cyanopropyl. NO = nitrophenylpropyl. PGC = porous graphitized carbon

Fraction	**solvent (s)**	**Applied column(s)**	**Eluting compounds**
1	Hx	CN-NO-PGC	Alkanes
2	Hx	CN-NO-PGC	alkanes, sulphur, PCBs with 2 or 4 chlorines in *ortho*-position, PCNs with 3 chlorine atoms
3	Hx	NO-PGC	naphtalene, biphenyl, PCBs with 1 or 2chlorines in *ortho*-position, PCNs with 3 to 5 chlorine atoms
4	Hx:Toluol 60:40	PGC	
5	HxToluol	PGC	non-*ortho*-chlorinated PCBs, PCDDs/Fs
6	Hx:DCM 95:5	NO	small-seized PAHs like acenapthylene with more than two aromatic rings
7	Hx:DCM 95:5	NO	PAHs with three aromatic rings (anthracene)
8	Hx:DCM 95:5	NO	PAHs with four aromatic rings (pyrene)
9	Hx:DCM 95:5	NO	PAHs with four aromatic rings (chrysene)
10	Hx:DCM 95:5	NO	PAHs with five aromatic rings (benzo[a]pyrene)
11	Hx:DCM 95:5	NO	PAHs with six aromatic rings (benzo[ghi]pyrene)
12	Hx:DCM 95:5	NO	PAHs with seven aromatic rings (coronene)
13	Hx	CN	mainly mononitro-PAHs
14	↓	CN	(hydroxy-)quinones, keto-, dinitro-, hydroxyl-PAHs, N-heterocykles with rising polarity
15	DCM	CN	
16	↓	CN	
17	ACN	CN	2-hydroxyanthraquinone
18		CN	more polar compounds

3.3.5 H295R Steroidogenesis Assay

H295R human adrenocortical carcinoma cells were obtained from the American Type Culture Collection (ATCC CRL-2128; ATCC, Manassas, VA, USA) and grown as described previously (Hilscherova et al., 2004). Cells were cultured in 100 mm^2 Petri Dishes with 12.5 ml of supplemented medium at 37^oC with a 5% CO_2 atmosphere. Briefly, the cells were grown in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient mixture (DMEM/F12) (Sigma D-2906; Sigma, St. Louis, MO, USA) supplemented with 1.2 g/L Na_2CO_3, 10 mL/L of ITS+ Premix (BD Bioscience; 354352), and 25 mL/L of BD Nu-Serum (BD Bioscience; 355100) unless specified differently.

Experiments were conducted in 24-well cell culture plates (COSTAR, Bucks, UK) with a cell concentration of 300,000 cells/mL. One mL of cell suspension was added to each well and the cells were allowed to attach for 24 h. After the attachment period, the medium was changed and the experiment was initiated. Cells were exposed to sediment extracts and fractions for 48 h in 24-well plates. DMSO was used as carrier solvent and did not exceed 0.1% v/v. For each raw sediment extract, test plates included five concentrations and a solvent control (SC), in quadruplicate. Each of the 18 fractions was dosed at only one concentration of 20 mg/ml sediment equivalents, in triplicate. At the end of each experiment, cell viability was evaluated and the culture medium was transferred to an Eppendorf tube and stored at -80 ºC prior to analysis for hormones.

Cell viability was evaluated with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) bioassay (Mosman, 1983).

Frozen medium from exposures was thawed on ice, and hormones were extracted twice with diethyl ether (5 mL) in glass tubes, and phase separation was achieved by centrifugation at 2,000 x g for 10 min. The solvent was evaporated under a stream of nitrogen, and the residue was dissolved in ELISA assay buffer and was either immediately measured or frozen at -80 °C for later analysis. Hormones in culture medium were measured by competitive ELISA using the manufacturers recommendations (Cayman Chemical Company, Ann Arbor, MI; Testosterone [Cat # 582701], 17ß-Estradiol [Cat # 582251]). Extracts of culture medium were diluted 1:75 for testosterone prior to use in the ELISA.

3.3.6 Statisics

All samples were analyzed using the statistical software SPSS Statistics 17.0 (SPSS Inc., Chicago, IL). A one-way Analysis of Variance (ANOVA) was used to test for differences among samples for cytotoxicity, E2 and T data for both raw extracts and fractions. Normal distribution was assumed by analysis of a normality plot. Homogeneity was assumed by inspection of a boxplot. The parametric post hoc Dunnetts test was used to compare doses and fractions to control. Differences were considered significant at $p < 0.05$.

3.4 Results

3.4.1 Raw sediment extract

3.4.1.1 Cytotoxicity

Cytotoxicity was observed at greater concentrations for all raw sediment extracts except Lauchert 2004 (Lau2004). Significant declines in cell viability occurred at 20 mg SEQ/mL at the sites of Lauchert 2006 (Lau2006), and Sigmaringen (Sig2006), and at greater or equal to 10 mg SEQ/mL at Oepfingen (Opf2006) (Figure 3.2). In contrast, the Lau2004 raw sediment extract significantly increased cell growth at 5 and 10 mg SEQ/ml and no significant declines in cell viability was observed at any concentration.

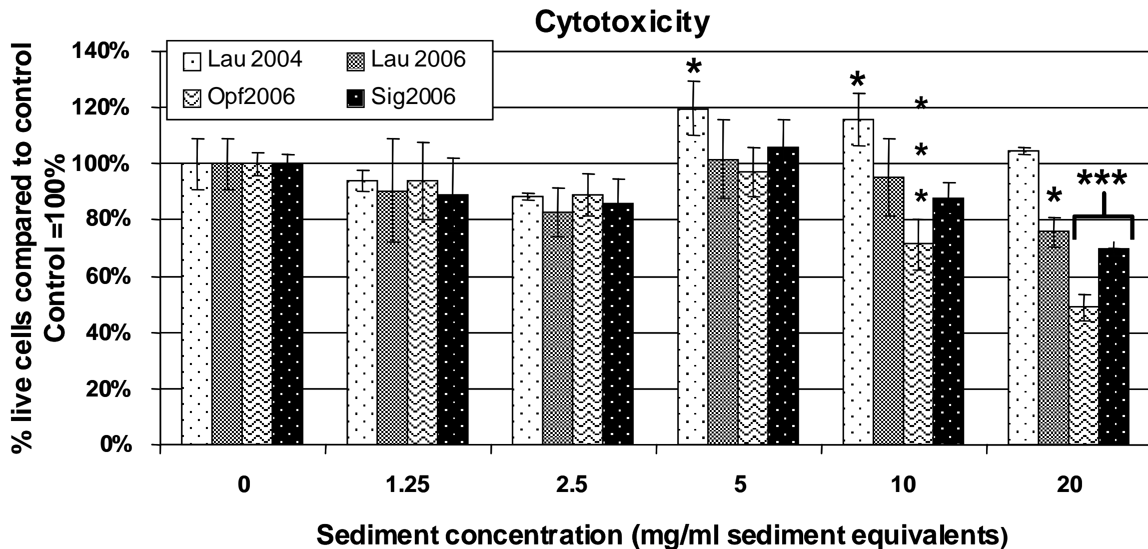


Figure 3.2. Cytotoxicity of raw sediment extracts Sigmaringen (Sig2006), Opfingen (Opf2006), and Lauchert sampled in 2004 (Lau2004) and Lauchert sampled in 2006 (Lau2006) in the H295R cell line. Cells were treated for 48 h with the indicated concentrations of raw sediment extracts. Cytotoxicity data is expressed as % live cells compared to solvent controls (100% = No cytotoxicity). Values represent the mean ± sd. * = Significant differences reported relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol = $p < 0.05$; 2 symbols = $p<0.01$; three symbols = $p < 0.001$.

3.4.1.2 Estradiol

All raw sediment extracts significantly induced E2 production in H295R cells. Sediments from Lau2006 and Opf2006 caused a significant induction of E2 production at all concentrations tested while sediments collected from Lau2004 and Sig2006 altered E2 production at concentrations greater or equal to 2.5 mg SEQ/mL (Figure 3.3). The greatest effects on E2 production were observed for extracts of sediments from Opf2006 and Sig2006 with maximum E2 production being more than 4-fold greater than those in the controls. Extract concentrations required to induce a maximum response were 2.5 and 5 mg SEQ/mL for Opf2006 and Sig2006, respectively. Maximum E2 production of cells exposed to Lau2006 raw sediment extract was 2.4-fold relative to the controls and was observed at 5 mg SEQ/mL. Least induction of E2 production (1.9-fold from controls) was observed after exposure of cells to 10 mg SEQ/mL of unfractionated extract of Lau2004 sediment.

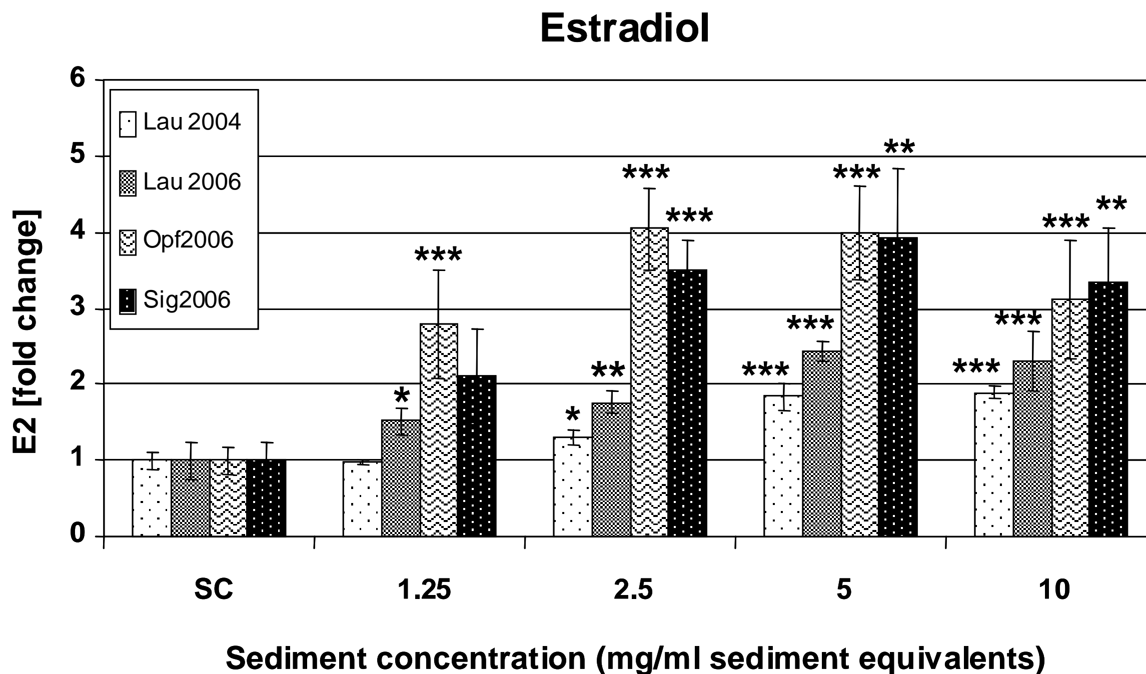


Figure 3.3. Effects of the exposure of H295R cells with raw sediment extracts Sigmaringen (Sig2006), Opfingen (Opf2006), and Lauchert sampled in 2004 (Lau2004) and Lauchert sampled in 2006 (Lau2006) on estradiol (E2). Cells were treated for 48 h with the indicated concentrations of raw sediment extracts. Estradiol is expressed as fold changes compared to solvent controls (SC = 1). Values represent the mean ± sd. * = Significant differences reported relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol = $p < 0.05$; 2 symbols = $p<0.01$; three symbols = $p < 0.001$.

3.4.1.3 Testosterone

Only exposure to Opf2006 and Lau2004 sediment extracts produced significant changes in T production (Figure 3.4). Significant increases in 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 (1.5-fold from controls). No significant changes in T production were observed for Lau2006 and Sig2006 at any concentration and for Opf2006 at the greatest concentration tested.

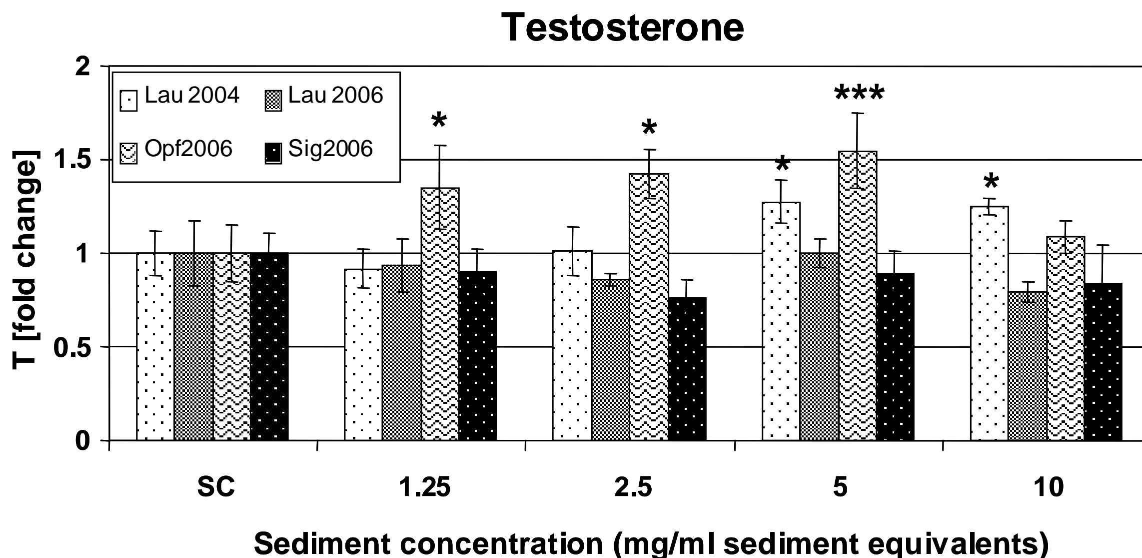


Figure 3.4. Effects of the exposure of H295R cells with raw sediment extracts Sigmaringen (Sig2006), Opfingen (Opf2006), and Lauchert sampled in 2004 (Lau2004) and Lauchert sampled in 2006 (Lau2006) on Testosterone (T). Cells were treated for 48 h with the indicated concentrations of raw sediment extracts. Testosterone is expressed as fold changes compared to solvent controls (SC = 1). Values represent the mean ± sd. * = Significant differences reported relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol = $p < 0.05$; 2 symbols = $p<0.01$; three symbols = $p < 0.001$.

3.4.2 **Fractionation samples**

At least one fraction of extracts of sediments from at all sites significantly affected E2 and T production. In sample Lau2004, E2 significantly increased in fractions 10, 14 and 15. Furthermore, only fraction 15 and the 1-18 combined samples resulted in a significant increase in T (Figure 3.5). No other Lau2004 fraction significantly affected E2 or T production compared to control. In sample Lau2006, fraction 7 significantly reduced T production although only slightly. In contrast, fractions 10, 11, 13, 14, 15 and 16 increased E2 production by up to 3-fold (Figure 3.6). No other Lau2006 fractions

significantly affected E2 or T production compared to control. Opf2006 fraction 7 significantly reduced T production and increased E2 production compared to controls (Figure 3.7). Exposure to Opf2006 fraction 18 significantly increased T production. In addition, E2 production increased compared to control in fractions 14, 15, 17. No other Opf2006 fraction significantly affected E2 or T production compared to control. Similarly to Opf2006 fraction 7, Sig fraction 7 significantly reduced T production and increased E2 production compare to control (Figure 3.8). Exposure to Sig2006 fraction 10 significantly increased T production compared to controls. In addition, production of E2 was greater when cells were exposed to fractions 9, 10, 13, 15 compared to control. No other Sig fraction affected E2 or T production compared to control.

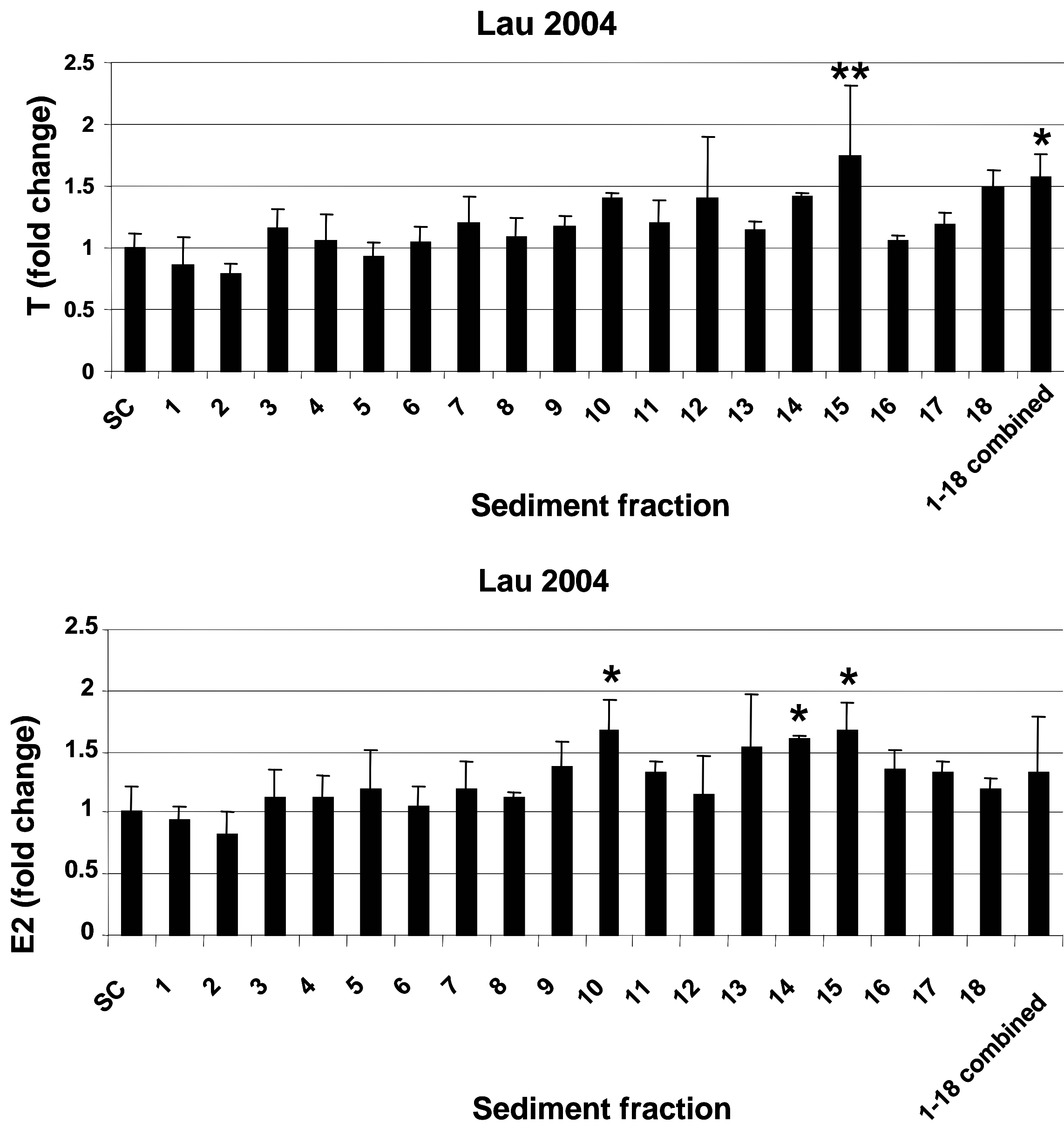


Figure 3.5. Effects of the exposure of H295R cells on 18 fractions from Lauchert 2004 (Lau2004) sediment samples on Testosterone (T) and Estradiol (E2). Cells were treated for 48 h with 20mg SEQ/ml of each of the 18 fractions in triplicate. T and E2 is expressed as fold changes compared to solvent controls (SC = 1). Values represent the mean ± sd. * = Significant differences reported relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol = $p < 0.05$; 2 symbols = $p<0.01$; three symbols = $p < 0.001$.

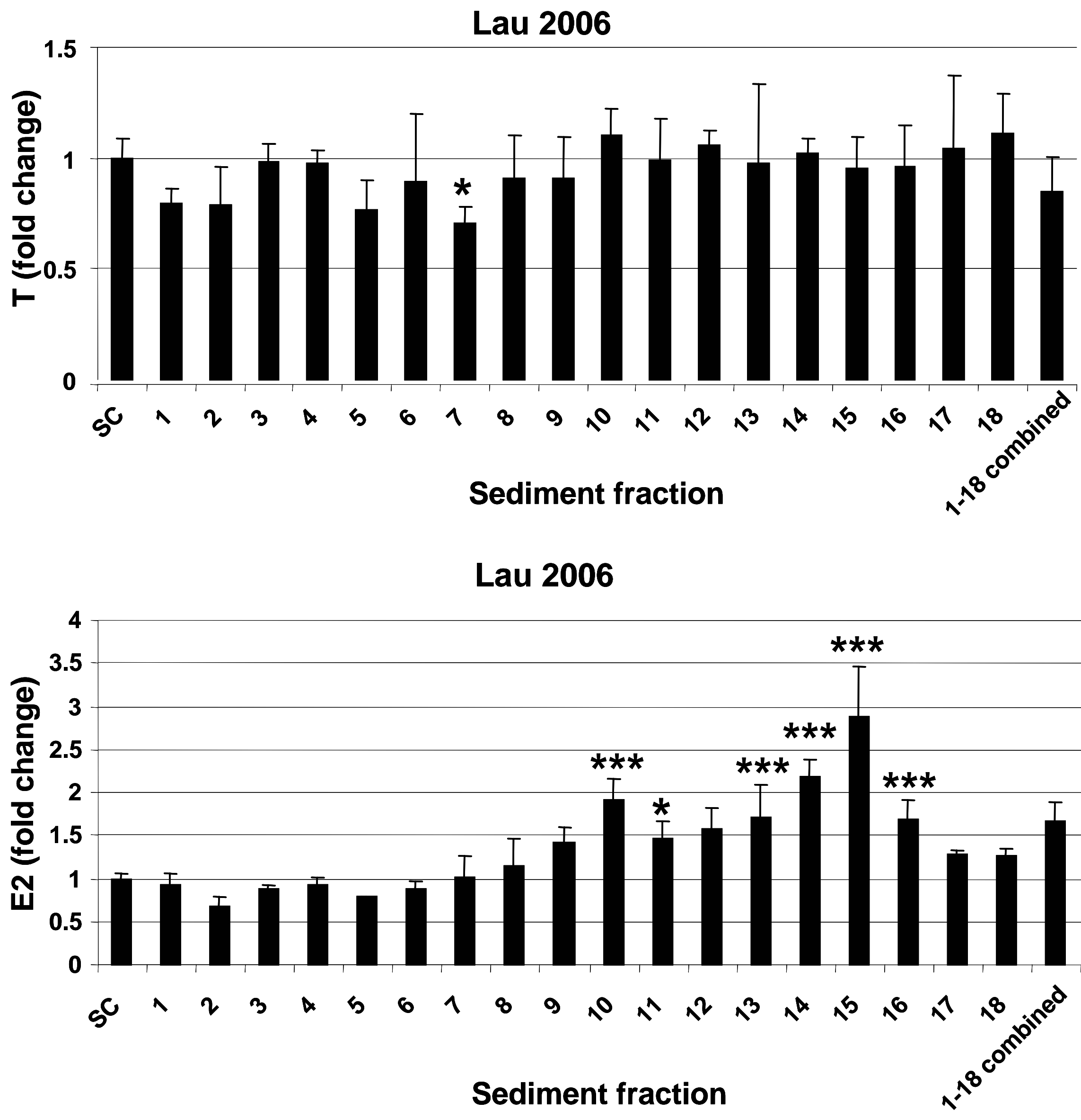


Figure 3.6. Effects of the exposure of H295R cells on 18 fractions from Lauchert 2006 (Lau2006) sediment samples on Testosterone (T) and Estradiol (E2). Cells were treated for 48 h with 20mg SEQ/ml of each of the 18 fractions in triplicate. T and E2 is expressed as fold changes compared to solvent controls (SC = 1). Values represent the mean ± sd. * = Significant differences reported relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol = $p < 0.05$; 2 symbols = $p<0.01$; three symbols = $p < 0.001$.

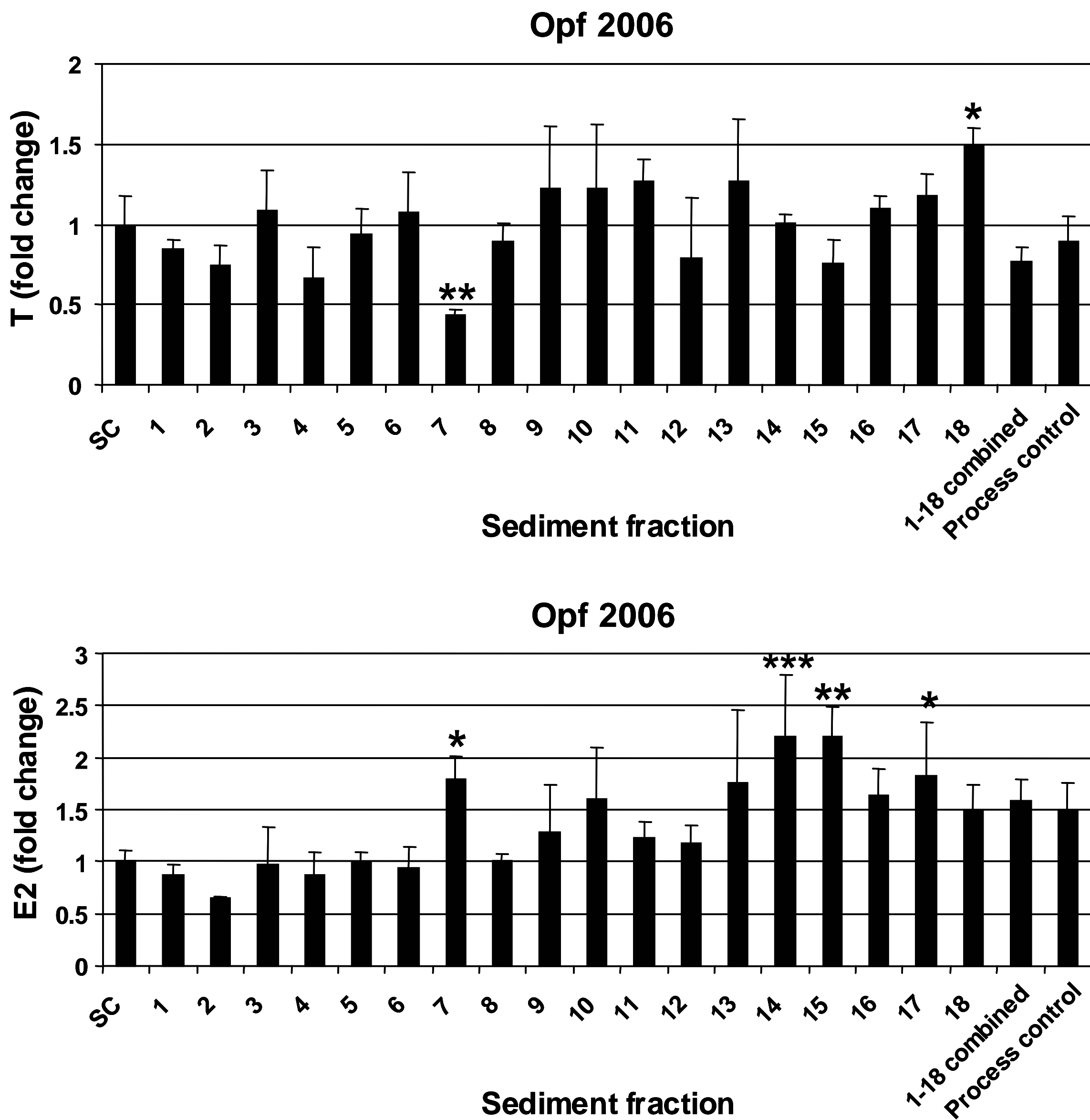


Figure 3.7. Effects of the exposure of H295R cells on 18 fractions from Opfingen (Opf2006) sediment samples on Testosterone (T) and Estradiol (E2). Cells were treated for 48 h with 20mg SEQ/ml of each of the 18 fractions in triplicate. T and E2 is expressed as fold changes compared to solvent controls (SC = 1). Values represent the mean ± sd. * = Significant differences reported relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol = $p < 0.05$; 2 symbols = $p<0.01$; three symbols = $p < 0.001$.

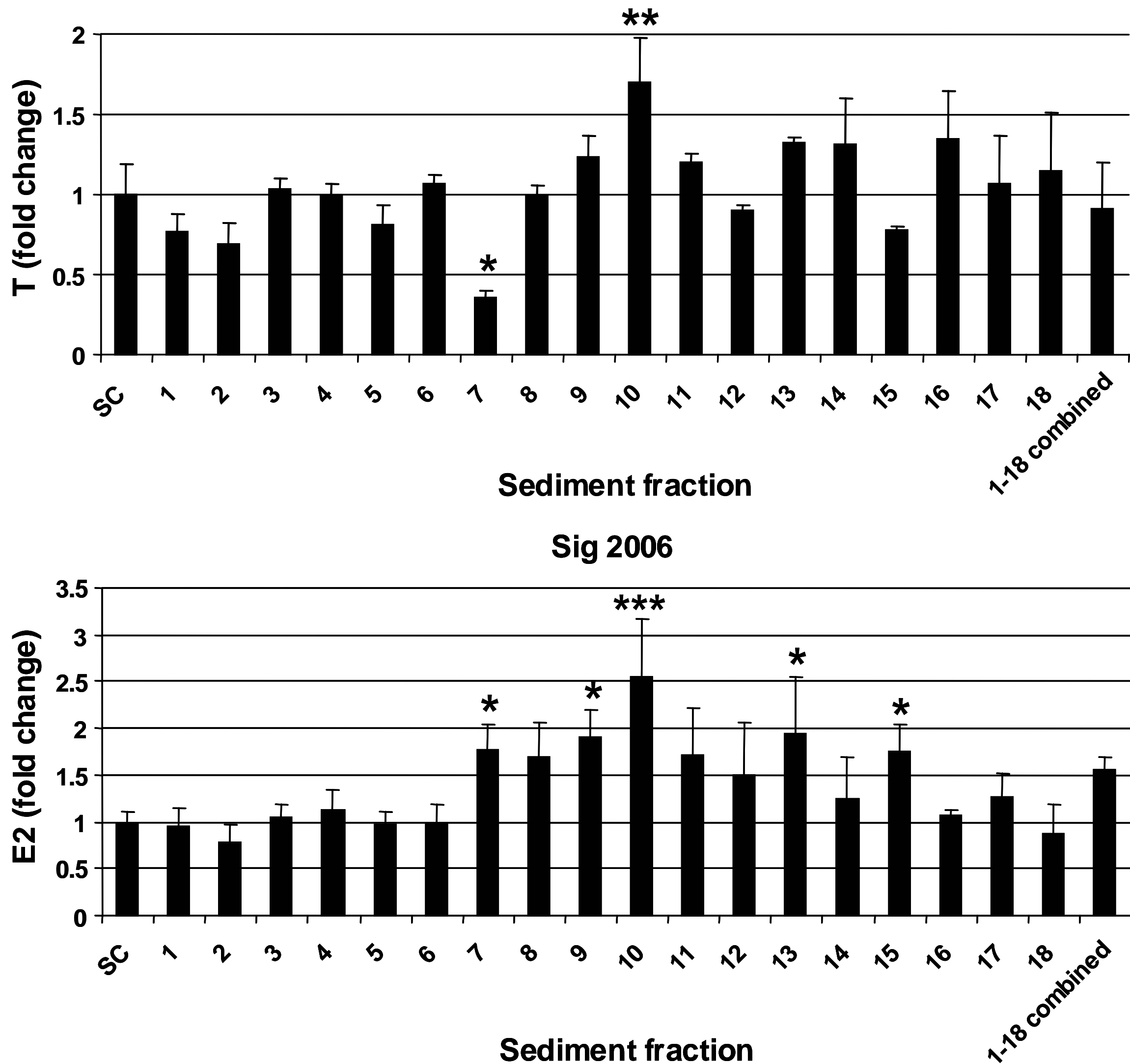


Figure 3.8. Effects of the exposure of H295R cells on 18 fractions from Sigmaringen (Sig2006) sediment samples on Testosterone (T) and Estradiol (E2). Cells were treated for 48 h with 20mg SEQ/ml of each of the 18 fractions in triplicate. T and E2 is expressed as fold changes compared to solvent controls (SC = 1). Values represent the mean ± sd. * = Significant differences reported relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol = $p < 0.05$; 2 symbols = $p<0.01$; three symbols = $p < 0.001$.

3.5 Discussion

In this study we demonstrated that un-fractionated extracts of Upper Danube River raw sediments can disrupt both E2 and T production in H295R cells. Raw sediment extracts Opf2006 and Sig2006 caused greater effects with greater cytotoxicity and greater E2 production observed at these sites than did extracts of sediments from Lau2004 and Lau2006. Furthermore, fractionation of these samples allowed identification of the chemicals most likely to have caused the toxicity or hormone modulation. For example, most of the greater E2 production observed in the un-fractionated Sig2006 extract was accounted for by fractions 7, 9, 10, 13 and 15. However, greater E2 production observed in Opf2006 appeared to be driven primarily by chemicals present in fractions 7, 14-15 and 17. For the less potent extracts of sediments from Lau2004, most of the greater E2 production was associated with fractions 10, 14 and 15. In general, the addition of the response of the fractions corresponded well with what was measured in the unfractionated extracts. The one exception was sample Lau2006 where a greater than additive effect of the fractions was observed. This was probably due to an interaction between some of the fractions. Furthermore, fraction 15 increased E2 production 3-fold, the most out of any fraction at any of the sites.

E2 production was disrupted at lesser extract concentrations and to a greater extent than T production for all raw sediment samples. Therefore, changes in E2 production seem to be a more sensitive endpoint compared to T. This trend was also observed when comparing the results obtained during the fractionation experiments; significantly greater E2 production compared to the controls was measured in a larger

number of fractions than that observed for T. Also, the magnitude of change was greater for E2 than for T. One reason for this could be that H295R cells produce greater quantities of T compared to E2, and therefore, slight increases in absolute E2 production will result in relatively large changes when expressed relative to the controls.

Fractionation of the raw sediment extracts 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. 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. However, significantly greater T was only observed in Lau2004 fraction 15 and not at any of the other sites. Many different types of chemicals can be found in fraction 15 that could account for the increase in E2 production, including 17β-estradiol, estrone and stigmasterol. However, Grund et al. (submitted 2009) reported E2 and ethinylestradiol (EE2) in the raw extracts that were less than the detection limit of the utilized analytical method and unlikely to be the whole cause of the greater E2 production observed in the cells (Table 3.2). Further evidence that the greater E2 production in fraction 15 was not caused by E2 or EE2 is that these chemicals would also be found in fraction 16, and no change in E2 or T production was noted after exposure of cells to this fraction except for a slight increase of E2 production at the Lau2006 site.

Bisphenol A (BpA) and nonylphenol (NP) were also measured in unfractionated extracts but their concentrations were less than those that were previously reported to cause effects in H295R cells (unpublished data; Table 3.2). It is unclear whether there could have been interactions between chemicals that resulted in more than additive

(synergistic) effects. Fraction 14 was another active fraction for which greater E2 production was observed at three of the four sites. It is assumed that this fraction contains similar active chemicals than fraction 15 such as BpA, NP or natural or synthetic hormones. Other types of chemicals that can be found in fraction 14 are phthalates and plant sterols. One chemical that has previously been shown as an endocrine disruptor and that would also elute with fractions 14 and 15 is bis(2-ethylhexyl)phthalate (DEHP) (Chikae et al., 2004; Laskey and Berman, 1993). Plant sterols, in particular β-sitosterol, have been shown to bind to the estrogen receptor and cause greater vitellogenin concentrations in zebrafish and rainbow trout (Tremblay and Van der Kraak, 1998; Nakari and Erkomaa, 2003). Furthermore, exposure of extracted wood sterols in environmentally relevant concentrations to zebrafish caused a significant change in the sex ratio of the zebrafish towards females in the F2 generation (Nakari and Erkomaa, 2003).

Table 3.2. Total concentrations of target estrogens in sediment extracts from the upper Danube River measured by LC-MS/MS (-ESI) and their relative estrogenic potencies (REPs) determined with the yeast estrogen screen (YES) in previous studies. Modified from Grund et al. Submitted 2009.

[ng/g SEQ]	NP		BPA		E1		EE2		E2
	a	b	a	b	a	b	a	b	a/b
Sigmaringen	6.5	4.1	15	11	0.13	0.05	<LOQ	<LOQ	<LOQ
Lauchert	1.4E+02	8.4E+02	1.2	1.2	0.15	0.05	<LOQ	<LOQ	<LOQ
Oepfingen	<LOQ	1.4	6.2	2.6	0.10	0.08	<LOQ	<LOQ	<LOQ
PrCo	26	2.2E+01	1.8	1.6	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
REPs	1.1E-05*		1.2E04**		1.3E-01*		7.3E-01*		1*

SEQ = sediment equivalent. a = sample extracted with acetone:hexane (Ac/Hx, 1:1 v/v). b = sample extracted with methanol (MeOH). < LOQ = less than limit of quantification. PrCo = procedural control. REP = relative estrogenic potency according to *Schultis & Metzger (2004) and **Beck et al. (2006), respectively.

In general, the greater E2 production in fractions 14 and 15 is probably caused by an additive effect of all the chemicals and not just one of the chemicals acting alone. Estrone, E2, EE2, NP and BpA were all measured in the raw extracts and most of these chemicals would be found in fraction 14 and 15 but none of them were in sufficient concentration to cause the observed effects alone.

Fraction 10 is also of concern because greater E2 production compared to control was observed in three of the four sited studied. Fraction 10 contains PAHs with five aromatic rings (i.e. benzo[a]pyrene and benzo[a]fluoranthene). PAHs can act as antiestrogens in *in vitro* assays and can be a reason for the greater E2 production in fraction 10 (Arcaro et al., 1999; Chaloupka et al., 1992). Grund et al., unpublished measured 22 PAHs in fractions 9-15 for Opf2006, Sig2006 and Lau2006 (Table 3.3) and found the greatest sum concentration of the 22 PAHs in fraction 10. Furthermore, fractions 10 of sediments collected at Sig2006 and Lau2006 each contained 4 times the amount of PAHs when compared to that of Opf2006 fraction 10. Correspondingly, Opf2006 fraction 10 did not significantly increase E2 in the H295R cells.

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. Additionally, exposure to Opf2006 and Sig2006 fraction 10 caused a significantly greater E2 production. Fraction 7 contained PAHs with three aromatic rings similar to anthracene and phenylnapthalenes. Phenylnapthalenes, in particular, have been shown to inhibit 17β hydroxysteroid dehydrogenase, the enzyme that converts androstenedione (ASD) to T (Frotscher et al., 2008). Therefore, the lesser T production caused by the exposure of fraction 7 could be

accounted for, in part, by phenylnapthalenes. It is unclear what chemicals in this fraction could cause the greater E2 production but it is possible that a different chemical through a different mode of action caused the greater E2 than what caused the reduction of T.

Given that exposure to fractions 1-6 did not result in any changes in E2 or T production at any of the sites studied indicates that chemicals such as many PCBs, PCN, TCDD and TCDF that elute with these fractions can be ruled out as a cause of the observed effects. Other chemicals that can be found in Fractions 1-6 are alkanes, DDT and its metabolites, and therefore, these compounds are also unlikely to have caused the observed changes in E2 and T. Two additional fractions that did not elicit an effect on the production of T and E2 by H295R cells were fraction 8 and 12. Fraction 8 contains PAHs with four aromatic rings (i.e. pyrene), and fraction 12 contains PAHs with seven aromatic rings (i.e. coronene).

In conclusion, all raw sediment extracts increased E2 production in the H295R cells to a greater or lesser extent, with Opf2006 and Sig2006 revealing more marked changes than the reference site at Lau2004. Changes in E2 and T production seemed to be consistently associated with certain fractions. One group of chemicals that could be of relevance with respect to the observed alterations in E2 production are five aromatic ringed PAHs, which elute with fraction 10, which increased E2 production in three of the four samples. Furthermore, fractions 14-15 containing steroids, sterols and phthalates increased E2 production at all sites by up to three-fold. While none of these chemicals have been previously associated with effects on the synthesis of E2, this indicated that some of the strongest inducers of E2 are represented by polar compounds. Fraction 7 was also of concern because in three of the four sites lesser T concentrations were observed.

This fraction can contain PAHs with three aromatic rings similar to anthracene and phenylnapthalenes (a known 17β hydroxysteroid dehydrogenase inhibitor). In contrast, chemicals such as PCBs, TCDD, PCN, and DDTs could be ruled out as causative agents for the observed toxicity because the fraction containing these compounds did not reveal any effects on hormone production.

Many of these chemicals have been linked to toxicity in fish and therefore cannot be ruled out as a possible cause in the decline of fish populations observed in the Upper Danube River. For example, DEHP has been found to increase hatching time and decrease body weight in Japanese medaka (*Oryzias latipes*) and disrupt T, E2, 11-ketotestosterone and Progesterone production in common carp (*Cyprinus carpio*) (Chikae et al., 2004; Han et al., 2009).

However, in general there is still a great deal of uncertainty regarding the specific chemicals that were responsible for the changes observed on the hormone production in H295R cells. Therefore, future work should expand on a more thorough chemical analysis of the here identified fractions for a wider variety of potential EDCs. The study presented here aided in the identification of biological active fractions, which allows narrowing of the target analytes to groups of chemicals with certain physico-chemical properties. It furthermore indicated that there are a number of unknown chemicals in the environment that have the potential to interfere with biological relevant processes such as the synthesis of sex steroid hormones and cannot be ruled out as a cause in the decline of fish populations on the Upper Danube River, and that warrant consideration in current risk-assessment strategies.

Table 3.3. Total concentrations of selected polycyclic aromatic compounds given for selected fractions of AMD extracts of sediment samples from the Upper Danube River measured by GC-MS. Modified from Grund et al. In prep.

[ng/g SEQ]	DL	Sigmaringen						Lauchert 2006						Oepfingen					
		F9	F10	F11	F13	F14	F15	F9	F10	F11	F12	F14	F15	F9	F10	F11	F13	F14	F15
Benzo[*a*]anthracene[b]	1.32	**74.5**	<DL	<DL	<DL	<DL	<DL	**67.6**	<DL	<DL	<DL	<DL	<DL	**27.7**	<DL	<DL	<DL	<DL	<DL
Chrysene[b]	1.32	**78.1**	<DL	<DL	<DL	<DL	<DL	**80.2**	<DL	<DL	<DL	<DL	<DL	**37.5**	<DL	<DL	<DL	<DL	<DL
Benzo[*k*]fluoranthene[b]	0.39	<DL	**110.8**	0.94	<DL	<DL	<DL	<DL	**145.1**	<DL	0.65	<DL	<DL	<DL	**31.2**	<DL	0.47	<DL	<DL
Benzo[*a*]pyrene[b]	0.39	<DL	**55.2**	1.32	<DL	3.27	<DL	<DL	**71.1**	<DL	<DL	<DL	<DL	<DL	**16.7**	<DL	<DL	<DL	<DL
Benzo[*g,h,i*]perylene[b]	0.09	<DL	**23.8**	**23.8**	1.75	<DL	<DL	<DL	0.22	**52.3**	2.64	<DL	<DL	<DL	<DL	**22.7**	1.55	<DL	<DL
Dibenzo[*a,h*]anthracene[b]	3.21	<DL	9.82	8.32	<DL	<DL	<DL	<DL	<DL	**16.3**	4.01	<DL	<DL	<DL	<DL	5.31	4.04	<DL	<DL
Indeno[*1,2,3-cd]*pyrene[b]	0.09	<DL	**19.2**	**21.0**	0.83	<DL	<DL	<DL	<DL	**45.1**	1.25	<DL	<DL	<DL	<DL	**21.4**	0.80	<DL	<DL
Benzo[*j*]fluoranthene[b]	0.39	<DL	**109.1**	0.70	0.45	<DL	<DL	<DL	**146.9**	<DL	0.79	<DL	<DL	<DL	**29.8**	<DL	0.57	<DL	<DL
9,10-Anthracen-dione	2.43	<DL	<DL	5.53	<DL	**27.1**	<DL	<DL	<DL	<DL	<DL	**34.5**	<DL	<DL	<DL	<DL	<DL	**19.11**	<DL
Triphenylphosphat	0.09	<DL	<DL	3.43	<DL	<DL	1.96	<DL	<DL	<DL	<DL	<DL	5.68	<DL	<DL	<DL	<DL	<DL	**15.6**
Sum single fraction [A]		162.2	328.0	89.3	4.48	40.7	10.21	148.7	363.4	113.7	15.5	58.2	8.94	66.5	77.6	49.4	8.42	24.8	18.2
		634.8						708.6						244.9					

[A] Sum of 22 PAHs measured. Only 10 PAHs are shown with concentrations greater than 10 ng/g SEQ (bold numbers). [b] U.S.EPA priority PAHs (Callahan et al. 1979). F = fraction. <DL = below detection limit. SEQ = sediment equivalent.

CHAPTER 4

4.0 ASSESSMENT OF TOXICITY OF UPPER DANUBE RIVER SEDIMENTS USING A COMBINATION OF CHEMICAL FRACTIONATION, THE *DANIO RERIO* EMBRYO ASSAY AND THE AMES FLUCTUATION TEST.

4.1 Abstract

There have been declines in fish stocks in the Upper Danube River since the early 1990s. It has been hypothesized that, among other causes, these declines may be associated with contamination of sediments in the affected areas. Here, we report on the results of an effect directed analysis (EDA) study conducted to determine the toxicity of extracts from sediments of the Danube River by means of the *Danio rerio* embryo assay and the Ames fluctuation assay to assess teratogenicity/embryo mortality and mutagenicity, respectively. For the sediment samples that revealed toxicity, fractionation of each sample was performed by separating compounds according to their polarity, planarity, and the size of the aromatic ring system. A total of eighteen fractions for each sediment sample were tested to assess which group of chemicals caused the original toxicity. Specifically, in the *Danio rerio* assay, Opfingen and Sigmaringen soxhlet extracted sediments caused 100% embryo mortality at a concentration of 33.3 mg sediment equivalent (SEQ)/ml, but none of the 18 fractions produced any observable toxicity at concentrations up to 100 mg SEQ/ml. In the Ames fluctuation assay, significant mutagenic activity was measured in accelerated membrane-assisted cleaned up sediment extracts and in some of the fractions of samples Opfingen, Sigmaringen and Lauchert. Fraction 10, which contains PAH's with five aromatic rings, produced a significant mutagenic response in all sediment samples measured only in S9 bio-activated

samples. Furthermore, fraction 15 which contains hydroxyl PAHs produced a significant mutagenic response in all sediment samples measured only in non bio-activated samples. These results show how EDA can be affective in determining the toxicity of sediments and determining which group or groups of chemicals caused the original toxicity.

4.2 Introduction

The worlds river systems provide fresh water to for drinking, irrigation for agriculture, many recreational activities and support thousands of species. However, these waterways have also been used for transport and disposal of wastes, such that they have become polluted though out history. Possible sources of contamination vary from municipal wastewater, which includes many chemicals including urine and feces, detergents cosmetics, fragrances, personal care products and, pharmaceuticals industry (i.e. PCBs, dioxins, and metals), agricultural runoff (i.e. pesticides and fertilizers), to storm water runoff from urban areas (i.e. salts, oil, and antifreeze). Sediments, that are a sink for a large number of chemicals, have been shown to elicit a variety of toxicities including metal-related effects, endocrine disruption, genotoxicity, mutagenicity, teratogenicity and dioxin-like effects. For example, sediment samples from the Upper Danube River that were analyzed in six separate assays were found to have considerable geno-toxic, cytotoxic, mutagenic, embryo-toxic and estrogenic effects. Due to the often complex nature of sediment contamination that is the result of the simultaneous presence of a diverse number of chemicals, it is often difficult to associate the observed biological activity of a sample with the exposure to certain substances. Multiple lines of evidence approach to elucidate the many different types of sediment toxicity has been discussed in

detail by Chapman and Hollert (2006). According to their findings, it is important to use multiple bioassays covering a variety of different biological endpoints to avoid missing significant effects. Keiter et al. (2006), for example, who analyzed sediment samples taken from the Danube River in Germany using a multiple lines of evidence approach using six separate bioassays found a variety of different types of significant effects including genotoxicity, cytotoxicity, mutagenicity, teratogenicity and estrogenicity.

The Danube River is the second largest river in Europe and drains 817,000 km^2 from 19 different countries. Despite increasing water quality, fish catches in the Upper Danube River have been decreasing since 1990, particularly grayling and sturgeon (Wurm, 2001; Keiter et al., 2006; Hensel and Holcik, 1997). Various theories have been suggested to explain the population declines of these species in the Danube River, including habitat destruction, dam construction, over-fishing and pollution. To date, many studies have assessed toxicity of sediment samples taken from the Danube river and have found several sites where known pollutants have been measured and produced significant toxicity including mutagenicity and teratogenicity effects (Otte et al., 2008; Keiter et al., 2006; Keiter et al., 2008; Maljevic and Balac, 2007).

According to the Toxic Substances Control Act in the United States over 70,000 industrial organic chemicals currently exist. For most 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 (Ankley et al., 1998). Measurement and toxicity testing for all chemicals that currently exist would be too costly and time consuming. Other means to assess which toxic chemicals are found in sediments and if they are found in relevant concentrations are needed.

Effect-directed analysis (EDA) is a method that can evaluate toxicity of single chemicals in complex matrixes (i.e. sediments) (Brack, 2003). This procedure utilizes a combination of bioassays, fractionation techniques and chemical analysis. After a certain sediment sample has been shown to generate a particular effect in a bioassay the source of that toxicity is still unknown. Fractionation of that sediment sample into many different chemical groups based on each chemicals physical properties and running these fractions on selected bioassays will narrow down what group of chemicals caused the original toxicity. Further chemical analysis of the particular fraction where toxicity was observed will pinpoint exactly what chemicals are present in the fraction. This information can be used to find the source of the contaminants (i.e. industry or agriculture) or decide on remedial efforts if needed. A new fractionation technique that uses an on-line fractionation procedure on coupled HPLC columns in only one run is a good candidate to use in EDA because this process decreases the risks of chemical fractionation losses and artifact formation compared to other similar fractionation techniques (Lübcke-von Varel et al., 2008).

Mutagenic and teratogenic effects can be measured using different types of assays including the Ames fluctuation assay to measure mutagenic effects and the *Danio rerio* embryo assay to measure teratogenic effects. The *D. rerio* embryo assay is a useful tool to measure teratogenic effects because *D. rerio* embryos have been shown to be sensitive to toxicants during early life-stages, and have a transparent chorion so lethal and sub-lethal effects are easily monitored in the egg (Hollert et al., 2003; Keiter et al., 2006). Furthermore, *D. rerio* are easily obtained, cultured, and can reach sexual maturity in only 3 months. They can also produce between 50 and 200 eggs every 2-3 days that develop

rapidly (approximately 96 hours). The Ames fluctuation assay has been shown to be a useful tool for the assessment of 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. Sediment toxicity assays that use *Salmonella* are well characterized and routinely used in genotoxicity testing of sediments: alone in 2004, 41.1% of all genotoxicity assays with sediments were conducted using this bacterium (Chen and White, 2004). In addition, different types of mutations can be measured depending on the type of *Salmonella* strain used; TA100 *Salmonella* strain measures base pair substitutions and TA 98 *Salmonella* strain measures frameshift mutations. Metabolic activation or de-activation of toxicants can also be evaluated by adding rat liver microsomes (S9) to the assay.

The aim of the current study was to use a new fractionation technique developed by Lübcke-von Varel (2008) to characterize four sediment extracts from the upper Danube River that had previously been shown to cause toxicity in various bioassays regarding their genotoxic and teratogenic potentials (Keiter et al., 2006; Keiter et al., 2008; Otte et al., 2008). First, the raw sediment extracts were analyzed in two assays; the *D. rerio* embryo assay and the Ames fluctuation assay. Second, each raw sediment extract was fractioned into 18 separate chemical groups and the toxicity of each fraction was characterized in the *Danio rerio* embryo assay and the Ames fluctuation assay.

4.3 Materials and Methods

4.3.1 Sampling

In 2006, near-surface bottom sediment samples were collected at two locations along the Upper Danube River (Sigmaringen (Sig2006) and Lake Oepfingen (Opf2006)) as well as at one tributary stream just upstream of its confluence with the Danube River (Lauchert (Lau2006)). Samples were collected by means of a van-Veen-gripper or a stainless steel shovel. Furthermore, a Lauchert sediment sample collected in 2004 (Lau2004) was included as an uncontaminated reference site based on the results of previous studies (Keiter et al. 2008, Seitz et al. 2008). All 2006 sampling sites were chosen in accordance with a suspected gradient of sediment contamination at these locations (Keiter et al. 2006; Keiter et al. 2008; Seitz et al. 2008; Boettcher et al. submitted), and because of their exposure to sewage treatment plants effluents, respectively (Fig. 4.1).

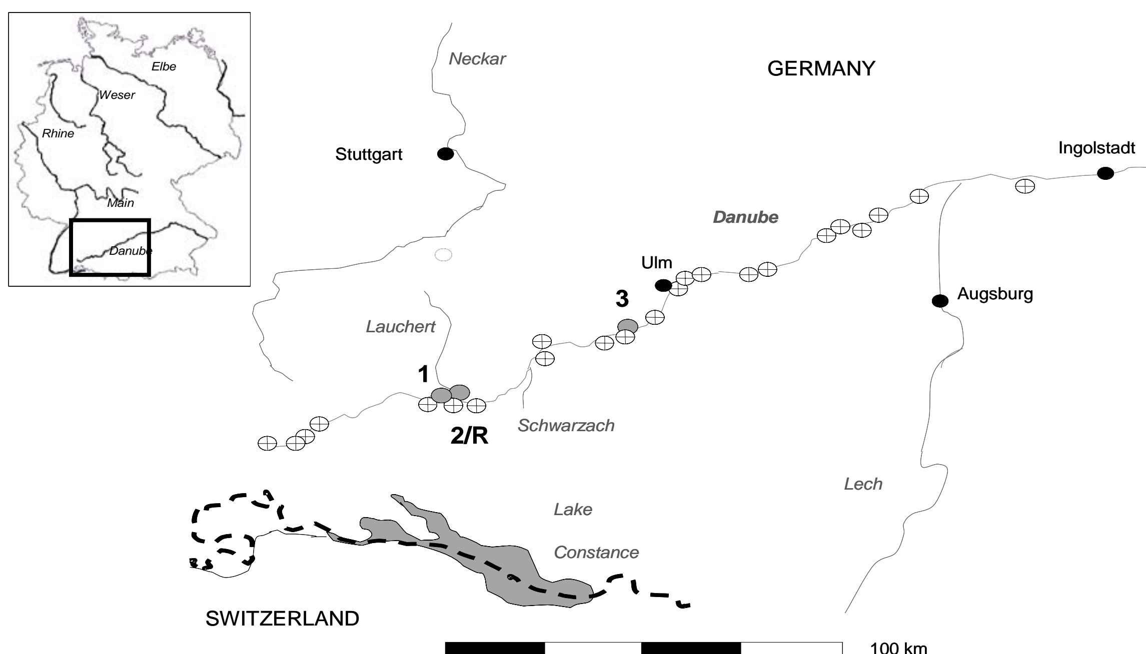


Figure 4.1. Sampling sites along the Upper Danube River. 1 = Sigmaringen, 2 = Lauchert (tributary), 3 = Oepfingen. ⊕ Sewage treatment plants (> 10,000 residents according to LFW (2005). R = reference sediment sample collected at site Lauchert in 2004 (Keiter et al. 2008).

4.3.2 Soxhlet-extraction of sediment samples

Sediment samples were pooled, homogenized, freeze dried and sieved (mesh size 1.25 mm) immediately after return to the laboratory. Dried and sieved sediments were Soxhlet extracted for 14 h with dichloromethane (DCM; p.a.; Merck, Darmstadt, Germany) and acetone (p.a.; AppliChem, Darmstadt, Germany)) (3:1 v/v, 400 mL) according to the method described by (Hollert et al., 2005). After extraction, the extracts were concentrated first by using a rotary evaporator and then by a gentle stream of nitrogen. Elemental sulphur was removed by copper treatment. Residues from each sample were re-dissolved in 1 mL dimethyl sulfoxide (DMSO; Fluka, Buchs, Switzerland) for the

H295R assay and in 1mL hexane (Hx; p.a.; Merck) and acetone (7:3 v/v) toluene (Fluka) for following clean up procedure. To investigate possible biological and chemical interference due to the solvents, all experiments included a procedural control (PrCo) that was extracted in the same manner as the sediment samples.

4.3.3 Accelerated membrane-assisted Clean-up of sediment extracts

A new accelerated membrane-assisted clean-up (AMAC) technique was used to purify the complex matrix of the sediment extracts according to an optimized protocol described previously (Streck et al. 2008). The solvents acetone, Hx, toluene and methanol and DCM (Suprasolv or LiChrosolv grade) were obtained from Merck (Darmstadt, Germany). Briefly, an aliquot of the raw extract equivalent to 20 g of sediment (20 g sediment equivalents, SEQ) was transferred to dialysis membranes (commercially available polyethylene tubes) and dialyzed using an ASE 200 device (Dionex, Sunnyvale, CA). The temperature, pressure, number and duration of cycles were chosen as described previously (Lübcke-von Varel et al. 2008). Dialysis extracts were collected in glass ASE vials and capped with a PTFE-coated screw cap. After evaporating the extracts to dryness, the residue was weighted and re-dissolved in DMSO for EROD assays and in 1mL Hx:DCM (9:1 v/v) to a final concentration of 25 g SEQ $mL^{-1}$for fractionation procedure.

4.3.4 Fractionation

Fractionation was performed using a recently developed automated on-line fractionation procedure for polycyclic aromatic compounds in sediment extracts on three coupled

normal-phase high-performance liquid chromatography columns (Table 4.1; Lübcke-von Varel et al. (2008)). 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 (CN) with hexane (Hx) as mobile phase, while non-polar substances are flushed to the nitrophenylpropyl silica (NO) and porous graphitized carbon (PGC) stationary phases. To separate PAHs with more than two aromatic rings from the more polar PACs such as nitro- and keto-PACs, the CN-column is switched off-line. Flushing of the NO and PGC phases with Hx continues and the remaining chlorinated diaromatic compounds elute from NO to the PGC column. Afterwards a sequential fractionated elution from each of the columns begins, starting with the separation of chlorinated diaromatic compounds on PGC in forward and back-flush mode using Hx and toluene as mobile phases. The NO phase is then eluted with Hx:DCM (95:5). Finally, the CN column is eluted with Hx, DCM and acetonitrile. After passing through the detector the eluent is collected by the fraction collector into 18 glass bottles. After fractionation, the fractions of each sample were evaporated to dryness and the residue was re-dissolved in DMSO for EROD assays and in toluene to a concentration of 5 g SEQmL^{-1} for GC–MS analyses.

Table 4.1 Solvents, columns used to separate specified compounds into each fraction.

Fraction	solvent (s)	Applied column(s)	Eluting compounds
1	Hx	CN-NO-PGC	alkanes
2	Hx	CN-NO-PGC	alkanes, sulphur, PCBs with 2 or 4 chlorines in *ortho*-position, PCNs with 3 chlorine atoms
3	Hx	NO-PGC	naphtalene, biphenyl, PCBs with 1 or 2chlorines in *ortho*-position, PCNs with 3 to
4	Hx:Toluol 60:40	PGC	5 chlorine atoms
5	HxToluol	PGC	non-*ortho*-chlorinated PCBs, PCDDs/Fs
6	Hx:DCM 95:5	NO	small-seized PAHs like acenapthylene with more than two aromatic rings
7	Hx:DCM 95:5	NO	PAHs with three aromatic rings (anthracene)
8	Hx:DCM 95:5	NO	PAHs with four aromatic rings (pyrene)
9	Hx:DCM 95:5	NO	PAHs with four aromatic rings (chrysene)
10	Hx:DCM 95:5	NO	PAHs with five aromatic rings (benzo[a]pyrene)
11	Hx:DCM 95:5	NO	PAHs with six aromatic rings (benzo[ghi]pyrene)
12	Hx:DCM 95:5	NO	PAHs with seven aromatic rings (coronene)
13	Hx	CN	mainly mononitro-PAHs
14	↓	CN	(hydroxy-)quinones, keto-, dinitro-,
15	DCM	CN	hydroxyl-PAHs, N-heterocykles with rising
16	↓	CN	polarity
17	ACN	CN	2-hydroxyanthraquinone
18		CN	more polar compounds

Flow rates are 10 ml min^{-1} for fractions 1 to 5 and 20 ml min^{-1} for fractions 6 to 18, respectively. Columns connected in series are hyphenated. Hx = hexane. DCM = dichloromethane. ACN = acetonitrile. CN = cyanopropyl. NO = nitrophenylpropyl. PGC = porous graphitized carbon

4.3.5 Ames fluctuation Assay

4.3.5.1 ***Salmonella typhimurium*** **Bacteria Culture** and TA 98 *Salmonella* strain

Salmonella bacteria strain TA98 (measures frameshift mutations) or TA100 (measures base pair substitutions), neither of which can produce histidine, was cultured in 10ml growth medium and 50µg/ml ampicillin. Specifically, growth medium, ampicillin and either TA98 or TA100 bacteria strain was added to a 100ml flask and placed into a water bath at 37°C. The flask was shaken overnight at 160 rpm for 10 hours to allow bacteria to grow.

4.3.5.2 Experimental design

All AMAC extracts and fractions were run using both bacteria strains TA98 and TA100 and with and without metabolic enzymes (S9) for each bacteria strain. Opf2006, Sig2006, Lau2006 and Lau2004 sediment AMAC extracts were run in six dilutions (12.5 - 400 mg/ml) with a 2% DMSO solvent control (SC) and a positive control (PC). All dilutions, SC and PC were run in triplicate. Sediment fractions were run at 400mg/ml sediment equivalents in triplicate. Different PCs were used depending on bacteria strain and if S9 mix was used.

<u>Positive controls</u>

1. 4-nitro-o-phenyldiamine (0.05mg/ml) was used with TA98 bacteria strain without S9
2. Nitrofurantoin (0.06mg/ml) was used with TA100 bacteria strain without S9
3. 2-aminoanthracene (0.025mg/ml) was used with TA98 and TA100 bacteria strains with S9

Briefly, in 24 well plates, *Salmonella* was diluted in an exposure medium and exposed to either AMAC sediment extracts, sediment fractions, PC or a SC (one sample or dilution

per well) and incubated for 90 min at 37°C. After 90 min, a purple indicator medium was added to all wells. 800 µl from each well of the 24-well plate was transferred into 16 wells of a 384-well plate in triplicate for a total of 48 wells per sample. The 384-well plate was incubated at 37°C for 48 h to allow any bacteria that had been back mutated and able to produce histidine to grow. After 48 h, if a back mutation occurred and the bacteria reproduced then the media turned from purple to yellow. The number of yellow wells were counted per 16 wells and compared to the solvent control.

4.3.6 *Danio rerio* Embryo Assay

Adult *Danio rerio* were stimulated to breed with the addition of artificial plants in the evening hours. Breeding took place within one hour after the lights were set to be turned on in the morning (All lights were on a 16h light and 8h dark cycle). Eggs were collected within one hour post fertilization and fertilized eggs were separated out under a microscope. Fertilized eggs were exposed to four or five dilutions of Opf2006, Sig2006, Lau2006 and Lau2004 Soxhlet extracted sediment and AMAC extracted sediment. For the fractionation samples, only Opf2006 and Sig2006 fractions were analyzed because no toxicity was observed at any concentration in Lau2006 and Lau2004 AMAC extracts. All fractionation samples were run at 100mg/ml sediment equivalents.

In brief, sediment extracts or fractions were diluted in nanopure water containing various ions. Ten eggs were added to the extract dilution or fraction solution in a test tube. One egg and 100µl of the solution was removed and placed in one well of a 96-well plate, this was repeated for the other nine eggs and all samples. Soxhlet extracted sediments and

fractions were all ran in replicate exposures. The 96-well plates were covered and placed in an incubator at 21 °C for 48 h. After 48 h, four lethal endpoints were analyzed.

Lethal endpoints included: 1. No heart beat; 2. No detachment of tail; 3. Retarded somite formation; 4. Coagulation of embryo.

4.3.7 Statistics

All samples were analyzed using the statistical software SPSS Statistics 17.0 (SPSS Inc., Chicago, IL). Homogeneity was accessed by the Levene test. A one-way Analysis of Variance (ANOVA) was run to analyze raw extracts in the Ames fluctuation assay. The parametric post hoc Dunnetts test was used to compare doses of raw extract to control. Where homogeneity of variance failed, the Mann-Whitney U test was performed to analyze differences of all the fractionation samples from controls in the Ames fluctuation assay. LC50 values for the soxhlet and AMAC extracts determined by the *D. rerio* assay were calculated using TOXCALC software (Michigan State University) and calculated using the binomial test.

4.4 Results

4.4.1 Ames Fluctuation Assay

4.4.1.1 Accelerated membrane-assisted clean-up (AMAC) raw extracts

Exposure to AMAC extracts of sediments collected at Opf2006, Sig2006 and Lau2006 caused a significant increase in revertants at the greatest sediment concentration only in the TA98 stain with the bioactivation enzymes S9 (Fig 4.2). The Lau2004 AMAC extract did not induce any revertants at any concentration and in any of the bacteria

strains with or without S9 (Fig 4.2 – 4.4). Furthermore, none of the four AMAC extracts caused a significant increase in revertants in the TA100 bacteria strain both with and without S9 (Fig 4.3, 4.4). In the TA98 bacteria strain with S9 exposure, Sig2006 induced revertants starting at 50 mg SEQ/ml, Opf2006 induced revertants starting at 100 mg SEQ/ml, and Lau2006 induced revertants at the highest concentration of 400 mg SEQ/ml (Fig 4.2).

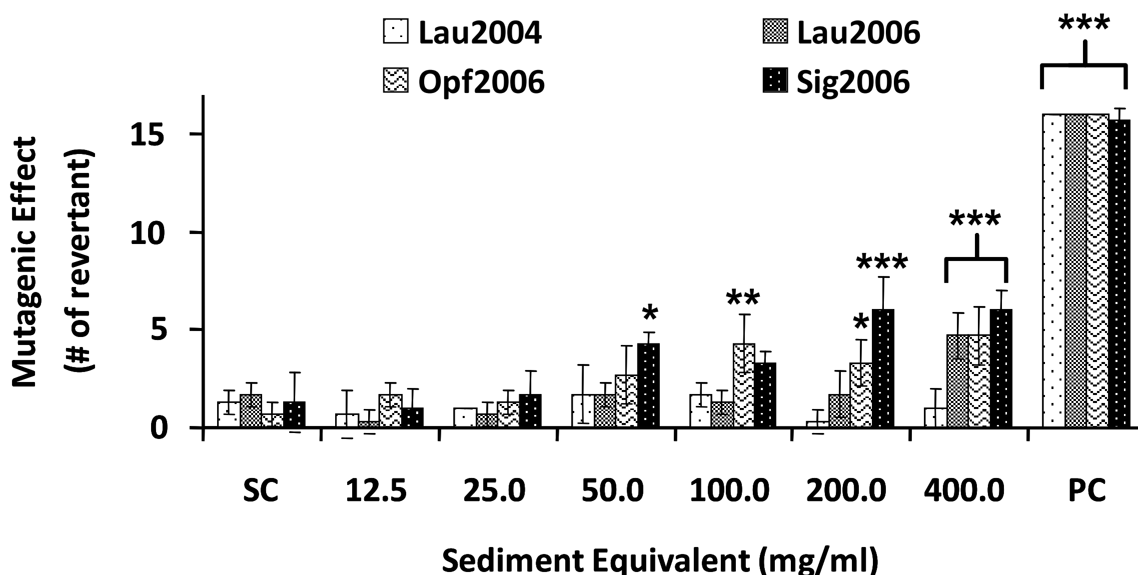


Figure 4.2. Mutagenic Activity of raw sediment extracts Sigmaringen (Sig2006), Opfingen (Opf2006), and Lauchert sampled in 2004 (Lau2004) and Lauchert sampled in 2006 (Lau2006). PC = Positive Control. Measured by the Ames Fluctuation Assay using TA98 bacteria with bioactivation enzymes (S9). Mutagenic activity is expressed as # of revertants. Values represent the mean ± sd. * = Significant differences reported relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol = $p < 0.05$; 2 symbols = $p<0.01$; three symbols = $p < 0.001$.

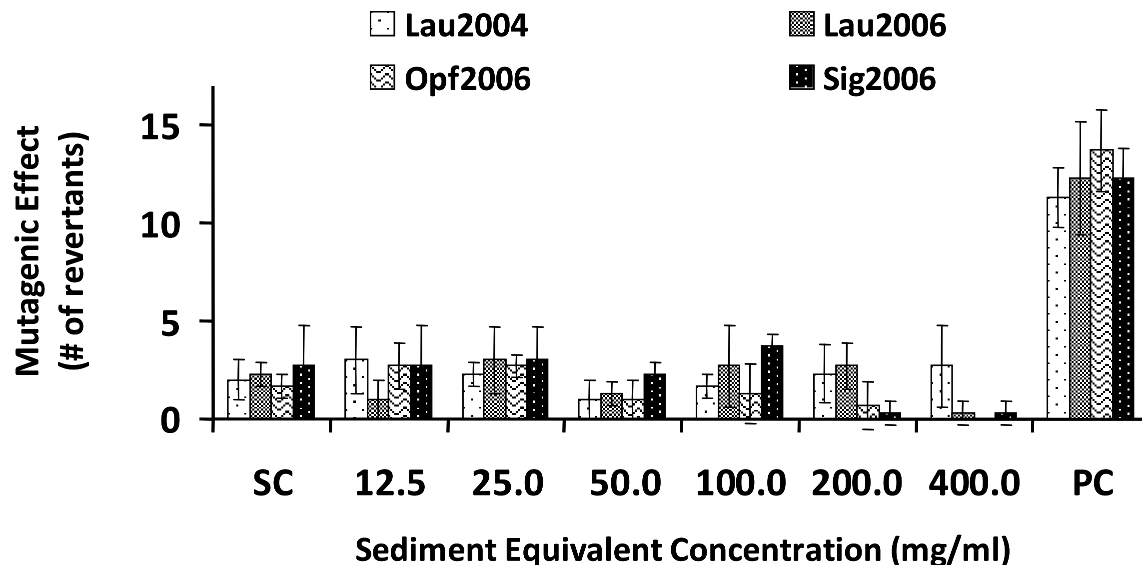


Figure 4.3. Mutagenic Activity of raw sediment extracts Sigmaringen (Sig2006), Opfingen (Opf2006), and Lauchert sampled in 2004 (Lau2004) and Lauchert sampled in 2006 (Lau2006). PC = Positive Control. Measured by the Ames Fluctuation Assay using TA100 bacteria with bioactivation enzymes (S9). Mutagenic activity is expressed as # of revertants. Values represent the mean ± sd. * = Significant differences reported relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol = $p < 0.05$; 2 symbols = $p<0.01$; three symbols = $p < 0.001$.

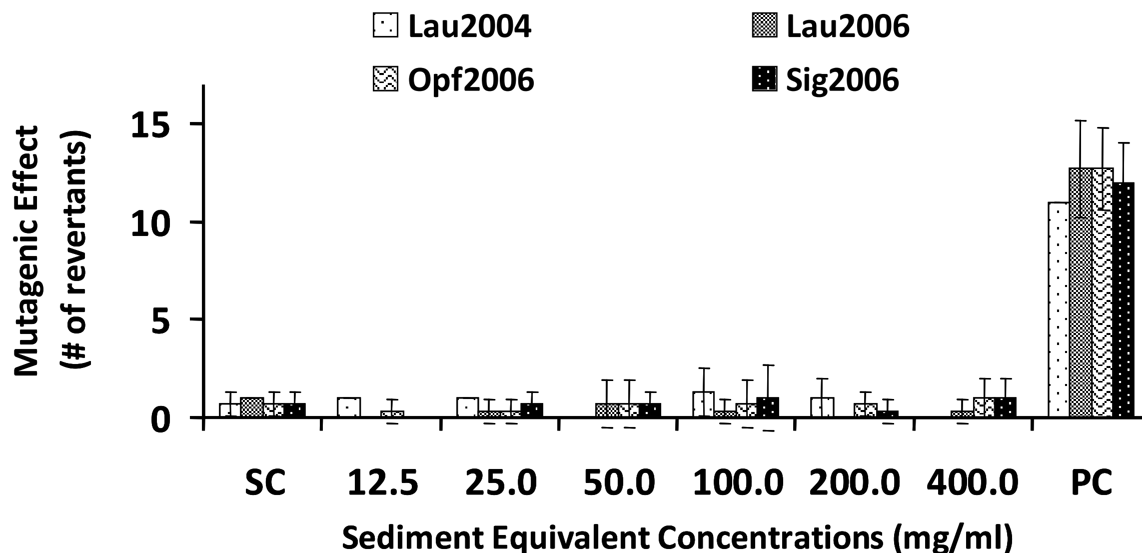


Figure 4.4. Mutagenic Activity of raw sediment extracts Sigmaringen (Sig2006), Opfingen (Opf2006), and Lauchert sampled in 2004 (Lau2004) and Lauchert sampled in 2006 (Lau2006). PC = Positive Control. Measured by the Ames Fluctuation Assay using TA100 bacteria without bioactivation enzymes (S9). Mutagenic activity is expressed as # of revertants. Values represent the mean ± sd. * = Significant differences reported relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol = $p < 0.05$; 2 symbols = $p<0.01$; three symbols = $p < 0.001$.

4.4.1.2 Fractions

Significant mutagenic effects were revealed in the TA98 bacteria strain with S9 when exposed to Sig2006 fractions 9,10,11,15 and 16 (Fig 4.5). Fractions 8, 10 and 11 of sediments collected at the same site caused significantly greater revertants when using the TA100 bacteria strain with S9. In addition, fractions 3 and 15 caused significantly greater revertants when using the TA98 bacteria strain without S9. No effects were shown in any of the Sig2006 fractions when using the TA100 bacteria strain without S9 (Fig 4.5).

Opf2006 fraction 10 caused significantly greater revertants in both TA98 and TA100 bacteria strains with S9. Moreover, fraction 13 induced significantly greater revertants when using the TA100 bacteria strain with S9. When using the TA98 bacteria strain without S9 fractions 15 and 16 significantly induced a mutagenic response. No effects were observed in any of the Opf2006 fractions when using the TA100 bacteria strain without S9 (Fig 4.5).

Lau2006 fractions 10, 11, 12 and 15 significantly induced a mutagenic response using the TA98 bacteria strain with S9. Using the TA100 bacteria strain, Lau2006 fractions 8, 9 and 10 significantly induced a mutagenic response. Furthermore, Lau2006 fractions 9, 14 and 15 significantly induced a mutagenic effect in the TA98 bacteria strain without S9. No effects were shown in any of the Lau2006 fractions when using the TA100 bacteria strain without S9 (Fig 4.5).

No effects occurred in any of the fractions of sediments collected at Lau2004 when using the TA100 bacteria strain without S9, and the TA98 bacteria strain with S9. Significant mutagenic effects were shown in the TA100 bacteria strain with S9 when

exposed to Lau2004 fractions 4, 8, 9, 10, 11. In addition, fractions 15 and 17 caused significantly greater revertants when using the TA98 bacteria strain without S9 (Fig 4.5).

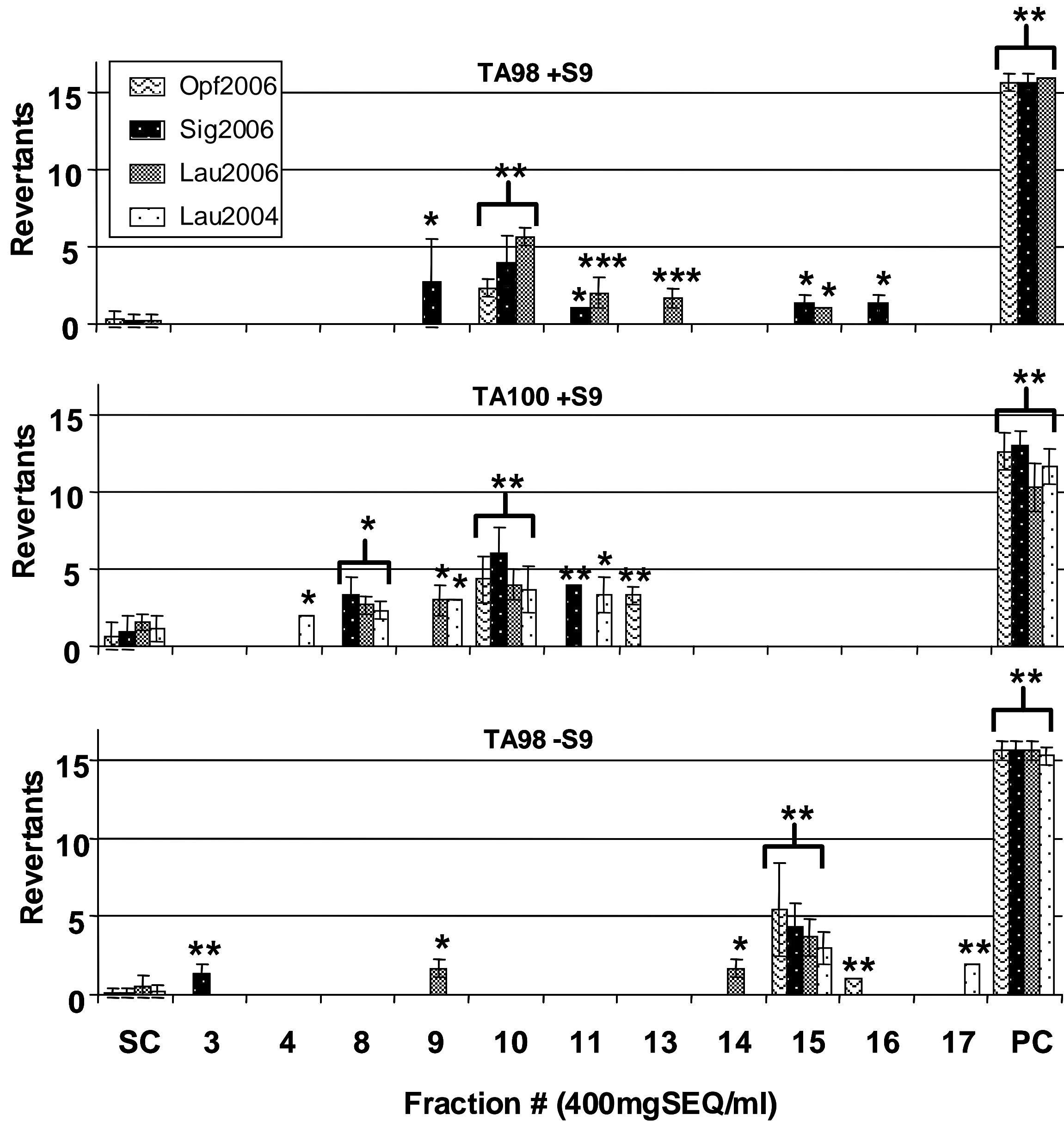


Figure 4.5. Mutagenic Activity of 18 fractions of Sigmaringen (Sig), Opfingen (Opf), and Lauchert sampled in 2004 (Lau2004) and Lauchert sampled in 2006 (Lau2006). Measured by the Ames Fluctuation Assay using TA100 and TA98 bacteria with and without bioactivation enzymes (S9). TA100 without S9 is not shown because no mutagenic effects were observed in any of the fractions. PC = Positive Control. Mutagenic activity is expressed as # of revertants. Values represent the mean ± sd. * = Significant differences reported relative to the solvent control. Multiple symbols indicate different significant levels: 1 symbol = $p < 0.05$; 2 symbols = $p<0.01$; three symbols = $p < 0.001$.

4.4.2 *Danio rerio* Assay

Soxhlet extracted sediment from Opf2006, Sig2006 and Lau2006 caused mortality in *D. rerio* embryos in a dose dependent manner. Opf2006 and Sig2006 were the most toxic with an LC50 of 18.9 mg SEQ/ml and 21.5 mg SEQ/ml, respectively. Sediments collected at Lau2006 were less toxic with a LC50 of 58.6 mg SEQ/ml (Table 4.2).

Similar to the soxhlet extracted samples, AMAC extracts of samples collected at Opf2006 and Sig2006 were the most toxic to *D. rerio* embryos with LC50s of 75.6 mg SEQ/ml and 100 mg SEQ/ml, respectively. Lau2006 and Lau2004 did not produce any toxicity at any concentration measured. Therefore, a LC50 could not be calculated for these samples (Table 4.2).

Only Sig and Opf fractions were run in the *D. rerio* assay because no mortality was observed in the AMAC extracts for Lau2004 and Lau2006 (Fig 4.6, 4.7). A general trend of decreasing toxicity with increasing sample manipulation was noticed in the *Danio rerio* assay. For example, the soxhlet extracted sediments produced greater toxicity in the *D. rerio* assay (Fig 4.6) compared to the AMAC extracts (Fig 4.7). None of the fractions of sediments from any of the sites caused a significant increase in mortality compared to the solvent control (Fig 4.8).

Table 4.2. LC50 values for soxhlet and accelerated membrane-assisted clean-up (AMAC) raw sediment extracts in the *Danio rerio* Assay. NA= Not Available. CBC = Cannot Be Determined because no mortality was observed in any of the concentrations.

Site ID	**LC50** Soxhlet Extracted Raw Sediment	**LC50** AMAC Raw Sediment Extracts
Opf2006	18.9 mgSEQ/ml	75.6 mgSEQ/ml
Sig2006	21.5 mgSEQ/ml	100 mgSEQ/ml
Lau2006	58.6 mgSEQ/ml	CBD (>100 mgSEQ/ml)
Lau2004	NA	CBD (>100 mgSEQ/ml)

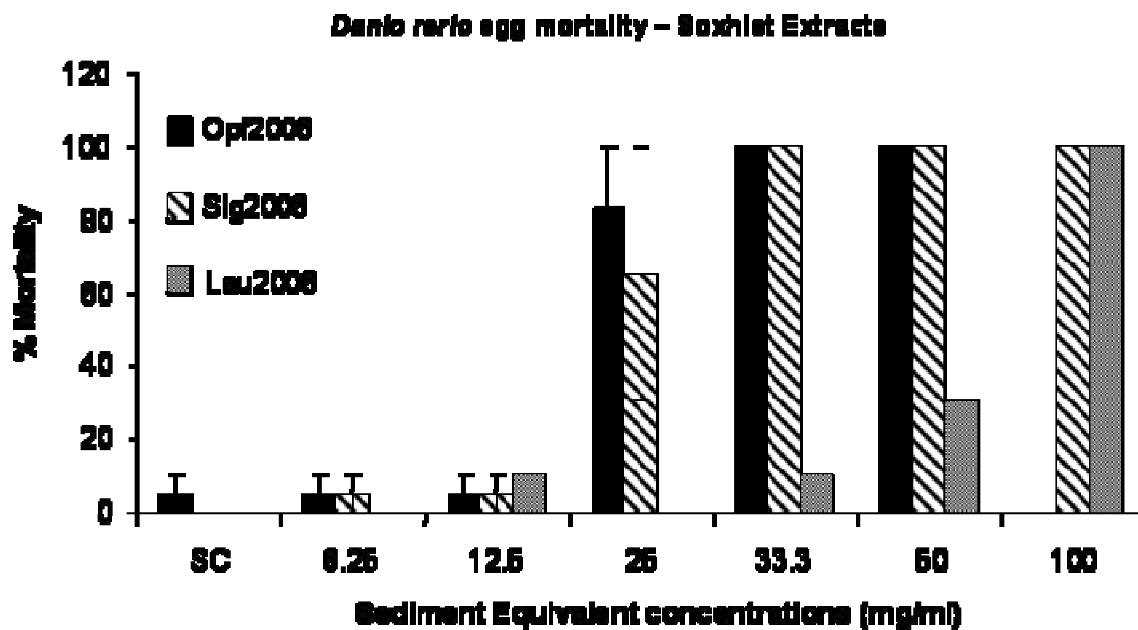


Figure 4.6. *Danio rerio* embryo mortality of soxhlet extracted sediment. Sampled at Sigmaringen (Sig2006), Opfingen (Opf2006), and Lauchert sampled in 2004 (Lau2004) and Lauchert sampled in 2006 (Lau2006). SC = Solvent Control.

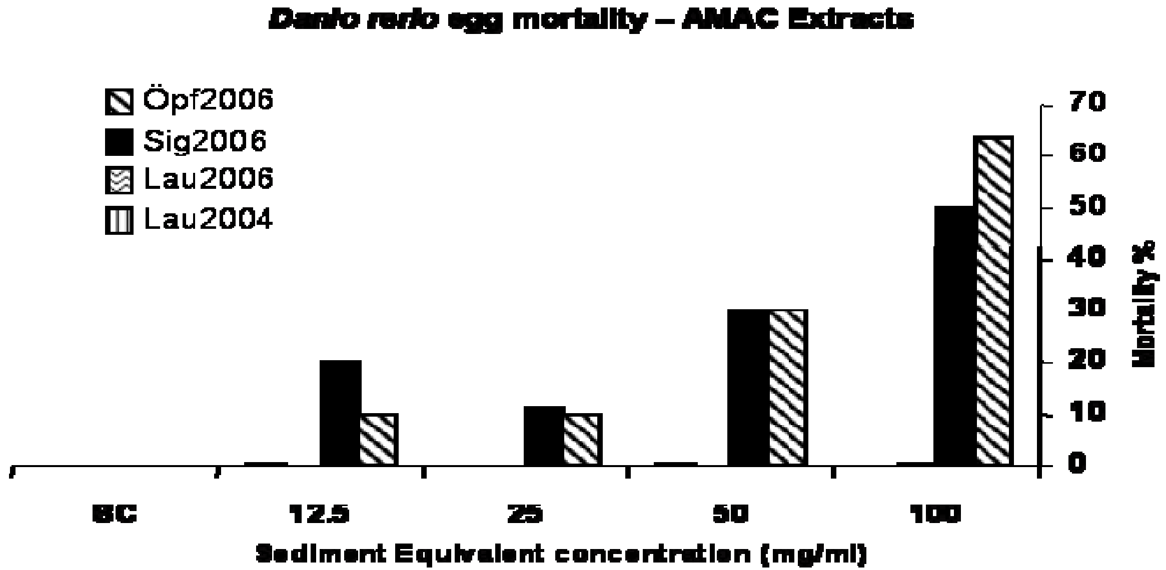
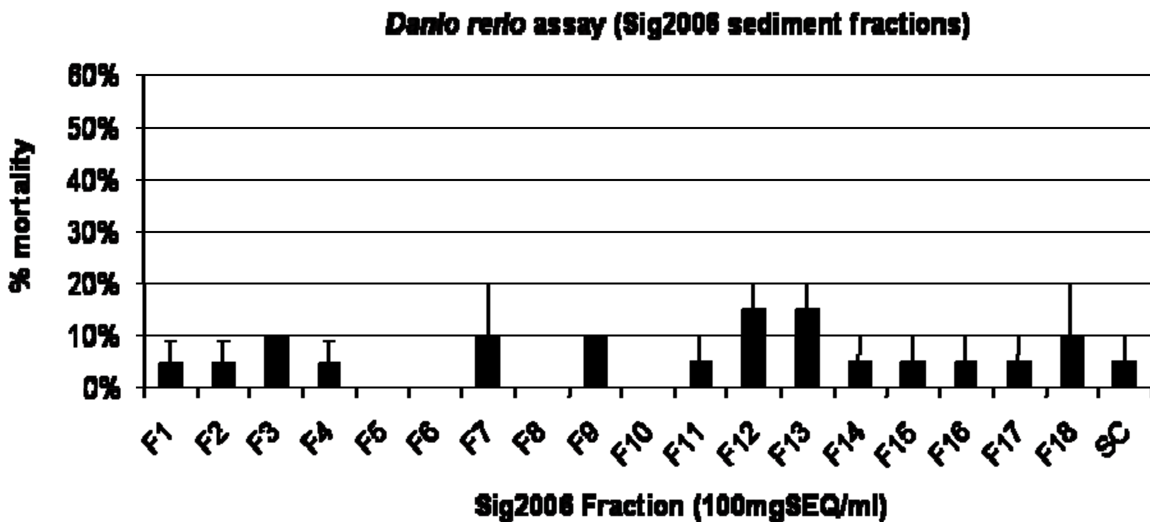


Figure 4.7. *Danio rerio* embryo mortality of accelerated membrane-assisted cleaned-up (AMAC) sediment. Sampled at Sigmaringen (Sig2006), Opfingen (Opf2006), and Lauchert sampled in 2004 (Lau2004) and Lauchert sampled in 2006 (Lau2006). SC = Solvent Control.



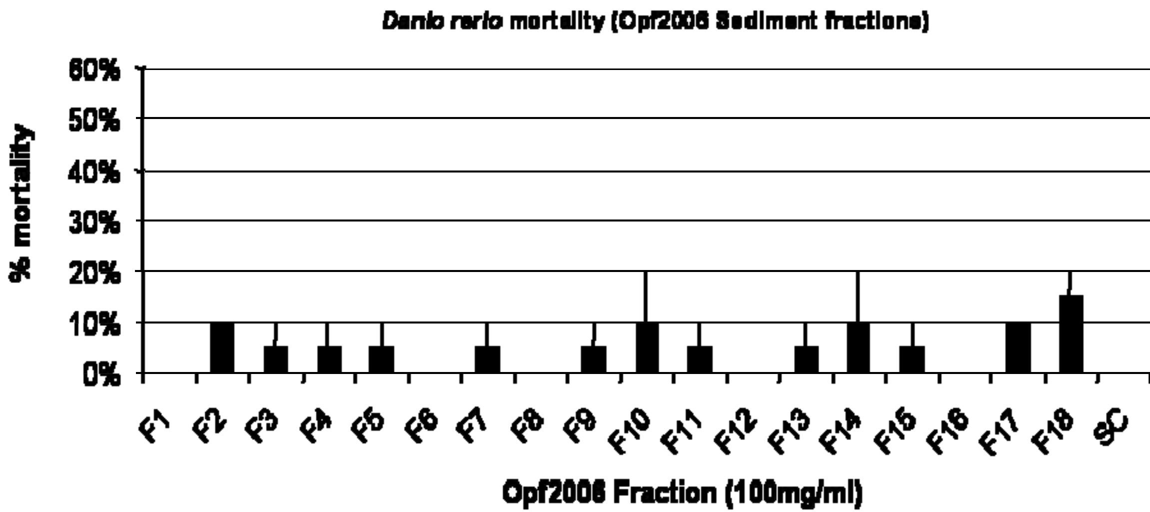


Figure 4.8. Danio rerio embryo mortality of 18 fractions of sediment sampled at Sigmaringen (Sig2006) and Opfingen (Opf2006). SC = Solvent Control.

4.5 Discussion

Sediments from the Upper Danube River in Germany have been well studied and characterized during the past years. These studies have revealed a variety of significant toxic effects as determined by different types of biological tests including mutagenic and teratogenic responses, and effects on steroidogenesis (Keiter et al., 2006; Keiter et al.,

2008; Otte et al., 2008; Grund et al., submitted 2009). Three locations that have been shown to be characterized by sediments that induced significant toxic effects were Sigmaringen, Opfingen and Lauchert. Sediments collected at these sites were e.g. shown to significantly induce EROD activity in RTL-W1 cells when compared to other sediments (Keiter et al., 2008; Grund et al., in prep). In this study, raw extracts of sediments from the same location showed significant toxicity with the exception of Lau2006 and Lau2004 in the *D. rerio* assay and in the Ames fluctuation assay. The greatest toxicity was observed at the Opf2006 site in both assays. Sig2006 was the second most toxic sample and Lau2006 was the third most toxic sediment extract in both assays. Additionally, Lau2004 was the least toxic of the sediment extracts in both assays.

The pattern observed in the current study is similar to other studies that have shown that Sigmaringen and Opfingen sediments were generally more toxic than those collected at Lauchert. For example, Keiter et al. (2008) measured EROD activity in 10 Upper Danube River sediment extracts and found the Opfingen sediment extract induced EROD activity by the highest amount of all the sediment extracts studied and Lauchert sediments were among the least toxic samples. This same pattern was also observed by Seitz et al. (2008), who measured genotoxicity using the comet assay.

The studies by Seitz et al. (2008) and Keiter et al. (2008) used sediment samples that were collected in 2004. In 2006, new samples were collected from the same sites and reanalyzed in various EROD assays (Otte et al., 2008; Grund et al., in prep). When bio-TEQs derived from RTL cells were compared between sites and years it was found that the Lauchert and Sigmaringen sediments collected in 2004 were significantly less toxic than the samples collected in 2006. In the current study, we observed a similar

trend in the Ames fluctuation assay when we compared the Lau2004 extracts with the Lau2006 extracts. It is unclear why an increase in toxicity was observed after these two years and more research is needed to explain this. Furthermore, in the *D. rerio* assay both the Lau2004 and Lau2006 AMAC extracts did not cause increased mortality at any concentration and were considerably less toxic than the Opf2006 and Sig2006 AMAC sediments.

In a comparison between the Soxhlet extracted sediments and the AMAC extracts when run in the *D. rerio* assay, the Soxhlet extracted sediments were noticeably more toxic than the AMAC cleaned up samples. The AMAC procedure removes large lipids and lipid-like matrix components that can interfere with chemical analysis of environmental samples (Streck et al., 2008). The AMAC procedure was shown to have good recoveries of 10 spiked compounds, so the loss of these chemicals is an unlikely cause for the reduction in toxicity (Streck et al., 2008). However, many other chemicals were not subjected to recovery analysis in this extraction. Thus, it cannot be excluded that the reduced toxicity that was measured in the AMAC extracts might be because of the removal some of the biological active compounds.

Further separation of Opf2006 and Sig2006 sediment extracts into 18 fractions completely eliminated toxicity of all samples in the *D. rerio* assay. This indicates additive or synergistic interactions between chemicals present in different fractions. Additive and synergistic effects have been reported for a number of chemicals. For example, Schmitz et al. (1996) found a synergistic effect with PCB's when exposed to H4IIE cells in a mixture compared to each PCB separate.

In contrast to the *D. rerio* assay, AMAC sediment extracts were less toxic than the fractions when analyzed in the Ames fluctuation assay. For example, no significant mutagenic response was observed in the AMAC extracts at any site in the Ames fluctuation assay using TA100 bacteria and S9, but a significant response was observed for some of the fractions. Potentiation or antagonistic interactions between the chemicals in the different fractions could result in the decreased mutagenicity of the AMAC extracts. For example, a previous study found that hexachlorobiphenyl could decrease the toxicity of TCDD (Brown et al., 1994) and a similar type of antagonism could have occurred in the AMAC extract. Furthermore, this type of toxicity was only noted in the TA100 bacteria with bioactivation enzyme S9 and not in the TA100 bacteria without S9, where no effects were observed for the AMAC extracts and the fractions at any of the sites.

Fraction 10 for all of the sites was most affected in both TA98 and TA100 bacteria strains when bioactivation enzymes (S9) were added, but no mutagenic activity was observed in fraction 10 when S9 was not added. Thus the lack of toxicity observed in the Ames fluctuation assay without addition of bioactivation enzyme suggests that the chemical(s) of concern has to be bioactivated to cause a significant mutagenic effect. In addition, fraction 10 is where the greatest concentration of PAHs can be found out of the 18 fractions (Grund et al., in prep) and many PAHs need to be bioactivated to produce toxicity. It is very likely that the PAHs in fraction 10 were driving the increased mutagenic response. In contrast, Grund et al. (in prep), found that total PAHs caused between 3 and 14 % of EROD induction measured in RTL-W1 cells.

A spike in mutagenic activity in fractions 8, 9 and 11 was observed in the TA100 bacteria strain with S9 among all sites compared to the same fractions run in the TA98 bacteria with S9. Therefore, it is possible that the bioactivated chemicals in fractions 8, 9 and 11 generate base pair substitutions more readily than frameshift mutations, whereas fraction 10 generated both types of mutations.

In a few cases, mutagenic effects were observed without bioactivation enyzymes. Fraction 15 produced significant mutagenic effects at all sites in the TA98 bacteria strain without S9. Fraction 15 contained more polar compounds like hydroxyl quinones and hydroxyl-PAHs, which would not need to be bioactivated to be toxic. However, the other bacterial strain used in this study (TA100 without S9) did not produce a mutagenic response in any fraction or AMAC extract.

In general, fractions 8, 10 and 15 produced a consistent mutagenic response among sites and would be important fractions to study further. These fractions have been shown to be of importance in other studies using different assays and/or different sediments. Grund et al. (in prep) found that fractions 9-11 and 13-15 from the same sediments produced the greatest EROD induction in RTL-W1 cells. Other research examined endocrine disrupting properties within the same fractions and mutagenic and AhR-mediated activity from fractions from the River Elbe and found the strongest effects in fractions 9-11 and in fraction 15 (Higley et al., in prep; Lübeke-von Varel et al., in prep;). This is in accordance with the results of this study except most of the mutagenic activity was in fractions 8, 10 and 15.

While the Ames fluctuation assay is a very useful tool to assess mutagenic effects, it has limitations that must be taken into consideration. For example, very cytotoxic

samples can produce false negatives because the bacteria are killed and cannot back mutate and grow. Furthermore, some chemicals target only certain types of frame-shift mutations and might be missed because the assay is specific to only certain types of mutations. A previous study analyzed caffeine for its mutagenic effects and found that caffeine targeted runs of greater than five GC base pairs in *E. coli*. The same paper concluded that caffeine was missed as a possible mutagen in the Ames test because the Ames test only measured frame-shift mutations with runs of five GC base pairs (Pons and Muller, 1990). One other important point to note is that the Ames fluctuation assay does not distinguish between a chemical that causes a replication error and a chemical that inhibits repair if a mutation does happen. While this may not be particularly important when screening sediments, it becomes more significant when trying to determine the mechanism of action of a mutagen.

In conclusion, the results reported here corresponded well with results of other studies that found similar toxicity in the same fractions but using different assays or sediments (Grund et al., in prep; Higley et al., in prep; Lübeke-von Varel et al., in prep). Future studies should investigate the toxicity of the active fractions observed in this study using extensive chemical analysis. Furthermore, many chemicals found in these fractions do not have sufficient toxicity data and should be investigated using toxicity testing. These results cannot rule out sediments as a source for pollution and a cause for the decline in several fish populations in the Upper Danube River.

CHAPTER 5

5.0 OVERALL DISSCUSSION

Overall, the four objectives set out in Chapter 1 of this thesis were accomplished. First, a proof of concept of the H295R Assay was performed and it was determined to be a sufficient assay to test for endocrine disrupting chemicals. Secondly, utilization of the H295R assay, the Ames fluctuation assay, *D. rerio* embryo assay and a new fractionation technique to analyze Upper Danube River sediments were performed.

The study described in Chapter 2 characterized seven endocrine disrupting chemicals with regard to their potential to interfere with estradiol, testosterone and combined, direct and indirect aromatase activity in H295R cells. It was found that the H295R cell line is responsive to endocrine disrupting chemicals and would be a good candidate for sediment testing. Additionally, for most of the chemicals analyzed, E2, T, and direct and combined aromatase activity were good predictors of the mode of action of the chemical, with indirect aromatase activity being a less precise endpoint because feedback loops made it difficult to predict the chemicals true effects.

Results provided in Chapter 3 demonstrated that all raw extracts tested in the H295R assay caused an increase in estradiol production up to 4-fold from controls. Testosterone production increased slightly from controls in only two of the raw extract samples. Of the 18 fractions, fractions 7, 10 and 15 increased estradiol in at least three of the samples studied. Furthermore, fraction 7 significantly decreased testosterone production compared to controls in three of the four sediment samples.

Additionally, in the *Danio rerio* assay, two raw sediment extracts killed 100% of *Danio rerio* embryos at a concentration of 33.3 mg sediment equivalents (SEQ)/ml, but

none of the 18 fractions of these samples produced any measured toxicity at a concentration of 100 mg SEQ/ml. In the Ames fluctuation assay, significant mutagenic activity was measured in raw sediment extracts and in the fractions. Fraction 10 produced a significant mutagenic response in all sediment samples measured only in S9 bio-activated samples. Furthermore, fraction 15 produced a significant mutagenic response in all sediment samples measured only in non bio-activated samples.

Taken as a whole, these results show the value of using effect-directed analysis, which utilizes multiple bioassays and fractionation techniques to assess toxicity of sediments that covers a variety of different biological endpoints that are caused by unknown chemicals. Furthermore, the utilization of fractionation to pin-point groups of chemicals that caused toxicity in sediments is a valuable tool but other procedures are also needed because there is still a great deal of uncertainty regarding the specific chemicals that were responsible for the observed toxicity of the sediments in the H295R, *D. rerio* and Ames fluctuation Assays. For example, further assessment of the exposure to specific chemicals can be achieved by removing certain contaminants or factors to rule them out as the possible cause of the original toxicity. In addition, future work should expand on a more thorough chemical analysis of the here identified active fractions for a wider variety of potential toxic chemicals. Also, many chemicals found in these fractions do not have sufficient toxicity data and should be investigated further using toxicity testing.

In conclusion, the here presented study aided in the identification of biological active fractions, which allows narrowing the target analytes to groups of chemicals with certain physico-chemical properties. It furthermore indicated that there are a number of

unknown chemicals in the environment that have the potential to interfere with biological relevant processes and that warrant consideration in current risk-assessment strategies.

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