# Establishment of cause-effect relationships between observed biological effects and environmental stressors

## Dissertation

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**Matthias Grote**

aus Hamburg

**Universität Hamburg**

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1. Gutachter: Prof. Dr. H. Hühnerfuß
2. Gutachter: Prof. Dr. G. Schüürmann

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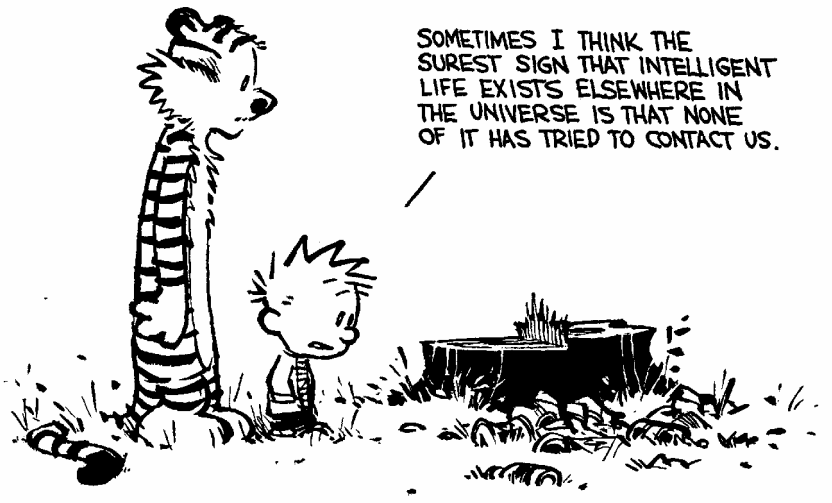
SOMETIMES I THINK THE SUREST SIGN THAT INTELLIGENT LIFE EXISTS ELSEWHERE IN THE UNIVERSE IS THAT NONE OF IT HAS TRIED TO CONTACT US.

# Contents

## Abbreviations used

| | |
|---|---|
| $^1O_2$ | singlet state oxygen |
| $^3O_2$ | triplet state oxygen |
| $ALT_{50}$ | adjusted medium lethal times |
| BCF | bioconcentration factor |
| BRO | Brofjorden (sampling site on the Swedish West coast) |
| BSAF | biota-sediment accumulation factor |
| BTF | Bitterfeld |
| CA | concentration addition |
| CAS RN | Chemical Abstract Service Registry Number |
| *D. magna* | *Daphnia magna* |
| DCM | dichloromethane |
| DMSO | dimethylsulfoxyde |
| EC | effect concentration |
| $EC_{50}$ | median effective concentration |
| $ED_{50}$ | median effective dose, (except in citation of Brack *et al* (1999): medium effective dilution) |
| EDA | effect-directed analysis |
| GC/MS | gas chromatography / mass spectrometry |
| HOMO | highest occupied molecular orbital |
| HPLC | high performance liquid chromatography |
| IA | independent action |
| ICQ | index of confirmation quality |
| IPQ | index of prediction quality |
| $K_{OW}$ | partitioning coefficient octanol / water |
| $LC_{50}$ | median lethal concentration |
| $LT_{50}$ | median lethal time |
| LUMO | lowest unoccupied molecular orbital |
| n.a. | not analysed |
| n.q. | not quantified |
| OSPAR | Oslo-Paris-Commission |
| PAH | polycyclic aromatic hydrocarbon |

| | |
|---|---|
| PAR | photosynthesis active radiation |
| PMF | photomodification factor |
| POPs | persistent organic pollutants |
| PSF | photosensitisation factor |
| PWF | photoactivated toxicity weighting function |
| QSAR | quantitative structure activity relationship |
| RPA | relative photodynamic activity |
| RPE | relative phototoxic efficacy |
| RPF | relative potency factor |
| SEq | sediment equivalents |
| SIM | single / selected ion mode |
| SM | synthetic mixture |
| $T_e$ | excess toxicity |
| TIC | total ion current |
| TIE | toxicity identification evaluation |
| TU | toxic unit |
| TUS | toxic unit sum |
| UFZ | Umweltforschungszentrum Leipzig-Halle |
| US-EPA | United States – Environmental Protection Agency |
| UV | ultra violet (radiation) |
| *V. fischeri* | *Vibrio fischeri* |

# Chapter I

## General Introduction

The establishment of cause-effect relationships between observed biological effects and responsible chemicals and environmental stressors is a key challenge in ecotoxicology that comprises the identification of toxicants present in hazardous environmental samples and the quantification of effect contributions of these toxicants to the observed adverse effects. In the environment toxicants commonly occur in mixtures rather than as single compounds. Therefore, a thorough understanding of combined effects of toxicants mixtures is needed for a quantitative toxicity assessment.

### *Effect-directed analysis*

#### General Approach

The hazardous potential of environmental samples can be assessed by different approaches. Conventional chemical analysis focuses either on target-screening, i.e., the quantification of pre-selected priority pollutants (e.g., persistent organic pollutants (POPs) or compounds from the monitoring list of the Oslo-Paris-Commission (OSPAR)), or on the identification of a broad spectrum of substances via non-target screening. However, results from chemical analyses cannot be directly used for the judgement on the actual hazard exerted by the sample. Information on the biological activity of analysed compounds is needed, which often is not available. Furthermore,

the risk of overlooking additional unknown hazardous compounds, not covered by the chosen analytical method, prevails. The use of biotests is an established alternative approach for hazard assessment of complex environmental samples. This approach provides an integrative parameter for the presence of stressors affecting the applied test organism. Knowledge of the identity of biologically active compounds is not necessary, and possible combined effects induced by the mixture are reflected by the results. However, biotesting alone does not provide information on the identity of compounds causing the observed effects. Thus, exclusive application of one of these two approaches is inappropriate for establishing causal links between observed biological effects and responsible chemicals.

Effect-directed analysis (EDA) may overcome the limitation of applying either chemical analyses or biological testing alone. This approach combines chemical analyses with preparative fractionation techniques and biological efficiency testing, in order to identify relevant compounds in biologically active environmental samples. The underlying general idea is very simple: If the cause of an ecotoxicological effect induced by a complex environmental sample is unknown, the EDA procedure can be used to separate whole sample toxicity into chemically defined fractions involving one or several fractionation steps which in turn characterise the properties of the components (Figure 1.1). Chemical analysis can then be performed in the biologically active fractions which are less complex and commonly more amenable to chemical analysis than the original sample. This facilitates the identification of substances responsible for the observed effects in the toxic fractions. In a confirmation step, the suspected cause-effect relationship between the identified compounds and the biological effects has to be proven.

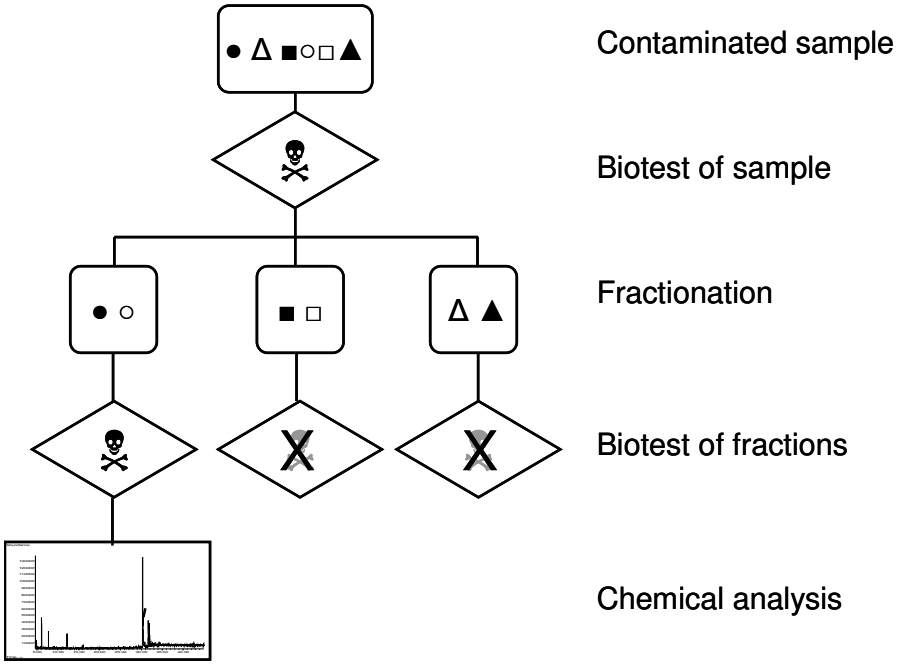


*Figure 1.1: Principle scheme for effect-directed analysis. Different symbols (●○Δ▲□■) stand for different substances in the sample. The symbol '☠' denotes an observed toxic effect in the bioassay.*

**State of the Art of effect-directed analysis**

First studies using the bioassay- or effect-directed analysis approach in ecotoxicology date back to the early 1980s and are reviewed by Schuetzle and Lewtas (1986). However, it took until the late 1980s that the United States Environmental Protection Agency (US-EPA) laid ground for a wider acceptance of the approach by the publication of a detailed protocol on the identification of causes of toxicity in aqueous samples (Mount and Anderson-Carnahan 1988; Mount and Anderson-Carnahan 1989; Mount 1989). This carefully designed and frequently used framework named toxicity evaluation identification (TIE) procedure was originally developed for effluents. It strives primarily to identify possible toxicity removal techniques which are applicable to wastewater treatment. The principles of this specific methodology are outlined below as they laid the basis for subsequent compound-focused work.

The TIE-procedure consists of three basic phases, Characterisation (Mount and Anderson-Carnahan 1988), Identification (Mount and Anderson-

Carnahan 1989) and Confirmation (Mount 1989). Phase I, the Characterisation procedure, is conducted in order to characterise the nature of the observed toxicity in the environmental sample, e.g., toxicity caused by metal contamination as opposed to organic compounds. In Phase II the identification of suspect toxicants is the goal. In Phase III, the confirmation procedure, it is examined, whether the identified substances can be held responsible for the observed toxicity.

Since then, the spectrum of investigated environmental samples as well as applied chemical and biological methods have continuously been extended. Other types of samples, e.g., extracts of sediment impacted by industrial discharges (Brack *et al.* 1999) or by motorway runoff (Maltby *et al.* 1995), as well as sediment pore waters (Kosian *et al.* 1998) have been subject to toxicant identification by EDA. This development opened new perspectives, as EDA was no longer restricted to toxic aqueous samples, but other types of samples containing toxicants could be investigated. Compounds present in the sample can be rendered bioavailable through extraction followed by condensation to biologically active concentrations. The investigation of these samples often has a different focus than TIE studies on aqueous samples, which are commonly aiming at possible improvements of remediation techniques for waste water treatment. Studies on organic extracts, however, aim to assess the toxic potential of the sample. The respective compounds may not be bioavailable when sorbed to the sediment, however, may impose problems when remobilised or ingested by organisms. So far, most studies using EDA on organic sediment extracts focused on highly contaminated samples. However, many contaminants are ubiquitously distributed. Thus, it is interesting to investigate whether toxicants can be identified in extracts from samples, which cannot be considered as highly contaminated, by using the EDA approach. Accordingly, the aim of chapter 2 of the present thesis was to answer the following question:

- Can the methodology of EDA, currently used for the assessment of hazardous samples, be applied to identify potentially hazardous

compounds in samples which cannot be considered highly contaminated?

For this purpose effect-directed analysis was conducted in order to identify potentially relevant toxicants from a marine sediment. A biotest battery, comprising acute and chronic endpoints in the unicellular green algae *Scendesmus vacuolatus* and the marine bacteria *Vibrio fischeri*, was applied to extracts and fractionated samples, in order to discriminate between toxic and non-toxic fractions.

**Confirmation of toxicants**

For the establishment of cause-effect relationships the identification of biologically active compounds in the environmental sample is indispensable, however, it is not sufficient. A subsequent confirmation step has to be conducted, in order to evaluate how much of the observed toxicity in the environmental sample can be attributed to the identified toxicants. If just one toxicant is suspected, this is not complicated. However, when several toxicants are suspected to contribute to the sample's effect, the confirmation step is not a straightforward task.

The presence of several toxicants in hazardous environmental samples appears to be common rather than exceptional. Ho *et al*. (2002) concluded in a review on TIE studies, conducted on marine sediments and dredged materials, that within a single sample "there are usually multiple causes of toxicity detected". This goes in line with studies conducted on other samples including motorway runoff (Boxall and Maltby 1997), effluents from a wood drying plant (Svenson *et al.* 2000) or fresh water sediment from an industrialised area (Brack *et al.* 1999) for which observed effects induced by the sample were attributed to mixtures of toxicants. The central question addressed in chapter 3 is:

- How can cause-effect relationships be confirmed when mixtures of hazardous compounds are identified?

For this purpose results from two EDA studies (chapter 2 and Brack *et al*. (1999)) are taken as a basis to develop a methodology for mixture confirmation. The proposed method comprises comparison of the sample's toxicity to calculated combined effects of identified toxicants according to different predictive models and to tested synthetic mixtures. Results of the different approaches are used to derive an effect level dependent quantitative measure for confirmation.

## *Confounding factors in environmental hazard assessment*

When aiming to establish cause-effect relationships one has to be aware of possible confounding factors which may alter the assessment. Environmental factors have been demonstrated to be able to alter the toxicity of compounds as well as of environmental samples (Sprague 1995). Explicitly, the influence of varying the sample's pH is used to characterise toxicants in the TIE protocol. For example, ammonia is transferred to the less toxic ammonium ion at acidic conditions. Sprague (1995) summarises "factors that modify toxicity" such as variation in pH, salinity, hardness and content of suspended or dissolved organic matter. Limited evidence can be found for the influence of temperature and dissolved oxygen concentrations (Sprague 1995). The milieu factors that may affect the toxicity of a distinct environmental sample depend on the properties of the respective compounds.

In the samples investigated in this thesis polycyclic aromatic hydrocarbons (PAHs) were identified and confirmed as major contributors to the observed biological effects induced by the samples. The toxicity of many PAH compounds has been reported to be dependent on the light conditions. This phenomenon known as light enhanced toxicity or phototoxicity has been observed for various PAH compounds in bacteria (El-Alawi *et al.* 2002), animals (Newsted and Giesy 1987) and plants (Huang *et al.* 1997). It is attributed to the ability of some PAH compounds to form excited states through the absorption of light in the visible or ultra violet region. It alters the toxicity of the parent compound by either metabolisation or formation of

reactive oxygen species. A review on influence of light on toxic effects of PAHs is given by Arfsten *et al*. (1996).

In chapters 2 and 3, the assessment of algal toxicity of the environmental samples studied by EDA was based on test protocols designed for optimal conditions for algal reproduction. These test conditions comprised illumination which provided photosynthesis active radiation (PAR), produced by fluorescent tubes. These tubes emit a discontinuous light spectrum that qualitatively differs from natural sun light, which is characterised by a continuous light spectrum including ultra-violet (UV) radiation. It is uncertain whether the use of realistic light conditions would affect the toxicity of the investigated samples as PAHs were found to be only partially responsible for the observed toxicity. Furthermore, it is unclear whether a light influence on toxicity of the samples quantitatively alters the judgement on the toxicant confirmation. Accordingly, the question addressed in chapter 4 is:

- How can environmental factors confound the assessment of cause-effect relationships concerning the identification and confirmation of toxicants?

The influence of environmental factors on the confirmation step in EDA and on quantification of contributions of identified compounds is exemplarily investigated by the inclusion of natural light conditions in the toxicity assessment. Samples and identified toxicants, used for the development of the quantitative measure for confirmation in chapter 3, are tested for their algal toxicity under simulated sun light. The influence of the light conditions on the combined effects of identified toxicants is assessed. Furthermore, the question is addressed whether light dependence of toxicity of environmental samples is a common phenomenon, or whether it was a rather exceptional case, only observable in distinct samples with a special contamination pattern.

## *Modelling of photoenhanced toxicity*

Photoenhanced toxicity of PAHs is assumed to be due to their chemical properties, which allow absorption of light in the ultraviolet (UV) regions (280 – 400 nm) and partially in the visible region (400 – 800 nm). Absorption of photons results in formation of excited PAH species which can either induce formation of reactive oxygen species or PAH photometabolites. Different models to predict phototoxic behaviour of PAH have been described, aiming to identify compounds exerting phototoxic behaviour in *Daphnia magna* (Mekenyan *et al.* 1994), to rank different PAH compounds according to their phototoxic activity (Morgan and Warshawsky 1977; Oris and Giesy 1987; Newsted and Giesy 1987), or to predict phototoxicity for specific compounds under distinct light conditions quantitatively (Ankley *et al.* 1995; Huang *et al.* 1997; Krylov *et al.* 1997).

The question addressed in chapter 5 is:

- Can the photoenhanced algal toxicity of the identified PAH compounds be modelled in order to signal the relevance of light as confounding factor?

For this purpose, the algal toxicity of the identified PAH compounds is measured under three different light conditions. Previously described models for photoenhanced PAH toxicity are reviewed and their applicability to the light dependence of algal toxicity caused by compounds identified herein is assessed.

# Chapter 2

## Identification of toxicants from a marine sediment using effect-directed analysis*

### *2.1 Introduction*

As stated in chapter 1, so far, most EDA studies were conducted to identify causes of toxicity in different environmental samples which either exerted acute toxicity or were suspected of having high levels of contamination. The former included effluents from chemical production plants (Jin *et al.* 1999; Yang *et al.* 1999), wood dying plants (Svenson *et al.* 2000) and waste water treatment sites (Rowland *et al.* 2000), the latter included sediment pore waters (Kosian *et al.* 1998; Ho *et al.* 1997) motorway runoff (Boxall and Maltby 1997) and sediments from a highly industrialised area (Brack *et al.* 1999). Depending on the nature of the analysed sample, different toxicants were identified comprising inorganic compounds like zinc (Bailey *et al.* 1999), manganese (Boucher and Watzin 1999) or ammonia (Gupta and Karuppiah 1996), and natural and anthropogenic organic compounds like unsaturated fatty acids and monoterpenes (Svenson *et al.* 2000), tannins and lignins (Bailey *et al.* 1999), PAHs (Boxall and Maltby 1997) (Brack *et al.* 1999), substituted and hetero-PAHs (Kosian *et al.* 1998), chlorobenzene and

* Parts of this chapter are in press as publication in *Environmental Toxicology*

nitrobenzene (Yang *et al.* 1999), benzopyrone and phenol (Jin *et al.* 1999) and pesticides (Werner *et al.* 2000; Bailey *et al.* 2000).

In the near future European pollutant monitoring programs will be modified towards a broader range of compounds in order to fulfil the requirements of the water framework directive of the European Union. Watershed specific monitoring lists of compounds of major importance in the respective watershed will have to be created. Thus far, it is not clear how these lists will be composed or what rational will be used to determine whether a certain pollutant is of specific importance in the aquatic environment of concern. As EDA is one approach for the identification of toxic compounds, it has the potential of being used to complement to the creation of these lists. However, it is not clear, whether the EDA methodology is capable of identifying toxicants from less contaminated samples. For example, in samples which are not directly impacted by human activities, naturally occurring compounds as well as ubiquitously distributed environmental contaminants can be expected to be present. In this connection, sediments are a subject of particular interest, as they act as a sink for - especially lipophilic - contaminants and can thus be considered as an integrating archive of preceding inputs. For the assessment of the toxic potential of these sediment samples, accumulated compounds can be rendered bioavailable through extraction and concentration in order to induce observable effects in bioassays. As it is not clear what types of contaminants are to be expected, it is advisable to use a biotest battery as detector for toxicity, covering different toxic endpoints and organisms (Brack 2003). Especially for the investigation of samples not imposing obvious problems, the restriction to one biotest system bears the risk of overlooking important compounds.

The aim of this study was to investigate whether the currently available methodology for effect-directed analysis procedures which is commonly applied to hazardous samples is also capable of identifying potentially relevant toxicants from a site, which cannot be considered highly

contaminated. For this purpose, a marine sediment from Brofjorden on the Swedish west coast was sampled. The toxic potency of the organic sediment extract was characterised using a biotest battery, including luminescent bacteria and green algae, in order to represent different trophic levels and metabolic capacities. Different effect types are included such as acute inhibition of bacterial energy metabolism, chronic inhibition of bacterial reproduction and chronic toxicity on proliferation of photoautotrophic organisms. The sample was subjected to preparative chromatographic fractionation until chemical analysis was successful in identifying toxicants.

## *2.2 Material and Methods*

The effect-directed fractionation procedure used in this study has been based on consecutive steps: (1) Extraction of sediment samples, (2) detection of biological effects in the sediment extracts; (3) chromatographic fractionations, (4) detection of biological effects in the fractions; (5) identification and quantification of components of toxic fractions; and (6) confirmation of toxicants by applying biotests to the identified single substances as pure compounds.

### 2.2.1 Sampling and extraction

Sediment samples were collected at 11 different sites in the Brofjorden area close to the Malmön peninsular shore on the Swedish west coast. This area was chosen, because it represents a typical site between the North and the Baltic Sea with various diffuse inputs of contaminants from sea traffic and a nearby refinery. Therefore, a mixture of different substances at comparably low concentrations was expected to be present. A detailed description or the sampling procedure and sample preparation is given in chapter 6.1.1, 6.1.2, and 6.1.3. The samples were freeze-dried. The combined sample was characterised by a total organic carbon (TOC) content of 3.68%. Subsequently, the sample was Soxhlet-extracted by dichloromethane for 24 h. The aim of this study was not the exact quantification of specific compounds in the sediments, but an effect-directed identification of dominant

toxicants which were not known at the beginning of the work. Thus, the extraction method was not optimised for specific compounds but rather chosen to extract a broad spectrum of compounds. The extracts were then concentrated to approximately 200 mL and shaken with 5 g of fine copper powder activated with concentrated HCl over night in order to remove sulphur which impairs biological testing of extracts (6.1.3).

In this study, concentrations of sediment extracts and fractions are denoted as sediment equivalent (SEq) in the test medium (g / L). One gram of SEq / L corresponds to the amount of compounds extracted from 1 g dry sediment and dissolved in 1 L test medium.

### 2.2.2 Fractionation

The extract was fractionated in a two-step procedure. Fractions are marked as Fi.j with i signifying the fraction number in the primary fractionation step, and j representing the fraction number in the second step. In the primary fractionation step compounds present in the extract were separated according to their polarity into five fractions (F1-F5) using open column chromatography. A secondary fractionation step was performed for the fraction F2, which was known to contain PAH compounds. For this purpose preparative HPLC was applied using a fractionation scheme designed to separate compounds with different numbers of aromatic rings into distinct fractions (F2.1-F2.10). The fractionation procedures are described in detail in chapter 6.2.1.

### 2.2.3 GC-MS Analysis

The identification and quantification of compounds in the fractions was performed with a gas chromatograph equipped with a mass selective detector (GC-MS). For the identification of unknown compounds, the mass spectrometer was used in scan mode. Compounds were identified applying a spectra library (NIST/EPA/NIH Mass Spectral Library, National Institute of Standards and Technology, USA). The identity of compounds suspected to cause observed toxicity was confirmed comparing spectra and retention time with standard compounds. Identified compounds were quantified in selected-

ion mode using external standards. A detailed description of the analytical methods can be found in 6.2.2.

### 2.2.4 Effect detection

For the detection of biological effects in the extract and fractions, a biotest battery was applied consisting of one chronic algal biotest and two bacterial test systems. Algal toxicity was measured as inhibition of reproduction of the unicellular green algae *Scenedesmus vacuolatus* after one generation cycle of 24 hours. Acute bacterial toxicity was measured as inhibition of bioluminescence in the marine bacterium *Vibrio fischeri* after an incubation time of 30 minutes. It is related to the energy metabolism of the organism. The same organism was additionally used to assess chronic bacterial toxicity measured as inhibition of reproduction after 8 h. The bioassays are described in 6.3.

Samples obtained from extraction or fractionation were transferred to dimethylsulfoxyde (DMSO). These solutions were applied in a concentration of 0.1% to the biotest. This concentration of DMSO does not produce a detectable effect when exclusively present in the test systems (cf. 6.3). Controls were conducted with and without DMSO in each experiment.

In order to account for procedural losses the original extract was reconstituted from the five fractions F1 – F5 and tested again in the algal biotest. The same was performed with the subfractions F2.1 – F2.10 in order to reconstitute F2. In both cases the observed dose-response curves were in very good agreement with those obtained from the original extract or fraction. Thus, it can be concluded that no significant procedural losses of algal toxic compounds occurred during fractionation.

### 2.2.5 Preliminary confirmation of toxicants

For confirmation of toxicants, compounds identified in the fractions exerting effects were purchased as pure substances in the highest available purity from Aldrich (Steinheim, Germany), Riedel de Haen (Seelze,

Germany), Merck (Duesseldorf, Germany), or Sigma (Deisenhofen, Germany) and tested separately in approximately tenfold concentration as compared to analysed concentrations in the fraction. Compounds not inducing effects in this concentration were considered not to contribute significantly to the effect of the sample. Compounds causing effects were confirmed as potentially relevant toxicants in the studied environmental sample. Individual $EC_{50}$ values of the confirmed toxicants were estimated from their $K_{OW}$ values assuming base line toxicity using the following equation (Altenburger *et al.* 2004):

$$\log EC_{50,baseline}\ [mol/L] = -0.863 * \log K_{OW} - 0.897 \qquad \textit{[eq. 1]}$$

Values for log $K_{OW}$ were estimated using the KOWWIN V1.66 software (EPISuite, US-EPA). The estimated $EC_{50}$ values were used to calculated toxic units (TU) of the compounds:

$$TU = \frac{C_{analysed}}{EC_{50,baseline}} \qquad \textit{[eq. 2]}$$

where $C_{analysed}$ is the analytically determined concentration of the compound. This confirmation step is rather rough and should be understood as preliminary. A more detailed and in depth study on the confirmation of the identified toxicants with special focus of the mixture aspect is given in chapter 3 and Grote *et al*. (2005).

## *2.3 Results*

### 2.3.1 Sediment extracts

The sediment extract from Brofjorden, Sweden inhibits luminescence of the marine bacteria *Vibrio fischeri* (Figure 2.1) as well as the reproduction of the green algae *Scenedesmus vacuolatus* (Figure 2.2) in the algae test system after removal of elemental sulphur.

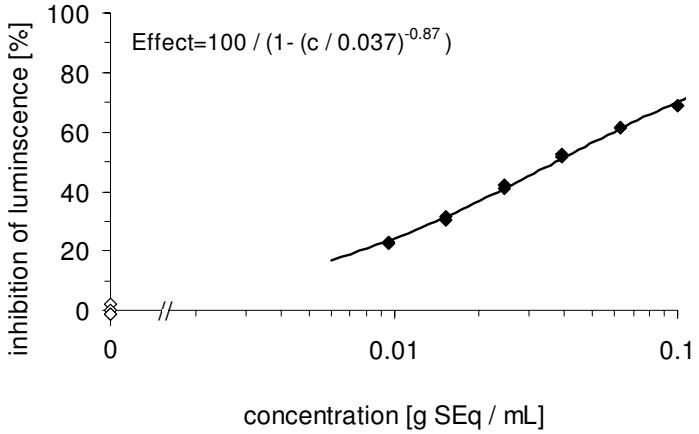


*Figure 2.1: Inhibition of bacterial luminescence induced by the sediment extract from Brofjorden, Sweden. Response of controls is depicted as open symbols. Additionally, the modelled concentration-response function is given.*

The highest tested concentration (0.1 g SEq / mL) inhibits bacterial luminescence by 70 % and algal reproduction by 60 %. A concentration-dependent response can be observed in both test systems. For luminescence inhibition an $EC_{50}$ value of 37 mg SEq / mL can be estimated from this relationship. This compares well to a previously observed $EC_{50}$ value for an extract from Baltic Sea sediments of 46 mg SEq / mL (Reineke 2003) in the same test system. However, this value is considerably higher than an $EC_{50}$ value of a river sediment extract from the highly industrialised area of Bitterfeld, Germany, where 0.12 mg SEq / mL was observed in the bioassay testing for luminescence inhibition of *Vibrio fischeri* (Brack *et al.* 1999).

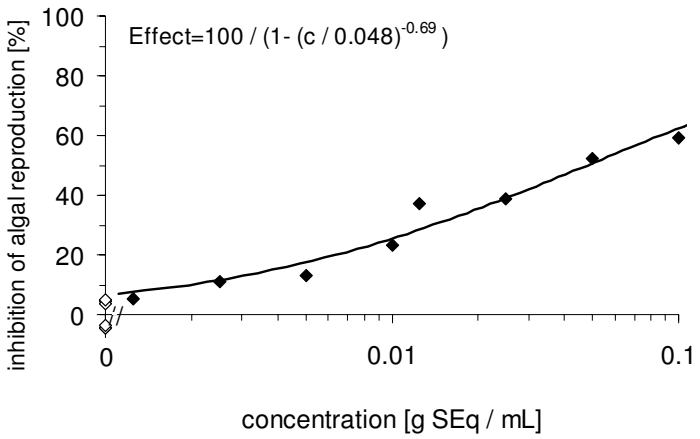


*Figure 2.2: Inhibition of algal reproduction induced by the sediment extract of Brofjorden, Sweden. Response of controls is depicted as open symbols. Additionally, the modelled concentration-response function is given.*

As the extract induces effects in two different test systems, it is suspected that toxicants are present. For the establishment of cause-effect relationships, the identification of toxicants responsible for the observed effect is required. Figure 2.3 depicts the GC/MS chromatogram of the sample which induced effects in the biotests. Hundreds of peaks of different compounds can be detected in the sediment extract with this analytical method. The identification, let alone the quantification of all compounds in this complex environmental mixture would be extremely laborious. However, even if all compounds in the samples were identified and quantified, no establishment of a cause-effect relationship would be possible due to the lack of toxicological data of the majority of substances.

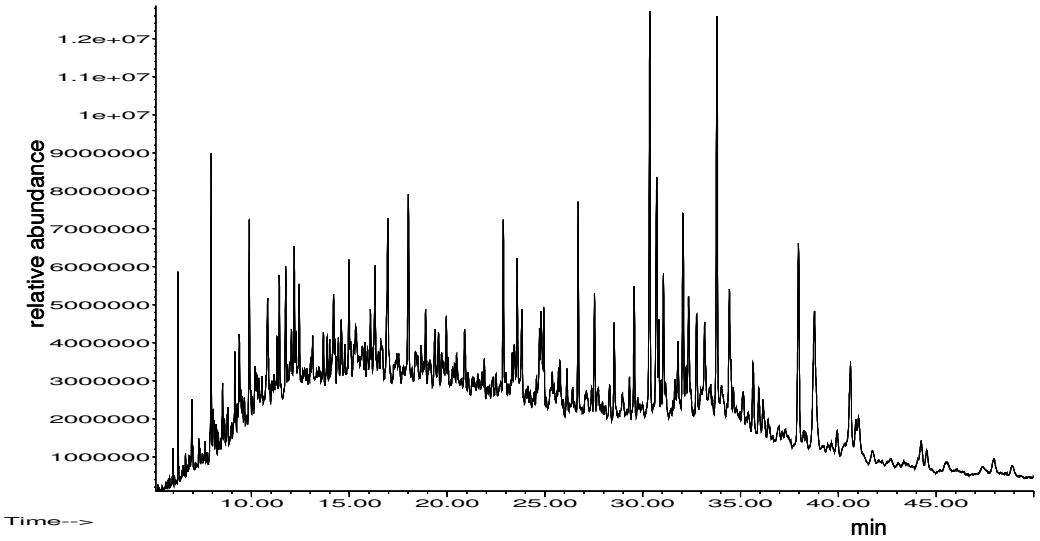


*Figure 2.3: GC/MS chromatogram of sediment extract from Brofjorden, Sweden. Abundance refers to the Total Ion Current (TIC).*

This chromatogram clearly illustrates the limitations of chemical analytical methods for the establishment of cause-effect relationships when used exclusively. Without an *a priori* knowledge of possibly responsible toxicants it is impossible to attribute observed effects to analysed pollutants.

### 2.3.2 Primary fractions

Testing of the fractions from fractions revealed that highest effects on algae reproduction (Figure 2.4) are exerted by fraction 2 (F2). Lower effects are induced by F3, while all other fractions induce no or only slight effects.

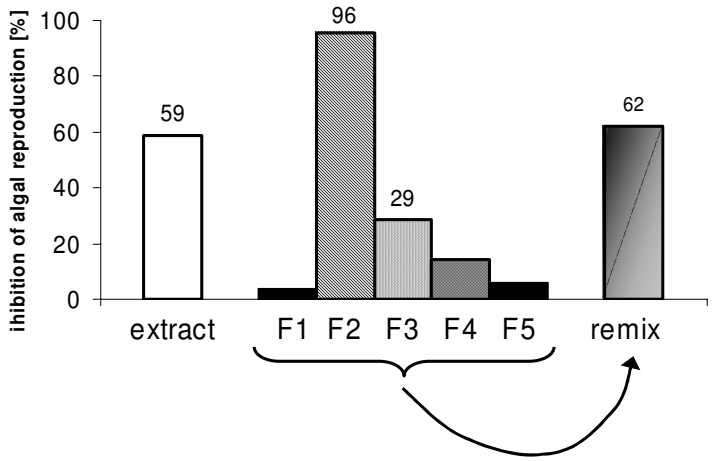


*Figure 2.4: Inhibition of algal reproduction induced by the sediment extract from Brofjorden, Sweden, and primary fractions at concentration of 0.1 g SEq / mL. Additionally, the inhibition exerted by the remix of the fractions F1 - F5 is depicted.*

The high effects induced by F2 were unexpected as they were higher than the effect exerted by the whole extract. The whole extract contains all compounds present in F2 in same concentrations, and additionally contains compounds present in the other fractions. In order to clarify whether this phenomenon was due to a procedural artefact, the fractions F1 to F5 were remixed and tested in the bioassay. The observed effect of 62% was very close to the effect of the original extract and clearly lower than the effect of F2 alone (Figure 2.4). Also the observed dose-response curve was in very good agreement with that obtained from the original extract. Thus, it can be concluded that the elevated toxicity of F2 was not due to procedural artefacts, i.e., introduction of additional toxicants from solvents used.

Primary fractions caused no or very low effects on luminescence of *Vibrio fischeri* after short-term exposure (Figure 2.5). This may be considered as an indication that effects to this organism induced by the extract were exerted via an unspecific narcotic mode of action. Toxicity may thus be attributed to the mixture of all compounds present, rather than to distinct substances. As the total concentration of compounds is reduced in each fraction as compared to the whole extract, compounds may not be present in concentrations high

enough to induce narcosis. The expected combined effects of F1-F5 calculated by assuming independent action of the constituents of 32% inhibition are lower than the toxicity of the extract. This can be understood as an indication for a similar mode of action which typically results in higher mixture effects than assumed from independent action.

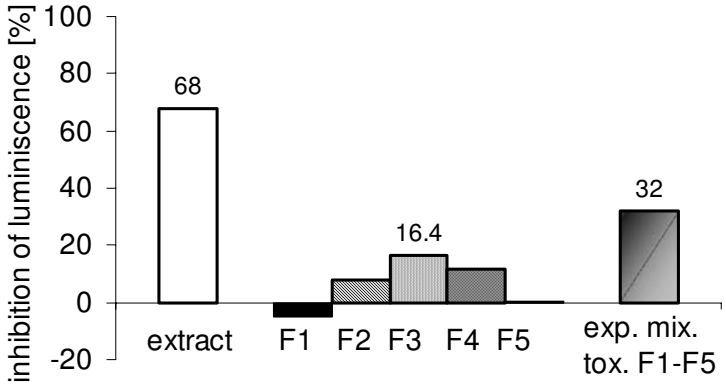


*Figure 2.5: Inhibition of bacterial luminescence caused by the sediment extract from Brofjorden, Sweden, and primary fractions at a concentration of 0.1 g SEq / mL. (The negative inhibition exhibited by F1 is within the variance of controls.) Additionally, the expected mixture toxicity of F1 – F5 is depicted based on a prediction according to the model of independent action.*

In addition to the acute toxicity test using the luminescence inhibition in *Vibrio fischeri*, a reproduction inhibition test was conducted with the same organism, in order to comprise possible more specific effects at chronic exposure. Results for the extract and all fractions are depicted in Figure 2.6.

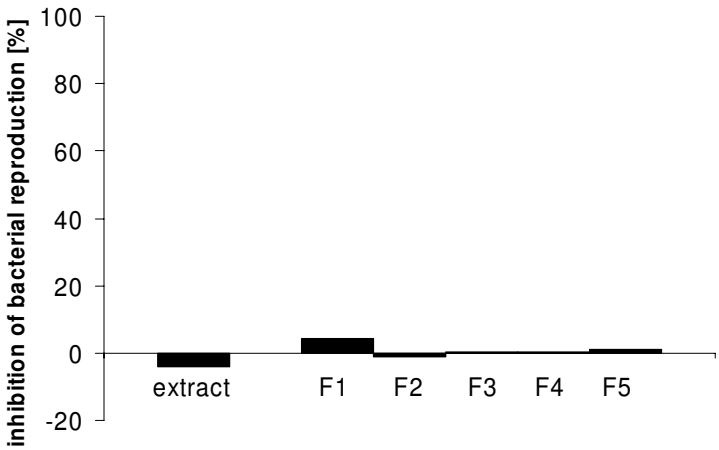


*Figure 2.6: Effects on reproduction of Vibrio fischeri after incubation of 8 hours exerted by concentrations of 0.1 g SEq / mL.*

Neither the extract nor the fractions caused observable inhibitory effects on the reproduction of *Vibrio fischeri*. This is surprising as the extract inhibited luminescence in this organism when applied in the same concentration. As luminescence is related to the energy metabolism of the organism, one could thus expect that disruption of this energy metabolism would result in a decrease in reproduction. One reason for this phenomenon may be sought in the different test media used. While for the short-term toxicity test a purely inorganic medium was used, the medium for the long term toxicity test additionally contained 1,2,3-propanetriol (glycerol), peptone and yeast extract, in order to provide a nutrition source for the bacteria. It is possible that lipophilic toxicants present in the samples bind to the organic matrix and are thus no longer bioavailable for the organisms.

As for the two organism used as detectors for toxicity in the effect-directed analysis only the bioassay with *Scenedesmus vacuolatus* was capable of detecting and differentiating effects in the extract as well as of identifying toxic fractions, this test was used for further investigation.

### 2.3.3 Secondary fractions

As previously explained, fraction 2, the most toxic fraction in algae, was subject to further fractionation. Effects caused by different concentrations of

subfractions of F2 are plotted in Figure 2.7. Each of the subfractions was less toxic than fraction 2. However, by remixing the ten subfractions (F2.1-F2.10) the toxicity of F2 could be reestablished. We thus believe that no toxicity was lost during the fractionation.

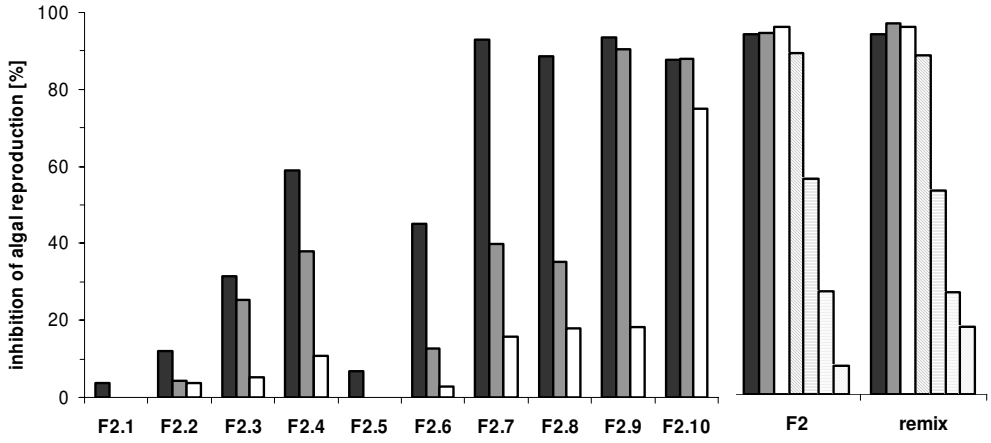


*Figure 2.7: Effects on algal reproduction induced by subfractions of F2. (■) 0.1, (■) 0.05, (□) 0.025 g SEq / mL test solution. For the original fraction F2 and the created remix of F2.1-F2.10 effects of additional dilutions (dilution factor 2) are shown.*

The fraction F2.10 was the most toxic subfraction of F2. It caused high effects on the algal reproduction when present in concentrations of 0.025 g SEq / mL. Furthermore, fractions F2.7, F2.8 and F2.9 exerted high effects (>90%) when present in 0.1 g SEq / mL. Fractions F2.3, F2.4 and F2.6 inhibit algal reproduction by 30 to 60 % in the same concentration. The other fractions (F2.1, F2.2 and F2.5) did not induce effects in the test system.

Strong absorbance of wavelengths of 252 nm was observed for fractions eluting first (F2.1-F2.3) from the column (Figure 6.3). However, only minor toxicity was observed for these fractions. Thus a large percentage of the compounds present in F2 may be responsible for a small percentage of the effects, while the relatively lower number of compounds in the later eluting fractions (Figure 6.3) may be responsible for a greater percentage of the effects. Alternatively, the toxic compounds may absorb at different wavelengths.

The subsequent analysis using GC/MS reveals that complexity observed for the chromatograms of the whole extract (Figure 2.3) is significantly reduced when analysing the secondary fractions. Exemplarily, the chromatogram of the fraction F2.7 is depicted in Figure 2.8.

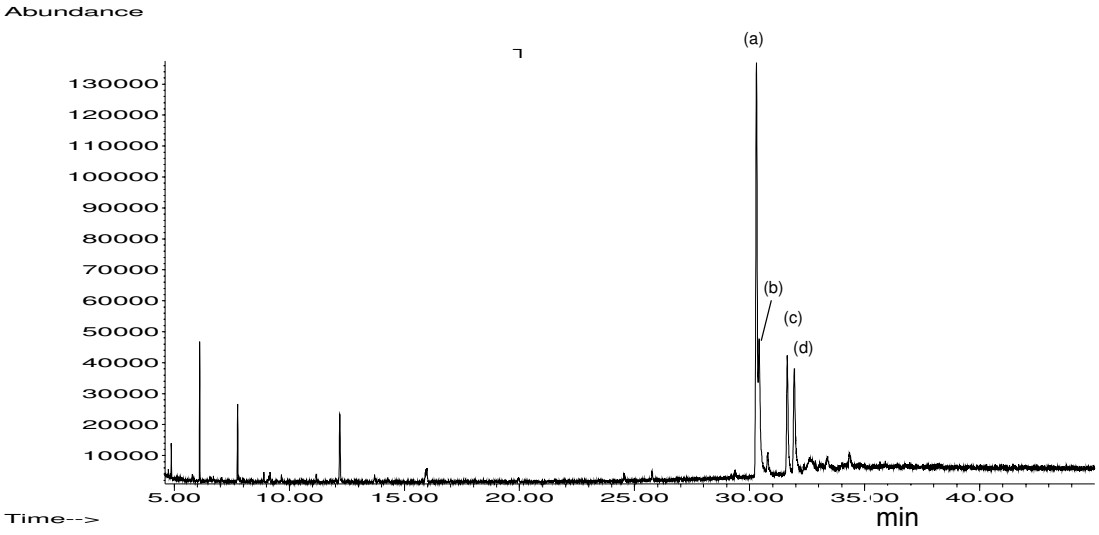


*Figure 2.8: Total Ion Current (TIC) Chromatogram of F2.7 (subfraction of F2) with four identified substances (a) Benzo[b]fluoranthene, (b) Benzo[k]fluoranthene, (c) Benzo[e]pyrene, (d) Benzo[a]pyrene.*

Apart from minor unidentified peaks, the four major peaks detected in F2.7 were identified as five-ring PAHs (Figure 2.8). Quantification of identified compounds in all fractions was performed with standard compounds using GC/MS. Results of quantification are summarised in Table 2.1.

*Table 2.1: Analysed concentrations in subfractions of F2 (µg / mL). (1 mL of the extract contains the equivalent of 100 g of extracted sediment.)*

| | F2.1 | F2.2 | F2.3 | F2.4 | F2.5 | F2.6 | F2.7 | F2.8 | F2.9 | F2.10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Naphthalene | | 0.31 | | | | | | | | |
| Biphenyl | | 0.32 | | | | | | | | |
| Acenaphthylen | | 0.06 | | | | | | | | |
| Acenaphthen | | 0.14 | | | | | | | | |
| Fluorene | | 0.47 | | | | | | | | |
| Phenanthrene | | 0.03 | 3.44 | | | | | | | |
| Anthracene | | 0.10 | 4.97 | | | | | | | |
| Methylphenanthrene | | | 4.42 | | | | | | | |
| Fluoranthene | | | 0.07 | 4.94 | | | | | | |
| Pyrene | | | 0.97 | 2.39 | | | | | | |
| Benzo[*a*]antracene | | | | 0.02 | | 2.36 | | | | |
| Chrysene | | | | 0.14 | | 2.08 | | | | |
| Benzo[*b*]fluoranthene | | | | | | | 8.14 | 0.015 | | |
| Benzo[*k*]fluoranthene | | | | | | | 2.68 | 0.003 | | |
| Benzo[*e*]pyrene | | | | | | | 2.14 | 1.12 | | |
| Benzo[*a*]pyrene | | | | | | | 3.09 | | | |
| Perylene | | | | | | | | 1.50 | | |
| Indeno[1,2,3-*cd*]pyrene | | | | | | | | 0.011 | 7.18 | |
| Dibenz[*a,h*]anthracene | | | | | | | | | 0.84 | |
| Benzo[*ghi*]perylene | | | | | | | | | 5.29 | |
| Dibenzo[*a,l*]pyrene | | | | | | | | | | n.q. |
| Coronene | | | | | | | | | | n.q. |

n.q.: not quantified

Maximum concentrations of fractions tested in the bioassay were 1 mL extract / 1 L test solution (equivalent to 0.1%). Thus concentration given herein can be read as µg / L for concentrations of compounds present in the biotests of the respective fractions.

The fractionation scheme was capable of separating the PAH compounds into distinct fractions. Compounds with smaller aromatic ring systems eluted in the foremost fractions, whereas compounds with larger ring systems were detected in the later eluting fractions. Most compounds were detected in either one fraction or in two adjacent fractions. In the latter case the majority of the compound was analysed in one of the fractions, while only traces of the compound were detected in the adjacent fractions. A comparably short time window in the fractionation scheme was chosen for F2.5 (Figure 6.3) in order to isolate benzo[*ghi*]fluoranthene, a compound which was previously reported as the PAH compound with the highest contribution to the observed effect of a sediment extract from Bitterfeld (Brack *et al.* 1999; Altenburger *et al.* 2004).

However, neither this compound was detected in the fractionated sample from Brofjorden, nor did the fraction F2.5 exert toxicity.

### 2.3.4 Preliminary confirmation of toxicants

Compounds identified in the fractions were tested individually as pure compounds in approximately tenfold concentration as they were detected in the fractions. Inhibition of algal reproduction induced by the identified compounds is depicted in Figure 2.9.

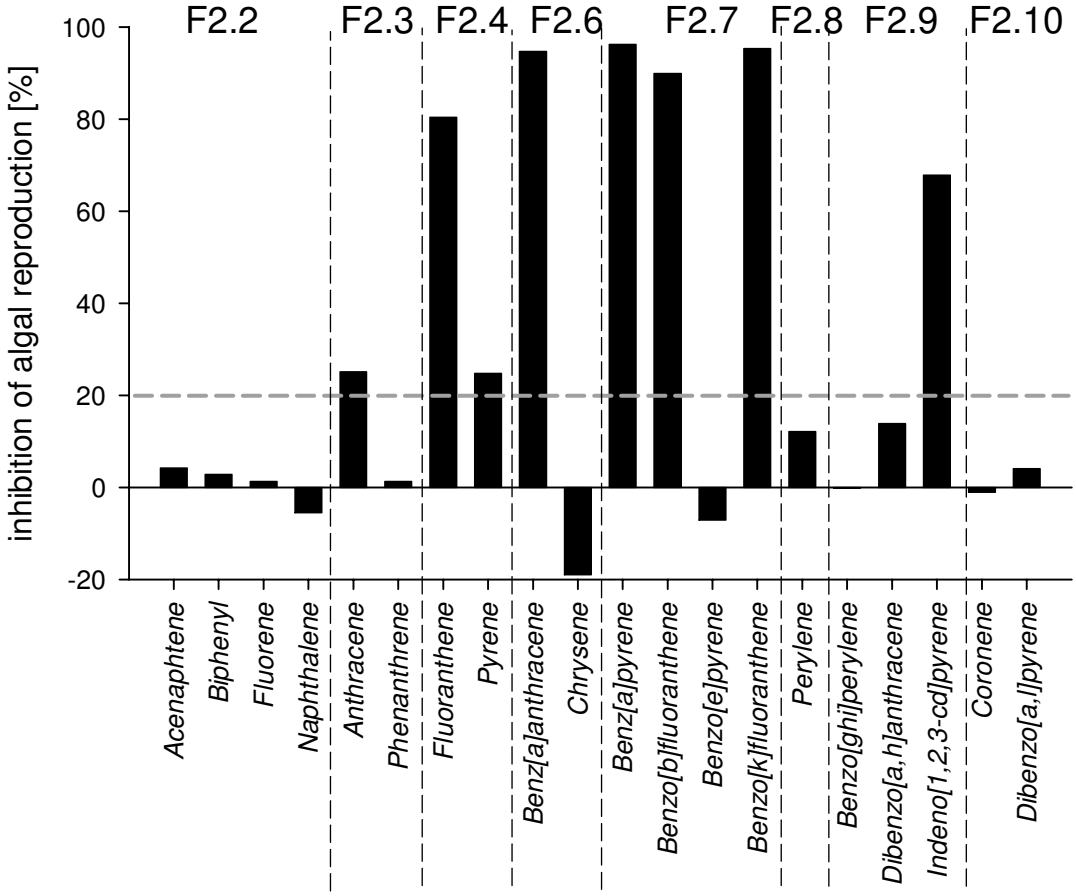


*Figure 2.9: Inhibition of algal reproduction caused by compounds identified in the subfractions of F2. Maximal test concentrations were one order of magnitude higher than analysed in the fractions. Dashed vertical lines indicate in which subfraction the compounds were detected. In F2.5 no compounds were identified.*

Compounds not causing effects in this concentration were considered not to contribute significantly to the effect of the sample. Conversely, compounds exerting effects were considered as potentially relevant toxicants in the studied environmental sample as they possibly contribute at least partially to the sample's and fractions' effects.

Only 8 out of the 20 tested compounds inhibit algal reproduction in the tested concentrations. As expected, compounds analysed in the non-toxic fraction F2.2 do not exert effects in the algal test system. In the toxic fraction F2.4, both identified compounds fluoranthene and pyrene appear to contribute to the fraction's effect. In the other toxic fractions, not all detected compounds cause effects when tested separately. Of the compounds identified in F2.3, only anthracene inhibits algal reproduction, while phenanthrene does not. In F2.7, four compounds were quantified three of which exert algal toxicity. Of the three compounds detected in F2.9, only indeno[1,2,3-*cd*]pyrene inhibits the algal reproduction. The toxicity of F2.8 and F2.10 remains unresolved as the compounds identified in these fractions did not exert effects in the test system. In summary, 8 compounds were identified as potentially relevant toxicants from the sediment sample. In order to estimate the relevance of the detected concentration of identified toxicants, their individual $EC_{50}$ values were estimated [eq. 1] assuming a narcotic mode of action. Toxic units (TU) of the individual compounds were calculated from analysed concentrations and estimated $EC_{50}$ values [eq. 2] and are depicted in Figure 2.10.

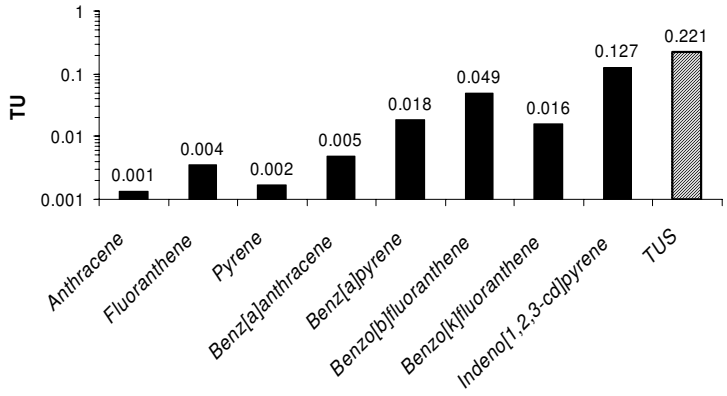


*Figure 2.10: Toxic Units (TU) of the identified toxicants in the samples. (TU = analysed concentration / $EC_{50,baseline}$). TUS is the toxic unit sum of all confirmed compounds.*

The depicted toxic unit sum (TUS) is the expected combined toxicity of the identified toxicants assuming all compounds act via a common narcotic mode of action. The TUS value of 0.22 denotes that the mixture of the eight identified compounds is present at 22% of its expected $EC_{50}$ value. This is in contrast to the observed effects of the extract and the fraction F2 which exerted effects of more than 50% in the present concentration. However, it is not known, if the assumptions concerning a narcotic mode of action and concentration additive behaviour are realistic for the given system.

## *2.4 Discussion*

The aim of this study was to investigate whether the methodology of EDA is capable of identifying potentially relevant toxicants from a sediment sample which cannot be considered highly contaminated. It was demonstrated that compounds present in the sediments can be extracted and thus be made bioavailable for different test species used as effect-detectors. Toxicants were separated from non-toxic compounds by preparative fractionation. Eight compounds were identified from toxic fractions of the organic extract and confirmed as contributors to the effects observed in the algae test system. Therefore, it can be concluded that the EDA methodology can be successfully applied to samples not inducing obvious problems. This is consistent with the

results of a simultaneously conducted study, investigating different extracts from marine sediment samples using EDA (Biselli *et al.* 2004; Kammann *et al.* 2004b). All toxicants identified in the present study are polycyclic aromatic hydrocarbons (PAHs) which are considered as ubiquitously occurring environmental pollutants. No specifically acting chemicals were detected unlike in a previous EDA study on organic sediment extracts from the highly industrialised area of Bitterfeld, Germany where *N*-phenyl-2-naphthylamin, prometryn and parathion-methyl were identified as contributors to observed effects (Brack *et al.* 1999). In the previously mentioned study on EDA in sediment extracts from the North and the Baltic Sea, different brominated phenols and indoles were identified and held partially responsible for observed biological effects (Kammann *et al.* 2004b; Reineke *et al.* 2005). It is unclear whether these compounds exert their toxicity via a specific mode of action or via an unspecific narcotic mode of action (Reineke *et al.* 2005).

Concentrations of PAH compounds detected in the investigated sediment are comparably low. Summing up concentrations of the 16 EPA-PAHs, leads to a PAH concentration of 450 µg / kg dry weight sediment or 12.2 µg / g TOC. Keeping in mind that the individual PAH compounds are characterised by different toxic potentials, the sum of these 16 compounds may be of little toxicological value; however, it forms a basis for comparison to previously published results. The concentration of 12.2 µg / g TOC lies at the lower end of the range previously detected in seven North Sea and Baltic Sea sediments (11.3 – 179 µg / g TOC) (Biselli *et al.* 2004). Due to the lack of comparable data on a TOC basis, results have to be compared on dry weight basis to other studies. In a recent review on sources and fate of PAHs in marine environments (Latimer and Zheng 2003) concentrations ranging from 4.9 to 40400 µg / kg dry weight sediment with median values of 900 µg / kg dry weight sediment were reported for the 288 marine estuary sediment samples. A similar concentration range of PAH contamination was observed for 63 Baltic Sea sediment samples with concentrations ranging from 3 to 30000 µg / kg dry weight sediment and a medium concentration of 2450

µg / kg dry weight sediment (Baumard *et al.* 1999). When comparing results, it can be stated that concentrations detected in the Brofjorden sample were lower than in two thirds of reported investigated sediments reported by Latimer and Zheng (2003) and Baumard *et al*. (1999). Thus the investigated sample can be considered as of low contamination.

From the three bioassays used as detectors for toxicity in the extracts and fractions, only the chronic algal bioassay testing for inhibition of reproduction of *Scenedesmus vacuolatus* was capable of discriminating toxic from non-toxic fractions. This result stresses the recommendation of applying several biotests in EDA studies (Brack 2003) as not all bioassays may be suitable to detect toxicity in the sample of interest. This may be due to the fact that after fractionation the effects observed in the extract are spread over several subfractions, or substances present in the toxic fractions are not amenable to chemical analysis (Kammann *et al.* 2004b). Furthermore, identified toxicants may be found not to be responsible for the observed effects (Kammann *et al.* 2004a).

In this study, bioassays for acute and chronic toxicity to *Vibrio fischeri* failed to identify toxic fractions. This goes in line with a previous study performing EDA aiming to identify toxicants in river water (Reineke *et al.* 2002). The initial toxicity of the whole sample observed using *Vibrio fischeri* was distributed over several fractions which all exerted low toxicity. In all of these fractions some compounds could be chemically analysed, however no toxicants explaining the observed effects were identified (Reineke *et al.* 2002). Brack (2003) states in a review on EDA studies that the bioassay using acute toxicity to *Vibrio fischeri* often fails to identify specific toxicants in complex environmental samples. However, more recently, the *V. fischeri* test system was successfully applied to identify 4-bromophenol as contributor to observed toxic effects in extracts of North Sea sediment samples (Reineke *et al.* 2005).

The fact that no chronic toxicity to *Vibrio fischeri* was observed at concentrations which inhibited luminescence by 68% (acute toxicity) was

surprising. It may have been related to reduced bioavailabilty of the toxic compounds due to the inclusion of organic matter (yeast, 1,2,3-propanetriol (glycerol) and peptone) in the test medium. Alternatively, short term disturbance of energy metabolisms may not lead to long term effects. Backhaus *et al*. (1997) reported in a study comparing bioassays for acute and chronic toxicity to *Vibrio fischeri* that depending on the compounds tested, the chronic toxicity test detected either higher or lower toxicity compared to the acute test.

For the algal toxicity, higher toxicity was induced by the fraction F2 than by the extract. The reason for this phenomenon remains unclear. A possible explanation could be a specific interaction between compounds present in F2 or an antagonism between the latter compound and compounds in the other fractions. Another possible explanation could account for modification of physical or physico-chemical properties, i.e., the modification of solubility of distinct toxicants caused by the presence of other compounds or the modification of light conditions. An influence of changes in pH which was previously reported to affect toxicity of 2,3,4-trichlorophenol (Walter 2001) can be excluded as pH was measured to be constant (6.9 ± 0.2). The discrepancy between toxicity of the extract and F2 laid ground for further investigations assessing the influence of light conditions on samples and identified toxicants (chapter 4).

For confirmation of toxicants, estimated $EC_{50}$ values were used assuming a narcotic mode of action. However, it is not clear whether this assumption holds true for the identified compounds. Furthermore, the toxicity of the mixture of identified toxicants was calculated by toxic unit summation. However, it is unclear whether the underlying assumption is met that combined effects are predictable by the model of concentration addition. Therefore, in order to establish a causal link between the observed effects and the identified compounds, a quantification of contributions of individual compounds and of the mixture is needed. The confirmation of toxicants is subject to subsequent investigations using information on the concentration-

response relationships of the individual compounds and an assessment of possible combined effects induced by the mixture of identified compounds (chapter 3).

In summary, it may be said that the EDA methodology can in principle be applied to samples which are not highly contaminated. The study illustrates possible problems, which may be encountered when using EDA. Not all bioassays used are successful in identifying toxicants. This stresses the need of using a biotest battery, in order to cover a broad range of possible toxic effects. Furthermore, identification of toxicants was not successful in all toxic fractions. Toxicity observed in the sample could not be completely attributed to the identified compounds. Results of F2.10 are unsatisfactory as this was the most toxic fraction after secondary fractionation, however no substances explaining the observed effects could be identified. The identified PAHs coronene and dibenzo[*a*,*l*]pyrene were not toxic in the screening experiment, probably due to their low solubility in the media. No other substances were found with the given instrumentation. The observed effect may be due to substances not amenable to GC/MS detection because of insufficient volatility or high polarity. Thus unresolved toxicity remained. This is a problem which is often encountered when performing EDA on environmental samples (Brack 2003). When aiming to identify other compounds contributing to the observed effects, further experimental work applying different analytical methods may be necessary.

# Chapter 3

# Confirmation of cause-effect relationships using effect-directed analysis for complex environmental samples*

## *3.1 Introduction*

Effect-directed analysis (EDA) is one approach aiming to establish a causal link between chemical substances and biological effects in environmental samples. The state of the art of this approach was recently reviewed by Brack (2003) with focus on organic contaminants, Ho *et al.*(2002) with respect to sediments, and dredged materials and Burgess (2000) with focus on marine waters. When compounds are identified in the toxic fractions, a confirmation step is needed, in order to provide evidence for the causal link between these compounds and the samples' toxicity. For this purpose, two different approaches are suggested: testing a synthetic mixture or calculation of an expected mixture toxicity.

Sample toxicity has been compared to toxicity of synthetic mixtures by different authors for either whole samples (Svenson *et al.* 2000) or subfractions of the original sample (Brack *et al.* 1999). For toxicity observed in

* Parts of this chapter are published as Grote M, Brack W, Walter HA, and Altenburger R. (2005) *Environmental Toxicology and Chemistry* 24:1420-1427.

*Daphnia magna* and the microalgae *Scenedesmus vacuolatus,* Brack *et al*. (1999) compared the medium effective dilution ($ED_{50}$) of different subfractions of the sample to the corresponding values for synthetic mixtures containing up to six of the identified compounds. Similarly, Svenson *et al*. (2000) determined the medium effective concentration ($EC_{50}$) of inhibition of nitrification in *Nitrobacter* by a synthetic mixture of six dominant toxicants. This value was found to be in accordance with that of the original wastewater sample.

As an alternative approach, the comparison of the sample's toxicity with a calculated expectation value of the mixture toxicity is used. Burkhard and Durhan (1991) calculated toxic units (TU) from analytical concentrations and medium lethal concentrations ($LC_{50}$) of toxicants measured in *Ceriodaphnia dubia*. Toxic units of the three identified toxicants were summed and compared with the TUs observed in the effluent.

Some authors also combine both approaches. Boxall and Maltby (1997) tested the toxicants identified in motorway runoff individually and in mixtures with the amphipod *Gammarus pulex*. Subsequently, they compared the observed mixture toxicity and the sum of TUs to the sample's toxicity.

The TU summation bases on the concentration addition (CA) model for the prediction of mixture toxicity, which assumes similar modes of action for all compounds in the mixture. It has been shown that this model is a suitable tool for the prediction of combined effects of mixtures of strictly similarly and specifically acting substances in different ecotoxicological test systems (Faust *et al.* 2000) as well as for substances with an unspecific, narcotic mode of action (Hermens and Leeuwangh 1982; Hermens *et al.* 1984). However, it was also demonstrated that for mixtures of substances with dissimilar modes of action, the model of independent action (IA) better predicts combined effects, whereas CA overestimates the combined effects (Faust *et al.* 2000).

For mixtures of environmental chemicals the modes of toxic action are commonly unknown. Thus, it is not clear, whether expected combined effects

of mixtures calculated using the TU methodology are appropriate to be applied for the confirmation step in EDA procedures. Furthermore, these calculations are commonly performed for one effect level only. However, concentration-response curves are reported to differ in shape and slope. The heterogeneity of these parameters may result in different conclusions depending on the regarded effect level. Banks *et al*. (2003) found concentration additive behaviour for the toxicity of a mixture of copper and diazinon to *Ceriodaphnia dubia* for higher effect levels, while for low and median effect levels CA overestimated the joint action of the mixture.

Although the importance of the confirmation step is generally recognised (Brack 2003), only few studies can be found in the literature which specially focus on the problem of confirmation in EDA. In the USEPA guidance document on the confirmation step in toxicity identification evaluation (TIE) (Mount *et al.* 1993), the authors propose seven different steps to be conducted and subsequently to use the weight of evidence in order to confirm that the causes of toxicity are correctly identified. In one of these steps, the correlation approach, which especially applies when mixture of toxicants are present, the authors recommend to utilise TU calculation. However, being aware of problems arising from application of TU calculations of non-additive compounds, they state that great care must be taken to understand the interactions of the toxicants. They conclude that in case the toxic effects of the compounds "are strictly non-additive, only the major one (the one present in the most TUs) should be included in the data set" (Mount *et al.* 1993) and thus be used for the comparison to the sample's toxicity. However, they do not provide guidance, how to determine non-additivity of the compounds' toxicity.

The aim of this study is to advance the methodology for the confirmation of mixtures in EDA by incorporating the available knowledge on predictability of combined effects of mixtures. For this purpose, the confirmation step was conducted for mixtures identified in two different environmental samples using

concentration-response curves of observed mixture toxicity and expected combined effects calculated on the basis of CA and IA.

## *3.2 Materials and Methods*

### 3.2.1 Investigated samples

Two extracts from sediments sampled at one freshwater site (Bitterfeld, Germany) and one marine site (Brofjorden, Sweden) were used for the development of the confirmation methodology. These samples were chosen, in order to study one highly and one moderately contaminated site. The results for the effect-directed analysis of the freshwater sediment taken in the highly industrialised area of Bitterfeld, Germany, were previously reported (Brack *et al.* 1999). The procedure conducted for the toxicant identification in the marine sediment from Brofjorden on the Swedish west coast is described in chapter 2.

### 3.2.2 Biotest system

As biotest system the chronic algal toxicity assay was used, which was successfully applied to identify toxicants in sediment extracts from Brofjorden, Sweden (chapter 2) and Bitterfeld, Germany (Brack *et al.* 1999). The toxic endpoint was the inhibition of the cellular reproduction of the unicellular green algae *Scenedesmus vacuolatus* during one generation cycle lasting 24 h (cf. 6.3.1). Samples were tested in geometric dilution series with a constant final DMSO concentration of 0.1 % in the test solution. Spacing between test concentrations was flexibly adapted to the steepness of each concentration–response curve, in order to cover the range between $EC_{10}$ and $EC_{90}$.

### 3.2.3 Test solutions

Extracts of sediments as well as identified compounds were tested individually in the algal test system. Additionally, synthetic mixtures of identified toxicants composed according to their analysed concentrations

were tested in the same test system. Mixture ratios resulting from analytical data are listed in Table 3.1.

Concentrations of mixtures are given as the sum of the concentrations [µmol / L] of the individual compounds. Concentrations referring to the sediment extracts are denoted as sediment equivalents (SEq). One g SEq / L corresponds to the amount of compounds extracted from 1 g dry sediment and dissolved in 1 L medium.

### 3.2.4 Fit of experimental data

Experimental data of the tested pure compounds, mixtures and extracts were fitted using a two parameter Hill function [eq. 3] which assumes logistic distribution of the data.

$$E = \frac{1}{1 + (\frac{c}{EC50})^{-P}} \qquad \textit{[eq. 3]}$$

where E denotes the fractional effect ($0 \leq E \leq 1$) and c the concentration. The parameters of the models ($EC_{50}$ and slope P) were estimated by a least-square approach using the software SigmaPlot 4.0 (SPSS, Chicago, IL, USA).

### 3.2.5 Calculation of expected mixture toxicity

Expected mixture toxicities were calculated from the concentration-response relationships of the single compounds and the ratio at which they are present in the mixture using the models of concentration addition and independent action as described in chapter 6.4.2. The obtained concentration-response curves refer to the sum of the individual concentrations of the identified toxicants. For a comparison with the original sample these concentrations were transformed to SEq / L, by division of the obtained values through the total toxicant concentration in 1 g sediment as given in Table 3.1.

### 3.2.6 Confirmation of toxicant mixtures using toxic units

For the commonly applied confirmation procedure using the TU approach, the observed extract toxicity as well as the analysed concentrations of suspect toxicants are transformed into toxic units which can then be compared. The $EC_{50}$ values of the extracts were used to determine the TU value of each extract ($TU_{Extract}$) using the following equation:

$$TU_{Extract} = \frac{1}{EC_{50}} \cdot 1\ g\ SEq / L \qquad \textit{[eq. 4]}$$

The predicted number of toxic units was calculated by summing up the TUs of the individual compound in the sediment (ΣTU) using [eq. 5], where $c_i$ is the concentration of the suspect toxicant i, $EC_{50,i}$ is the $EC_{50}$ value of the suspect toxicant i, and n is the number of toxicants.

$$\Sigma TU = \sum_{i=1}^{n} \frac{c_i}{EC_{50,i}} \qquad \textit{[eq. 5]}$$

The ratio of ΣTU to $TU_{Extract}$ is used as a measure of how much of the observed extract toxicity in terms of effect concentrations can be attributed to the identified toxicants.

### 3.2.7 Confirmation of mixtures using the Index of Confirmation Quality

As an alternative approach, complete concentration-response curves of expected and observed mixture toxicity were used for the confirmation. For comparison of sample's toxicity and mixture toxicity the index of confirmation quality is used (ICQ). The ICQ is the ratio of the mixture toxicity of the identified compounds and the sample's toxicity at one effect level. It can be calculated for the range of effect levels of interest.

$$ICQx_C = \frac{ECx_{Extract}}{ECx_C} \qquad \textit{[eq. 6]}$$

where x indicates the effect level; the index C denotes the considered mixture toxicity which can either be an expected toxicity according to one of the applied models, CA or IA, or the observed toxicity of the synthetic mixture (SM). The $ICQ_{50,CA}$ would thus stand for the ratio of the mixture toxicity expected from concentration addition to the extract's toxicity at the $EC_{50}$.

## *3.3 Results*

### 3.3.1 Effect-directed analysis

The results of the EDA performed for the sediments from Bitterfeld (Brack *et al.* 1999) and Brofjorden (chapter 2) were previously reported. Results of the identification and quantification of toxicants are given in Table 3.1. Toxicant concentrations in the Brofjorden sediment were typically one to two orders of magnitude lower than in Bitterfeld. This finding reflects that Bitterfeld was a major chemical production site during the last 100 years, whereas Brofjorden is less impacted by human activity.

### 3.3.2 Toxicity of extracts, single substances and mixtures

Results of the biological tests of the sediment extracts, the identified compounds and the synthetic mixtures are presented in Table 3.2. The extract of the Bitterfeld sediment is far more toxic than the extract of the Brofjorden sediment, with $EC_{50}$ values of 0.23 g SEq / L and 2.8 g SEq / L respectively. The relatively high toxicity of the sediment extract from Bitterfeld reflects the impact of the former chemical production site.

*Table 3.1: Concentrations of toxicants in the sediments of Brofjorden (top) and Bitterfeld (bottom) as determined in the toxic fractions. Additionally, the resulting fractional composition of the created synthetic mixtures (based on molar concentrations) are provided.*

| | Analysed concentration in Brofjorden sediment | | Fraction of compound in synthetic mixture $p_i$ |
|---|---|---|---|
| | [µg / kg] | [µmol / kg] | |
| Anthracene | 50.7 | 0.28 | 0.175 |
| Benzo[*a*]anthracene | 23.7 | 0.10 | 0.064 |
| Benzo[*a*]pyrene | 32.7 | 0.13 | 0.080 |
| Fluoranthene | 50.1 | 0.25 | 0.153 |
| Benzo[*b*]fluoranthene | 81.6 | 0.32 | 0.200 |
| Benzo[*k*]fluoranthene | 26.8 | 0.10 | 0.066 |
| Indeno[1,2,3-*cd*]pyrene | 71.9 | 0.26 | 0.160 |
| Pyrene | 33.6 | 0.17 | 0.102 |

cMix = $\Sigma c_i$ = 0.001622 µmol = 1 g SEq

| | Analysed concentration in Bitterfeld sediment | | Fraction of compound in synthetic mixture $p_i$ |
|---|---|---|---|
| | [mg / kg] | [µmol / kg] | |
| Anthracene | 1.1 | 6.2 | 0.026 |
| Benzo[*a*]anthracene | 0.35 | 1.5 | 0.006 |
| Benzo[*ghi*]fluoranthene | 2.47 | 10.9 | 0.046 |
| Fluoranthene | 3.8 | 18.8 | 0.079 |
| *N*-Phenyl-2-naphthylamin | 15.8 | 72.1 | 0.304 |
| Methyl-parathion | 1.6 | 6.1 | 0.026 |
| Phenanthrene | 5.9 | 33.1 | 0.140 |
| 2-Phenyl-naphthalene | 5.0 | 24.5 | 0.103 |
| Prometryn | 6.34 | 26.3 | 0.111 |
| Pyrene | 7.6 | 37.6 | 0.159 |

cMix = $\Sigma c_i$ = 0.2370 µmol = 1 g SEq

Algal toxicity of the identified compounds span over almost four orders of magnitude with indeno[1,2,3-*cd*]pyrene being the most toxic and methyl parathion the least toxic with $EC_{50}$ values of 0.007 and 29.4 µmol / L respectively. Also the slopes of the concentration-response curves are

heterogeneous. Benzo[*ghi*]fluoranthene shows the steepest curve whereas anthracene the shallowest one.

*Table 3.2: Concentration-response relationships of single substances, mixtures and extracts for reproduction inhibition in the unicellular green algae Scenedesmus vacuolatus*

| Substance [a] / mixture | N (C)[b] | $EC_{50}$ [µmol / L] | Standard Error | p | Standard Error |
|---|---|---|---|---|---|
| Indeno[1,2,3-*cd*]pyrene | 31(20) | 0.0042 | 0.0001 | 6.11 | 0.50 |
| Benzo[*a*]pyrene | 26(34) | 0.0070 | 0.0004 | 2.51 | 0.27 |
| Benzo[*k*]fluoranthene | 33(23) | 0.0181 | 0.0005 | 2.74 | 0.19 |
| Benzo[*ghi*]fluoranthene | 25(23) | 0.0437 | 0.0007 | 6.87 | 0.73 |
| Benzo[*a*]anthracene | 15(11) | 0.0579 | 0.0013 | 2.57 | 2.41 |
| Prometryn | 36(6) | 0.0656 | 0.0017 | 2.89 | 0.19 |
| Benzo[*b*]fluoranthene | 27(29) | 0.0884 | 0.0064 | 0.94 | 0.07 |
| *N*-Phenyl-2-naphthylamine | 10(11) | 0.1599 | 0.0019 | 8.19 | 0.69 |
| Fluoranthene | 10(10) | 0.1681 | 0.0049 | 4.78 | 0.64 |
| Pyrene | 55(44) | 0.2459 | 0.0043 | 6.32 | 0.71 |
| 2-Phenyl-naphthalene | 8(10) | 0.3516 | 0.0095 | 7.34 | 1.26 |
| Anthracene | 33(31) | 2.84 | 1.50 | 0.61 | 0.19 |
| Phenanthrene | 31(12) | 3.3612 | 0.0851 | 1.62 | 0.07 |
| Methyl parathion | 36(6) | 29.37 | 0.72 | 3.07 | 0.22 |
| Synth. mixture Bitterfeld[c] | 48(12) | 0.3521 | 0.1951 | 2.6054 | 0.1951 |
| Synth. mixture Brofjorden[c] | 48(12) | 0.0161 | 0.0002 | 7.8485 | 0.6215 |
| Sediment extract Bitterfeld[d] | 10(6) | 0.2384 | 0.0337 | 1.6597 | 0.3439 |
| Sediment extract Brofjorden[d] | 10(6) | 2.8564 | 0.2644 | 1.3938 | 0.1592 |

All experimental data were fitted using the Hill function [eq. 3]. [a]) Substances ordered for decreasing toxicity. [b]) Number of data points used for the fit (number of controls given in brackets). [c]) Parameters refer to total mixture concentration. (for mixture composition see Table 3.1). Concentration-response curves can be transformed to g SEq / L by dividing the parameter $EC_{50}$ by the total mixture concentration (Table 3.1), [d]) Parameters refer to the concentration given in g SEq / L.

### 3.3.3 The challenge of toxicant confirmation

In Figure 3.1, the concentration-response curves of the two investigated sediment extracts are shown. Additionally, plotted concentration-response curves illustrate the effects exerted by the individual compounds. At the $EC_{90}$

of the extract from the Bitterfeld sediment (0.9 g SEq / L), the concentration of prometryn alone would only cause an effect of 7%. A concentration of 2.9 g SEq / L would be necessary to obtain a concentration of *N*-phenyl-2–naphthylamin provoking 90 % effect.

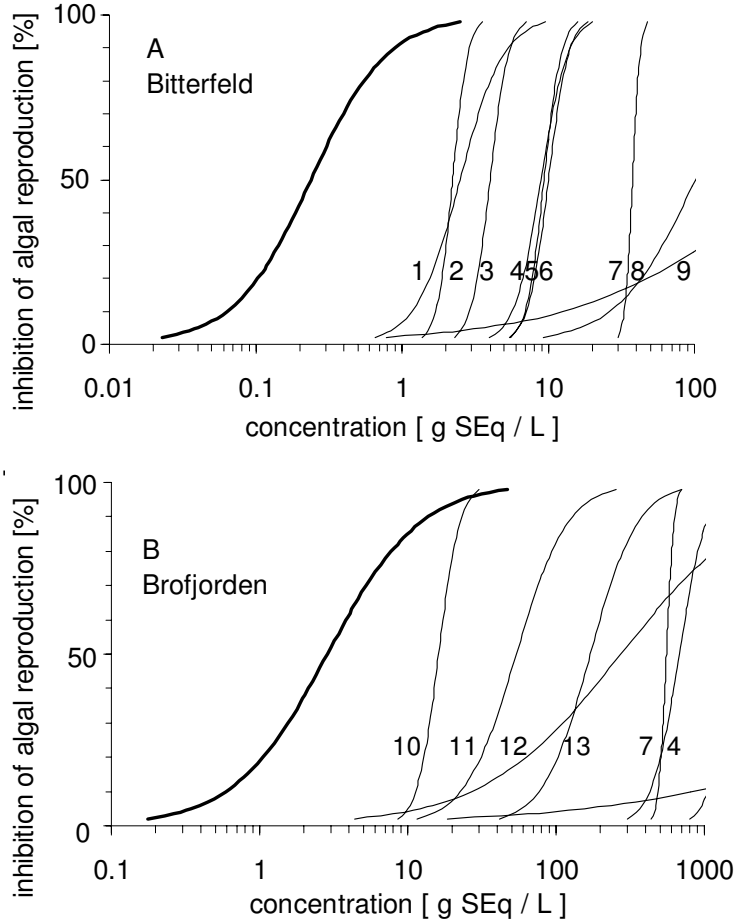


*Figure 3.1: Inhibition of algal reproduction of the extract of the sediment (bold line) taken on the freshwater site in Bitterfeld (A) and the marine site Brofjorden (B). The thin solid lines show the toxicity, which the identified toxicants would provoke if they were present individually. The modelled concentration-response curves refer to concentrations, at which the identified toxicants are present in the extract (for models, functions and parameters see Table 3.2). The numbers refer to the substances as follows: prometryn (1), N-phenyl-2-naphthylamine (2), benzo[ghi]fluoranthene (3), fluoranthene (4), 2-phenylnaphthalene (5), pyrene (6), benzo[a]anthracene (7), phenanthrene (8), anthracene (9), indeno[1,2,3-cd]pyrene (10), benzo[a]pyrene (11), benzo[b]fluoranthene (12), benzo[k]fluoranthene (13). The concentration response curve of methyl parathion is located above the depicted concentration scale.*

This figure illustrates the basic challenge of confirmation in EDA, as one only can state that the extract is more toxic than each individual compound. It is not clear, whether the extract toxicity can be explained by the mixture of the identified toxicants or to what extent. It is, therefore, crucial to assess the combined effect of the mixture of the occurring toxicants.

### 3.3.4 Mixture confirmation using toxic units

A commonly used approach to calculate an expectated mixture toxicity is the TU summation. The TUs of the identified individual compounds are calculated and summed (ΣTU) for each sediment extract. The obtained value can then be compared to the TU observed for the extracts (Table 3.3).

*Table 3.3: Toxic Units of identified toxicants in the sediments*

| | TU of identified toxicants in 1 g sediment | |
|---|---|---|
| | Brofjorden sediment | Bitterfeld sediment |
| Anthracene | 0.0001 | 0.0022 |
| Benzo[*a*]anthracene | 0.0018 | 0.0260 |
| Benzo[*ghi*]fluoranthene | - | 0.2431 |
| Fluoranthene | 0.0015 | 0.1090 |
| *N*-Phenyl-2-naphthylamin | - | 0.4506 |
| Methyl parathion | - | 0.0002 |
| Phenanthrene | - | 0.0099 |
| 2-Phenyl-naphthalene | - | 0.0688 |
| Prometryn | - | 0.3785 |
| Pyrene | 0.0007 | 0.1542 |
| Benzo[*a*]pyrene | 0.0185 | - |
| Benzo[*b*]fluoranthene | 0.0037 | - |
| Benzo[*k*]fluoranthene | 0.0059 | - |
| Indeno[1,2,3-*cd*]pyrene | 0.0620 | - |
| Σ TU | 0.094 | 1.442 |
| Observed $TU_{Extract}$ | 0.350 | 4.194 |
| Ratio ΣTU / $TU_{Extract}$ | 0.27 | 0.34 |

In the Brofjorden sediment extract, 0.35 TU are observed. However, by summing up the TUs of the identified toxicants in the extract, only 0.094 TUs

are obtained. The sum of TUs is dominated by indeno[1,2,3-*cd*]pyrene, which is present in a concentration equalling 0.062 TUs.

For the extract of the Bitterfeld sediment, 4.19 TUs are calculated, whereas the TUs of the identified compounds add up to 1.44 TUs. In this case the TU sum is not dominated by one substance. *N*-phenyl-2-naphthylamine contributes 0.45 and prometryn 0.38 TUs to the ΣTU. Other toxicants are present in 0.0002 to 0.24 TUs.

The commonly calculated quotient of the ΣTU and the $TU_{Extract}$ leads to a ratio which is used as a quantitative measure of how much of the originally observed toxicity of the environmental sample can be attributed to the identified toxicants. In the Brofjorden and the Bitterfeld sediment extracts, identified toxicants account for 27% and 34% of the toxicity of the original sample in terms of effect concentrations using the TU approach, respectively.

### 3.3.5 Mixture confirmation using the Index of Confirmation Quality

The ICQ values were calculated for the different expected combined effects of the models CA and IA and for the observed toxicity of the synthetic mixture. In Figure 3.2, the ICQ values are presented for effect levels between 10 and 90 %. The vertical line at the value of 1 indicates the extract toxicity, which is the reference. This toxicity has to be explained. In case that the concentration-response curve for the observed sample's toxicity and the expected or observed mixture toxicity were identical, the ICQ value would equal 1 on all effect levels. Values below 1 indicate unresolved toxicity.

The ICQ values, which can be considered as a measure for the distance between the extract toxicity and the mixture toxicity of the identified toxicants, vary depending on the effect level and on the mixture toxicity used for the calculation. When comparing the predictions by the two models CA and IA, it has to be stated that for both investigated cases, the calculated $ICQ_{IA}$ values are lower than $ICQ_{CA}$ values for the same effect level. For the Bitterfeld extract, the $ICQ_{CA}$ values vary between 0.15 and 0.8, whereas the $ICQ_{IA}$ values vary from 0.06 to 0.35. This difference reflects the factor of 2.5 to 2.8

between the predictions of the different models. For the Brofjorden extract, the difference between predictions of the two models is smaller compared with the Bitterfeld extract. They differ by a factor of 1.3-1.8 with $ICQ_{CA}$ values from 0.11 to 0.78 and $ICQ_{IA}$ values from 0.06 to 0.60.

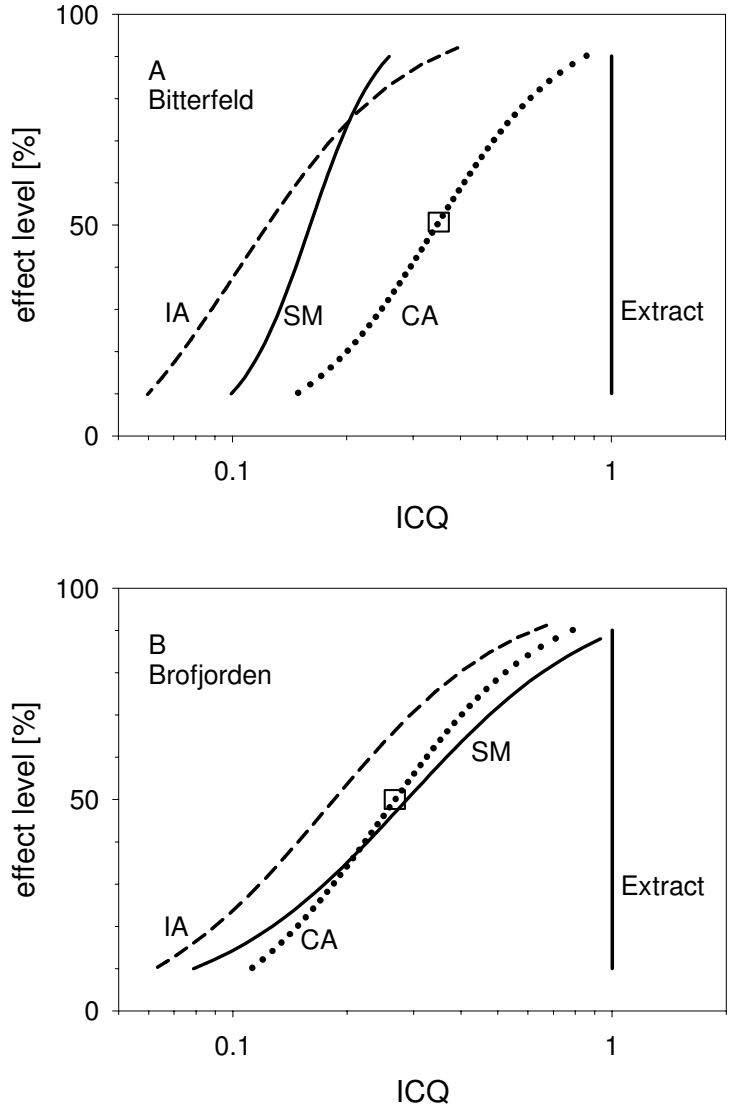


*Figure 3.2: Representation of the Index of Confirmation Quality (ICQ) for effect levels between 10 and 90% (A: Bitterfeld, B Brofjorden). The vertical line at 1 indicates the extract toxicity which is used as a reference and set to 1 for all effect levels. ICQ values are given for the measured toxicity of the synthetic mixture (SM) (solid line); expectated combined effects of identified toxicants according to concentration addition (CA) (dotted line) and independent action (IA) (dashed line). The little square highlights the previously calculated $\Sigma TU$ / $TU_{Extract}$ value.*

For illustration, that the previously calculated value for the ratio ΣTU / $TU_{Extract}$ (Table 3.3) is included in the presentation. It is equivalent to the $ICQ50_{CA}$ value, the ICQ value for CA at the 50% effect level. Note that the ICQ is, therefore, not in contradiction to the established TU approach, but can be considered as an extension to different effect levels and other prediction models.

In addition to the expected mixture toxicities, Figure 3.2 shows the result of the test of the synthetic mixture containing the toxicants as they occur in the extracts. For the extract from Brofjorden, the differences between the $ICQ_{SM}$ and $ICQ_{CA}$ values are small. This indicates that CA predicts the mixture effect of compounds identified in the Brofjorden sediment extract rather well. This is reasonable, as all identified toxicants are PAHs and thus might have a similar mode of toxic action in the algal test system. In case of the Bitterfeld extract, the $ICQ_{SM}$ values are closer to the $ICQ_{IA}$ than to the $ICQ_{CA}$, which indicates that IA provides the better prediction of the combined effect of the mixture. This is again not surprising, as substances of very different structures and thus possibly heterogeneous modes of action were detected in the extract from the Bitterfeld sediment.

## *3.4 Discussion*

The aim of this study was to improve the methodology for the confirmation of mixtures of toxicants in effect-directed analysis procedures by using the available knowledge on the calculation of expected combined effects. In this study confirmation of mixtures is addressed by comparison of the sample's toxicity with the toxicity of the mixture of the identified toxicants. The toxicity of the sample is usually known, as it is the starting point of the effect-directed fractionation procedure; however, the toxicity mixture of the identified toxicants has to be assessed. For this purpose three different approaches have been applied in this study, which included two models and one experimental assessment of the mixture toxicity.

The discussion considers the different approaches for the confirmation of identified compounds in effect-directed analysis: TU summation, mixture toxicity prediction with CA and IA and mixture testing. The approaches will be discussed with respect to accuracy, sources of systematic errors, precision, experimental requirements (expenses, time) and possibilities for inference.

As mentioned above, the key question of confirmation is how much of the sample's toxicity can be attributed to the identified toxicants. In order to calculate the contribution of the mixture of these toxicants to the overall toxicity of the investigated sample, it is essential to know what effects are provoked by this mixture. A straightforward approach to assess this toxicity is to prepare a synthetic mixture and test it in the used bioassay. Although this approach has several drawbacks, e.g., the additional experimental efforts, it can be considered to give a correct result which is only biased by experimental variance. Its usefulness as a confirmation approach will be discussed later. In this study, it is additionally used as a reference for the evaluation of the accuracy of approaches based on modelling.

### 3.4.1 Accuracy of the toxic unit summation approach

The TU approach is widely used. However, it has to be clarified, whether it provides reasonably accurate results and whether it can be used as a default approach for the confirmation step. It was shown that the $EC_{50}$ of the synthetic mixture of the identified toxicants of the Brofjorden sediment was in good agreement with the expectation value according to TU summation. However, for the other studied case (Bitterfeld), the corresponding expectation value overestimated the toxicity of the synthetic mixture by a factor of 2. This result indicates that the widely used TU summation may not be universally applicable to the confirmation step in EDA procedures. As an alternative approach for cases where "toxicants are (…) non-additive", it was proposed to include the TU value of the "major toxicant (the one present in the most TUs)" only in the confirmation step (Mount *et al.* 1993). However, for the sample from Bitterfeld, the toxicity of the synthetic mixture is clearly higher

than the effect of the most toxic compound in the mixture, in this case *N*-phenyl-2-naphthylamin. Considering just this substance to contribute to the mixture effect would result in an underestimation of the mixture toxicity by a factor of 2.

The result of less than “concentration additive behaviour” of the mixture composed of compounds identified in the Bitterfeld sediment may be explainable in the case that not all substances in the mixture act via a common mode of action. It has been reported that CA gives good predictions for mixtures of similarly acting compounds, but it tends to overestimate the combined effects of mixtures of dissimilarly acting compounds (Faust *et al.* 2000). In this case, little is known about the individual modes of action of the identified toxicants, e.g. *N*-phenyl-2-naphthylamine or benzo[*ghi*]fluoranthene to *Scenedesmus vacuolatus*. It is thus not clear, whether it is reasonable to assume either similar or dissimilar modes of action for these compounds. However, the less than “concentration additive behaviour” of the mixture toxicity may be regarded as an indication that distinctly different modes of actions are present.

In environmental samples, the presence of substances with dissimilar modes of action seems to be a common situation rather than an exception. Ho *et al*. (2002) summarise 13 studies on sediment toxicant identification by classifying the identified causes of toxicity into organics, metals and ammonia. They conclude that within a single sample usually multiple causes of toxicity prevail; not just one chemical class is active. This goes in line with Mount *et al*. (1993) who state in the guidance document on the confirmation step that TIE studies conducted at the Environmental Research Lab Duluth have shown that “often toxicants are not additive”.

Interestingly, EDA studies that address confirmation by TU summation and testing of a synthetic mixture often find that TU summation predicts a higher toxicity than what is actually observed in the synthetic mixture or the sample. Boxall and Maltby (1997) identified three PAHs as cause of toxicity to *Gammarus pulex* in motorway runoff. They observed 2.6 TUs by testing the

synthetic mixture, whereas 3.6 TUs were expected from summing up TU values of the three identified toxic PAHs. Svenson *et al*. (2000) identified four fatty acids and two monoterpenes to be responsible for the inhibitory effects on the nitrification activity of the bacteria *Nitrobacter* in wastewater from a plant for drying wood-derived fuel. The toxicity of the synthetic mixture composed of six dominant toxicants agreed well with the toxicity of the original sample. However, at the $EC_{50}$ of the sample, they calculated a sum of toxic units of the identified toxicants of 1.96. This value has to be understood as less than “concentration additive behaviour” of the single compounds, as a TU sum of 1 would have to be expected for “concentration additive behaviour”. Burkhard and Durhan (1991) identified three insecticides in effluents to cause toxicity to *Ceriodaphnia dubia*. They found TUs of the three identified toxicants to add up to 4.13, however, this value was higher than the original toxicity of the effluent of 2.87 TUs. Kosian *et al*. (1998) found a sum of 3.3 TUs in the six fractions obtained from a sediment pore water sample with the freshwater oligocheate *Lumbriculus variegatus*. This was more than the 1.4 TUs observed in the original sample.

It may thus be concluded that there are several indications that CA may systematically overestimate the toxicity of mixtures of compounds identified in EDA procedures as a result of dissimilar modes of action of the components. Therefore, one has to be aware that application of TU for confirmation of mixtures of compounds with unclear mode of action implies that one uses a tool which is possibly systematically biased.

As TU summation has gained wide acceptance in aquatic toxicology, it is of interest to know what this systematic bias implies for the confirmation step. For many purposes an overestimation of the combined effects of a mixture is not a matter of concern. For example in risk assessment, an overestimation of the expected toxicity results in an additional safety. However, when aiming to explain the observed toxicity of an environmental sample, approaches for the prediction of mixture toxicity basing on concentration addition, e.g. TU summation, might overestimate the combined effects of the identified

toxicants. They, therefore, bear the risk of overlooking additional toxicants that may be present in the sample. This can be illustrated for the extract from the Bitterfeld sediment. In this case, TU summation overestimated the toxicity of the synthetic mixture by a factor of two. Thus using the TU sum for the confirmation would result in an underestimation of the unresolved toxicity.

### 3.4.2 Expected mixture toxicity assuming similar and dissimilar modes of action

In this study the mixture toxicity was in one case predictable by concentration addition, in the other case by independent action. It is plausible that indeed both cases occur, as in the environment mixtures of compounds with similar as well as with dissimilar modes of action may be present. When similarly and dissimilarly acting compounds are present simultaneously, one would expect an intermediate toxicity, that is to say less than expected from CA and more than expected from IA. However, neither higher toxicity than predicted from CA nor lower toxicity than predicted from IA would be expected for non-interacting compounds. Thus, a prediction window spanned between the expected concentration-response curves according to CA and IA can be proposed which thus may be used for the confirmation step in EDA procedures. By inclusion of independent action as an alternative non-interaction model in addition to the commonly used TU approach, one may account for unknown modes of action of the identified toxicants in the confirmation step. In the guidance document on TIE – Phase III (Mount *et al.* 1993), the authors state that “if substances appear to be partially additive, then very careful work is required to properly add TUs”. However, no guidance is provided how to determine “partial additivity” of the compounds or how to “properly add TUs” in such a case. The calculation of ICQ values using mixture effects expected from CA and IA may be considered as an approach to address this open point.

It would be interesting to evaluate, whether also in the above mentioned studies (Svenson *et al.* 2000; Burkhard and Durhan 1991; Boxall and Maltby

1997), which overestimated the mixture toxicity by using CA, the calculation of a prediction using IA would have been better suited to describe the experimental data. However, for these calculations whole concentration-response curves of the samples as well as of the single compounds are required, which are typically not reported.

Apart from the assumptions on the mode of action, the TU summation approach has another limitation, as it is calculated for one effect level only, commonly the $EC_{50}$. However, concentration-response curves are reported to differ in shape and slope. The heterogeneity of these parameters may result in different conclusions on confirmation depending on the regarded effect level. In this study, the distances between the mixture toxicity and the extract toxicity are smaller for higher effect levels than for lower effect levels. Thus, the judgement on confirmation varies between effect levels. This can also be expressed quantitatively, as for the Brofjorden extract it was shown that on the $EC_{50}$ level the factor between the sample's toxicity and the toxicity of the synthetic mixture ($ICQ50_{SM}$) is about 0.27. However, on the $EC_{10}$ level ($ICQ10_{SM}$) it is only 0.08, whereas on the $EC_{90}$ level ($ICQ90_{SM}$) it is almost 1. The latter could be interpreted as complete confirmation, while on the $EC_{10}$ level only a minor contribution to the extract toxicity might be assumed. Thus from the same experimental data, different conclusions could be drawn, depending on the effect level considered. A reason for such behaviour may be sought in multiple modes of action of components. While at low concentrations, specific modes of action prevail, which in effect are dissimilar, at higher concentrations an unspecific narcotic mode of action might lead to "concentration additive behaviour". When using only one approach such as a TU sum on the $EC_{50}$ level, only one value is taken as a basis for the judgement on confirmation. Intuitively, it may not be appealing to exchange a convenient value ($\Sigma TU / TU_{Extract}$) by a range of values which are more difficult to interpret and seem to add to ambiguity. However, it has to be stressed that one has to be aware that this ambiguity is already inherent in the data, but it can just not be realised by performing TU summation. I, therefore, propose a

confirmation on a range rather than on a single effect level, as this helps in judging on the consistency of the assessment.

### 3.4.3 Experimental requirements and possibilities for inference

One major disadvantage of the seemingly simple approach of testing a synthetic mixture containing the same amount of identified toxicants as the original sample is that additional experiments are required. This is becoming important when several samples are investigated. For each sample, a specific mixture would have to be composed and tested. Additionally, the possibilities for inference of this approach are very low. The result may be correct and relevant for one specific case; however, general conclusions regarding other mixture ratios cannot be drawn.

One advantage of the approach using IA and CA for the calculation of whole concentration-response curves is that the experimental design and amount of data needed are the same as compared to the TU summation approach. Commonly, concentration-response curves are fitted to the experimental data in order to estimate an $EC_{50}$. When confirmation is performed with whole concentration-response curves, this reduction to one value is not needed; rather the whole curve may be used for the calculations. Calculations are more extensive compared with commonly performed TU summation; however, they can easily be automated with common table calculation software. Once established, they require only few additional efforts. However, the use of toxicity data from the literature might be limited as concentration-response curves are needed which are often not reported.

### 3.4.4 Variance

The variance of the low-effect levels may become important, especially when the model of independent action is used to calculate expectated combined effects, as for this model absolute effects are used for the calculation. Therefore, special efforts for accurate quantification of low effect concentrations might be needed (Scholze *et al.* 2001). However, one should be aware that joint toxicity quantification is only one of several sources of

uncertainty in EDA studies. Additional variance may be due to matrix effects, incomplete recoveries of fractionation steps, losses due to incomplete dissolution or evaporation during solvent exchanges and the often low quantitative accuracy of non-target analysis.

### 3.4.5 Conclusions

For the confirmation step in EDA studies TU summation should only be used, when strong indication is given that toxicants act via a similar mode of action. Even then it is only a point estimate which does not allow the evaluation of the consistency of the data for other than the considered effect level.

Therefore, in cases where modes of toxic action are unclear or evaluation of consistency is the aim, to calculate expected combined effects using CA and IA for an effect level range, e.g., $EC_{10}$ to $EC_{90}$ can contribute to overcome the inherent limitation of the TU approach. The ICQ introduced here is a suitable way to transfer the data into an easily readable form. Quantification of confirmation can thus be addressed for different effect levels and unknown modes of action.

# Chapter 4

## Light as a confounding factor for toxicity assessment of complex contaminated sediments*

### *4.1 Introduction*

As shown in chapter 3, algal toxicity caused by a marine sediment extract from Brofjorden, Sweden, was partially attributed to a mixture of 8 identified PAHs. Algal toxicity induced by a sediment extract form Spittelwasser, Bitterfeld, was partially attributed to a mixture of 10 compounds, six of which are PAHs.

The toxicity of many PAH compounds has been reported to be dependent on the light conditions. This phenomenon known as “light enhanced toxicity” or phototoxicity has been reported for many PAHs in bacteria (Arfsten *et al.* 1994; El-Alawi *et al.* 2002), crustacea (Newsted and Giesy 1987), fish (Oris and Giesy 1987) and the higher plant *Lemna gibba* (Huang *et al.* 1997). It is attributed for the ability of some compounds to form excited states through the absorption of light in the visible or ultra violet region. These excited states are amenable to chemical reactions. Two different mechanisms are discussed to be responsible for light enhanced toxicity. Oxidation reactions of the excited PAHs with dissolved oxygen may occur, resulting in metabolites with different chemical properties and often altered bioactivities. This mechanism

* Parts of this chapter are in press as publication in *Environmental Toxicology and Chemistry*

called photomodification has been demonstrated to be relevant to photoinduced toxicity of some PAH compounds to *Lemna gibba* (Huang *et al.* 1995; Huang *et al.* 1997) and *Vibrio fischeri* (Brack *et al.* 2003). In contrast, photosensitisation describes the reaction where the excited PAH molecule transfers its energy to dissolved oxygen which results in the formation of singlet-state oxygen which is capable of oxygenating many different biomolecules, altering their chemical structure, and consequently modifying their normal function (Larson and Berenbaum 1988).

The assessment of algal toxicity of environmental samples and single compounds is typically based on test protocols, designed for optimal conditions for algal reproduction. These test conditions often comprise illumination which provides photosynthesis active radiation (PAR), produced by fluorescent tubes emitting a discontinuous light spectrum. However, these illumination conditions differ qualitatively from natural light sources, i.e., natural sun light, which is characterised by a continuous light spectrum including ultra-violet (UV) radiation.

Although phototoxicity has been demonstrated for single PAH compounds, it is not clear whether it is relevant to the toxicity assessment of environmental samples. The presence of substances exerting photoenhanced toxicity in an environmental sample may be considered as an indication that the use of realistic illumination conditions (i.e., simulated sun light) might affect the toxicity assessment of the sample. However, as PAHs are often present in mixtures with low concentrations of individual compounds and are often accompanied with other toxicants, it is not clear whether phototoxic effects are observable in environmental samples or whether they are typically superimposed by other factors.

As concluded in chapter 3, environmental exposure typically is characterized by the presence of toxicant mixtures rather than single compounds. For the prediction of combined effects of chemical mixtures two models have been proposed. Concentration addition provides good predictions of combined effects of compounds with similar modes of action,

whereas independent action provides good predictions for mixtures of compounds with dissimilar modes of action (Faust *et al.* 2000). Phototoxicity cannot be considered as a distinct mechanism of action. As formation of metabolites through photomodification as well as oxidative damage induced by photosensitisation of the PAH may contribute to toxicity of each mixture constituent, it is not clear whether the combined mixture effect is predictable by one of the proposed models CA or IA, or whether it exerts even synergistic behaviour and is not predictable at all.

The aim of this study was to assess the influence of the use of more realistic light conditions on the toxic potency of two environmental samples, previously investigated using effect-directed analysis as references for a low contaminated site and a site with complex contamination from former chemical production. The consequences of light dependence of toxicity of samples and toxicants on the confirmation step are to be assessed. The proposed models CA and IA were to be tested for their suitability to predict combined effects of mixtures of phototoxic compounds identified from the sediment extracts under realistic light conditions. Furthermore, it was aimed to address the question, whether light enhanced toxicity of environmental samples commonly occurs, or whether it can be considered as an exceptional phenomenon, due to special contamination patterns in the investigated samples.

For this purpose the test system using *Scenedesmus vacuolatus* was modified, in order to conduct reproduction tests under variable light conditions. Algal toxicity of sediment extracts was assessed under standard growth light and simulated sun light. In order to validate the predictive power of the models, identified compounds were tested individually and in mixtures under simulated sun light. Results were compared to expected combined effects calculated from CA and IA. Finally, light dependent toxicity of sediment extracts from different sites in the river Elbe and its tributaries Mulde and Spittelwasser was assessed.

## 4.2 Materials and Methods

### 4.2.1 Algal toxicity test using different illumination sources

Algal toxicity was measured using the previously described test system. The toxic endpoint was the inhibition of the cellular reproduction of the unicellular green algae *Scenedesmus vacuolatus* during one generation cycle lasting 24 h according to the procedure described in 6.3.1. Samples were tested in geometric dilution series with a constant final DMSO concentration of 0.1 % in the test solution. Spacing between test concentrations was flexibly adapted to the steepness of each concentration–response curve, in order to cover the range between $EC_{10}$ and $EC_{90}$.

Three different light conditions were chosen, in order to assess light influence on the algal toxicity of the samples and identified toxicants. One illumination condition was the standard light condition used for the bioassay as established in our laboratory protocol. This illumination was originally designed for optimal algal growth and is characterised by a discontinuous spectrum with peaks at 430, 550 and 610 nm. The alternative light conditions used were chosen in order to obtain an environmentally more realistic light regime. For this purpose a lamp was used which is designed to mimic natural sun light. Additionally, as a reference case aiming to minimise the influence of light on the toxicity of environmental samples, a filter was added to the same lamp which completely eliminated the UV and reduced the visible region. This was the lowest irradiation which still allowed a sufficient cell reproduction. A complete elimination of light was obviously not possible, as algae need light for photosynthesis to cover their basic needs of energy supply. Details on the different light conditions are provided in 6.3.1.

### 4.2.2 Test solutions

*Environmental samples*

Samples investigated using EDA have previously been described (Brack *et al.* 1999; Grote *et al.* 2005). These two sediment extracts from

Spittelwasser, Bitterfeld, Germany and Brofjorden, Sweden were tested under three different light conditions. Additionally, 13 sediment extracts from transect sites from the river Elbe and its tributaries Mulde and Spittelwasser sampled in march and April 2003 (Grote *et al.* 2004) were tested under standard light conditions and UV-filtered light. Furthermore, a preparative fractionation of these samples using open column chromatography was conducted in order to separate PAH from other compounds present in the extracts. For this purpose samples were fractionated as described in chapter 2.2.2. The fraction 2 (F2) containing PAH compounds was tested under the same conditions as the extracts.

All concentrations referring to the sediment extracts are denoted as sediment equivalents (SEq). One g SEq / L corresponds to the amount of compounds extracted from 1 g dry sediment and dissolved in 1 L medium.

*Single compounds*

Dilution series of all identified toxicants from Spittelwasser (Brack *et al.* 1999) and Brofjorden (chapter 2) were tested under simulated sun light, additionally to the tests previously conducted under standard growth light (chapter 2). Concentration response curves were modelled using the Hill model assuming logistic distribution of the data [eq. 3].

*Mixtures*

Mixture testing was designed to:

- test the prediction models for their applicability to mixtures of phototoxic compounds
- assess the contribution of the identified compounds to the observed phototoxic effects induced by the environmental samples.

For testing the applicability of the prediction models, compounds identified in Brofjorden were composed to a mixture in the ratio of their individual $EC_{10}$ values, thus achieving comparable contributions of the different compounds in the low effect range (equitoxic mixture ratio). For the assessment of

contributions of identified toxicants to the toxicity induced by the samples, compounds were composed to a synthetic mixture at ratios as detected in the sediment extracts (sediment concentration ratio). These results were also used for the toxicant confirmation. Fractional composition of all mixtures tested is summarised in Table 4.1.

*Table 4.1: Fractional composition ($p_i$) of tested mixtures on a molar basis. Mixtures composed from identified toxicants from Brofjorden and Bitterfeld in either equitoxic ratio or at the ratio identified in the sediment (Sed. conc. ratio). Values denote the fraction of the compound in the total mixture concentration.*

| | abbreviation | Brofjorden equitoxic mixture ratio | Brofjorden Sed. conc. ratio | Bitterfeld Sed. conc. ratio |
|---|---|---|---|---|
| Anthracene | ANT | 0.42 | 0.18 | 0.026 |
| Benzo[*a*]anthracene | B[a]A | 0.08 | 0.06 | 0.006 |
| Benzo[*a*]pyrene | B[a]P | 0.012 | 0.08 | - |
| Benzo[*b*]fluoranthene | B[b]F | 0.04 | 0.20 | - |
| Benzo[*ghi*]fluoranthene | B[ghi]F | - | - | 0.046 |
| Benzo[*k*]fluoranthene | B[k]F | 0.09 | 0.07 | - |
| Fluoranthene | FLU | 0.20 | 0.15 | 0.079 |
| Indeno[1,2,3-*cd*]pyrene | IP | 0.01 | 0.16 | - |
| Methyl-parathion | PARAT | - | - | 0.026 |
| *N*-Phenyl-2-naphthylamin | PNA | - | - | 0.304 |
| 2-Phenyl-naphthalene | 2-PN | - | - | 0.103 |
| Phenanthrene | PHE | - | - | 0.140 |
| Prometryn | PROM | - | - | 0.111 |
| Pyrene | PYR | 0.15 | 0.10 | 0.159 |

### 4.2.3 Prediction of combined effect of mixtures

Predictions of combined effects of the compounds were calculated using the models of CA and IA as described in 6.4.2. For the comparison of predictions derived from the reference models to observed combined effects of mixtures, the index of prediction quality (IPQ) is used (Altenburger *et al.* 1996). It is calculated according to:

for $EC_{predicted} > EC_{observed}$, $IPQ = (EC_{predicted}/EC_{observed}) - 1$, and

for $EC_{predicted} < EC_{observed}$, $IPQ = - (EC_{observed}/EC_{predicted}) + 1)$. *[eq. 7]*

This measure provides an easily interpretable linear scale for judgement of accuracy of predictions. The IPQ can be used for both prediction models. In the case that only CA is regarded, the IPQ is equivalent to Marking's additivity index (Marking 1985) and the cTEI (corrected toxicity enhancement factor) from Warne and Hawker (1995).

For confirmation of toxicants identified through effect-directed analysis, contribution of the mixture of identified compounds to the observed effects induced by the studied samples had to be assessed. For this purpose, the index of confirmation quality (ICQ) was used as described in chapter 3. It provides an effect level dependent quantitative measure for confirmation.

## *4.3 Results*

### 4.3.1 Influence of light conditions on toxicity of sediment extracts

Two sediment extracts, for which EDA revealed PAHs as major contributors to toxicity, were investigated under three different light conditions. Concentration-response relationships are shown in Figure 4.1. Functions of modelled curves are given in Table 4.2. Toxicity of the two sediment extracts strongly depended on the light conditions. When tested under simulated sun light, an increase in toxicity by a factor of 10 to 15 can be observed for Brofjorden and Bitterfeld sediment extracts, compared to toxicity observed under standard growth light. When tested under UV-filtered light, both environmental samples induced much weaker toxicity than under standard growth light. For the sediment extract from Bitterfeld, an inhibition of algal reproduction of no more than 50% was observed for concentrations of up to 0.01 g SEq / L. For the Brofjorden sediment extract, no inhibition of algal reproduction was observed for concentration of up to 0.1 g SEq / L.

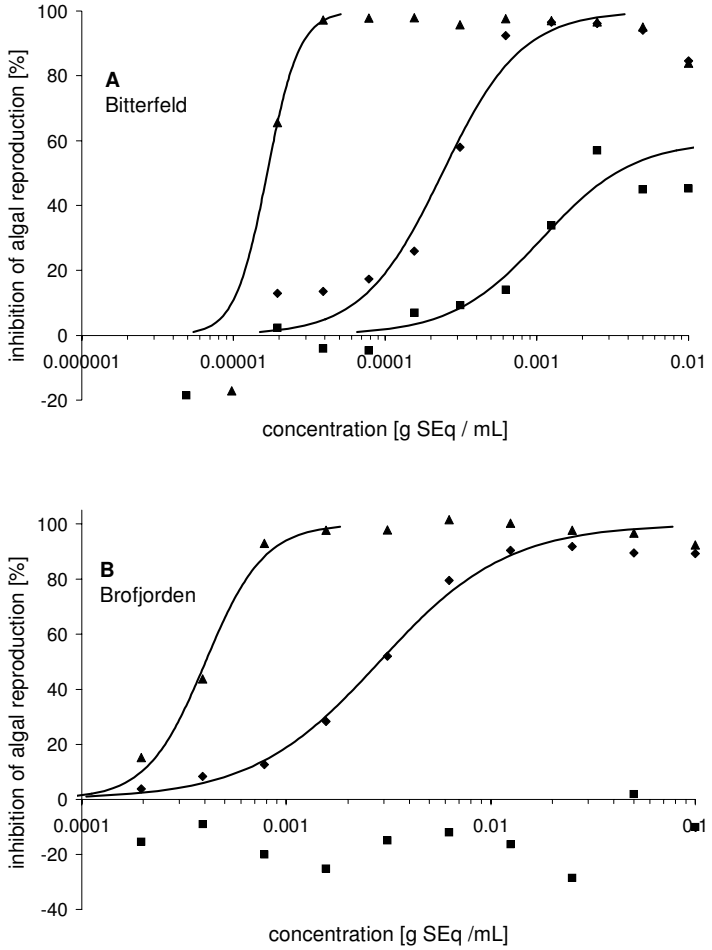


*Figure 4.1: Toxicity of investigated extracts under simulated sun light (▲), standard growth light (♦) and UV-filtered light (■): Spittelwasser, Bitterfeld (A), Brofjorden, Sweden (B). Functions of fitted concentration-response relationships are given in Table 4.2.*

The results demonstrate that the toxicity of the samples strongly depends on the light condition, although identified toxicants, suspect to induce photoenhanced toxicity, only partially contributed to the extract toxicity under standard growth light. Interestingly, the Brofjorden extract, in which exclusively PAH compounds were identified as toxicants under standard light conditions, did not cause effects when tested under UV-filtered light. The Bitterfeld extract, in which additionally to PAHs specifically acting compounds like prometryn and *N*-phenyl-2-naphthylamine were identified as contributors to toxicity, caused effects under UV-filtered light. This indicates the presence

of toxicants exerting their effect via a mode of action independent from the light conditions.

### 4.3.2 Influence of light conditions on toxicity of individual toxicants

In order to clarify, whether this increased toxicity under simulated sun light was due to just one or several of the identified compounds, or whether possibly other toxicants not yet identified were also contributing to the extracts' toxicity, the influence of simulated sun on the toxicity of the identified toxicants was investigated. Exemplarily, experimental data for three compounds are depicted in Figure 4.2. Concentration-response relationships for all identified toxicants in Brofjorden and Bitterfeld sediment extracts are reported in Table 4.2.

The toxicity of all identified PAH compounds (except phenanthrene and 2-phenylnaphthalene) was increased when tested under simulated sun light. The pronouncement of this effect varied between PAH compounds. For indeno[1,2,3-*cd*]pyrene the $EC_{50}$ decreased from 4.2 nmol / L under standard growth light condition to 2.4 nmol / L under simulated sunlight. The toxicity of anthracene shifted by more than one order of magnitude. $EC_{50}$ values of 2.84 µmol / L and 0.10 µmol / L were observed under standard growth light and simulated sun light, respectively. The order of toxicity of compounds changed under simulated sun light compared to standard growth light. While under standard light benzo[*b*]fluoranthene was less toxic than benzo[*k*]fluoranthene, it induced higher toxicity under simulated sun light.

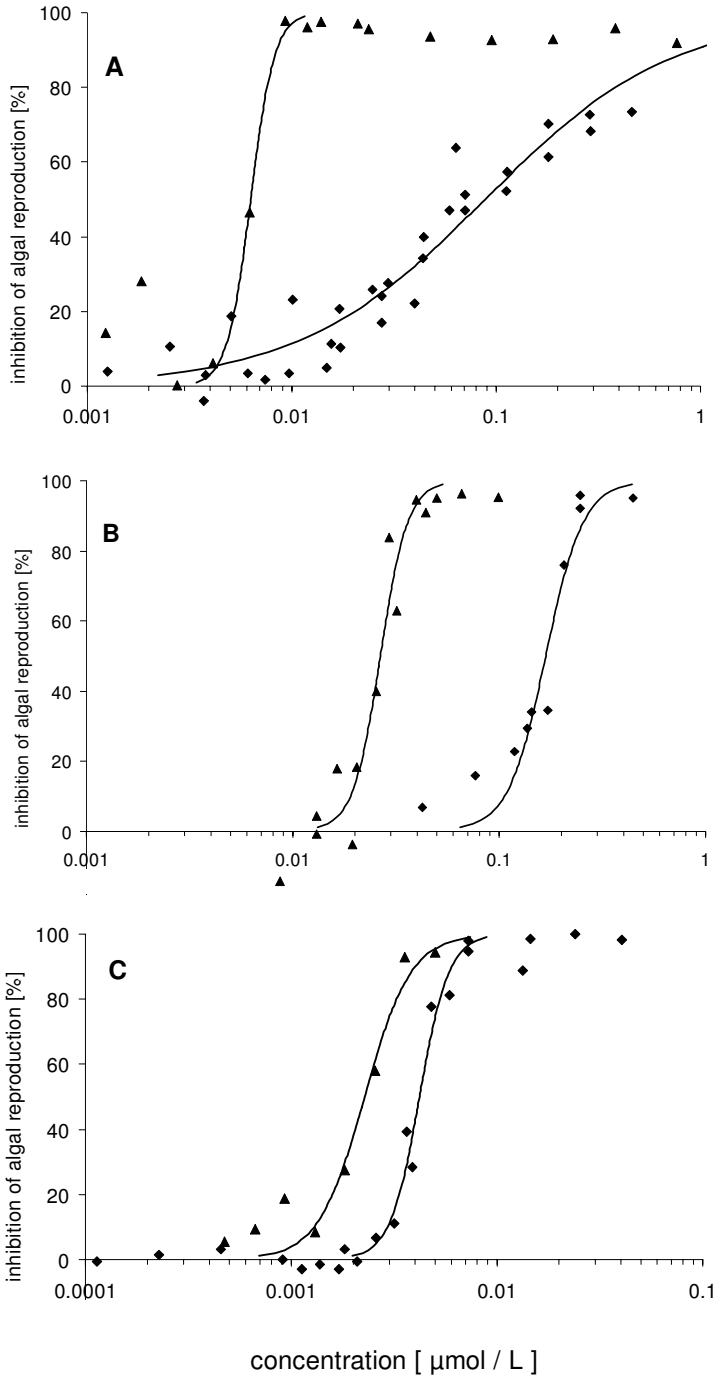


*Figure 4.2: Inhibition of algal reproduction induced by benzo[b]fluoranthene (A), fluoranthene (B) and indeno[1,2,3-cd]pyrene (C) under simulated sun light (▲) and standard growth light (♦). Functions of fitted concentration-response relationships are given in Table 4.2.*

*Table 4.2: Concentration-response relationships of compounds identified as major toxicants in Brofjorden or Bitterfeld sediment extracts, synthetic mixtures and extracts under standard growth light and simulated sun light.*

| | Standard growth light[d] | | Simulated sun light | |
|---|---|---|---|---|
| | $EC_{50}$ [µmol / L] | P | $EC_{50}$ [µmol / L] | P |
| Anthracene[a,b] | 2.84[c] | 0.61 | 0.104 | 3.78 |
| Benzo[*a*]anthracene[a,b] | 0.0579 | 2.57 | 0.0115 | 5.61 |
| Benzo[*a*]pyrene[b] | 0.0070 | 2.52 | 0.0025 | 3.14 |
| Benzo[*b*]fluoranthene[b] | 0.088 | 0.94 | 0.0063 | 7.52 |
| Benzo[*ghi*]fluoranthene[a] | 0.0437 | 6.87 | 0.0102 | 5.20 |
| Benzo[*k*]fluoranthene[b] | 0.018 | 2.75 | 0.0132 | 7.71 |
| Fluoranthene[a,b] | 0.1681 | 4.78 | 0.027 | 6.58 |
| Indeno[1,2,3-*cd*]pyrene[b] | 0.0042 | 6.11 | 0.0023 | 3.87 |
| *N*-Phenyl-2-naphthylamin[a] | 0.099 | 5.56 | 0.25 | 4.91 |
| Parathion-methyl[a] | 29.4 | 3.07 | 21.1 | 2.87 |
| Phenanthrene[a] | 3.34 | 1.62 | 3.79 | 1.99 |
| Prometryn[a] | 0.066 | 2.89 | 0.051 | 2.61 |
| Pyrene[a,b] | 0.25 | 6.33 | 0.035 | 3.70 |
| 2-Phenylnaphthalene[a] | 0.28 | 1.89 | 0.36 | 4.21 |
| | | | | |
| Brofjorden equitoxic mix[e] | - | - | 0.021 | 7.84 |
| Brofjorden sed. conc. ratio mix[e] | 0.016 | 7.85 | 0.0076 | 5.19 |
| Bitterfeld sed. conc. ratio mix[e] | 0.352 | 2.61 | 0.062 | 4.70 |
| | | | | |
| Brofjorden sediment extract[f] | 2.86 | 1.3938 | 0.035 | 2.89 |
| Bitterfeld sediment extract[f] | 0.24 | 1.6597 | 0.015 | 3.02 |

Experimental data were fitted using a logistic fit model [eq. 3]. [a,b]) Compounds present in the mixtures composed from toxicants identified in the [a]Bitterfeld, [b]Brofjorden sediment extracts [c]) In this case the $EC_{50}$-value was constrained to a previously determined value using a best-fit approach (Altenburger *et al.* 2004). [d]) Results of these experiments have previously been reported (chapter 3). Values given here are based on nominal concentrations. They are reported in order to facilitate comparison to values observed under simulated sun light condition. [e]) Parameters refer to total mixture concentration. (for mixture composition see Table 4.1). [f]) Parameters refer to the concentration given in g SEq / L.

The toxicity of the non-PAH compounds prometryn and parathion-methyl, and of the PAHs phenanthrene and 2-phenylnaphthalene was not significantly altered by simulated sun light. Therefore, it can be concluded that the increased toxicity, observed under simulated sun light, was due to properties of distinct compounds and not result of an increased sensitivity of the algae exposed to the different light conditions. *N*-phenyl-2-naphthylamin was slightly less toxic under simulated sun light compared to standard growth light. One possible explanation could be the light induced degradation of the compound, however this hypothesis remains to be verified using chemical analysis.

As inferred from Figure 4.2(A) the use of simulated sun light conditions not only increased the toxicity of the PAH compounds, but may additionally influence the slopes of the concentration-response relationships of some compounds. This phenomenon is illustrated in Figure 4.3, where the ratio of the $EC_{90}$ to the $EC_{10}$ is plotted, a measure for the steepness of a concentration-response curve.

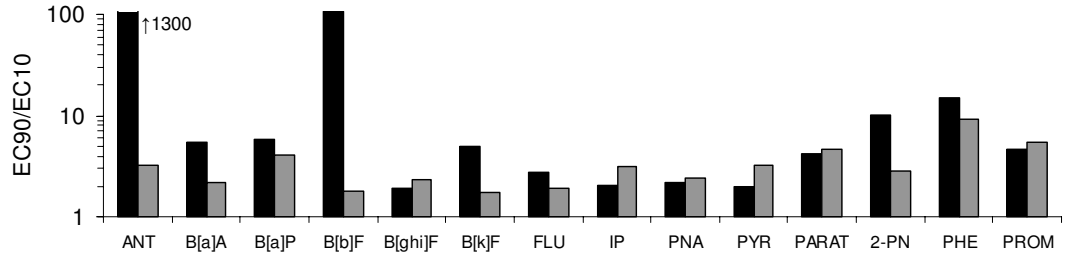


*Figure 4.3: Ratio of $EC_{90}/EC_{10}$ of compounds exerting photoenhanced toxicity observed under standard growth light (■) and simulated sun light (■). High value stands for shallow concentration-response relationships, low values for steep relationships. (For abbreviations see Table 4.1.)*

It turns out that for anthracene and benzo[*b*]fluoranthene a drastic increase in the steepness of the concentration-response relationship was observed. Also the slopes calculated for benzo[*a*]pyrene and benzo[*k*]fluoranthene are steeper under simulated sun light.

### 4.3.3 Predictability of photoenhanced mixture toxicity

In order to investigate, whether the models for mixture toxicity prediction are capable of assessing combined effects of phototoxic compounds, different mixtures were investigated. In order to test, if the models work in principle, one equitoxic mixture was created from toxicants identified in the Brofjorden sediment extract. In this mixture, compounds can be expected to contribute to the combined effects in an approximately similar extent. Observed toxicity induced by the equitoxic mixture is presented in Figure 4.4.

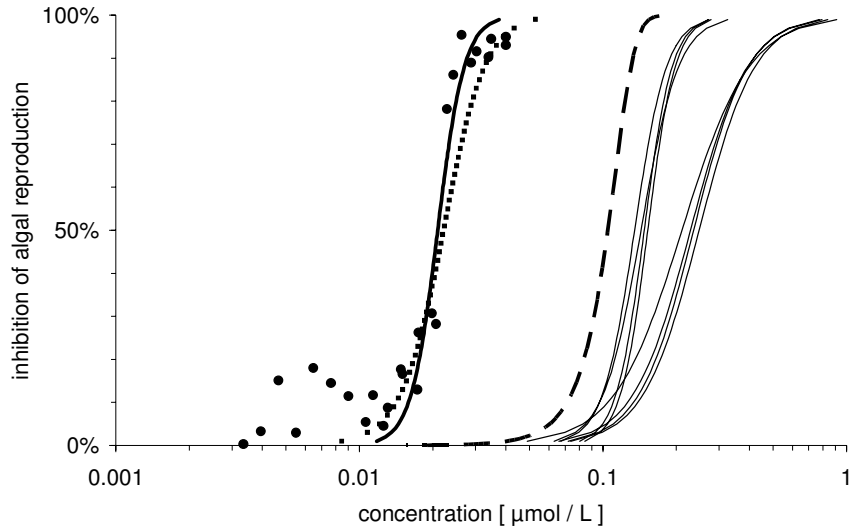


*Figure 4.4: Expected and observed toxicity of the mixture of compounds identified in the Brofjorden sediment extract, mixed at equitoxic ratio. Experimental data (●), fitted experimental data (──), expected toxicity according to CA (▪▪▪), expected toxicity according to IA (– –) and toxicity of the individual compounds (effects produced by the individual compounds present in the mixture) (——) are shown. The concentration axis refers to the total mixture concentration. For concentrations of individual compounds total mixture concentration has to be multiplied by $p_i$ (Table 4.1).*

The results reveal that the combined effects of the equitoxic mixture are almost exactly predicted by the CA model. The prediction by independent action clearly underestimates the combined effects. Interestingly, the whole concentration-response relationship of the mixture can be observed in a concentration range where the respective individual compounds cause no

observable effects. In order to illustrate this phenomenon, the effects induced by the single compounds at the $EC_{90}$ of the mixture are plotted in Figure 4.5.

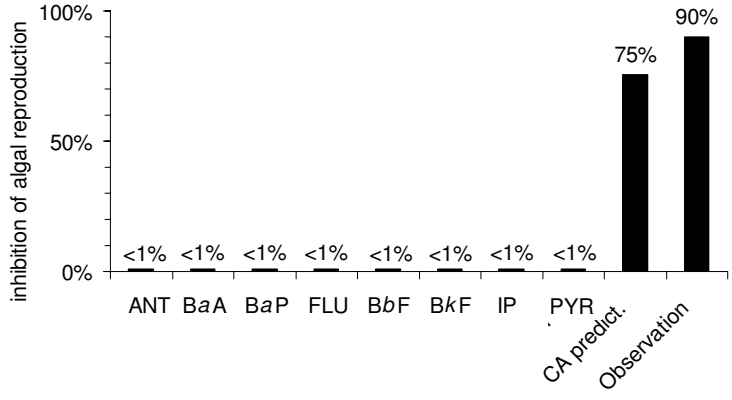


*Figure 4.5: Individual effects of the single compounds of the equitoxic mixture of compounds identified from Brofjorden sediment extract at the mixture concentration exerting 90% effect. Calculation of the individual effects is based on the fitted concentration-response functions. Additionally, the observed and modelled mixture effects are shown. (For abbreviations see Table 4.1.)*

It can be seen that at the observed mixture concentration exerting 90% effect, each of the individual compounds would induce very low effects if present singly. It should be noted that these low individual effects were calculated from the fitted concentration-response relationships of the single compounds as they can commonly not be observed. Estimation of low effect concentrations is a difficult task and may require further modelling efforts (Scholze *et al.* 2001), however, these values illustrate that concentrations of the single compounds would not induce an observable effect. Contrarily, the combined effect predicted by the model of concentration addition (75% inhibition of reproduction) is in reasonable agreement with the observation of 90% inhibition.

In order to test, whether the models for the prediction of combined effects are also capable of predicting effects of more realistic mixtures, compounds were mixed in ratios as they were analysed in the sediment extracts (Table 4.1). Results of the mixture testing are presented in Figure 4.6.

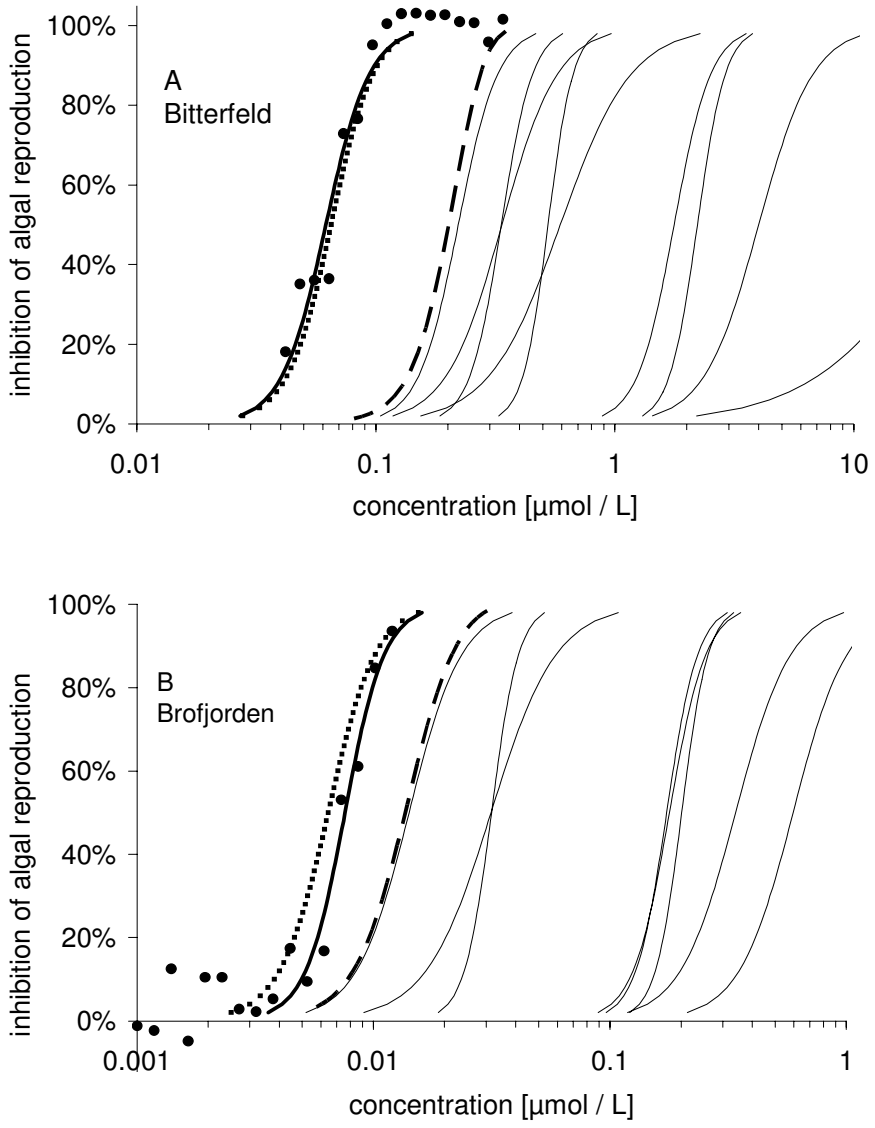


*Figure 4.6: Expected and observed toxicity of mixtures composed according to ratios at which toxicants were identified in the sediment extracts. (A Bitterfeld, B Brofjorden). Experimental data (●), fitted experimental data (——), expected toxicity according to CA (▪▪▪), expected toxicity according to IA (– –)and toxicity of the individual compounds (effects produced by the individual compounds present in the mixture) (——) are shown. The concentration axis refers to the total mixture concentration. For concentrations of individual compounds total mixture concentration has to be multiplied by $p_i$ (Table 4.1).*

It can be inferred from Figure 4.6 that in the two tested mixtures, the expected contributions of the individual toxicants to the combined effects of the mixture are more heterogeneously distributed than in the equitoxic

mixture (Figure 4.4). Whereas for the equitoxic mixture it was aimed that all compounds contributed to the combined effect, in the latter case it is not clear which of the compounds significantly contribute. For the Bitterfeld mixture, compounds causing the highest individual effects in the mixture are benzo[*ghi*]fluoranthene, pyrene and fluoranthene. For the Brofjorden mixture, indeno[1,2,3-*cd*]pyrene, benzo[*a*]pyrene and benzo[*b*]fluoranthene are the compounds with the highest individual effects.

The observed toxicity of the mixture, created from substances identified in the Bitterfeld sediment extract, is again in very good agreement with the prediction according to concentration addition. The mixture created from compounds identified in the Brofjorden sediment is slightly less toxic than what was calculated from the CA model. However, the prediction using the IA model clearly underestimates the combined effects of the mixture.

In order to illustrate the suitability of the prediction models for the assessment of combined effects of the different tested mixtures of phototoxic compounds, the index of prediction quality [eq. 7] is plotted in Figure 4.7. The IPQ of each mixture is not one value, but it depends on the effect level considered. In all three mixtures tested for their inhibition of algal reproduction under simulated sun light, combined effects were better predictable by the model of concentration addition than by the model of independent action. The latter underestimated the combined effects of all tested mixtures, however, the extent of underestimation varied between the mixtures. IPQ values for the IA prediction for the Brofjorden mixture at sediment concentration ratio vary between 0.6 and 1. For the equitoxic mixture ratio, IPQ values between 3.9 and 4.4 can be observed which is equivalent to an underestimation of combined effect by a factor of 5.

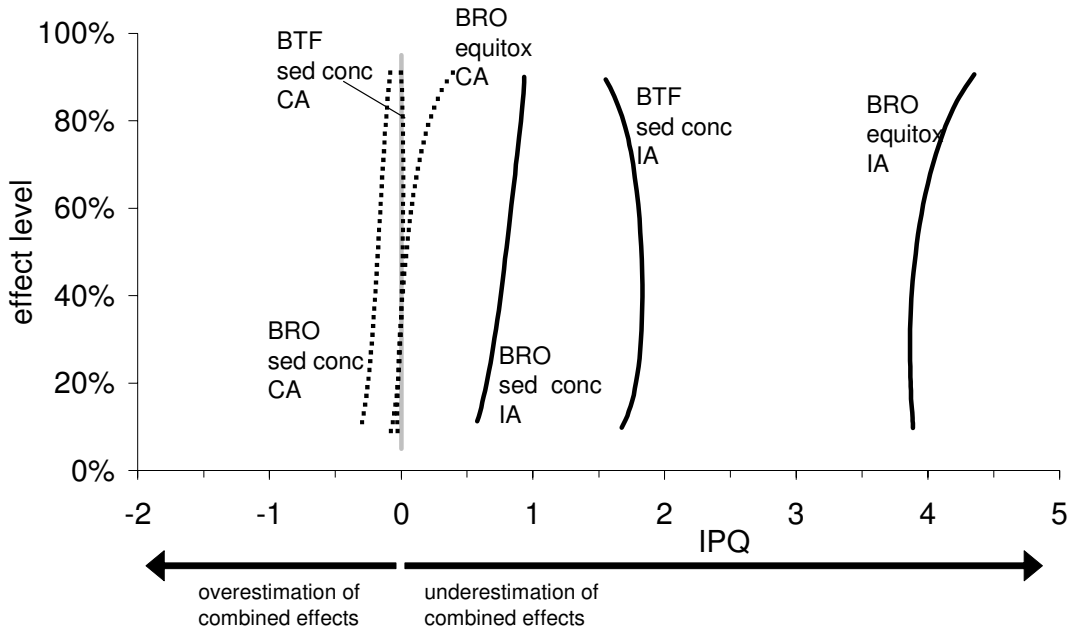


*Figure 4.7: Index of Prediction Quality (IPQ) for all three tested mixtures. The straight grey line at zero is the observed toxicity of each specific mixture. IPQ values of CA predictions are plotted in dotted lines, values from IA predictions in solid lines. Plotted mixtures are Brofjorden equitoxic ratio (BRO equitox), Brofjorden sediment concentration ratio (BRO sed conc) and Bitterfeld sediment concentration ratio (BTF sed conc). Exact predictions of combined effects would result in an IPQ = 0 on all effect levels. IPQ-Values < 0 indicate an overestimation, values > 0 an underestimation of combined effects.*

### 4.3.4 Impact of photoenhanced mixture toxicity on confirmation of toxicants

The influence of simulated sun light on the toxicity of the sediment extracts, on the identified toxicants and on the mixtures of these compounds has been demonstrated. It was, therefore, interesting to know, whether these observed increases affect the confirmation of toxicants in effect-directed analyses. In Figure 4.8, the index of confirmation quality (ICQ) is depicted for the two investigated sediment extracts.

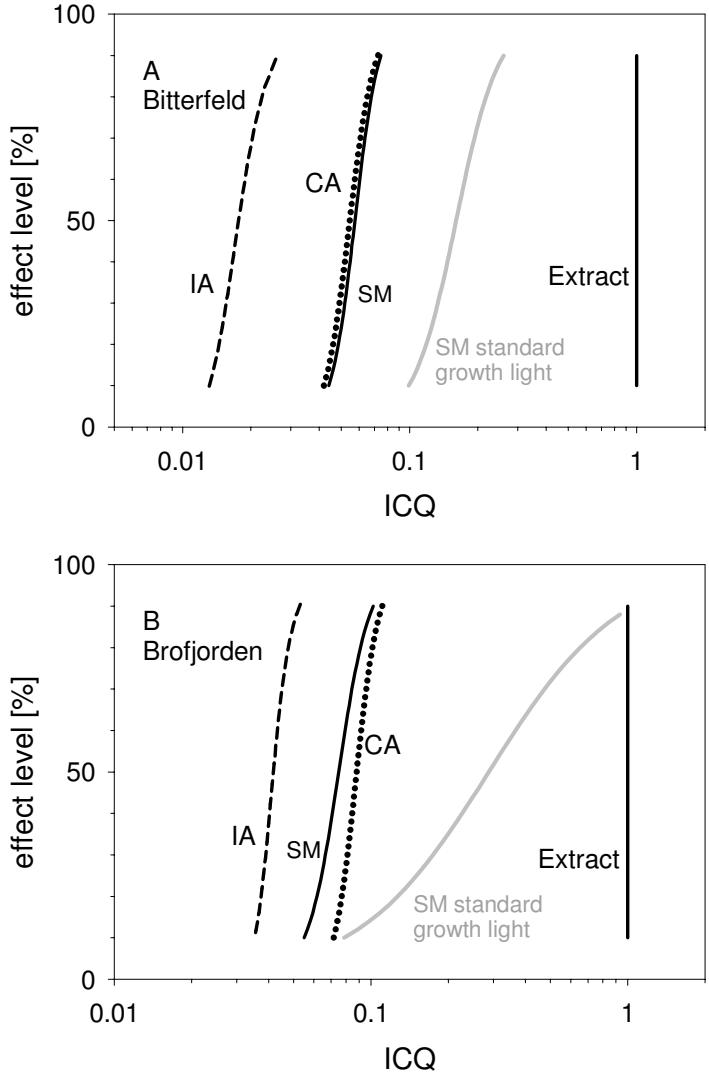


*Figure 4.8: Index of Confirmation Quality (ICQ) for effect levels between 10 and 90% for the investigated sediment samples (A: Bitterfeld, B: Brofjorden). The vertical line at 1 indicates the extract toxicity which is used as a reference and set to 1 for all effect levels. ICQ values are given for the measured toxicity of the synthetic mixture SM (solid line), expected combined effects of identified toxicants according to concentration addition CA (dotted line) and independent action IA (dashed line). Additionally, the ICQ values of the synthetic mixtures obtained under standard algal growth light are plotted (grey solid line) (cf. chapter 3).*

Lower ICQ values were obtained when simulated sun light instead of standard growth light is used for the assessment. For the sample extracted from the Bitterfeld sediment, $ICQ_{SM}$-values from 0.1 to 0.3 were observed for the synthetic mixture tested under standard algal growth light, whereas values

between 0.045 up to 0.08 were observed for the same sample tested under simulated sun light. For the Brofjorden sediment extract, $ICQ_{SM}$-values from 0.08 to 1 were determined for the mixture, tested under standard algal growth light. For the same sample, $ICQ_{SM}$-values from 0.06 to 0.09 were observed under simulated sun light. A decrease of the ICQ-value corresponds to an increase of unaccounted toxicity. This is due to the fact that the increase in toxicity of the extract induced by the light conditions is more pronounced than the increase in toxicity of the mixture of identified toxicants. That is to say that although the toxicity of the identified toxicants increases, less of the extract toxicity can be attributed to these substances under simulated sun light.

### 4.3.5 Influence of light conditions on effects of algal reproduction induced by 13 extracts from river Elbe sediments

Light dependence of toxicity was observed for two different environmental samples. In order to test, whether this observed light dependency of extract toxicity was an exceptional case or whether it is a rather common phenomenon when testing sediment extracts, 13 sediment extracts from transect samples from the river Elbe and its tributaries Mulde and Spittelwasser were investigated. Observed inhibition of algal reproduction of sediment extracts tested under standard growth light and UV-filtered light is plotted in Figure 4.9 (A). Additionally, the toxicity of the fraction F2 containing the PAH compounds of the samples was tested in the same concentration. Results are shown in Figure 4.9 (B).

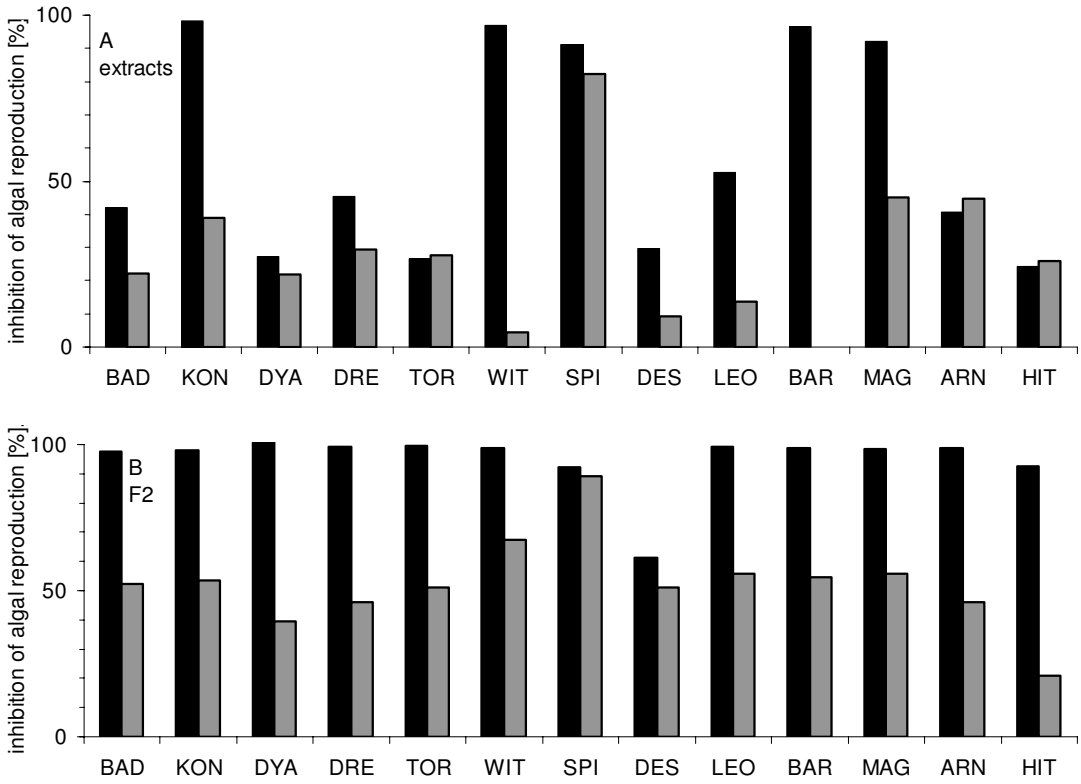


*Figure 4.9: Inhibition of algal reproduction exerted by sediment extracts (A) of transect samples from river Elbe and its tributaries Mulde and Spittelwasser at 4 g SEq / L test solution under standard growth light (■) and UV-filtered light conditions (■). Additionally, the inhibition of the most effective fraction (F2) containing PAH compounds is shown for the same concentrations and light conditions (B). (BAD: Bad Schandau, KON Königstein, DRE and DYA: Dresden, TOR: Torgau, WIT: Wittenberg, SPI: Spittelwasser (Tributary of river Mulde), DES: Dessau (river Mulde), LEO: Leopoldhafen, BAR: Barby, MAG: Magdeburg, ARN: Arneburg, HIT: Hitzacker). For details on samples and fractionation refer to Grote et al. (2004).*

It can be inferred from Figure 4.9 (A) that for 8 out of the 13 extracts under UV-filtered light significantly smaller effects were observed than under standard growth light. Most drastically, this phenomenon was observed for the samples WIT and BAR, which do not cause any effects under UV-filtered light conditions, but inhibit algal reproduction by 100% under standard growth light. A similar observation can be made when only the fraction 2 (F2) is regarded (Figure 4.9 B). This fraction contained the PAH compounds of the sample and thus the strongest light influence on toxicity can be expected for this fraction. The F2 fractions of all samples induce higher effects under

standard growth light than under UV-filtered light conditions. Interestingly, the fraction F2 causes higher effects than the extract using the same concentration for all investigated sediment extracts. This phenomenon was already observed for the extract from Brofjorden which was analysed by effect-directed analysis (chapter 2).

This result was unexpected, as intuitively one would expect that by removing components from a complex mixture its toxicity reduces or stays unaffected. However, one would not expect to increase toxicity by taking out components from the mixture, unless specific interaction occurs. In principle, interaction between compounds can take place either by physico-chemical interaction during exposition (modification of solubility, formation of complexes, etc.), during the toxicokinetic phase (modification of uptake rates) or by direct biological interaction (e.g., competition for binding sites at the receptor). No indication can be found for either of these specific modes of interaction. However, the extracts of all sediments were coloured. Depending on the content of humic substances colours from yellow to brown were observed. During the fractionation process, toxicants were separated from humic substances. One possible explanation for the increased toxicity of F2 compared to the extract might be that the humic substances present in the extract acted as a UV-filter by absorbing light of short wave lengths. By elimination of the UV-filtering substances, the PAH compounds might exert photoenhanced toxicity. This hypothesis for increased toxicity of F2 compared to the extract is supported by an observation made by Weinstein and Oris (1999) and Oris *et al*. (1990) who reported that humic acids reduce the photoinduced toxicity of fluoranthene and anthracene to fish and daphnia.

## *4.4 Discussion*

For the assessment of the toxic potency of sediment extracts to green algae, the illumination conditions play a key role. This was shown for the samples which previously have been investigated with EDA as well as for 8 out of 13 samples from different sites of the river Elbe basin. The influence of

light on sediment toxicity was previously described by Monson *et al*. (1995) who reported increased mortality of *Lumbriculus variegates* exposed *in situ* to contaminated sediments in natural sun light as compared to controls which were kept in the dark. Light can, therefore, be considered as a confounding factor in the assessment of sediment toxicity. Typically, this is attributed to the PAH contamination (Monson *et al.* 1995). However, also phototoxic behaviour of synthetic dyes, polyacetylenes and naphthols (Larson and Berenbaum 1988), sulfites (Eberlein-König *et al.* 1993) and azaarenes (Wiegman *et al.* 2001) has been reported. In this study, fractionation revealed that photoenhanced toxicity was most elevated in fraction 2 containing PAH compounds.

Combined effects of mixtures investigated here under simulated sun light were qualitatively well predicted by the concentration addition model. Previously, it has been demonstrated that the CA model predicts combined effects of mixtures of compounds exerting their toxicity via a similar mode of action, while the model of independent action better predicts combined effects of mixtures of compounds with dissimilar modes of action (Faust *et al.* 2000). It is thus surprising that CA predictions are in good agreement with the observations, as different modes of action are described for photoinduced toxicity. For toxicity exerted via photosensitisation, a similar mode of action of the different compounds is reasonable, as it is understood as a mechanism, where the excited PAH molecule initiates the formation of singlet oxygen which is believed to be the reactive toxic species. However, for many compounds photomodification reactions are reported to be relatively rapid. Krylov *et al*. (1997) determined half-lives of 2 and 5 hours for anthracene and benzo[*a*]anthracene exposed to simulated solar radiation respectively. These times are well below the illumination duration in the used test system. Complex mixtures of photomodification products may be formed from each PAH. In some cases more that 30 products were observed after irradiation of a single PAH (Krylov *et al.* 1997). Brack *et al*. (2003) identified and quantified 8 photomodification products from a suspension of anthracene, exposed to

simulated sun light. Thus, in a PAH mixture exposed to simulated sun light, one may expect the generation of a very complex mixture with changing concentrations of its constituents over time. The analysis of exact pathways of photomodification reactions, the corresponding kinetics and the determination of toxicity of photomodification products would be extremely laborious. However, the fact that the comparably simple model of CA is capable of describing the combined effect of these multiple mixtures quantitatively is surprising. CA is used in this case as a black box model not considering the underlying complex reaction pathways present. Recently, Ankley *et al*. (2003) pointed out that previously published results of experiments by Swartz *et al*. (1997) and Boese *et al*. (1999) were not inconsistent with the concept of concentration addition as a basis for predicting the photoenhanced toxicity of PAH mixtures, but those studies were not conducted in a manner suitable to assess the predictive power of the model quantitatively. Erickson *et al*. (1999) demonstrated that acute lethality of the oligochaete *Lumbriculus variegatus*, induced by binary PAH mixtures tested under UV light, can be predicted via a concentration addition model. In the present study, it was shown that this model also is suitable to predict combined effects of multiple mixture of PAH compounds on algae exposed to simulated sun light.

The equitoxic mixture is the optimal design in order to determine, which model provides the more accurate prediction, as one may assume that all 8 substances contribute to the combined effect of the mixture. Faust (1999) demonstrated through theoretical considerations that the factor between the predictions of the two models can at the maximum be equal to the number of mixture components. However, this maximum factor can only be obtained in case that all concentration-response curves of the mixture components are infinitely steep. As all observable curves have a finite steepness, the maximum factor between the conceptual predictions reduces. Therefore, a factor of 5 for a mixture of 8 substances is rather high, which reflects the relatively steep concentration-response curves observed for the PAHs under simulated sun light. As noted previously, at the $EC_{90}$ of the mixture, none of

the individual compounds causes a significant effect. This shows that compounds, present in concentrations far below their individual threshold concentration, may contribute to the combined effect of the mixture. Assuming that in the real environment even more phototoxic PAHs may be present simultaneously, the distance between the predictions may get even more pronounced. Although these results were not unexpected from a conceptual point of view, they clearly highlight the necessity of considering phototoxic PAHs in the environment as complex mixtures, rather than discrete chemicals.

Previously reported results of investigations of the same environmental samples revealed that identified toxicants only partially accounted for the observed sample's toxicity under standard growth light (chapter 3). In the Brofjorden sediment extract, 8 identified PAHs accounted for 34% of effects of the extract observed under standard growth light. In the Bitterfeld sediment extract, 10 identified compounds of which six were PAHs accounted for 16% of the observed extract toxicity (chapter 3). However, the majority of toxicants was not identified. It was therefore not clear, whether a suspected phototoxic behaviour of the identified PAHs in the sample, would affect the toxicity of the whole sample when exposed to simulated sun light or whether these effects would be superimposed by other factors. The increase in toxicity under simulated sun light and the decrease in UV-filtered light revealed that phototoxic compounds accounted for a large part of the sample's toxicity. Furthermore, the influence of simulated sun light on the toxicity of the samples as well as of the identified toxicants resulted in a quantitative alteration of the judgement on confirmation. Lower ICQ-values were observed, when simulated sun light was used for the assessment, as compared to values observed under standard growth light (chapter 3). This means that less of the observed sample's toxicity can be attributed to the identified toxicants. One reason for this may be the fact that the importance of the light conditions for the investigated samples was not known *a priori*. During the effect-directed analysis procedure, only standard algal growth light

was used for the toxicity assessment of fractionated samples. The subsequent study revealed the influence of simulated sun light conditions on the toxicity of the samples and the identified toxicants. Possibly, fractions containing substances only exerting their toxic effects under sun light conditions were classified as non-toxic and subsequently not further analysed. Therefore, toxicants present in these fractions might have been overlooked.

In this study sediment extracts were investigated which partially contained lipophilic compounds which were bound to organic matter under environmental conditions. Organisms such as algae may thus not be exposed to these substances under real environmental conditions. However, these compounds can be considered as potentially relevant, as they may accumulate in biological matrices or remobilise from the sediment under specific conditions.

In this study it was shown that light conditions may increase the toxicity of different environmental samples. This phenomenon was related to the content of PAH compounds in the samples. Effect on the reproduction of algae co-exposed to PAH mixtures and simulated sun light were quantitatively well predicted by the model of concentration addition from concentration-response relationships of the mixture components. However, as for the application of the CA model concentration-response relationships are required for each compound in the mixture for the individual light conditions, the development of models for prediction of photoenhanced toxicity are desirable in order to allow a predictive toxicity assessment for different light intensities and spectral compositions.

# Chapter 5

## Modelling photoinduced algal toxicity of polycyclic aromatic hydrocarbons*

### *5.1 Introduction*

The relevance of photoinduced algal toxicity of PAHs under realistic light conditions has been demonstrated in chapter 4. For phototoxic action two different reaction mechanisms are currently discussed: photosensitisation and photomodification (Figure 5.1).

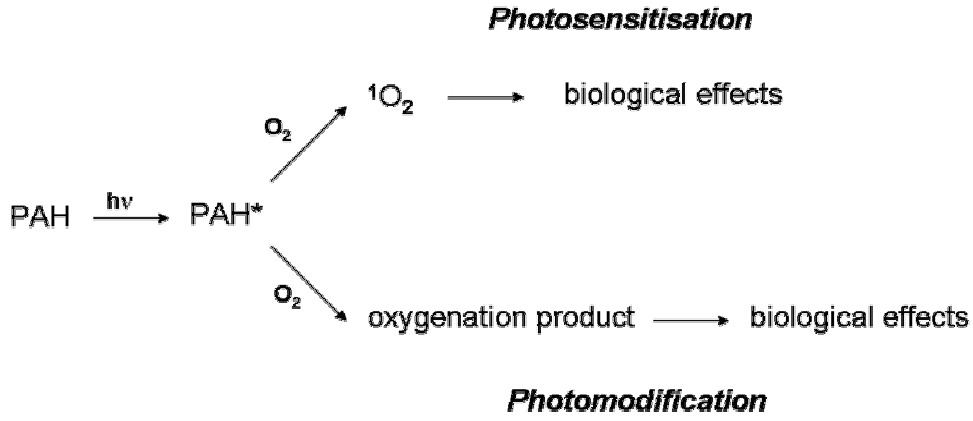


*Figure 5.1: Schematic illustration of the two proposed mechanisms of phototoxic action.*

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* Parts of this chapter are publishes as Grote M, Schüürmann G, Altenburger R (2005) *Environmental Science & Technology* 39:4141-4149

Photosensitisation reactions initiated by PAHs often proceed via the formation of singlet-state oxygen ($^1O_2$). This process begins with the sensitising PAH absorbing a photon, which elevates the PAH to an excited singlet state. The excited singlet-state PAH can undergo intersystem crossing to the excited triplet state where it can react with ground triplet-state oxygen ($^3O_2$) to form $^1O_2$. Due to its low energy barrier to reaction, $^1O_2$ is capable of oxygenating many different biomolecules, altering their chemical structure, and consequently modifying their normal function. Evidence for the role of oxygen in PAH phototoxicity has been demonstrated in different biological systems. In genetically engineered *Escherichia coli*, bacteria lacking catalase proficiency were more susceptible to toxic damage when co-exposed to PAHs and UV light than bacteria with catalase activity (Kagan *et al.* 1990; Tuveson *et al.* 1987). The hemolysis of human erythroctes induced by PAHs plus UV light was greatly reduced under anaerobic conditions as compared to aerobic conditions (Kagan *et al.* 1989; Kagan *et al.* 1990). Photomodification of PAH, usually via oxidation, results in the formation of new compounds that will exert bioactivities different from the parent compounds. Huang *et al*. (1997) demonstrated that the half life of anthracene under simulated solar radiation in a biotest system with *Lemna minor* is about 2 hours. Thus, the majority of the parent compound will be modified during the duration of a typical biotest. Consequently, the test result will rather represent a measure for toxicity of the mixture of transformation products than of the parent compound. Brack *et al*. (2003) identified different photometabolites in a suspension of irradiated anthracene. Anthracene-1,4-dione, a thus far unknown trace photometabolite, proved to be a very potent toxicant dominating the toxicity of photomodified anthracene solution to *Vibrio fischeri*.

Although the principle mechanisms producing photoinduced toxicity of PAHs are known, for most PAHs the exact mechanism of action remains to be clarified including susceptibility of the compound to either photoactivation or photomodification, kinetics of formation and degradation of photometabolites and toxicity of metabolites and activated species. Possibly,

a combination of the two described pathways of phototoxic action may be relevant to most PAH compounds.

When assessing the toxicity of PAHs, it is crucial to consider the influence of the light conditions in order to avoid an underestimation of toxicity. However, when aiming to assess the relevance of photoinduced PAH toxicity for environmental exposure conditions, it has to be taken into account that the light conditions are typically site specific. They depend on the geographic characteristics, e.g., the latitude, the altitude above sea level or water depth, and on temporarily changing factors, e.g., the weather, time of the day and time of the year. Therefore, every toxicity assessment of PAHs is highly site specific. Thus, an exact quantification and diagnosis of causes for PAH toxicity is laborious.

As an alternative, predictive approaches might be used, which aim to estimate elements of the toxicological behaviour of a compound from their structural properties. Models for estimating acute PAH phototoxicity in aquatic organisms were presented by Morgan and Warshawski (1977), Newsted and Giesy (1987), Oris and Giesy (1987), Mekenyan *et al*. (1994) and Huang *et al*. (1997), Krylov *et al*. (1997).

Morgan and Warshawsky (1977) investigated the photodynamic immobilisation of *Artemia salina* nauplii by 41 carcinogenic and non-carcinogenic PAHs. A fixed number of organisms was exposed to one concentration of the investigated compound and irradiated with monochromatic light of 366 nm for consecutive brief time intervals. The rate of immobilisation of *Artemia salina* nauplii exposed to PAH and UV light was determined to be a function of irradiation time and the amount of light absorbed by the compound. The authors introduced a quantum yield for immobilisation $\Phi_I$ which describes the relative number of organisms immobilised divided by the number of light quanta absorbed by the substance (Morgan and Warshawsky 1977):

$$\Phi_I = \frac{d(ANI)/dt}{I_a} = \frac{average\ number\ immobilsed / \min}{quanta\ absorbed / \min} \qquad [eq.\ 8]$$

The average number of nauplii immobilised (ANI) was thus described by the following equations (Morgan and Warshawsky 1977) taking into account the amount of light absorbed by the compound:

$$ANI = (I_0\, l\, \alpha\, \Phi_I)\ (2.303\ \varepsilon\ C\ t) + B \qquad [eq.\ 9]$$

where $I_0$ is the light intensity, $l$ is the path length of the nauplii, α is the fraction of the substance absorbed by the nauplii, $\Phi_I$ is the immobilisation quantum yield, ε is the molar absorptivity of the compound at 366 nm, C is the concentration of the compound placed in solution, t is the irradiation time, and B is a constant of integration (Morgan and Warshawsky 1977). Plots of ANI as a function of (2.303 ε C t) were described to be linear with slopes equal to ($I_0\, l\, \alpha\, \Phi_I$). The authors conclude that $\Phi_I$ of any sensitiser could, in principle, be obtained from the slope of the described relationship. However, due to difficulties in exact determination of quantities $I_0$, $l$ and α, the absolute value for $\Phi_I$ was not calculable. Therefore, a measure of relative photodynamic activity (RPA) was proposed. The RPA value for each compound was calculated by dividing the slopes of the immobilisation curves of the investigated compound by the corresponding slope of benz[*c*]acridine which was used as a reference compound (Morgan and Warshawsky 1977):

$$RPA = \frac{I_0 l \alpha \Phi_I}{I_0 l \alpha^r \Phi_I^r} = \frac{\alpha \Phi_I}{\alpha^r \Phi_I^r} \qquad [eq.\ 10]$$

where $\Phi_I$ and $\Phi_I^r$ are the quantum yields for immobilisation for the test compound and benz[*c*]acridine, respectively, and α and $\alpha^r$ are the corresponding fractions of compounds absorbed by the nauplii. As a result, RPA values for the 41 investigated compounds were presented. Benz[*a*]anthracene with an RPA value of 9.8 was the substance with the highest photodynamic activity. For 9 compounds RPA values of 0 were calculated, which corresponds to no observed photodynamic activity (Morgan and Warshawsky 1977).

Newsted and Giesy (1987) investigated the photoinduced acute toxicity of 20 PAHs to *Daphnia magna*. *D. magna* were co-exposed to PAH plus 120 µW/cm$^2$ UV-A and 25 µW/cm$^2$ UV-B. Unlike in the study by Morgan and Warshawsky (1977) a broader range of wavelengths instead of monochromatic light was used. Mortality times were recorded and reported as median lethal times ($LT_{50}$). A quantitative relationship was derived from those experiments that included the intensity of active irradiance that penetrated the organism, the irradiance absorbed by the PAH, the toxicant concentration in the organism and the PAH's potency. The potency (Φ) is "the efficiency of the observed lethality relative to the absorbed dose of irradiance" (Newsted and Giesy 1987) which is defined in a similar way as the immobilisation quantum yield by Morgan and Warshawsky (1977). The relative amount of mortality can be calculated from the following equation (Newsted and Giesy 1987):

$$\%mortality = \frac{\sum_{\lambda}^{n}(I_{\lambda}T_{\lambda})(\varepsilon_{\lambda}bC_{a})}{n}\Phi t + B = A\Phi t + B \qquad \textit{[eq. 11]}$$

where A is the average number of quanta absorbed, λ is the integrated waveband for one of the four wavebands (UV-B, UV-A, VIS), $I_\lambda$ is the radiation intensity of band λ, $T_\lambda$ is the optical transmittance of the organism, $\varepsilon_\lambda$ is the molar extinction coefficient of the investigated compound at the wavelength λ, b is the path length of the organism, and $C_a$ is the molar concentration in the organism.

As all parameters used in the equation were measured or calculated from literature data, absolute values for the potency Φ can be presented for all investigated compounds. Additionally, RPA values were calculated in a similar manner as by Morgan and Warshawsky (1977) except that in this study benzo[*b*]anthracene was used as a reference compound and internal concentrations rather than ambient water concentrations were used.

Oris and Giesy (1987) studied the photoinduced toxicity of PAHs to larvae of the fish fathead minnow (*Pimephales promelas*). Six of the 12 compounds tested caused acute photo-induced toxicity. The efficacy value Φ of each

individual compound was determined from the slope of the time- mortality curve in an analogous way as described by Newsted and Giesy (1987) for the potency Φ. Instead of the RPA value, the authors calculated the relative potency factor (RPF), an index of the relative efficacy of a compound compared to the least efficacious of the compounds tested, in this case benzo[*a*]pyrene. Interestingly, benzo[*b*]anthracene which was used as a reference compound for photo-induced toxicity to *Daphnia magna* (Newsted and Giesy 1987) exerted only a marginal level of toxicity and was not used for further calculations.

All these models have in common that they have predictive character only for substances which have been tested in the test system used. Predictions can be derived for untested concentrations or untested light intensities. However, no extrapolations to untested substances or other organisms are possible.

Therefore, all authors try to relate the observed photoinduced toxicities to other properties of the compounds. Morgan and Warshawsky (1977) found a higher probability of carcinogenic PAHs to cause photoinduced toxicity than for non-carcinogenic. Newsted and Giesy (1987) found a correlation of the $LT_{50}$ values for *Daphnia magna* to the lowest triplet state energy of the PAH compound. Oris and Giesy (1987) described a model using phosphorescence lifetime and a molecular connectivity index to be capable of classifying tested substances in phototoxic or non-phototoxic compounds.

Mekenyan *et al*. (1994) reanalysed the data presented by Newsted and Giesy (1987). They found that PAHs exerting photoinduced toxicity to *D. magna* possessed a HOMO-LUMO (highest occupied molecular orbital – lowest unoccupied molecular orbital) gap of 7.2 ± 0.4 eV. They thus proposed to use the HOMO-LUMO gap “window” of 7.2 ± 0.4 eV as an indication for PAH phototoxicity in *D. magna*.

Huang *et al*. (1997) and Krylov *et al*. (1997) developed a model to describe the toxicity of 16 PAH to the higher plant *Lemna gibba* under

simulated solar radiation. As photoinduced toxicity is considered as a combination of two distinct mechanisms, photosensitisation and photo-modification, they aimed to develop a model accounting for these different mechanisms. They separated toxicity caused by photomodification products from toxicity induced through photosensitisation by the intact compounds by irradiation of the test solution prior to the incubation. This irradiation lasted until the test compound was largely photomodified. By comparison of observed toxicity of the intact compound to the toxicity of the photomodified compound the relative impact of the two mechanisms was quantified. They presented calculated values for photosensitisation factors (PSF) and photomodification factors (PMF) for each investigated PAH (Huang *et al.* 1997). The sum of PSF and PMF was demonstrated to correlate to a relative toxicity value which was calculated from the growth rate inhibition induced by a concentration of 2 mg / L of each compound.

El-Alawi *et al*. (2002) successfully applied the model of Huang *et al*. (1997) and Krylov *et al*. (1997) to describe the long-term toxicity (26 hours) of PAHs to *Vibrio fischeri* in simulated solar radiation. Relative toxicity observed at 0.5 g / L was shown to correlate to the sum of PSF and PMF. As reason for the use of effects determined at one concentration instead of effect concentrations at one effect level of the determined concentration-response relationship the authors state that the "QSAR model for *L. gibba* mathematically relates better to inhibition of an effect than to an $EC_{50}$" (El-Alawi *et al.* 2002). However, no explanation is given for the use concentrations of 2 g / L for the model in *L. gibba* (Huang *et al.* 1997) or 0.5 g / L in *V. fischeri* (El-Alawi *et al.* 2002). Nevertheless, the fact that the model is based on one concentration only, limits its applicability to other concentrations and other endpoints.

The aim of this study was to investigate the applicability of the proposed models to identify PAH compounds which exert photoinduced toxicity to algae. Furthermore, we tried to formulate models which quantitatively predict photoinduced toxicity to algae. For this purpose the toxicity of 14 PAHs to the

unicellular green alga *Scenedesmus vacuolatus* was studied under three different light conditions. Models described in the literature were modified, in order to be applicable to exposure conditions and the toxic endpoint used in the algae test system.

## *5.2 Material and Methods*

### 5.2.1 Selection of test chemicals

For the investigation of their phototoxic behaviour, 11 PAH compounds were selected which were previously identified as potentially relevant toxicants using effect-directed analysis in a freshwater sediment (Brack *et al.* 1999) and a marine sediment (chapter 2). Algal toxicity of the two investigated extracts proved to be dependent on the illumination conditions used in the bioassay (chapter 4). In addition, in order to test the suitability of the proposed HOMO-LUMO gap window of 6.8 – 7.6 eV as indication for photoinduced toxicity (Mekenyan *et al.* 1994), three compounds were included in the study which possess HOMO-LUMO gaps slightly smaller, slightly larger or just inside the proposed window. Information on identity of test compounds in provided in Table 5.1.

*Table 5.1: Identity, source and purity of substances used for test of single compounds and mixtures*

| Substance | CAS RN | Source | Purity [%] |
|---|---|---|---|
| Anthracene[a,b] | 120-12-7 | Aldrich | 99 |
| Benzo[*a*]anthracene[a,b] | 56-55-3 | Aldrich | 99 |
| Benzo[*a*]pyrene[b] | 50-32-8 | Aldrich | 97 |
| Benzo[*b*]fluoranthene[b] | 205-99-2 | Promochem | 99 |
| Benzo[*ghi*]fluoranthene[a] | 203-12-3 | Promochem | 98 |
| Benzo[*ghi*]perylene[c] | 191-24-2 | Aldrich | 98 |
| Benzo[*k*]fluoranthene[b] | 207-08-9 | Aldrich | 98 |
| Chrysene[c] | 218-01-9 | Riedel-deHaën | 97 |
| Fluoranthene[a,b] | 206-44-0 | Aldrich | 98 |
| Indeno[1,2,3-*cd*]pyrene[b] | 193-39-5 | Promochem | 99 |
| Perylene[c] | 198-55-0 | Fluka | >99 |
| Phenanthrene[a] | 85-01-8 | Aldrich | 99 |
| 2-Phenylnaphthalene[a] | 612-94-2 | AccuStandard | 99 |
| Pyrene[a,b] | 129-00-0 | Fluka | 99 |

[a)] toxicant identified in Bitterfeld sediment, [b)] toxicant identified in Brofjorden sediment,
[c)] compound included due to HOMO-LUMO gap

### 5.2.2 Biotest system

As biotest system the previously described algal bioassay using synchronous cultures of the unicellular green alga *Scenedesmus vacuolatus* was applied. All compounds were tested under three different light conditions. In addition to the standard growth light conditions, tests were conducted under simulated sun light, i.e., under realistic environmental conditions, and under UV-filtered light, in order to minimise light influence on the toxicity. Details on the test protocol and on the light conditions are provided in 6.3.1. Samples were tested in geometric dilution series with a constant final DMSO concentration of 0.1 % in the test solution. Spacing between test concentrations was flexibly adapted to the steepness of each concentration–response curve, in order to cover the range between $EC_{10}$ and $EC_{90}$.

### 5.2.3 Calculation of base line toxicity and excess toxicity

It is possible for every organic compound to exert toxicity if applied in high concentrations. Especially for lipophilic organic compounds, toxicity can be expected by pure accumulation in the cell membranes and subsequent disturbance of their functions. This is referred to as narcotic mode of action or base line toxicity (Lipnick 1990). As the base line toxicity is closely related to lipophilic properties it can be estimated from the log $K_{OW}$. Values for log $K_{OW}$ were estimated using the KOWWIN V1.66 software (EPISuite, US-EPA). Expected base line toxicity of a compound to *Scenedesmus vacuolatus* were calculated by a simple QSAR (Altenburger *et al.* 2004) as given in [eq. 1].

The excess toxicity ($T_e$) is the quotient of a predicted toxicity (herein base line toxicity) to a measured toxicity (Lipnick 1990):

$$T_e = \frac{EC_{50,baseline}}{EC_{50,measured}} \qquad \textit{[eq. 12]}$$

It is a measure of how much more toxic a compound actually is, as compared to the assumption that it would only act via an unspecific narcotic mode of action. Based on a classification by Verhaar *et al*. (1992) reactive chemicals and specifically acting chemicals possess higher $T_e$ values than narcotics and polar narcotics. Verhaar *et al*. (1992) reported medium log $T_e$ values for polar narcotics of $\mu$=0.81 ($\sigma$=0.31). Thus $T_e > 27$ ($\mu+2\sigma$) can be considered as an indication that a compound either belongs to the group of reactive chemicals or exerts its toxicity via a specific mode of action.

### 5.2.4 Calculation of HOMO-LUMO-Gap

For calculation of the energies of the HOMO (highest occupied molecular orbital) and LUMO (lowest unoccupied molecular orbital), the following procedure was applied: Initial force-field geometry optimization of the compounds employing the SYBYL software (SYBYL software package, Version 6.8, Tripos Inc., St. Louis, MO, USA) was followed by semiempirical quantum chemical AM1 (Dewar *et al.* 1985) calculations as implemented in

MOPAC (MOPAC 93, Revision 2 (1994) Fujitsu Limited, Chiba, Japan, and Stewart Computational Chemistry, Colorado Springs, Colorado, USA), and the HOMO and LUMO energies refer to the optimized AM1 geometries of the compounds that were ultimately achived.

### 5.2.5 Application and modification of the model of Ankley *et al*. (1995) to single compound's toxicity

Ankley *et al*. (1995) proposed a model which allows the calculation of time-dependent mortality for *Lumbriculus variegatus* from tissue concentrations of fluoranthene and irradiation intensity of UV light. Assuming that the rates of elimination and damage repair are negligible over the test period, exposure concentration, light intensity and 'time to death' are inversely related to each other:

$$1 = \frac{k_3}{D_L} R_0 I t_D \qquad \textit{[eq. 13]}$$

where $k_3$ is a rate constant for accrual of damage, $D_L$ is the level of critical damage resulting in death, $R_0$ is the initial tissue concentration of the PAH, $I$ the light intensity and $t_D$ the 'time to death'. The model was successfully applied to two other compounds, pyrene and anthracene (Ankley *et al.* 1997).

In the algal test system used in this study instead of 'lethal time' inhibition of reproduction is measured. A constant exposure time of 24 hours equivalent to one generation cycle is used. The critical level of damage $D_L$ is replaced by the level of damage (of cellular functions) required to produce 50% of inhibition of reproduction $D_{I50\%}$. Analogously, the tissue concentration R0 can be replaced by the median effective dose ($ED_{50}$). Ankley *et al*. (1995) used three different light intensities of the same light source. In the present study not only the intensities of the light but also the spectral compositions were varied. The term $I$ was, therefore, replaced by a measure of absorption J, which was proposed by Krylov *et al*. (1997). J is the integral of overlap between the irradiation spectra and the molar absorbance and is equivalent to

the transition intensity. (The calculation of J is described below.) By replacing terms in Equation 13, the following equation is obtained:

$$1 = \frac{k_3 t}{D_{I50\%}} R_{I50\%} J = C R_{I50\%} J \qquad \textit{[eq. 14]}$$

where C is a constant combining $k_3$, $D_{i50\%}$ and t. $R_{I50\%}$ is the tissue concentration of the PAH inducing 50% effect and is equivalent to the median effective internal dose ($ED_{50}$) value. The equation can be written as:

$$\frac{1}{ED_{50}} = CJ \qquad \textit{[eq. 15]}$$

or

$$\log ED_{50} = - \log J - \log C \qquad \textit{[eq. 16]}$$

The median effective internal dose $ED_{50}$ may be estimated from the observed $EC_{50}$ values based on concentrations in the test medium and the bioconcentration factor (BCF) of the compound (McCarty 1986).

$$ED_{50} = EC_{50} * BCF \qquad \textit{[eq. 17]}$$

BCF values for the used test species were estimated using the following equation (Geyer *et al.* 1984):

$$\log BCF = 0.681 * \log Kow + 0.164 \qquad \textit{[eq. 18]}$$

From [eq. 16] the relation between log ($ED_{50}$) and log J, the absorbed amount of photons by the compound, can be expected to be linear with a slope of -1. The same holds true for log ($EC_{50}$), however, another intercept of the y-axis can be anticipated.

### 5.2.6 Calculation of transition intensities

In Figure 5.2 (A) the three illumination conditions used for the experiments are depicted for wave lengths from 300 to 460 nm. They differ in absolute intensity and in spectral composition. The absorbed amount of photons by a compound is dependent on the irradiation spectrum and on the molar

absorption of the compound. The transition intensity J was calculated as overlapping integral of irradiated spectrum and molar absorbance of a compound in the waveband of 300 – 460 nm according to Krylov *et al*. (1997):

$$J = \int_{300nm}^{460nm} \varepsilon_\lambda \cdot I_\lambda \cdot d\lambda \qquad \textit{[eq. 19]}$$

where $\varepsilon_\lambda$ [L / mol / cm] is the molar extinction coefficient at the wavelength λ [nm], $I_\lambda$ [E / $cm^2$ / s / nm] the irradiation intensity for each wavelength. The upper limit of 460 nm was chosen as this was the maximum wavelength at which some of the compounds absorbed energy. More recently, a similar equation has been published as photoactivated toxicity weighting function (PWF) (Diamond *et al.* 2000). However, the term PWF may be misleading, as it denotes a measure of absorbance which is not directly related to toxicity. Therefore, we prefer to use the more neutral term J. Absorbance spectra including their molar extinction coefficient ε were used as reported by Karcher *et al*. (1985). The overlap was calculated for steps of 2 nm intervals. As an illustration of the procedure the absorbance spectrum of one compound is exemplarily depicted in Figure 5.2 (B). The overlap of the irradiation and the absorption spectra is shown in Figure 5.2 (C). The transition intensity J is equivalent to the area under the curve.

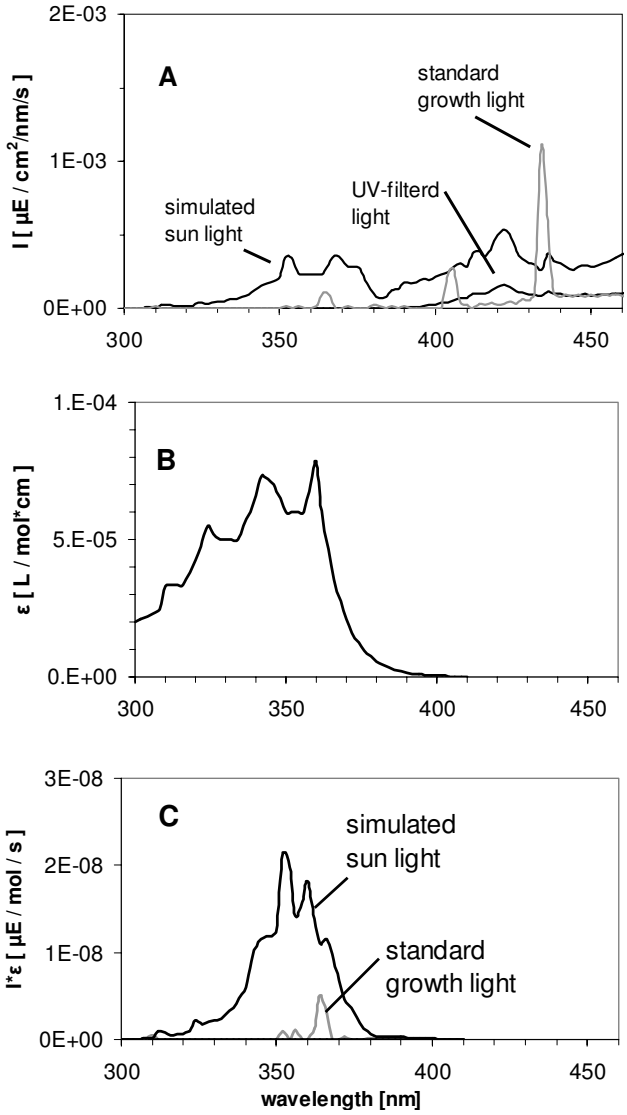


*Figure 5.2: Calculation of transition intensities: (A) Irradiation spectra of the three different light sources (extract from Figure 6.6), (B) molar extinction coefficient of fluoranthene for wavelengths from 300 to 460 nm, (C) overlap of irradiation and absorption spectra.*

It turned out that the transition intensity was large under simulated sun light conditions, compared to standard light conditions. Under UV-filtered light conditions practically no absorption took place for fluoranthene.

## *5.3 Results and Discussion*

### 5.3.1 Light-dependent algal toxicity of PAHs

The toxicity of the compounds that were examined depended on the light conditions used for the illumination of the biotest. Exemplarily, experimental data for the effects of three typical compounds on the reproduction of *Scenedesmus vacuolatus* after one generation cycle of 24 hours are shown in Figure 5.3. Functions of concentration-response relationships fitted to the experimental data are presented in Table 5.2. The depicted concentration dependent inhibition of algal reproduction reflects different degrees of dependence on light conditions. Some of the data have previously been described (chapter 2 and 3) but are included here in order to facilitate comparison to new data obtained under UV-filtered light conditions or to previously untested compounds.

All PAHs studied herein induced higher toxicity when tested under simulated sun light compared to standard light conditions. The latter still provoked higher toxicity than UV-filtered light conditions. Exceptions from this observation were phenanthrene and 2-phenylnaphthalene for which the toxicity was not dependent on the light conditions, and chrysene and perylene which did not exert an effect on the algal reproduction in simulated sun light in concentrations up to their water solubility limits. Therefore, for these compounds no tests were conducted for other light conditions. The influence of the light conditions was most pronounced for benzo[*b*]fluoranthene, benzo[*k*]fluoranthene and benzo[*a*]anthracene with $EC_{50}$ values of 6.2, 13.2 and 11.5 nmol / L under simulated sun light, respectively. These compounds were non-toxic in UV-filtered light. Light conditions only had a small impact on the toxicity of indeno[1,2,3-*cd*]pyrene. The $EC_{50}$ value shifted from 2.3 nmol / L in simulated sun light to 6.6 nmol / L in UV-filtered light.

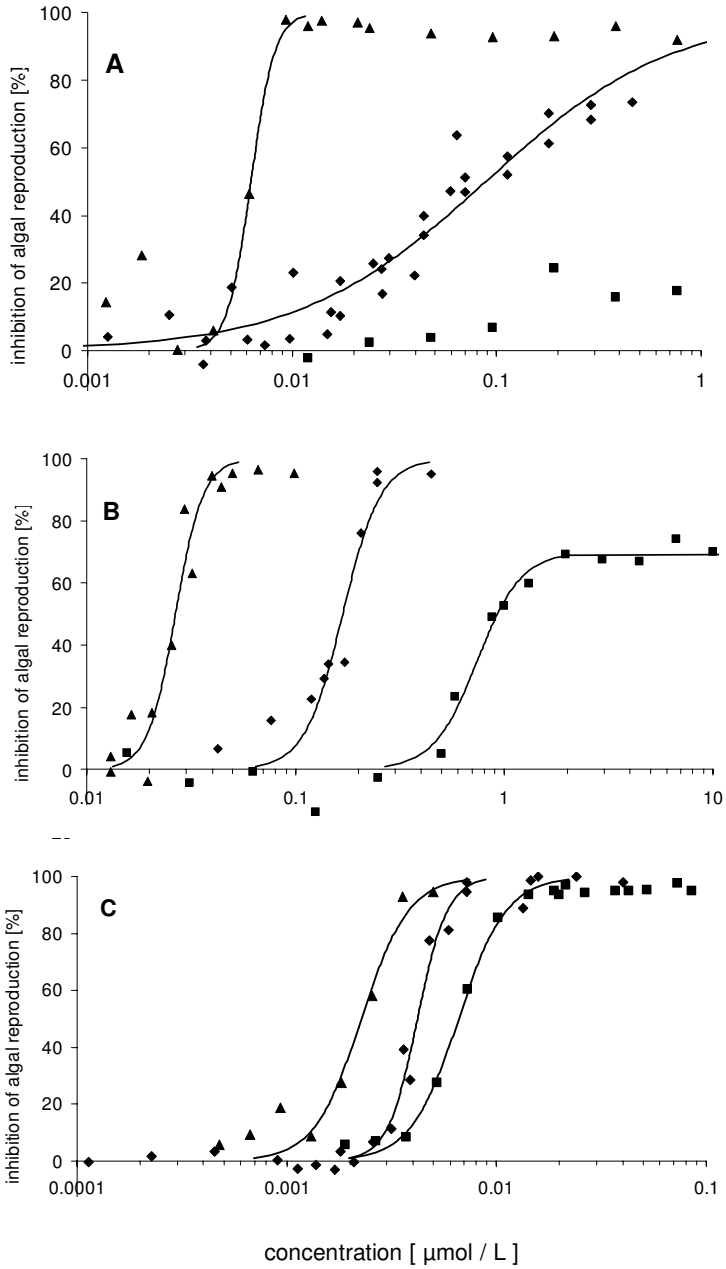


*Figure 5.3: Toxicity of representative compounds under simulated sun light (▲), standard growth light (♦) and UV-filtered light (■): benzo[b]fluoranthene (A), fluoranthene (B), indeno[1,2,3-cd]pyrene (C). Fitted concentration-response relationships are shown as solid lines (functions are given in Table 5.2.)*

The results depicted in Figure 5.3 illustrate the problems encountered when conducting experiments on toxicity. Not for all compounds tested, complete concentration-response curves can be modelled as maximum

effects of 100% are not achieved. A reason for this may be the sought in the limited water solubility of the lipophilic compounds. Maximum effects of 20% for benzo[*b*]fluoranthene and 70% for fluoranthene and pyrene observed under UV-filtered light may result from the constrained highest achievable concentration in the test medium.

*Table 5.2: Parameters of modelled concentration-response functions under three different illumination conditions.*

| | Simulated sun light | | Standard growth light | | UV-filtered light | |
|---|---|---|---|---|---|---|
| | $EC_{50}$ [µmol / L] | P | $EC_{50}$ [µmol / L] | P | $EC_{50}$ [µmol / L] | P |
| Anthracene | 0.104 | 3.78 | 2.84[a] | 0.61 | 13.7 | 0.33 |
| Benzo[*a*]anthracene | 0.0115 | 5.60 | 0.0579 | 2.57 | effects<20% | |
| Benzo[*a*]pyrene | 0.0025 | 3.14 | 0.0070 | 2.51 | 0.032 | 4.01 |
| Benzo[*b*]fluoranthene | 0.0063 | 7.52 | 0.088 | 0.944 | effects<20% | |
| Benzo[*ghi*]fluoranthene | 0.0102 | 5.20 | 0.0437 | 6.87 | 0.084 | 4.29 |
| Benzo[*ghi*]perylene | 0.0072 | 1.51 | not tested | | not tested | |
| Benzo[*k*]fluoranthene | 0.0132 | 7.71 | 0.018 | 2.74 | effects<20% | |
| Chrysene | no observed effects | | not tested | | not tested | |
| Fluoranthene | 0.027 | 6.58 | 0.1681 | 4.78 | 0.74[b] | 4.14 |
| Indeno[1,2,3-*cd*]pyrene | 0.0023 | 3.87 | 0.0042 | 6.11 | 0.0066 | 3.81 |
| Perylene | no observed effects | | not tested | | not tested | |
| Phenanthrene | 3.79 | 1.99 | 3.34 | 1.62 | 4.38 | 1.76 |
| 2-Phenylnaphthalene | 0.364 | 4.21 | 0.28 | 1.89 | 0.352 | 3.61 |
| Pyrene | 0.035 | 3.70 | 0.25 | 6.33 | 0.53[b] | 13.5 |

Experimental data were fitted using a logistic fit: $E = E_{Max} / (1- (c / EC_{50}) \text{ ^ } -P)$ [eq. 3], $E_{Max}$ was fixed to 100% unless complete inhibition of algal reproduction was obviously not reached. For fluoranthene and pyrene $E_{Max}$ of 70% was observed under UV-filtered light. [a]) in this case the $EC_{50}$ of the logistic function was constrained to a previously determined $EC_{50}$ value using a best fit approach (Altenburger *et al.* 2004). [b]) in this case this parameter equals the $EC_{35}$.

For the following considerations and calculations only the $EC_{50}$ values are considered. It should be taken into account that for each of the values concentration-response relationships were determined for a broad concentration range. The $EC_{50}$ value is thus only a derived value based on

severe reduction of the experimental results. The $EC_{50}$ is not capable of describing data obtained from experiments where a maximum effect of 20% was observed.

### 5.3.2 HOMO-LUMO gap as indicator for photoenhanced toxicity

It was aimed to investigate whether the computed HOMO-LUMO gap, proposed as an indicator for photoinduced toxicity in *Daphnia magna* (Mekenyan *et al.* 1994), is also suitable to distinguish phototoxic from non-phototoxic compounds for the green algae *Scenedemus vacuolatus*. As Mekenyan *et al*. (1994) used adjusted medium lethal times ($ALT_{50}$) as parameter of toxicity, a parameter qualitatively different from $EC_{50}$ values determined in the present study, two different approaches were chosen aiming to use the $EC_{50}$ values for the relation between the calculated molecular descriptor and the observed toxicity. In Figure 5.4 the enhancement of toxicity induced by the use of simulated sunlight compared to the toxicity in UV-filtered light is plotted. The toxicity enhancement is the ratio of the $EC_{50,\text{UV-filt. light}}$ to the $EC_{50,\text{sim. sun light}}$. The calculated HOMO-LUMO gaps are given in Table 5.3.

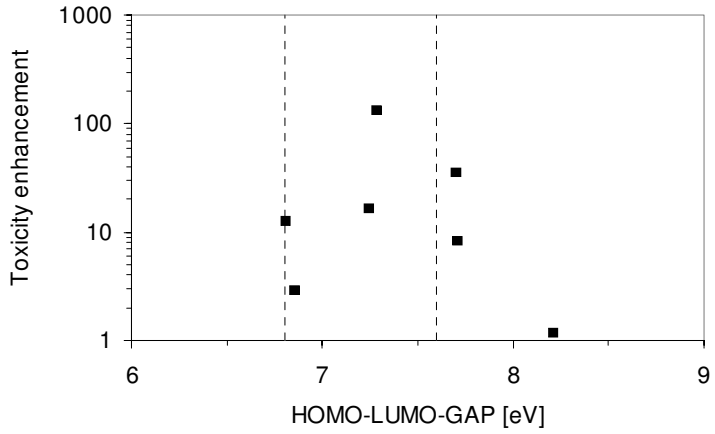


*Figure 5.4: Toxic enhancement provoked by simulated sun light. ($EC_{50,\text{UV filt light}}$ / $EC_{50,\text{sim. sun light}}$). Dashed lines show the HOMO-LUMO gap (7.2 ± 0.4 eV) where photo-induced toxicity is expected to occur for PAH compounds according to Mekenyan et al. (1994).*

The results displayed in Figure 5.4 show that substances exerting enhanced toxicity under simulated sun light fall into or fall at least near the proposed HOMO-LUMO gap window of 7.2 ± 0.4 eV (Mekenyan *et al.* 1994)

*Table 5.3: Calculated $EC_{50}$ of compounds using QSAR for base line toxicity [eq. 1] and resulting excess toxicity ($T_e$) for the different light conditions. Additionally calculated HOMO-LUMO gaps are reported.*

| | log $K_{OW}$ | BCF | $EC_{50,base\ line}$ [µmol/L] | $T_{e,sim.sun\ light}$ | $T_{e,stand.\ growth\ light}$ | $T_{e.UV-flit.\ light}$ | HOMO-LUMO gap [eV] |
|---|---|---|---|---|---|---|---|
| Anthracene | 4.35 | 3.13 | 21.9 | 210.7 | 7.7 | 1.6 | 7.28 |
| Benzo[*a*]anthracene | 5.52 | 3.92 | 2.14 | 187.1 | 37.1 | -* | 7.39 |
| Benzo[*a*]pyrene | 6.11 | 4.32 | 0.66 | 264.3 | 97.2 | 21.0 | 6.81 |
| Benzo[*b*]fluoranthene | 6.11 | 4.32 | 0.66 | 105.4 | 8.4 | -* | 7.60 |
| Benzo[*ghi*]fluoranthene | 5.52 | 3.92 | 2.15 | 210 | 25 | 25 | 7.71 |
| Benzo[*ghi*]perylene | 6.7 | 4.73 | 0.20 | 28.5 | n.t. | n.t. | 6.96 |
| Benzo[*k*]fluoranthene | 6.11 | 4.32 | 0.66 | 50.3 | 35.1 | -* | 7.39 |
| Chrysene | 5.52 | 3.92 | 2.14 | -* | n.t. | n.t. | 7.70 |
| Fluoranthene | 4.93 | 3.52 | 6.94 | 260.9 | 41.6 | 7.5 | 7.70 |
| Indeno[1,2,3-*cd*]pyrene | 6.7 | 4.73 | 0.20 | 90.5 | 48.8 | 31.1 | 6.85 |
| Perylene | 6.11 | 4.32 | 0.66 | -* | n.t. | n.t. | 6.70 |
| Phenanthrene | 4.35 | 3.13 | 21.9 | 5.8 | 6.6 | 5.0 | 8.21 |
| 2-Phenylnaphthalene | 4.93 | 3.52 | 6.94 | 19.1 | 24.6 | 19.6 | 8.16 |
| Pyrene | 4.93 | 3.52 | 6.94 | 199.1 | 28.5 | 12.2 | 7.24 |

*) no toxicity observed, n.t.: not tested

In this plot, only substances could be included with a measurable $EC_{50}$ for the two illumination conditions. However, some compounds did not induce an observable effect in the presence of UV-filtered light. On the other hand, as light is present even under the UV-filtered illumination conditions, it cannot be completely ruled out that photoinduced toxicity takes place at least for some of the compounds at these light conditions. Therefore, in order to include all tested compounds, a similar plot is presented in Figure 5.5 where the excess toxicity of the investigated compounds under simulated sun light is used instead of the toxicity enhancement. The excess toxicity is a measure for the surplus toxicity of a compound as compared to the assumption that it would

act via a purely narcotic mode of action (Lipnick 1990). Calculated excess toxicity values are given in Table 5.3.

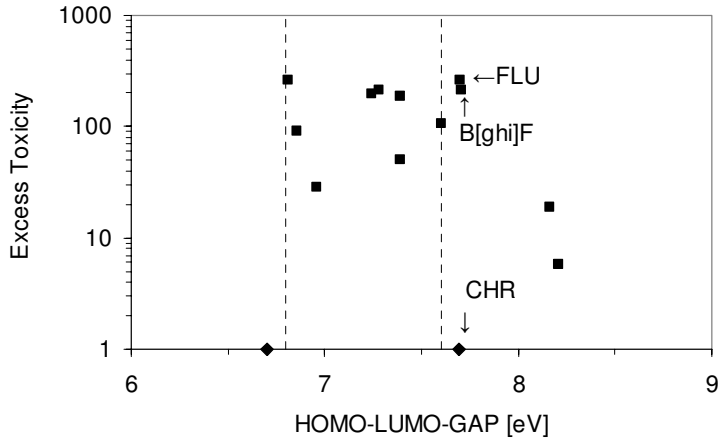


*Figure 5.5: Excess toxicity of compounds in simulated sun light. ($EC_{50,base\ line}$ / $EC_{50,sim.\ sun\ light}$) (■). For the two compounds that did not cause effects under simulated sun light, base line toxicity was assumed and used for the calculation (♦). Dashed lines show the HOMO-LUMO gap window (7.2 ± 0.4 eV), where photo-induced toxicity is expected to occur for PAH compounds according to Mekenyan et al. (1994). Chrysene (CHR), Fluoranthene (FLU), Benzo[ghi]fluoranthene (B[ghi]F).*

The results look similar to those shown in Figure 5.4. All compounds lying inside of the proposed HOMO-LUMO gap window of 7.2 ± 0.4 eV exert higher toxicity than assumed from narcosis. However, three substances with a nearly identical HOMO-LUMO gap exert a clearly different phototoxic behaviour. All three lying slightly outside the HOMO-LUMO gap window, fluoranthene (7.701 eV) and benzo[*ghi*]fluoranthene (7.708 eV) were 260 and 210 times more toxic than expected from a purely narcotic mode of action respectively, whereas chrysene (7.696 eV) did not exert toxicity even at concentrations close to its water solubility. A reason for this may be sought in differences in absorbance of the compounds. The long-wave-length transition at 349, 359 and 361 nm for benzo[*ghi*]fluoranthene, fluoranthene and chrysene, respectively, differ in intensity because this transition is an allowed process for benzo[*ghi*]fluoranthene and fluoranthene, whereas for chrysene it is forbidden. Interestingly, two of these substances were previously studied for their photoinduced toxicity to *Daphnia magna* (Newsted and Giesy 1987). In

this study which was the basis for the development of the HOMO-LUMO gap model (Mekenyan *et al.* 1994), chrysene exerted higher toxicity than fluoranthene. Phenanthrene (8.2 eV) and 2-phenylnaphthalene (8.4 eV) lying far outside the HOMO-LUMO gap window showed excess toxicity values of 6 and 23, which are somewhat higher than base line toxicity. However, they would not be classified as either reactive or specifically acting according to the classification scheme of Verhaar *et al.* (1992). Furthermore, as these values were not affected by the light conditions, it can be assumed that other modes of action may be involved.

In conclusion, the HOMO-LUMO gap window, originally proposed as an indicator for photoinduced toxicity to *Daphnia magna*, is also capable of identifying potentially phototoxic compounds to *Scenedesmus vacuolatus*. This may be surprising as Mekenyan *et al*. (1994) used a toxic endpoint (adjusted medium lethal time ($ALT_{50}$)) different from this study where the $EC_{50}$, the concentration provoking 50% of inhibition of algal reproduction, was used. The replacement of the log $ALT_{50}$ by either photoinduced toxicity enhancement or excess toxicity were capable of indicating phototoxic behaviour of the compounds when plotted against the HOMO-LUMO gap. Although the HOMO-LUMO gap window was successful to predict phototoxic behaviour qualitatively, it was not capable of quantifying the expected photoinduced toxicity. Mekenyan *et al*. (1994) observed a bell-shaped curve whose flanks were aimed to be described by two straight lines. However, neither a clear bell-shaped curve nor a quantitative relation between HOMO-LUMO gap and photoinduced toxicity enhancement or $T_e$ was observed in this study.

### 5.3.3 Quantitative modelling of light dependency of toxicity of the individual phototoxic compounds

The toxicity of the different PAH compounds depends on the light conditions to a varying extend. In order to make the impact of the light conditions comparable between the investigated compounds, the excess

toxicity is calculated for each light situation. Calculated $EC_{50,baseline}$ values and the $T_e$ values of the investigated compounds under the three different light conditions are shown in Table 5.3. For illustration the relative excess toxicity is plotted in Figure 5.6.

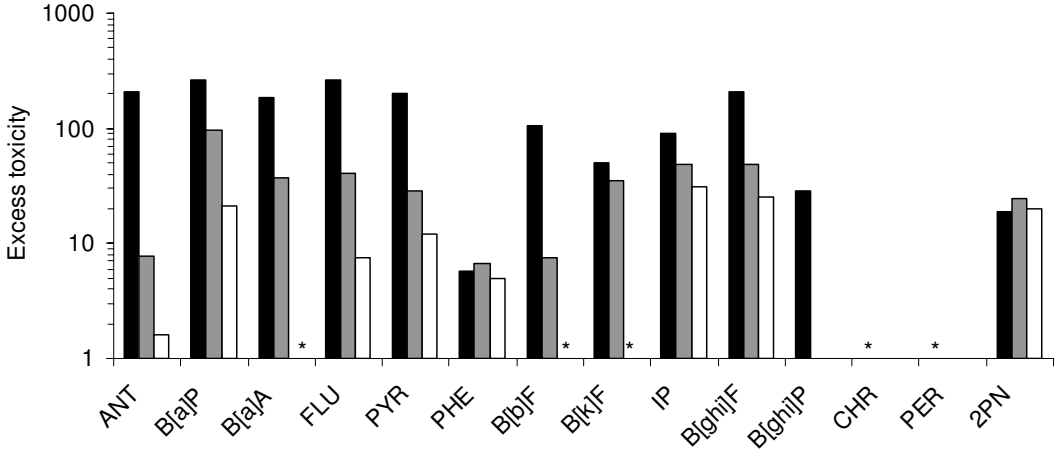


*Figure 5.6: Excess toxicity of compounds under three different light conditions. Simulated sun light (■), standard growth light: (■), UV-filtered light (□). Compounds marked with asterisk (*) did not induce observable effects.*

The influence of the light conditions on the toxicity of the compounds varies. Whereas no influence of the light conditions on the excess toxicity can be observed for phenanthrene and 2-phenylnaphthalene, compounds like benzo[*a*]anthracene and benzo[*b*]fluoranthene possess high excess toxicity values under simulated sun light ($T_e$ of 187 and 37), lower under standard light ($T_e$ of 102 and 8), and they are non-toxic under UV-filtered light. However, not all compounds show $T_e$ values < 10 for UV-filtered light which would be assumed from a purely narcotic mode of action. Compounds with the highest excess toxicity values under UV-filtered light are benzo[*a*]pyrene, indeno[1,2,3-*cd*]pyrene and benzo[*ghi*]fluoranthene ($T_e$ of 21; 31 and 25). These compounds absorb light within the visible range of the spectrum. It can, therefore, be assumed that for these compounds photoinduced toxicity may still be relevant under the UV-filtered light condition.

According to the model [eq. 16] for estimation of toxic effects from compound concentration and light intensity modified from Ankley *et al*. (1995) [eq. 13] a linear relation between log $EC_{50}$ and log J (transition intensity) can be assumed. The transition intensities J were calculated as overlap integral of the irradiated light and the molar absorption of the compounds [eq. 19]. The results of the integrated overlap are presented in Table 5.4. As 2-phenylnaphthalene does not absorb light in the region of the used irradiation source and its toxicity does not depend on the light conditions, this compound was excluded from further considerations.

*Table 5.4: Transition intensity J of irradiation spectra and molar absorption of the compounds calculated from Equation 19.*

| | $J_{sim.\ sun\ light}$ [µE / mol / s] | $J_{stand.\ growth\ light}$ [µE / mol / s] | $J_{UV\text{-}filt.\ light}$ [µE / mol / s] |
|---|---|---|---|
| Anthracene | 2.52E-07 | 1.04E-08 | 2.10E-10 |
| Benzo[*a*]anthracene | 1.78E-07 | 8.05E-09 | 1.59E-10 |
| Benzo[*a*]pyrene | 9.26E-07 | 9.36E-08 | 4.63E-09 |
| Benzo[*b*]fluoranthene | 4.27E-07 | 2.25E-08 | 3.17E-10 |
| Benzo[*ghi*]fluoranthene | 1.32E-07 | 1.13E-08 | 1.78E-09 |
| Benzo[*ghi*]perylene | 3.67E-07 | 5.96E-08 | 1.17E-09 |
| Benzo[*k*]fluoranthene | 5.11E-07 | 6.73E-08 | 2.67E-08 |
| Chrysene | 1.40E-08 | 2.04E-09 | 2.49E-10 |
| Fluoranthene | 2.46E-07 | 1.26E-08 | 2.06E-10 |
| Indeno[1,2,3-*cd*]pyrene | 1.24E-06 | 1.85E-07 | 1.16E-07 |
| Perylene | 9.76E-07 | 9.12E-07 | 2.31E-07 |
| Phenanthrene | 1.91E-09 | 2.46E-10 | 1.13E-11 |
| Pyrene | 1.96E-07 | 4.49E-09 | 6.62E-10 |

In order to test, whether for all investigated compounds the observed toxicity shows a linear correlation to the energy absorbed by the compound, the $EC_{50}$ was plotted against the transition intensity J for three selected compounds (Figure 5.7).

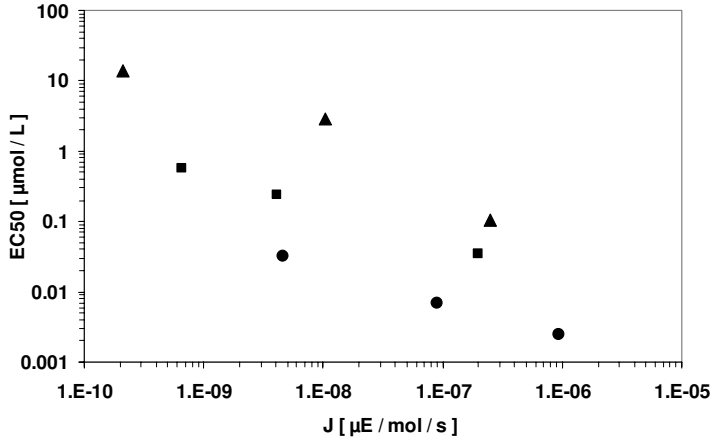


*Figure 5.7: Relation between measured $EC_{50}$ and overlap integral J of irradiation spectra and molar absorbance of compounds for selected compounds: anthracene (▲), pyrene (■) and benzo[a]pyrene (●)*

A linear relationship between the log $EC_{50}$ and the log J can be inferred from Figure 5.7 for the individual compounds. Additionally, the slopes of the compound-specific relationships appear to be similar, though the intercepts are different. One reason for this may be sought in the different lipophilicity of the compounds. In this representation ambient water concentrations were used. However, only compounds incorporated in the organism can exert toxicity. Thus referring exclusively to the ambient water concentration of a compound may be misleading when aiming to compare different substances for their photoenhanced effects. The uptake of chemicals from the ambient water by the organism is referred to as bioaccumulation, which is understood as a combined process of biomagnification and bioconcentration. The former occurs during the consumption of low trophic level aquatic biota as food by higher level biota. The latter occurs by passive diffusion from the ambient water into the organism. Bioconcentration is generally dominant in most situations and is the only possible uptake route for autotrophic organisms, such as algae (Connell 1998). It can be quantitatively described by the bioconcentration factor (BCF), a measure of relation of internal to ambient concentration at thermodynamical equilibrium. However, in order to use BCF values adequately for estimation of internal concentrations, thermodynamical

equilibrium has to be achieved which for many organisms needs long time to establish. Manthey *et al*. (1993) demonstrated for a series of phenylurea herbicides that equilibrium between ambient water concentrations and internal concentrations in the unicellular organism *Scenedesmus vacuolatus* is achieved within 30 minutes. Thus, internal doses inside the test organism used in the present study can be estimated by simple inclusion of the bioconcentration factor assuming steady-state conditions. The resulting $ED_{50}$ values, calculated from the measured $EC_{50}$ and the estimated BCF [eq. 18], are further on used in this study.

In order to investigate, if a similar relation between log $ED_{50}$ and log J can be observed between all investigated compounds, linear regressions were calculated for each the data sets of the six individual compounds with data for three different light conditions each. The result of the linear regressions between log $ED_{50}$ and log J are reported in Table 5.5.

*Table 5.5: Functions of relationships between log $ED_{50}$ and log J for different compounds.*

| | a* slope | b* y-axis intercept | $R^2$ |
|---|---|---|---|
| Anthracene | -0.678 | -2.152 | 0.934 |
| Benzo[*a*]pyrene | -0.480 | -1.188 | 0.997 |
| Benzo[*ghi*]fluoranthene | -0.496 | -1.453 | 0.985 |
| Fluoranthene | -0.496 | -1.273 | 0.987 |
| Indeno[1,2,3-*cd*]pyrene | -0.412 | -0.361 | 0.937 |
| Pyrene | -0.492 | -1.233 | 0.999 |

*Data were fitted using the equation: log $ED_{50}$ = a * log J + b

The complete data set is depicted in Figure 5.8, including all calculated $ED_{50}$ values and the six linear regressions. For three compounds (benzo[*a*]anthracene, benzo[*b*]fluoranthene and benzo[*k*]fluoranthene) no regression calculation was performed, as only two data points were available. Nevertheless, the data points of these compounds are included in the plot. The regression analysis reveals that all compounds show a clear linear relationship between the two parameters considered (Table 5.5). The slopes

vary from -0.412 to -0.678. The average slope ā is -0.51 (± 0.09). The relationships are more or less parallel (Figure 5.8). The variance between the relationships of the individual compounds is reduced when using estimated internal concentrations instead of ambient water concentrations (Figure 5.7). However, as still more than one order of magnitude lies between some of the relationships, factors other than absorbed energy and PAH tissue concentration seem to be important for the quantitative description of PAH phototoxicity.

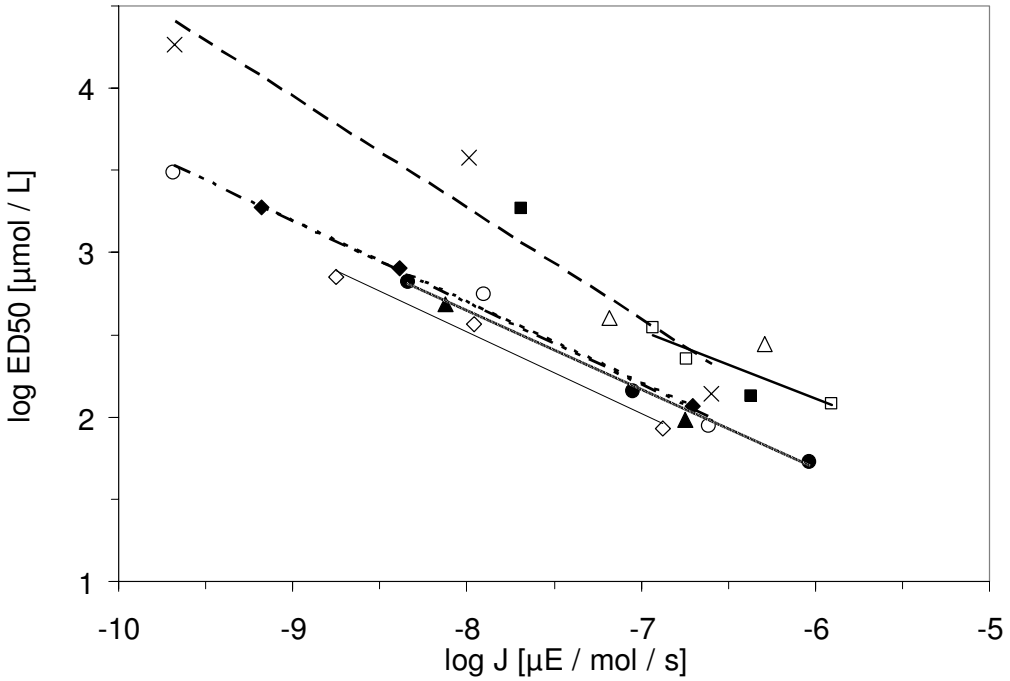


*Figure 5.8: Relationship between log $ED_{50}$ and log J (transition intensity) for all investigated compounds exerting phototoxic behaviour. For data points and regressions see Table 5.4 and Table 5.5. Anthracene (x), benzo[a]pyrene (●), benzo[a]anthracene (▲), fluoranthene (○), pyrene (♦), benzo[b]fluoranthene (■), benzo[k]fluoranthene (Δ), indeno[1,2,3-cd]pyrene (□), benzo[ghi]fluoranthene (◊)*

### 5.3.4 Relative phototoxic efficacy

So far the internal concentration and the amount of absorbed photons were included in the regression. However, there is no reason to assume that all PAHs act identically when present inside the organism and having

absorbed a certain amount of energy. The electron excited through the absorption of a photon has several possibilities to react. Triplet-state formation – one possible reaction pathway – is believed to play a key role in the process of photosensitising. The quantum yield of this reaction differs between different PAH compounds. Secondly, the lifetime of these excited triplet-state species can vary from compound to compound. Thus, the possibility of one photon absorbed to produce singlet oxygen is dependent on the compounds' properties. A deeper insight into the reactions involved in this reaction mechanism is beyond the aim of this study. However, it has previously been proposed to use a pragmatic straightforward quantum yield Φ of toxicity (Morgan and Warshawsky 1977; Oris and Giesy 1987; Newsted and Giesy 1987), in order to describe the distinct behaviour of different compounds. This procedure does not require detailed information on the processes involved, but uses the experimental data to derive comparable values to characterise the relative potency of the compounds.

In order to be able to compare different compounds, Morgan and Warshawsky (1977), Newsted and Giesy (1987) and Oris and Giesy (1987) calculated relative photodynamic activities (RPA) or relative potency factors (RPF) from slopes of time-effect relationships, observed for one concentration under one irradiation condition. In this study, complete concentration-response relationships have been determined under three different light conditions. Assuming the mechanism of phototoxic action to be similar for all compounds – only the extent of conversion of absorbed energy into toxicity would differ – the relation between log $ED_{50}$ and log J * Φ should result in a linear relationship for all compounds. This assumption of a linear relationship was thus used to determine relative phototoxic efficacy values (RPE) of the compounds. The term RPE was chosen to avoid confusion with previously proposed RPA and RPF values which are calculated in a different way. The relative efficacy RPE of the compounds was iteratively varied until a straight line with maximised $R^2$ was obtained. The relative efficacy of the most efficient compound (benzo[*ghi*]fluoranthene) was set to 1 and all other values

were adjusted accordingly. The resulting relationship is presented in Figure 5.9.

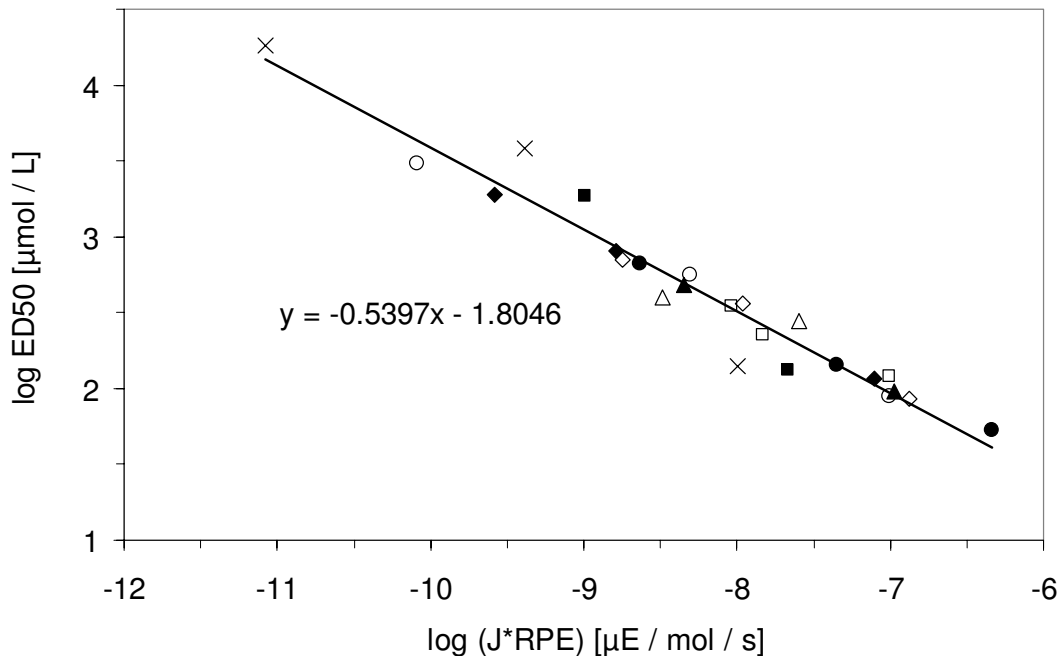


*Figure 5.9: Regression through the complete data set when relative efficacies for photoinduced toxicity (RPE) are assumed according to Table 5.6. Anthracene (x), benzo[a]pyrene (●), benzo[a]anthracene (▲), fluoranthene (○), pyrene (♦), benzo[b]fluoranthene (■), benzo[k]fluoranthene (Δ), indeno[1,2,3-cd]pyrene (□), benzo[ghi]fluoranthene (◊)*

Results of the iterative approximation of RPE values of the individual compounds are given in Table 5.6. Using these values, the following equation can now be proposed for the prediction of phototoxicity of PAHs to *Scenedesmus vacuolatus*:

$$\log ED_{50} [\,\mu mol / L\,] = -0.5397 \log J * RPE - 1.8046 \qquad \textit{[eq. 20]}$$

The slope of this relationship (-0.5397) is close to the medium slope of the individual compounds (ā: -0.51). Maximum RPE values for phenanthrene, benzo[*ghi*]perylene, perylene and chrysene given in Table 5.6 were estimated from [eq. 20] using $J_{sim.\ sun\ light}$ and either $EC_{50,sim.\ sun\ light}$ or $EC_{50,baseline}$ in case that no effects were observed. Assuming [eq. 20] holds true for these

compounds, higher RPE values would have resulted in higher toxicity than observed.

*Table 5.6: Estimated Relative Phototoxic Efficacy (RPE) values for photoinduced toxicity of the different investigated compounds. Additionally, literature values for quantum yields for triplet state formation ($\Phi_T$) and singlet oxygen production ($\Phi_{singlet\ oxygen}$) are presented.*

| | Relative phototoxic efficacy (RPE) | $\Phi_T$ | $\Phi_{singlet\ oxygen}$ |
|---|---|---|---|
| Anthracene | 0.04 | 0.6[c] | 0.31[d] |
| Benzo[*a*]anthracene | 0.6 | 0.8[c] | 0.75[d] |
| Benzo[*a*]pyrene | 0.5 | 0.4[c] | |
| Benzo[*b*]fluoranthene | 0.05 | | |
| Benzo[*ghi*]fluoranthene | 1 | | |
| Benzo[*ghi*]perylene | ≤0.02[a] | 0.53[d] | |
| Benzo[*k*]fluoranthene | 0.05 | | |
| Chrysene | ≤0.0004[b] | 0.85[d] | 0.9[d] |
| Fluoranthene | 0.4 | 0.6[c] | 0.5[d] |
| Indeno[1,2,3-*cd*]pyrene | 0.08 | | |
| Perylene | ≤0.00002[b] | 0.009[d] | 0.29[d] |
| Phenanthrene | ≤0.03[a] | 0.8[c] | 0.5[d] |
| Pyrene | 0.4 | 0.27[c] | 0.6[d] |

[a]) values were calculated from $ED_{50,sim.\ sun\ light}$ and $J_{sim.\ sun\ light}$ using [eq. 20] [b]) values were calculated from $ED_{50,baseline}$ and $J_{sim.\ sun\ light}$ using [eq. 20] [c]) from Huang *et al*. (1997) [d]) from Dabestani and Ivanov (1999)

Care should be taken, in order not to misinterpret the relative efficacy RPE as relative phototoxic strength. This value is a relative measure of how much of the absorbed energy of the bioaccumulated compounds is actually converted into damage of cellular functions. For example, although only possessing a low relative efficacy value of 0.08, indeno[1,2,3-*cd*]pyrene is the most toxic compound (in terms of $EC_{50}$) due to its high lipophilicity (and resulting bioconcentration) and its high light absorption.

The relative efficacy values of the investigated compounds vary by several orders of magnitude. As benzo[*ghi*]fluoranthene was the most effective compound to convert absorbed photons into damage of cellular

functions, its relative efficacy was set to one. Benzo[*a*]antracene also exerted a high efficacy. Anthracene had a comparably low relative efficiency (0.025). This may also reflect the relatively fast photodegradation of this substance. Huang *et al* (1997) and Brack *et al*. (2003) reported high rate constants for photomodification of anthracene exposed to simulated solar radiation.

### 5.3.5 Comparison of RPE values to previously published values

Formation of excited triplet-state PAH species and formation of singlet oxygen are believed to play a key role for photoenhanced PAH toxicity (Ankley *et al.* 2003). For some of the studied compounds, literature values for quantum yields of these processes are available and are included in Table 5.6. However, when comparing these values to the experimentally determined RPE values, no obvious relationship is apparent (Figure 5.10).

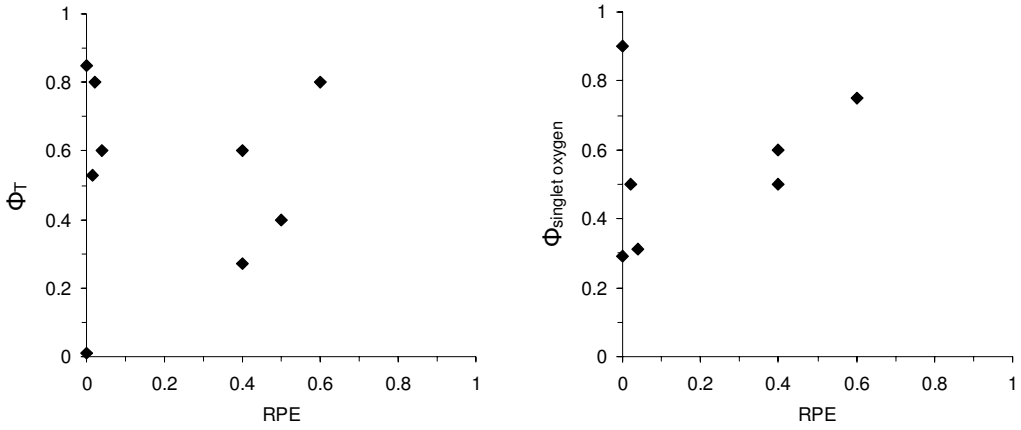


*Figure 5.10: Comparison of RPE values to quantum yields for triplet state formation ($\Phi_T$) and singlet oxygen production ($\Phi_{singlet\ oxygen}$) (Table 5.6)*

Previous experimental studies presented as a result values for relative photodynamic activity RPA (Morgan and Warshawsky 1977; Newsted and Giesy 1987), relative potency factor RPF (Oris and Giesy 1987) or photosensitising and photomodification factors (PSF, PMF) (Huang *et al.* 1997; Krylov *et al.* 1997). Literature data for the different parameters for the compounds tested in this study are summarised and compared to relative phototoxic efficacy RPE values determined herein in Table 5.7.

*Table 5.7: Comparison of relative efficacy values of PAHs investigated herein to published values*

| | RPE[a]) this study | RPA[b]) Morgan and Warshawsky 1977 | RPF[c]) Oris and Giesy 1987 | RPA[b]) Newsted and Giesy 1987 | PSF[d])+ PMF[e]) Huang *et al*. 1997 |
|---|---|---|---|---|---|
| | *Scenedesmus vacuolatus* | *Artemia salina* | *Pimephales promelas* | *Daphnia magna* | *Lemna gibba* |
| Anthracene | 0.04 | | 21.5 | 1.06 | 1.019 |
| Benzo[*a*]anthracene | 0.6 | 9.8 | 16.4 | 0.45 | 0.407 |
| Benzo[*a*]pyrene | 0.5 | 0.55 | 1 | 1.83 | 0.076 |
| Benzo[*b*]fluoranthene | 0.05 | | | | 0.042 |
| Benzo[*ghi*]fluoranthene | 1 | 0.96 | | | |
| Benzo[*ghi*]perylene | ≤0.02 | 0.02 | no mortality[f] | 1.45 | 0.049 |
| Benzo[*k*]fluoranthene | 0.05 | 0.08 | | 0.3 | |
| Chrysene | ≤0.0004 | 0.2 | | 0.44 | 0.036 |
| Fluoranthene | 0.4 | 0.15 | | 0.42 | 0.199 |
| Indeno[1,2,3-*cd*]pyrene | 0.08 | 0.19 | | | |
| Perylene | ≤0.00002 | 0.04 | no mortality[f] | 0.13 | |
| Phenanthrene | ≤0.03 | | no mortality[f] | no mortality[f] | 0.063 |
| Pyrene | 0.4 | 0.72 | 100.1 | 1.64 | 0.072 |

[a]) relative phototoxic efficacy, [b]) relative photodynamic activity, [c]) relative potency factor, [d]) photosensitation factor, [e]) photomodification factor, [f]) In these experiments no mortality was observed, thus no RPA / RPF values were determined. For correlation purposes values were set to zero.

Apart from a weak correlation between the RPE values presented here and the RPA values published by Morgan and Warshawsky (1977) (n=10, $R^2 = 0.46$), no correlation can be observed between the RPE values presented in this study and previously published values for RPA, RPF or PSF+PMF. Neither can any correlation be found between the previously published values. This is somewhat surprising. The absolute difference in sensitivity of different species towards phototoxic activity can easily be explained by different protective mechanisms. However, if phototoxic action is caused by a common mode of action, one would expect that the relative efficacy of the compounds compared to each other would be similar. This, however, seems not to be the case. Benzo[*a*]pyrene which showed medium phototoxic efficacy to algae in this study was the most effective compound in

the study on *Daphnia magna* (Newsted and Giesy 1987) and the compound with the weakest phototoxic activity towards the fathead minnow (Oris and Giesy 1987).

One reason for this discrepancy may be sought in either the inclusion or exclusion of bioaccumulation of the compound in the tissue. For calculations of RPA values, Morgan and Warshawsky (1977) did not consider differences in bioaccumulation of the compounds. In the studies of Newsted and Giesy (1987) and Oris and Giesy (1987) as well as in the present study, calculations of phototoxic activity were based on internal concentrations. Thus, differences in bioconcentration are not included in the relative phototoxic efficacy values.

Another reason for differences could be the exposure conditions varying between studies. They differed in light intensity and spectral composition of the irradiance. Furthermore, the test duration differed substantially. Whereas the test with *Lemna minor* was run for 8 days, fish larvae were exposed for 4 days, the algae in this study were irradiated for 14 hours, and the test with *Artemia salina* was run for just 30 minutes. The longer the test duration, the larger the impact of photomodification products will become. Huang *et al*. (1997) were the only ones to separate the toxicity caused by photosensitising from toxicity caused by photomodified PAHs. Still after an 8 day exposure for 9 out of 16 investigated PAHs the impact of photosensitising prevailed. Thus, it is reasonable to assume that the impact of photomodification in the algal test system is less pronounced.

### 5.3.6 Conclusion

Summing up, it may be said that the HOMO-LUMO gap can be used as a qualitative indication whether phototoxic behaviour can be expected for PAHs in different organism. For quantitative assessment of photoenhanced toxicity of a PAH compound to *Scenedesmus vacuolatus* a model can be proposed using internal concentration of the compound in the organism, irradiation spectra of the illumination source, the absorbance of the compounds and an empirically determined relative phototoxic efficacy of the compound. For the

development of a truly predictive model for phototoxicity of PAHs, a challenge for future research might be to derive values for the relative efficacy from molecular descriptors.

# Chapter 6

# Experimental and Modelling Section

## *6.1 Sampling and Sample Preparation*

### 6.1.1 Sampling

Sediment samples were collected at 11 sites in the Brofjorden close to the Malmön peninsular shore in Sweden (Figure 6.1). This area was chosen, because it represents a typical site between the North and the Baltic Sea with various inputs of contaminants from sea traffic and a nearby refinery. Therefore, a mixture of different substances at comparably low concentrations was expected to be present. The samples were taken from a depth of about 27-28 m in the area of N 58°20' 14-30", E 11°20' 65-75" on 14/06/2000. The sampling was carried out from a research vessel of the Kristineberg marine research station. Samples were collected with a grab (box) sampler (Jonasson and Olausson 1966) equipped with an inner metal container measuring 28 x 28 $cm^2$ x 50 cm. The sediment cores were lifted on board and the water column left above the samples was removed with plastic tubes. The upper sediment layer (approx. 2 – 4 cm) consisted of brown-green, well bioperturbated sediment, followed by a grey layer and then by a black layer at a depth of approx. 15 cm. Only the top layer was sampled by carefully lifting off these 4 cm with a metal plate. Samples from all 11 sites were combined, frozen and transported to the laboratory of the UFZ, Leipzig, for further treatment. There, samples were freeze-dried.

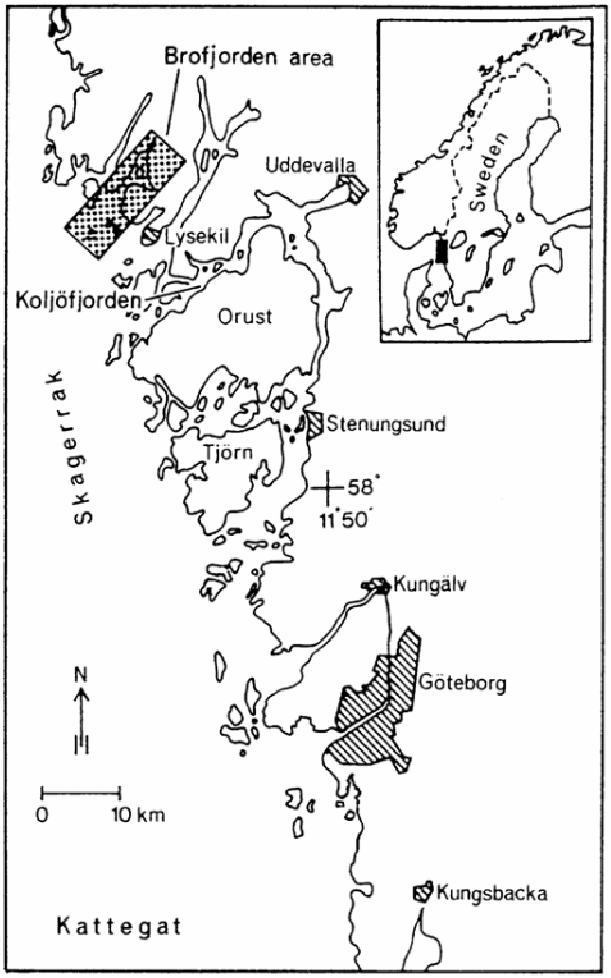


*Figure 6.1: Sampling site in Brofjorden, Sweden*

Content of total organic carbon (TOC) of the freeze-dried sediment sample was analysed by measuring the $CO_2$ formation of the sample exposed to an oxygen stream while heating to 580°C using a LECO RC 412 system.

### 6.1.2 Extraction

The freeze-dried samples were sieved through a 63 µm mesh. 200 g of the sieved sample were mixed with 200 g of fine sea sand and Soxhlet-extracted with approximately 2 L of dichloromethane for 24 h. The aim of this study was not the exact quantification of specific compounds in sediments, but an effect-directed identification of dominant toxicants which were not known at the beginning of the work. Thus, the extraction method was not

optimised for specific compounds, but it was rather chosen to extract a broad spectrum of compounds.

### 6.1.3 Sulphur Clean-up

The extracts were then concentrated to approximately 200 mL, shaken with 5 g of fine copper powder (99%, Baker, Deventer, The Netherlands) activated with concentrated HCl over night and filtered through glass fibre filters (GF/F, Whatman, Maidstone, England), in order to remove sulphur which impairs biological testing of extracts.

## *6.2 Chemical Methods*

### 6.2.1 Fractionation

The extract was fractionated in a two-step procedure. Primary fractionation was conducted using open column chromatography. If primary fractions were toxic and still too complex for toxicant identification and confirmation, a secondary fractionation step using normal-phase HPLC was applied. Fractions are marked as Fi.j with i signifying the fraction number in the primary fractionation step, and j representing the fraction number in the second step.

*Open Column Chromatography*

For the first fractionation step, neutral alumina N (activity I, 18 g, specific surface 150 $m^2$/g, ICN Biomedicals GmbH, Eschwege, Germany) was partially deactivated with 4.5% water. An aliquot of 20 mL of desulfurized sediment extract corresponding to 200 g of sediment was added to 18 g of deactivated alumina. By evaporation of the solvent in a rotation evaporator the sample was dried. The loaded alumina was then transferred onto 90 g of neat deactivated alumina in *n*-hexane in a glass column with a diameter of 3 cm. Compounds were eluted with solvents of increasing polarity, separating visible bands. F1, a colourless fraction was eluted with 72 mL *n*-hexane, followed by the slightly yellow-coloured fraction F2, eluted with 270 mL

*n*-hexane/dichloromethane (DCM) (90:10, v:v), followed by F3, an intensively yellow-coloured fraction, eluted with 270 mL dichloromethane. The next fraction was eluted with 270 mL methanol/acetic acid (99:1, v:v). Then 100 mL of 0.01 M HCl was added and the solution was washed 3 times with 25 mL *n*-hexane. The united organic phases are named F4. The pH of the aqueous solution was then elevated to 12 by addition of 1 M NaOH and then washed 3 times with 25 mL *n*-hexane again. F5 was composed of the united organic phases. All fractions were evaporated to dryness and redissolved in 20 mL *n*-hexane. As F3 was only partially soluble in *n*-hexane it was kept in DCM.

*HPLC*

A secondary fractionation step was performed for the fraction F2 which was known to contain PAH compounds. Therefore, a secondary fractionation procedure was chosen providing a reliable separation of aromatic compounds. For this purpose 28 standard PAH compounds with molecular weights from 152 to 300 g / mol were used to develop a fractionation scheme, designed to separate compounds with different numbers of rings into distinct fractions. Fractionation of F2 was performed on normal phase HPLC, using isocratic *n*-hexane/dichloromethane (95:5, v:v) at a flow of 19 mL / min. The system was equipped with a ET250/4 Nucleosil 100-5 $NO_2$ column and UV-VIS detection (BIO-TEK HPLC 535, Milan, Italy). UV-active compounds were detected at 252 nm. Injection volume was 5 mL. For development of the fractionation scheme, 28 standard PAH compounds with molecular weights from 152 to 300 g / mol were used. The fractionation scheme was designed to separate compounds with different numbers of rings into distinct fractions. Retention times of all standard compounds were determined on the analytical HPLC (Table 6.1). Subsequently, time windows for fractionation were defined.

*Table 6.1: Molecular weights (MW) and retention times ($R_t$) of standard compounds used for the development of the fractionation scheme on the analytical and preparative HPLC. In the right column fractions are given in which compounds are expected.*

| Standard compound | MW [g / mol] | $R_t$ (analytical) [min] | $R_t$ (preparative) [min] | fraction |
|---|---|---|---|---|
| Acenaphthene | 154 | 5.37 | 8.96 | F2.2 |
| 1-Phenylnaphthalene | 204 | 6.1 | 10.17 | |
| Fluorene | 166 | 6.25 | | |
| Acenaphtylene | 152 | 6.32 | | |
| Anthracene | 178 | 7.18 | | F2.3 |
| 1-Methylanthracene | 192 | 7.2 | 12.74 | |
| 4H-Cyclopenta[*def*]phenanthrene | 190 | 7.39 | | |
| Phenanthrene | 178 | 7.46 | 13.23 | |
| 2-Phenylnaphthalene | 204 | 7.54 | | |
| Pyrene | 202 | 9.07 | | F2.4 |
| 1-Methylpyrene | 216 | 9.31 | | |
| Fluoranthene | 202 | 9.5 | 17.81 | |
| 11H-Benz[*b*]fluorene | 216 | 9.69 | | |
| Benzo[*ghi*]fluoranthene | 226 | 11.41 | 21.89 | F2.5 |
| Benz[*a*]anthracene | 228 | 12.32 | | F2.6 |
| Chrysene | 228 | 12.75 | 24.81 | |
| Cylopenta[*cd*]pyrene | 226 | 13 | | |
| Triphenylene | 228 | 13.6 | | |
| Benzo[*k*]fluoranthene | 252 | 16.18 | | F2.7 |
| Benz[*j*]fluoranthene | 252 | 17.63 | | |
| Benzo[*a*]fluoranthene | 252 | 17.66 | 37.96 | |
| Benz[*e*]pyrene | 252 | 18.03 | | |
| Perylene | 252 | 18.78 | | F2.8 |
| Indeno[1,2,3-*cd*]fluoranthene | 276 | 21.82 | | F2.9 |
| Indeno[1,2,3-*cd*]pyrene | 276 | 22.87 | 52.16 | |
| Benz[*ghi*]perylene | 276 | 23.93 | | |
| Dibenzo[*a,h*]anthracene | 278 | 24.54 | | |
| Coronene | 300 | 31.29 | | F2.10 |

For selected compounds, $R_t$ values were measured on the preparative HPLC in order transfer the scheme to the HPLC used for the sample fractionation. The transformation of retention times from the analytical HPLC to the preparative HPLC was performed by linear regression (Figure 6.2). Using this regression time windows were transferred from the analytical system to the preparative HPLC.

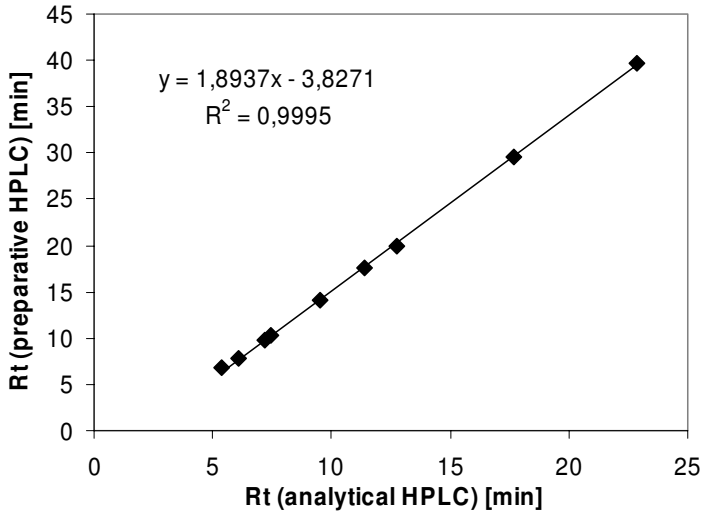


*Figure 6.2: Transfer of fractionation from analytical to preparative HPLC.*

Distinct time windows were defined from retention times of the standard compounds. Subsequently, ten secondary fractions were collected Figure 6.3: HPLC-Chromatogram for the fractionation of F2. Depicted molecular structures indicate where standard compounds, used during the development of the fractionation scheme, would elute.(Figure 6.3): F2.1 (4-7.30 min), F2.2 (7.30-11.55 min), F2.3 (11.55-16.11 min), F2.4 (16.11-21.24 min), F2.5 (21.25-23.09 min), F2.6 (23.10-31.39 min), F2.7 (31.40-38.09 min), F2.8 (38.10-46.09 min), F2.9 (46.30-65.59), F2.10 (66-100 min). All secondary fractions were evaporated to dryness and dissolved in *n*-hexane.

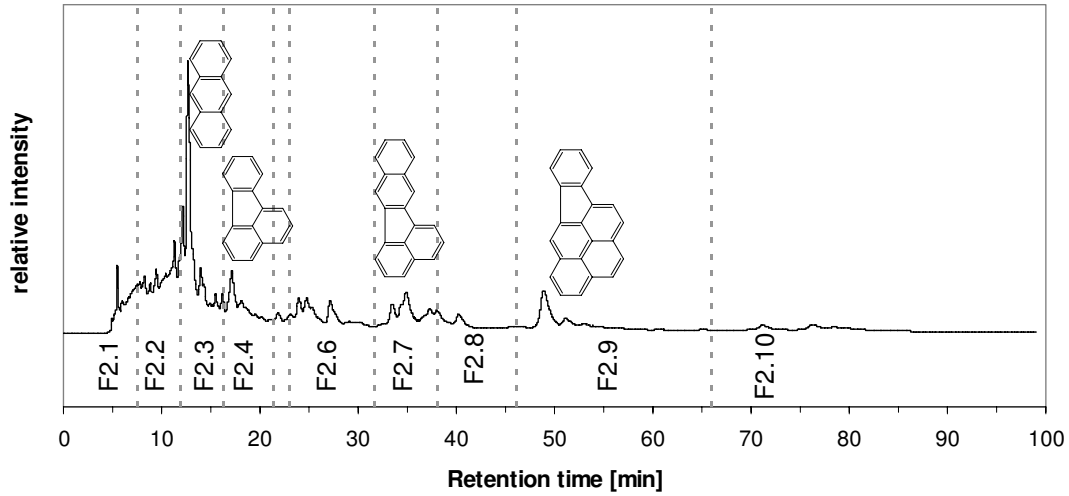


*Figure 6.3: HPLC-Chromatogram for the fractionation of F2. Depicted molecular structures indicate where standard compounds, used during the development of the fractionation scheme, would elute.*

*Procedural blanks*

Procedural blanks were conducted for the extraction and fractionation procedures, in order to account for possible artefacts induced through the procedure. None of the procedural blanks exerted toxicity in the test systems. It can thus be concluded that effects observed in the extracts and fractions were due to toxicants extracted from the sample.

### 6.2.2 Analytical Methods

*GC/MS*

The components of the fractions were separated by gas chromatography (model 5890 II, Hewlett Packard, Waldbronn, Germany) on a 5% diphenyl dimethyl polysiloxane capillary (model HP-5MS, Hewlett Packard; 30 m length 0.25 mm diameter, 0.25 µm film thickness) with helium as carrier gas, using a column head pressure of 76 hPa. Aliquots of 1 µL *n*-hexane solution were injected splitless. The injector temperature was 280°C. The column temperature was held at 60°C for 1 min and then increased with a rate of 30°C/min to 150°C, then increased with a rate of 6°C/min to 186°C and then to 280°C with a rate of 4°C/min. For the identification of unknown compounds,

the mass spectrometer (model 5971, Hewlett Packard; 70 eV, 280°C) was used in scan mode for ions of m/z 50 to 500. Compounds were identified using a spectra library (NIST/EPA/NIH Mass Spectral Library, National Institute of Standards and Technology, USA). The identity of compounds suspected to cause observed toxicity was confirmed comparing spectra and retention times with standard compounds. Identified compounds were quantified in selected-ion mode using external standards.

## *6.3 Biological Methods*

### 6.3.1 Algal Toxicity

Synchronous cultures of the unicellular green alga *Scenedesmus vacuolatus* were used as a test system. The parameter of toxicity was the inhibition of the cellular reproduction during one generation cycle lasting 24 hours according to the procedure decribed by Altenburger *et al*. (2004).

*Standard test protocol*

*Organism and culture conditions: Scenedesmus vacuolatus* (formerly *Chlorella fusca* var. *vacuolata*) Shih. et Krauss, strain 211-15, culture collection Pringsheim (Göttingen, Germany) was grown photoautotrophically at 28 ± 0.5 °C in a medium specified by Altenburger *et al*. (2004). Carbon supply was provided by addition of 1.5 mmol/L $NaHCO_3$ (final concentration) at the onset of the light phase. The culture vessels comprised tubes with an inner diameter of 2.6 cm (tubes 30x200 mm). Culture volumes were 10-60 mL. A stirring bar (20x5 mm), placed at the bottom of each tube kept the cells in suspension by stirring in 4 min intervals at a speed of about 250 rpm. The cultures were illuminated using a combination of two types of white fluorescent tubes (Osram L36W/41 Interna and Osram L36W/11 Daylight, Osram, Berlin, Germany) with an intensity of 13-18 $W/m^2$ (22-33 klux) and a photon flux density of about 370 µmol photons $m^{-2}$ $s^{-1}$ at the surface of the culture vessels. The cell density for these conditions was $7.5x10^4$ cells $mL^{-1}$.

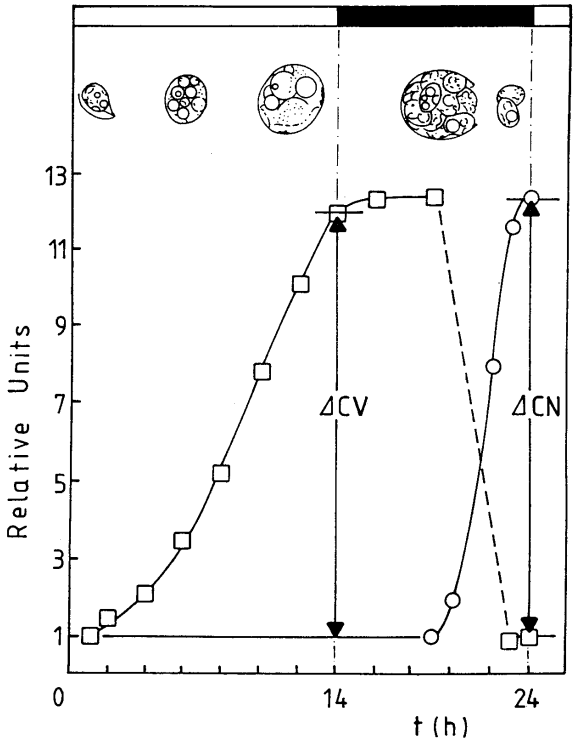


*Figure 6.4: Scheme of growth and development of synchronised Scenedesmus vacuolatus under standardised condition. □ cell volume (CV), ○ cell number (CN). The inhibition of cell reproduction (ΔCN) was used for quantification of algal toxicity. (Reproduced with permission from Altenburger et al. (1990))*

*Synchronisation*: The cells were synchronised by a light dark change of 14:10 h and a periodical dilution to a standard cell density before the onset of the light phase of the growth cycle ($t_0$). Synchronisation was checked routinely. For this purpose the homogeneity of the population of autospores was examined by analysing the cell size distribution at $t_0$.

*Application of test substances, sampling and determination of test parameters:* The dissolved test substances were added to the cultures at the beginning of the growth cycle ($t_0$). The concentration of DMSO (used as a co-solvent) in the medium did not exceed 0.1 % (v/v). This concentration proved to be of no measurable effect (Figure 6.5). The samples were taken from the cultures immediately after starting the experiment and at the end of the standard cycle ($t_{24}$). The inhibition of cell reproduction was calculated by

expressing the difference in cell count increase to the control as percent of the cell increase in the control cultures.

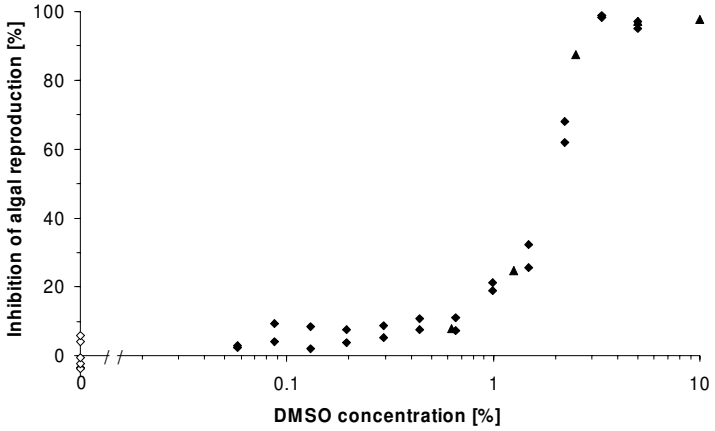


*Figure 6.5: Effect of DMSO on reproduction of Scenedesmus vacuolatus. Independent experiments are depicted with (♦) and (▲). Open symbols show the response of the controls.*

*Determination of cell number:* The cell number and the cell volume distribution were analysed, utilising a CASY II (Schärfe System, Reutlingen, Germany) with a tube having an aperture of 60 µm in diameter. Aliquots of algal suspension (1-10 mL) were diluted in either electrolyte CASYTON (Schärfe Sytems) or supplemented with NaCl to give a final concentration of 0.13 mol/L NaCl. The dilution was chosen to give 2500-15000 counts/50 µL (max. 5 % error by coincidence). The counting of cells was performed twice on duplicate samples. The system calibration was checked using latex particles of a defined size (15.6 fL, Coulter Calibration Standards).

*Test protocol for toxicity assessment under simulated sun light and UV-filtered light*

In addition to tests conducted according to the standard test protocol, toxicity was assessed under simulated sun light and UV-filtered simulated sun light. For these test conditions modifications of the standard test protocol were made. In order to minimise absorption of glass walls or water of the thermo-stabilising bath, test vessels were illuminated from the top. 30 mL Pyrex test tubes with a 2 cm diameter opening in the cap were used. For test

conducted under simulated sun light, the opening was filled with a polyacetate film Ultraphan 800 and test tubes were closed gas tight. In order to avoid too high light intensities and to be sure light solely entered through the opening, tubes were wrapped in aluminium. Tests under UV-filtered light were conducted accordingly; however a filter (Opalfilm, silber mittel R35, Haverkamp, Germany) was placed between the irradiation source and the test tubes.

*Characterisation of light conditions*

Three different light conditions were chosen for the execution of the algal biotest experiments. The spectral composition of all light sources was measured using a spectroradiometer IL2000 SpectroCube, (Polytec, Waldbronn, Germany). As the application of different light sources required a different geometry of the test vessels, the resulting absolute light intensity was determined inside of the test vessels using a spherical photosynthetic active radiation (PAR) sensor (QSL 101 UE, Quantum Solar Laboratory, Biospherical Instruments). The definite light conditions inside the test vessels were then calculated by normalising the spectral composition to the light intensity obtained from the spherical measurement. The standard light condition used for the bioassay as established in our laboratory protocol (Altenburger *et al.* 2004) were chosen as one illumination condition. These conditions were originally designed for optimal algal growth and consist of a combination of two types of fluorescent light tubes (L36W/41 Interna, L36W/11 daylight, Osram, Berlin, Germany) with an intensity of 13-18 W/m² (22-33 kLux) providing a photosynthetic active radiation of 400 µE/$m^2$/s in the middle of the test vessels. This illumination condition is characterised by a discontinuous spectrum with peaks at 430, 550 and 610 nm. The alternative light conditions were chosen in order to obtain an environmentally more realistic light regime. For this purpose the lamp SOL500 (Dr. Hoenle, Munich, Germany) was used which is designed to mimic natural sun light. This simulated sun light was characterised by a continuous spectrum with approximately 10 % UV-A radiation and intensities of 850 µE/$m^2$/s. These

illumination conditions correspond approximately to a solar spectrum recorded in Karlsruhe (Germany) in March at 12:00 PM on a cloudless day (data from Dr. Hoenle) except for a somewhat higher intensity of ultraviolet irradiation between 315 and 400 nm (UV-A) of the lamp. Additionally, as a reference case aiming to minimise the influence of light on the toxicity of environmental samples, a filter was added to the same lamp which completely eliminated the UV and reduced the visible region to a total intensity of 160 µE/$m^2$/s. This was the lowest irradiation which still allowed a sufficient cell reproduction. A complete elimination of light was obviously not possible, as algae need light for photosynthesis to cover their basic needs of energy supply. The different spectral compositions and intensities of the light conditions used are depicted in Figure 6.6.

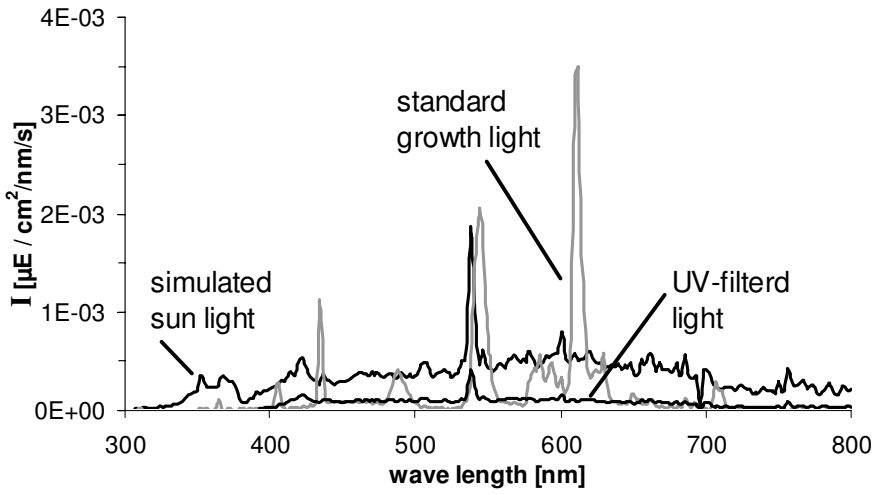


*Figure 6.6: Irradiation conditions used for the biotests.*

### 6.3.2 Bacterial Toxicity

*Luminescence inhibition in Vibrio fischeri (Acute Toxicity)*

The marine bacterium *V. fischeri* (NRRL-B-11177, Dr. Bruno Lange GmbH, Berlin, Germany) was used for testing the inhibition of bioluminescence which is related to the energy metabolism of the organism.

Bioluminescence was measured according to ISO 11348 (1998) in a saline medium (2% NaCl) at pH 7 and 15°C using the LUMIStox system (Dr. Bruno Lange GmbH, Berlin, Germany) at the beginning of the exposure ($t_0$) and after an incubation time of 30 minutes (t30). According to ISO 11348 the inhibition of bioluminescence is calculated as follows:

$$\%inhibition = 100 - (100 \cdot \frac{S_{t30} \cdot C_{t0}}{C_{t30} \cdot S_{t0}}) \qquad \textit{[eq. 21]}$$

where $S_{t0}$ is the bioluminescence of a sample at the beginning ($t_0$), $S_{t30}$ the bioluminescence after the incubation time of 30 minutes. $C_{t0}$ and $C_{t30}$ denote the arithmetic mean of the bioluminescence of all controls at $t_0$ and $t_{30}$ respectively.

Samples were applied dissolved in DMSO. The concentration of DMSO in the medium did not exceed 0.1 % (v/v). This concentration proved to cause no measurable effect (Figure 6.7).

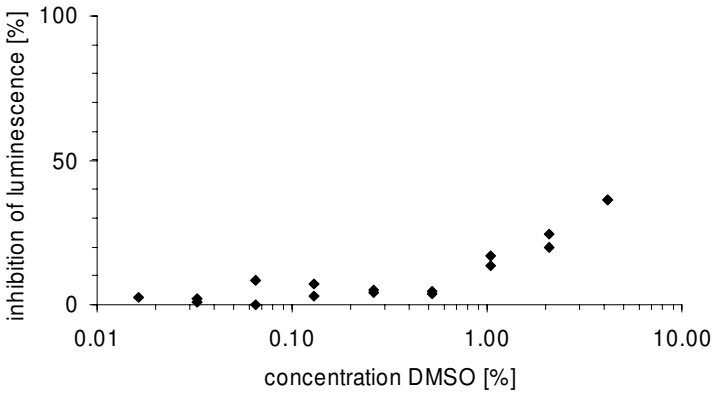


*Figure 6.7: Effects of DMSO in the luminescence inhibition test with Vibrio fischeri*

*Reproduction inhibition with Vibrio fischeri (Chronic Toxicity)*

The marine bacterium *V. fischeri* was also used for testing reproduction inhibition. The test media consists of 0.2 g / L $MgSO_4 \cdot 7H_2O$, 0.5 g / L $(NH_4)_2HPO_4$, 3 mL / L 1,2,3-propanetriol (glycerol), 5 g / L peptone, 0.5 g / L yeast extract, 30 g / L NaCl, 4.045 g / L $NaHPO_4 \cdot 2H_2O$, 2.79 g / L $KH_2PO_4$.

The optical density at 570 nm of the test solutions was measured at the beginning of the test ($t_0$) and at the end of the incubation of 8 hours ($t_8$). The relative inhibition is calculated according to:

$$\%inhibition = \frac{OD_{contr,t8} - OD_{sample,t8}}{OD_{contr,t8} - OD_{sample,t0}} \cdot 100 \qquad \textit{[eq. 22]}$$

## *6.4 Calculations*

### 6.4.1 Fitting of Concentration-response relationships

Experimental data of the single substances, mixtures and extracts tested under simulated sunlight and UV filtered light were fitted by the Hill model [eq. 23] which assumed a logistic distribution.

$$E = \frac{E_{Max}}{1 + (\frac{c}{X50})^{-P}} \qquad \textit{[eq. 23]}$$

where E is the fractional effect ($0 \leq E \leq 1$), $E_{Max}$ the maximal effect, c the concentration, X50 the concentration producing 50% of maximal effect (commonly $EC_{50}$) and P the slope parameter. $E_{Max}$ was fixed to 1 unless complete inhibition of algal reproduction was evidently not achieved.

### 6.4.2 Calculation of expected mixture toxicity

Expected mixture toxicities were calculated from the concentration-response relationships of the single compounds and the ratio at which they are present in the mixture. The concentration of each component in the mixture can be expressed as fraction of the total mixture concentration ($p_i$) which is defined as the sum the concentrations of the individual toxicants. Consequently, a total concentration of the mixture, at which a certain effect is generated, can be calculated using concentration addition according to (Faust *et al*. 2000):

$$ECx_{Mix,CA} = (\sum_{i=1}^{n} \frac{p_i}{ECx_i})^{-1} \qquad \textit{[eq. 24]}$$

where $ECx_{Mix,CA}$ is the total concentration of the mixture which is expected to provoke x % effect, $ECx_i$ the concentration of component i provoking the x% effect, when applied singly, and $p_i$ denotes the fraction of component i in the mixture. The calculation of total mixture concentrations for various effect levels leads to a complete iteration of an expected concentration-effect curve.

For independent action the following calculus applies (Faust *et al*. 2000):

$$E(c_{Mix}) = 1 - \prod_{i=1}^{n} (1 - E(c_i)) \qquad \textit{[eq. 25]}$$

The effect at the total concentration of the mixture, $E(c_{Mix})$, is based on the effects of the components, which they generate at concentrations, at which they are present in the mixture ($E(c_i)$). If the latter is expressed as fraction of the total mixture concentration, it holds:

$$E(c_{Mix}) = 1 - \prod_{i=1}^{n} (1 - E(p_i * c_{Mix})) \qquad \textit{[eq. 26]}$$

This allows the calculation of an effect expected according to the model of independent action, for any concentration of the mixture. The obtained concentration response curves refer to the sum of the individual concentrations of the identified toxicants.

# Summary

The establishment of cause-effect relationships between observed biological effects and environmental stressors is a key challenge in ecotoxicology. This challenge comprises the identification of toxicants present in biologically active environmental samples and the quantification of effect contributions of these toxicants to the observed adverse effects. Furthermore, the identification of milieu factors affecting toxicity of compounds and samples is needed, in order to transfer results obtained in the laboratory to realistic environmental situations. For the prediction of the influence of milieu factors on toxicity of toxicants, models are needed, in order to assess the toxicity for specific environmental situations quantitatively. This thesis addresses different aspects of these challenges in four consecutive chapters.

The aim of the first part of the study was to investigate whether it is possible to identify potentially relevant toxicants in samples with relatively low levels of contamination using effect-directed analysis (EDA). For this purpose compounds extracted from a marine sediment sample from the Swedish west coast were separated into distinct fractions, using two preparative chromatographic techniques. As detector for toxicity a biotest battery was applied, consisting of one algal and two bacterial bioassays, comprising acute and chronic endpoints. Chronic algal toxicity was a powerful tool to discriminate between toxic and non-toxic fractions, while acute and chronic bacterial toxicity tests failed to identify toxic fractions. Eight compounds were

identified as potentially relevant toxicants to algae by chemical analysis of toxic fractions as inferred from the algal bioassay tests: anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*a*]pyrene, benzo[*k*]fluoranthene and indeno[1,2,3-*cd*]pyrene.

After identification of compounds using EDA, a confirmation step is commonly performed in order to verify whether the identified compounds can be held responsible for the observed effects. The aim of the subsequent chapter was to advance the methodology for the confirmation of cause-effect relationships in cases where mixtures of compounds are identified in the investigated samples. Tools for the assessment of mixture toxicity accounting for unknown modes of action and heterogeneity of concentration-response curves were adapted to be used in the confirmation step. Two samples were investigated using effect-directed analysis. All identified compounds were tested individually as pure compounds as well as in mixtures at ratios equal to those found in the sediment extracts. The observed extract toxicity was then compared with the expected combined effects calculated according to the models of concentration addition and independent action as well as with the observed toxicity of the synthetic mixture. Drawbacks of the established approach using toxic unit summation were demonstrated. Instead, the Index of Confirmation Quality was introduced, an easy to read representation allowing a quantitative measure of confirmation over a range of different effect levels.

The majority of compounds identified and confirmed as relevant toxicants in chapter 2 and 3 were polycyclic aromatic hydrocarbons (PAHs). Toxicity of PAHs was previously reported to depend on light conditions. The aim of chapter 4 was to analyse the influence of simulated sun light on the toxicity of the previously investigated samples. Additionally, toxicity of identified toxicants and mixtures of these compounds was assessed under these environmentally realistic light conditions. It was shown that the toxicity of the samples, the PAH compounds and the created synthetic mixtures exert photoenhanced toxicity. The combined effects of the mixtures can be

predicted using the model of concentration addition. The confirmation step in EDA revealed that, that only a smaller part of the samples' toxicity can be attributed to the combined effects of the identified compounds under simulated sun light revealed than under standard growth light.

In chapter 5 previously proposed models for the assessment of photoinduced PAH toxicity were tested with regard to their applicability to the prediction of photoinduced toxicity to the green algae *Scenedesmus vacuolatus* qualitatively and quantitatively. For this purpose, 14 different PAH compounds were tested under up to three different light conditions for their effect on the algae reproduction. Illumination conditions comprised standard algae growth light, simulated sun light aiming to mimic environmental light conditions, and UV-filtered light, in order to minimise light influence on PAH toxicity. Models proposed for the prediction of photoinduced toxicity were modified, in order to account for different exposure conditions and toxic endpoints used in the bioassay tests. The present results show that the gap between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) proposed as indication for photoinduced toxicity to *Daphnia magna* (Mekenyan *et al.* 1994) can be used as a qualitative indication of a potential photoinduced toxicity to the green algae *Scenedesmus vacuolatus*. The impact of light conditions on PAH toxicity can be quantified by a linear model which allows the estimation of the $ED_{50}$ of a compound from the amount of absorbed photons and an empirically determined relative phototoxic efficacy value of the compound.

In conclusion, cause-effect relationships were established between identified toxic compounds and observed effects in an environmental sample as well as between the light conditions and the toxicity induced by environmental samples. Furthermore, the cause-effect relationship between the light absorbance and the algal toxicity of PAH compounds was quantitatively modelled.

## *Zusammenfassung*

Der Nachweis von kausalen Zusammenhängen zwischen beobachteten biologischen Effekten und Umweltstressoren ist eine wichtige Herausforderung in der Ökotoxikologie. Diese Herausforderung besteht zum einen in der Identifizierung von Schadstoffen in biologisch wirksamen Umweltproben, zum anderen auch in der Quantifizierung der Beiträge der identifizierten Substanzen zu den beobachteten Schadeffekten. Außerdem müssen die Umweltfaktoren identifiziert werden, die Toxizität der Substanzen und Proben beeinflussen, um Ergebnisse aus dem Labor ins Freiland übertragbar zu machen. Um Vorhersagen treffen zu können, wie stark ein Umweltfaktor die Toxizität bestimmter Schadstoffen beeinflussen kann, werden Modelle benötigt, die von den Ergebnissen einzelner Experimente abstrahieren. So können Aussagen über nicht getestete Situationen getroffen werden. Diese Arbeit behandelt verschiedene Aspekte der hier beschriebenen Herausforderungen in vier aufeinander aufbauenden Kapiteln.

Im ersten Teil der Arbeit geht es um die Frage, ob sich aus einem Sediment eines wenig belasteten Standortes an der schwedischen Westküste potentiell relevante Schadstoffe mit Hilfe Effekt-orientierter Analytik identifizieren lassen. Zu diesem Zweck wurden organische Substanzen aus einem marinen Sediment extrahiert und mit Hilfe einer zweistufigen präparativen chromatographischen Trennung anhand ihrer physiko-chemischen Eigenschaften aufgetrennt. Als Toxizitätsdetektor diente eine Biotestbatterie, bestehend aus einem pflanzlichen und zwei bakteriellen Testsystemen, die sowohl akute als auch chronische Endpunkte beinhalteten (Hemmung der Vermehrung der einzelligen Grünalge *Scenedesmus vacuolatus*, Lumineszenzhemmung und Hemmung der Vermehrung des Bakteriums *Vibrio fischeri*). Der Test auf chronische Algentoxizität erwies sich dabei als gut geeignet, um zwischen toxischen und nicht toxischen Fraktionen zu unterscheiden. Durch die Tests auf akute und chronische Bakterientoxizität konnten keine toxischen Fraktionen nachgewiesen werden. Insgesamt wurden acht potentiell für Algen relevante Substanzen identifiziert:

Anthracen, Fluoranthen, Pyren, Benzo[*a*]anthracen, Benzo[*b*]fluoranthene, Benzo[*a*]pyren, Benzo[*k*]fluor-anthene und Indeno[1,2,3-*cd*]pyren.

Nach der Identifizierung von Substanzen mit Effekt-orientierter Analytik wird gemeinhin ein Bestätigungsschritt durchgeführt, in dem geklärt werden soll, ob oder zu welchen Anteil die identifizierten Substanzen für die beobachteten Wirkungen verantwortlich sind. Das Ziel des nächsten Kapitels war es, die Methodik dieses Bestätigungsschritts für die Fälle weiterzuentwickeln, in denen Stoffmischungen identifiziert werden. Zu diesem Zweck wurden Tools für die Bewertung von Mischungstoxizität herangezogen, die sowohl unbekannten Wirkmechanismen als auch der Heterogenität von Konzentrations-Wirkungskurven Rechnung tragen. Als Grundlage für diese Entwicklung dienten zwei mit Effekt-orientierter Analytik untersuchte Umweltproben. Alle identifizierten Substanzen wurden sowohl einzeln, als auch in Mischungen - im Verhältnis ihrer Sedimentkonzentrationen - getestet. Die Extrakttoxizität wurde dann ins Verhältnis zu den beobachten und zu den nach den Vorhersagemodellen *Konzentrationsadditivität* und *Unabhängige Wirkung* berechneten Kombinationseffekten gesetzt. Anhand der Ergebnisse werden die Nachteile der häufig verwendeten Methode der Toxic Unit Summation diskutiert. Stattdessen, kann der hier entwickelte *Index of Confirmation Quality* verwendet werden, der eine Quantifizierung der Beiträge der Mischung zur Probentoxizität auf verschiedenen Effektniveaus erlaubt.

Ein Großteil der in den Umweltproben identifizierten Schadstoffe gehört zur Stoffklasse der Polycyclischen Aromatischen Kohlenwasserstoffe (PAHs). Die Toxizität von PAHs ist in vielen Studien als lichtabhängig beschrieben worden. Das Ziel des folgenden Kapitels war es zu untersuchen, ob die Verwendung von simuliertem Sonnenlicht, im Gegensatz zu Standardlaborlicht, einen Einfluss auf die Toxizität der zuvor untersuchten Proben hat. Zusätzlich wurden die identifizierten Einzelstoffe und Mischungen auf ihre Algentoxizität unter simuliertem Sonnenlicht getestet. Es kann gezeigt werden, dass die Toxizität sowohl der Umweltproben, als auch der Einzelstoffe und Mischungen, durch die veränderten Lichtbedingungen erhöht

ist. Die Kombinationseffekte lassen sich hierbei quantitativ gut mit dem Modell der *Konzentrationsadditivität* vorhersagen. Aus der Quantifizierung der Beiträge der Schadstoffe zu der beobachteten Toxizität der Probe ergibt sich, dass die Probentoxizität unter simuliertem Sonnenlicht zu einem geringeren Anteil durch die identifizierten Substanzen erklärt werden kann als unter Standardlaborlicht.

Im letzen Teil der Arbeit wird versucht, in der Literatur beschriebene Modelle für die Vorhersage von PAH-Phototoxizität für den Effekt auf die Grünalge *Scenedesmus vacuolatus* zu adaptieren. Zu diesem Zweck wurden 14 PAHs unter drei verschiedenen Lichtbedingungen auf ihre Hemmung der Algenreproduktion getestet. Die Lichtbedingungen beinhalteten zum einen das Standardwachstumslicht der Algenkultur, zum anderen simuliertes Sonnenlicht, um eine umweltrelevante Beleuchtung zu testen, und UV-gefiltertes Licht, um den Lichteinfluss auf die PAH-Toxizität zu minimieren. Die in der Literatur beschriebenen Modelle für die Vorhersage lichtinduzierter Toxizität wurden modifiziert, um den speziellen Expositionsbedingungen und dem verwendeten Endpunkt Rechnung zu tragen. Die Ergebnisse zeigen, dass sich der Abstand zwischen dem höchsten besetzten und dem niedrigsten unbesetzten Molekülorbital (HOMO-LUMO-Gap) als qualitativer Indikator für potentiell phototoxisches Verhalten der Substanzen in *Scenedemus vacuolatus* eignet. Der Lichteinfluss lässt sich quantitativ durch ein lineares Modell beschreiben, welches die mittlere effektive Dosis ($ED_{50}$) aus der Menge der absorbierten Photonen und einem empirisch ermittelten relativen phototoxischen Effektivitätswert schätzt.

Zusammenfassend lässt sich sagen, dass kausale Zusammenhänge sowohl zwischen den identifizierten toxischen Substanzen und den beobachten Effekten in den Umweltproben als auch zwischen dem Umweltfaktor Licht und den toxischen Effekten der Proben hergestellt werden konnten. Außerdem konnte ein direkter Zusammenhang zwischen der Menge an absorbiertem Licht und der Algentoxizität von PAHs nachgewiesen und quantitativ durch ein Modell beschrieben werden.

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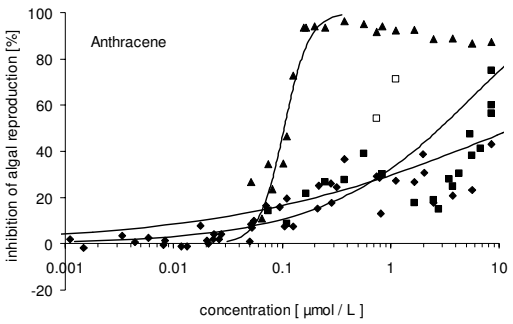
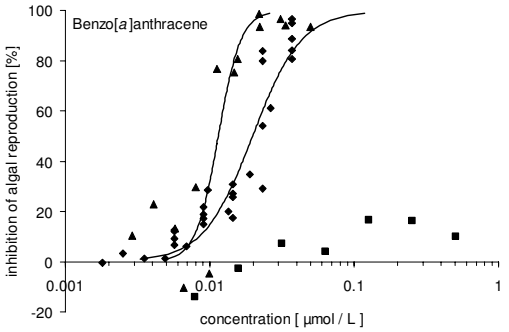
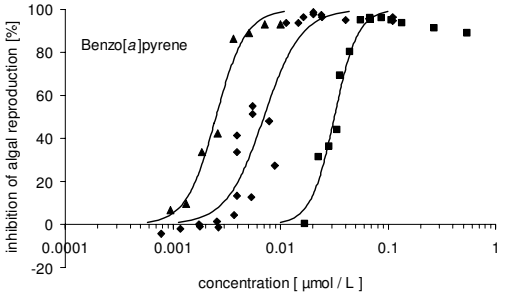
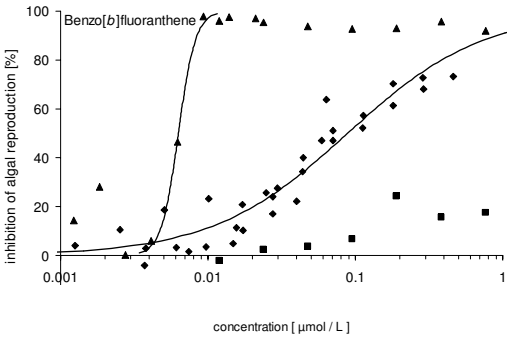
# *Appendix*

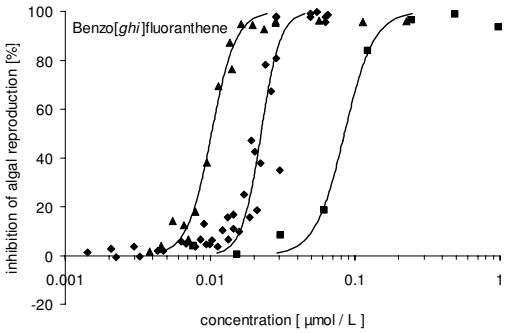
## *Concentration-response relationships observed under three different light conditions.*

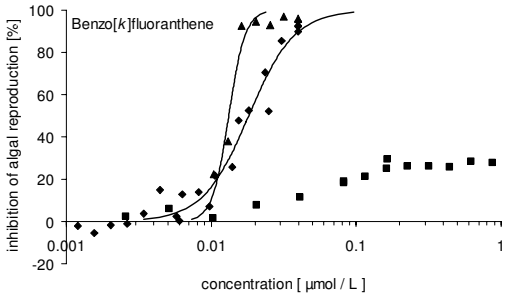


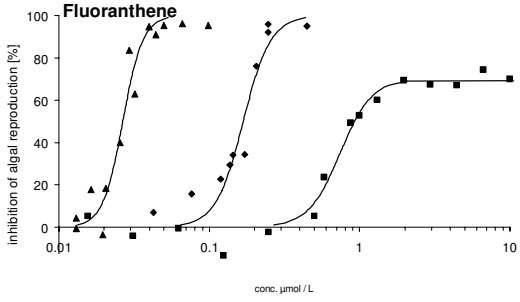



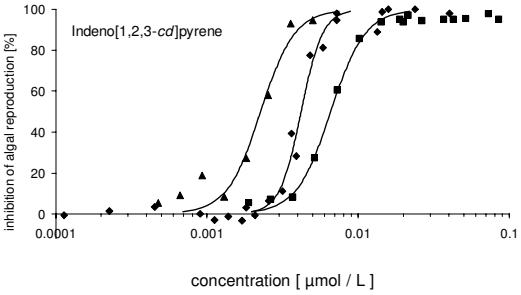



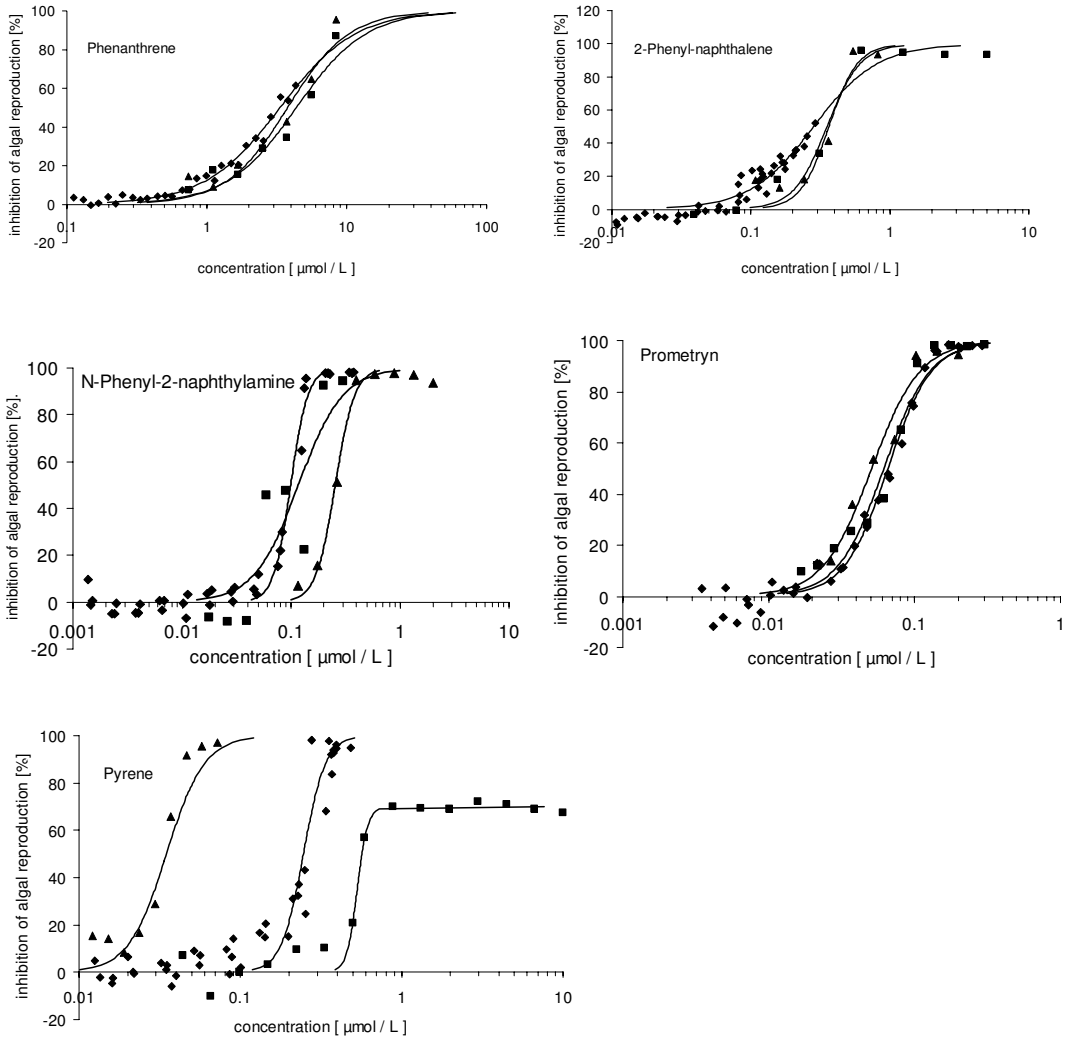






*Toxicity of tested compounds under simulated sun light (▲), standard growth light (♦) and UV-filtered light (■): Fitted concentration-response relationships are shown as solid lines (functions are given in Table 4.2 and Table 5.2.). Two data points in the measurement of anthracene toxicity under UV-filtered light were considered as outliers (□).*

## *Chemicals used and disposal*

| Substanz | Hersteller | Gefahr-symbol | R-Sätze | S-Sätze |
|---|---|---|---|---|
| **Anorganika** | | | | |
| Kupferpulver | | | | |
| NaOH | | C | 35 | 26-36/37/39-45 |
| HCl | | T, C | 23-35 | (1/2-)9-26-36/37/39-45 |
| $H_2SO_4$ | | C | 35 | (1/2-)26-30-45 |
| **Lösungsmittel** | | | | |
| Aceton | Merck, Darmstadt | F, Xi | 11 | 9-16-23,2-33 |
| DMSO | Merck, Darmstadt | Xi | 11 | |
| *n*-Hexan | Merck, Darmstadt | Xn, F, N | 48/20-62-65 | 36/37-62 |
| Cyclohexan | Merck, Darmstadt | Xn, N | 11 | 9-16-33 |
| Dichlormethan | Merck, Darmstadt | Xn | 40 | 23,2-24/25-36/37 |
| Essigsäure | | | | |
| Acetonitril | | | | |
| 11H-Benz[*b*]fluorene | | | | |
| 1-Methylanthracene | | | | |
| 1-Phenylnaphthalene | | | | |
| 2-Phenylnaphthalene | AccuStandard | | | |
| 4H-Cyclopenta[*def*]phenanthrene | | | | |
| Acenaphtene | | Xi | 36/37/38 | 53-45 |
| Acenaphtylene | | | | |
| Anthracene | Aldrich | | | |
| Benzo[*a*]anthracene | Aldrich | T | 45 | 53-45 |
| Benzo[*a*]fluoranthene | | | | |
| Benzo[*a*]pyrene | Aldrich | | | |

| | | | | |
|---|---|---|---|---|
| Benzo[*b*]fluoranthene | Promochem | T | 45 | 53-45 |
| Benzo[*a*]pyrene | | T, N | 45-46-60-61-50/53 | 53-45 |
| Benzo[*e*]pyrene | | | | |
| Benzo[*ghi*]fluoranthene | Promochem | - | - | - |
| Benzo[*ghi*]perylene | Aldrich | - | - | - |
| Benzo[*j*]fluoranthene | | - | - | - |
| Benzo[*k*]fluoranthene | Aldrich | T | 45 | 53-45 |
| Chrysene | Riedel-deHaën | | | |
| Coronene | | | | |
| Cylopenta[*cd*]pyrene | | | | |
| Dibenzo[*a,h*]anthracene | | T | 45 | 53-45 |
| Fluoranthene | Aldrich | Xn | 22 | |
| Fluorene | | | | |
| Indeno[1,2,3-*cd*]fluoranthene | | | | |
| Indeno[1,2,3-*cd*]pyrene | Promochem | | | |
| Perylene | Fluka | | | |
| Phenanthrene | Aldrich | N | 50/53 | 24/25-61 |
| Pyrene | Fluka | - | 53 | 24-61 |
| Triphenylene | | | | |
| Parathion-methyl | Riedel-deHaën | T+ | 24-28 | (1/2-)28-36/37-45 |
| Naphthalene | | Xn, N | 40-50/53 | 36/37-61 |
| *N*-Phenyl-2-naphthylamine | Aldrich | - | - | - |
| Prometryn | Riedel-deHaën | | | |

All compounds were disposed in accordance with the environmental guidelines established in the UFZ.

Publications

**peer-reviewed**

Grote M, Klinnert S, Bechmann W (2000) Comparison of degradation state and stability of different humic acids by means of chemolysis with tetramethylammonium hydroxide. *Journal of Environmental Monitoring* **2**:165-169.

Backhaus T, Altenburger R, Arrhenius A, Blanck H, Faust M, Finizio A, Gramatica P, Grote M, Junghans M, Meyer W, Pavan M, Porsbring T, Scholze M, Todeschini R, Vighi M, Walter H, Grimme LH (2003) The BEAM-project: prediction and assessment of mixture toxicities in the aquatic environment. *Continental Shelf Research* **23**:1757-1769.

Altenburger R, Walter H and Grote M, (2004) What contributes to the combined effect of a complex mixture? *Environmental Science & Technology* **38**:6353-6362

Grote, M, Brack, W, Walter, H, and Altenburger, R. (2005) Confirmation of cause-effect relationships using effect-directed analysis for complex environmental samples. *Environmental Toxicology and Chemistry* **24**:1420-1427.

Grote M, Schüürmann G, Altenburger R. (2005) Modelling photoinduced algal toxicity of polycyclic aromatic hydrocarbons. *Environmental Science & Technology* **39**:4141-4149.

Grote M, Brack W, Altenburger R. (in press) Identification of toxicants from a marine sediment sample using effect-directed fractionation *Environmental Toxicology.*

Grote M, Altenburger R, Brack W, Moschütz S, Mothes S, Michael C, Narten G-B, Paschke A, Schirmer K, Walter H, Wennrich R, Wenzel KD,

Schüürmann G, (in press) Ecotoxicological profiling of transect river Elbe sediments. *Acta hydrochimica hydrobiologica.*

Grote M, Brack W, Walter H; Altenburger R. (in press) Light as confounding factor for toxicity assessment in complex contaminated sediments *Environmental Toxicology and Chemistry*.

**not peer-reviewed**

Grote M, Walter H, Altenburger R (2003) Methodology to Identify Potential Exposure to Ecotoxicologically Relevant Mixtures, *Report on WP1* in Backhaus T, Altenburger R, Arrhenius A, Blanck H, Faust M, Finizio A, Gramatica P, Grote M, Junghans M, Meyer W, Pavan M, Porsbring T, Scholze M, Todeschini R, Vighi M, Walter H, Grimme LH: BEAM - Bridging Effect Assessment of Mixtures to Ecosystem Situations and Regulation (EVK1-1999-00055) Final Report

Grote M, Walter H, Altenburger R (2004) Toxizitätuntersuchungen von Elbsedimenten in Kontakttesten mit *Lemna minor* und *Danio rerio*. In 'Veranstaltungsband Statusseminar Sedimentkontakttests'. (Ed. U Feiler) (Bundesanstalt für Gewässerkunde, Koblenz: Berlin)

Grote M, Altenburger R, Brack W, Möckel C, Moschütz S, Mothes S, Michael C, Narten G-B, Paschke A, Schirmer K, Walter H, Wennrich R, Wenzel KD, Schüürmann G (2004) Identifizierung und Analyse des ökotoxischen Potentials von Flusssedimenten als Folge der durch Hochwasser mobilisierten Kontaminationen. In 'Endbericht des Ad-hoc-Verbundprojektes - Schadstoffbelastung nach dem Elbe-Hochwasser 2002'. (Ed. W Geller) pp. 314-329. (UFZ: Magdeburg)

## *Conference Contributions*

Grote M., Blanck H, Brack W., Altenburger R.; Identification of mixtures of potential environmental relevance in sediments, Platform Presentation 11th Annual Meeting of SETAC Europe, May 2001, Madrid, Spain

Grote M., Brack W., Altenburger R., Identifizierung von potentiell toxischen Mischungen aus einem Ostseesediment, Poster at the SETAC-GLB Conference, September. 2001, Berlin, Germany, rewarded '*Best Poster Award*'

Grote M., Brack W., Altenburger R., Biotest-directed fractionation and identification: a suitable tool for determination of realistic exposure situations? Platform Presentation 12th Annual Meeting of SETAC Europe, May 2002, Vienna, Austria

Grote M., Brack W., Altenburger R., Biotestgeleitete Fraktionierung und Identifizierung – Versuch einer Übersicht, Poster at the SETAC-GLB / GDCh Umweltchemie/Ökotoxikologie Conference, October. 2002, Braunschweig, Germany

Grote M., Altenburger R., Predictability of light enhanced PAH mixture toxicity. Poster 13th Annual Meeting of SETAC Europe, April 2003, Hamburg, Germany

Chairman of Session 'Modellierung in der Ökotoxikologie', SETAC GLB Conference, September 2003, Heidelberg,

Grote M, Brack W, Altenburger R, Confirmation of mixtures in effect-directed analysis, Poster, 15th Annual Meeting of SETAC Europe, April 2005, Lille, France

Chairman of Session 'Mischungstoxizität und multiple Stressoren', SETAC-GLB Conference, September 2005 Basel, Switzerland

## *Erklärungen*

Hiermit erkläre ich, dass ich bisher keine weiteren Promotionsversuche unternommen habe.

Leipzig, den

Ort, Datum Unterschrift

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbständig durchgeführt und keine anderen als die angegebenen Hilfsmittel verwendet habe.

Leipzig, den

Ort, Datum Unterschrift

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# Lebenslauf

**Persönliche Angaben**

| | |
|---|---|
| Geburtsdatum: | 28. Februar 1973 |
| Geburtsort: | Hamburg |

**Schulbildung**

| | |
|---|---|
| Aug. 1979 – Juni1983 | Grundschule in Hamburg and Elmshorn |
| Aug. 1983 – Mai1992 | Bismarck-Gymnasium, Elmshorn |
| Mai1992 | Abitur (Note: 1,9) |

**Universität**

| | |
|---|---|
| Okt. 1992 – März 1995 | Grundstudium der Chemie an der Universität Hamburg |
| Okt. 1994 | Diplomvorprüfung (Note 1,3) |
| April 1995 – März 1999 | Hauptstudium der Chemie an der Universität Potsdam |
| April 1996 – Okt. 1996 | Auslandstudium und Praktikum an der ECPM (Ecole de Chimie, Polymères et Materiaux) in Strasbourg, Frankreich |
| Juni 1998 – März 1999 | Diplomarbeit im Arbeitskreis Umweltchemie am Institut für Physikalische Chemie der Universität Potsdam |
| März 1999 | Studienabschluss als Diplomchemiker, Note: sehr gut |
| seit Oktober 2000 | Postgradualstudium 'Toxikologie und Umweltschutz' and der Universität Leipzig |
| seit Dezember 2002 | Promotionsstudent an der Universität Hamburg |

**Beruflicher Werdegang**

| | |
|---|---|
| seit März 2000 | Doktorand am UFZ - Leipzig im Department Chemische Ökotoxikologie |
| März 2000 – April 2003 | Mitarbeit im von der EU geförderten BEAM-Projekt |
| Mai 2003 – März 2004 | Wissenschaftlicher Mitarbeiter im Adhoc-Projekt Elbe (Elbehochwassers Sommer 2002) |