# Sediment ecotoxicology: Identification of hazard factors and ecotoxicological risks in the Tietê River Basin (Brazil)

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# Sediment ecotoxicology: Identification of hazard factors and ecotoxicological risks in the Tietê River Basin (Brazil)

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You and I have memories
longer than the road
that stretches out ahead...

*Lennon and McCartney*

we are going home ...

To my beloved husband George

to my lovely family

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# Summary

Sediments are a major issue for many scientists and authorities due to their potential to accumulate pollutants. Since pollutants may be made available under certain environmental conditions such as dredging or flood events, sediments can also become a source of diffuse contamination to the free water space. Their associated load of pollutants can be characterized particularly by its high complexity, which is hard to characterize based on chemical-analytic methods only. By combining ecotoxicological approaches using acute and mechanism-specic *in vitro* biotests, *in situ* investigations and chemical analyses, the present study aimed at comprehensively evaluating contaminated sediments from the Tietê River Basin (São Paulo State, Brazil). The Tietê River comprises several reservoirs along its course, which are widely used for providing drinking water, as a water source for agricultural irrigation and as recreation sites. The eight selected studied areas cover the entire length of the Tietê River from its spring to the river mouth and also include two important water bodies associated to this river, the Pinheiros River and Billings reservoir.

Several approaches involving different test organisms, various endpoints and different sediment phases (liquid as extract and solid as freeze-dried samples) were followed under laboratory conditions. Moreover, to be able to better extrapolate rsults from laboratory studies to the field situation, *in vitro* results were combined with *in situ* assessment. As a result, it is possible to confirm that sediments from the various sites at the Tietê River Basin are differently polluted with contaminants, which cause not only acute cytotoxicity, but also genotoxicity and AhR-mediated toxicity in fish cells as well as fish embryos. Furthermore, mutagenicity was recorded *in situ* in fish caught from the field.

Differences in cytotoxicity, genotoxicity and AhR-mediated toxic potentials between different locations could be identified by the neutral red, comet and EROD assays, respectively, with RTL-W1 cells exposed to acetonic sediment extracts. High correlations between genotoxicity *in vitro* and mutagenicity *in situ*/*in vivo* (micronucleus assay with fish blood erythrocytes)

were obtained, indicating the high ecological relevance of sediment genotoxicity for the situation in the field.

However, since organic extracts provide estimations of the *total* hazard potential, but neglect the *bioavailability* of sediment contaminants, there was still a need to develop more realistic, field-like exposure scenarios for sediment quality assessment, combining both bioavailability and *intact organisms* in order to improve the transferability of results and to better understand fate and behavior of water- and sediment-bound toxicants relevant for toxicity. Thus, in order to simulate *in situ* exposure conditions in a more realistic scenario, a sediment-contact fish embryo test was applied and changes in embryotoxicity were recorded in the whole river course.

Overall, bioassays results confirmed that most of the toxicity was due to the discharges of the metropolitan area of São Paulo, but also indicated that additional sources of pollutants occur along the whole river course, thus contributing to the degradation of each reservoir.

Results from effect-directed analyses of the most toxic sediment extracts (from Pinheiros and Billings sediments), combining fractionations of samples and chemical analyses suggested that polyclyclic aromatic compounds (PACs), such as priority and non-priority polycyclic aromatic hydrocarbons (PAHs), play an important role in the general toxicity.

The assessment of sediment quality is essential for the understanding of processes governing the fate and availability of pollutants, since sediments are the final compartments for storage and transformation of most pollutants discharged by anthropogenic activities. A comprehensive evaluation of the ecotoxicological situation of sediments requires different approaches and broad knowledge for interpreting results. As exemplified for the Tietê River basin, a battery of bioassays applied in combination with chemical analyses and effect-directed analysis represent suitable strategies to function as early warning systems not only for sediment pollution, but also for the health of the entire river system. The fractionation of the very toxic samples allowed the identification of specific groups of pollutants responsible for the toxicity, and the prioritization of individual fractions for subsequent effect-directed analysis. Effect-directed analysis should allow sequential reduction of the complexity of these environmental mixtures to toxyicant classes and, eventually, to individual toxicants.

# Kurzfassung

Auf Grund ihres hohen Potenzials zur Schadstoffakkumulation sind Sedimente von großer wissenschaftlicher und regulatorischer Bedeutung. Da diese Schadstoffe unter bestimmten Umweltbedingungen (Baggerungen, Hochwasser) wieder verfügbar werden können, stellen Sedimente auch eine Quelle diffuser Schadstoffbelastung in die freie Wassersäule dar. Die Sedimentgebundene Schadstofffracht zeichnet sich vor allem durch eine hohe Komplexität aus, die mit chemisch-analytischen Verfahren alleine nur unzureichend erfasst werden kann.

Durch die Kombination ökotoxikologischer Methoden, bestehend aus akuten und Mechanismus-spezifischen *In vitro*-Biotests, *In situ*-Untersuchungen und chemischer Analytik, zielte diese Studie auf eine umfassende Bewertung kontaminierter Sedimente des Tietê-Flusses (Brasilien, Bundesstaat São Paulo) ab. Entlang des Tietê befinden sich mehrere Stauhaltungen, die für Trinkwassergewinnung und, Bewässerungszwecke sowie als Naherholungsgebiet genutzt werden. Die acht in dieser Dissertation untersuchten Gebiete umfassen den Tietê auf seiner gesamten Länge (von der Quelle bis zur Mündung) ebenso, wie zwei wichtige Gewässer, die mit dem Tietê verbunden sind: den Nebenfluss Pinheiros und die Stauhaltung Billings.

Verschiedene Testorganismen, ökotoxikologische Endpunkte sowie gefriergetrocknetes und extrahiertes Sediment wurden im Labor verwendet. Außerdem wurden *in vitro*- Ergebnisse mit *In situ*-Untersuchungen kombiniert, um die Ergebnisse aus Laborstudien auf das Freiland zu extrapolieren. Dadurch konnte gezeigt werden, dass Sedimente des Tietê Schadstoffe enthalten, die mit Fischzellen neben Cytotoxizität, Gentoxizität und AhR-vermittelter Wirksamkeit auch Embryotoxizität bewirken können. Weiterhin wurde *in situ* mit Fischen aus dem Freiland eine mutagene Wirksamkeit der Sedimente nachgewiesen.

Durch die Exposition von RTL-W1-Zellen gegenüber acetonischen Sedimentextrakten der verschiedenen Flussabschnitte wurden mit Neutralrot-, Comet- und EROD-Assay unterschiedliche Cytotoxizität, Gentoxizität und AhR-vermittelte Wirksamkeit nachgewiesen. Enge Korrelationen zwischen

Gentoxizität *in vitro* und Mutagenität *in situ/in vivo* im Mikronukleus-Assay mit Erythrocyten aus Fischblut wurden ermittelt. Dies belegt die hohe ökologische Bedeutung gentoxischer Effekte durch Sedimente im Freiland.

Da organische Extrakte das gesamte Schädigungspotenzial von Sedimentproben abbilden, ohne die Bioverfügbarkeit zu berücksichtigen, galt es, realistischere Expositionsszenarien für die Bewertung von Sedimentqualität zu entwickeln. Diese sollten die Bioverfügbarkeit berücksichtigen und die Wirkung auf intakte Organismen zeigen, um so die Übertragbarkeit von Laborergebnissen zu verbessern. Weiterhin wird so ein besseres Verständnis von Verbleib und Verhalten von Wasser- und Sediment-gebundenen Schadstoffen ermöglicht, durch welche die Toxizität bewirkt wird. Daher wurde ein Sediment-Kontakttest angewandt und Veränderungen der Embryotoxizität über den gesamten Flussverlauf erfasst.

Die Ergebnisse der Biotests belegen, dass ein Großteil der Toxizität auf Abwässer der Metropolregion São Paulo zurückgeführt werden kann. Die Ergebnisse zeigen aber auch, dass die Schadstoffquellen sich im Flussverlauf ändern und erst in Summe zur Degradierung der Reservoire beitragen.

Die Ergebnisse der Effekt-dirigierten Analyse, die Fraktionierung und chemische Analyse von Sedimentextrakten mit den höchsten Schädigungspotentialen (Pinheiros und Billings) verbindet zeigen, dass polyzyklische aromatische Verbindungen (PACs) wie prioritäre und nicht-prioritäre polyzyklische aromatische Kohlenwasserstoffe (PAKs) einen wichtigen Beitrag zur allgemeinen Toxizität leisten.

Die Bewertung der Sedimentqualität ist für das Verständnis der Vorgänge, die das Schicksal und die Verfügbarkeit von Schadstoffen in Gewässer bestimmen, von grundlegender Bedeutung, da diese das Kompartiment sind in dem Schadstoffe aus anthropogenen Quellen ab- und zwischengelagert und transformiert werden. Eine umfassende Bewertung der ökotoxikologischen Belastung von Sedimenten erfordert ihrerseits variable Konzepte und umfassende Kenntnisse für die Interpretation der Ergebnisse.

Wie am Beispiel des Einzugsgebiets des Tietê gezeigt werden konnte, stellt die Kombination von Bioassays in Testbatterien, chemischer Analyse und Effekt-dirigierter Fraktionierung geeignete Werkzeuge für ein Frühwarnsystem dar, das nicht nur die Sedimentbelastung, sondern auch den allgemeinen Zustand des gesamten Flusssystems erfasst. Die Fraktionierung besonders toxischer Proben ermöglicht dabei die Identifizierung spezifischer Schadstoffgruppen, sowie die Priorisierung einzelner Fraktionen für spätere Effekt-dirigierte Analysen, um so letztlich möglichst Einzelsubstanzen zu identifizieren.

# Sumário

Devido ao seu potencial de acumulação de poluentes, sedimentos têm despertado interesse em muitos cientistas e autoridades. Como poluentes tornam-se disponíveis sob certas condições ambientais (tais como dragagem ou inundações), sedimentos são também fonte potencial de contaminação para corpos d'água. A carga de poluentes associada aos sedimentos é de natureza complexa dificultando sua caracterização apenas por meio de análises químicas. O presente estudo visa avaliar ecotoxicologicamente sedimentos contaminados da Bacia do Rio Tietê, por meio de bioensaios *in vitro*, de toxicidade aguda e mecanismos específicos, investigações *in situ* e análises químicas. O Rio Tietê possui vários reservatórios ao longo de seu curso que são amplamente utilizados para abastecimento de água potável, como fonte de água para irrigação agrícola e para recreação. As oito áreas selecionadas neste estudo abrangem toda a extensão do Rio Tietê, desde a nascente até sua foz. Incluem também dois importantes corpos d'água associados ao Rio Tietê, o Rio Pinheiros e o reservatório Billings.

Em laboratório, os sedimentos coletados na Bacia do Rio Tietê foram avaliados em diferentes fases (líquida com extratos de sedimento, e sólida com sedimentos liofilizados) por meio de diferentes biotestes envolvendo vários organismos teste. Além disso, para extrapolar os resultados obtidos em laboratório para a realidade no campo, esses resultados foram combinados à avaliação *in situ*. Os resultados confirmaram que os sedimentos avaliados estão poluídos com vários contaminantes que causam além de citotoxicidade aguda, genotoxicidade e toxicidade mediada por AhR em células permanentes derivadas de peixes, bem como embriotoxicidade. Além disso, mutagenicidade *in situ* foi evidenciada em peixes capturados no campo.

Diferentes níveis de citotoxicidade, genotoxicidade e toxicidade mediada por AhR foram identificados por meio dos bioensaios do Vermelho Neutro, Ensaio do Cometa e teste de EROD, respectivamente, em células permanentes RTL-W1 expostas aos extratos orgânicos de sedimentos. Uma alta correlação entre genotoxicidade *in vitro* e mutagenicidade *in situ* foi obtida por meio do teste do micronúcleo *in vivo* (com eritrócitos de peixes), indi-

cando a relevância ecológica do potencial genotóxico desses sedimentos para a realidade no campo.

Todavia, como extratos orgânicos fornecer estimativas do potencial de risco total, mas negligenciam a biodisponibilidade dos contaminantes associados à sedimentos, houve a necessidade de desenvolver um bioensaio em condições mais realistas, levando em conta biodisponibilidade e organismos inteiros, para compreender melhor o destino e comportamento dos poluentes associados à água e aos sedimentos. Deste modo, para simular as condições de exposição *in situ* aplicou-se um teste de contato de sedimento com embriões de peixes. Resultados mostraram diferentes níveis de embriotoxicidade em todo o curso do rio.

De modo geral, os resultados sugerem que a maioria da toxicidade é devido as descargas da região metropolitana de São Paulo, mas indicam que as fontes de poluentes ocorrem de forma diferente em todo o curso do rio, contribuindo para a degradação de cada reservatório. Os resultados das análises de efeito-dirigido dos extratos de sedimentos mais tóxicos (Pinheiros e Billings), sugeriram que compostos policíclicos aromáticos (CPA), tais como os hidrocarbonetos policíclicos aromáticos (HPAs) prioritários e não prioritários, desempenham um papel importante na toxicidade geral.

A avaliação da qualidade do sedimento é essencial para a compreensão dos processos que regem o destino e disponibilidade dos poluentes nos corpos d'água, já que sedimentos são o compartimento final de armazenagem e transformação da maior parte dos poluentes emitidos por atividades antrópicas. Uma avaliação detalhada da situação ecotoxicológica de sedimentos exige diferentes abordagens e conhecimentos gerais para interpretação dos resultados.

Como exemplificado para a bacia do Rio Tietê, a associação de uma bateria de bioensaios, análises químicas e análises de efeito dirigidas pode ser usada como sistema de alerta precoce, não só para a poluição de sedimentos, mas também para todo o sistema fluvial.

Grupos específicos de poluentes responsáveis pela toxicidade foram identificados por meio de fracionamento químico das amostras mais tóxicas, o que permitiu a prioritização das frações individuais para futuras análises de efeito dirigido. Análises de efeito dirigido podem reduzir a complexidade dessas misturas ambientais para classes de poluentes ou até mesmo para poluentes individuais.

# Chapter 1

# Introduction

## 1.1 Structure of this thesis

This doctoral thesis consists of six chapters. It begins with a general introduction (Chapter 1), which includes an overview on sediment ecotoxicology, the characterization of the areas studied and the methods applied to address the aims of this thesis. The next four chapters are formed by scientific article/manuscripts related to this research, which were written by the author of this thesis and co-workers (Chapters 2 to 5). Chapter 6 presents final conclusions, which can be drawn from the entire study.

Appendix A presents the development of the concept for this project. During the development of this doctoral work, the author contributed to two other scientific articles. These publications are presented (Appendices B and C), and were written by Susanne Jernbro and co-workers and Melanie Böttcher and co-workers, respectively. Appendix D presents a list of publications related to the development of this doctoral work.

All chapters have been designed as stand-alone manuscripts. The reader should thus be able to read one of the chapters and follow the conclusions without knowledge of the entire thesis.

## 1.2 Ecotoxicology

When sciences were being ascribed to deliver rational approaches to access and predict adverse effects of chemicals on the environment, a whole new branch called ecotoxicology emerged from the collaboration of several biological and chemical sub-disciplines(Altenburger, 2002). The term ecotoxicology was suggested for the first time in 1969, by the French toxicologist René Truhaut, who defined it as "the branch of toxicology concerned with

the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal, vegetable and microbial, in an integral context" (Truhaut, 1977). According to Fent (2001), ecotoxicology has been established in the last decades as an environmental science, evolving on the one hand from toxicology, and on the other hand from applied ecology or environmental chemistry. Therefore, ecotoxicology comprises the integration of ecology and toxicology (Chapman, 1995; Baird *et al.*, 1996, Fig. 1.1).

Ecotoxicology deals with the interactions between environmental chemicals and biota, thereby focusing on adverse effects at different levels of biological organization, from molecules up to populations and ecosystems (Fent, 2001, Fig. 1.2).

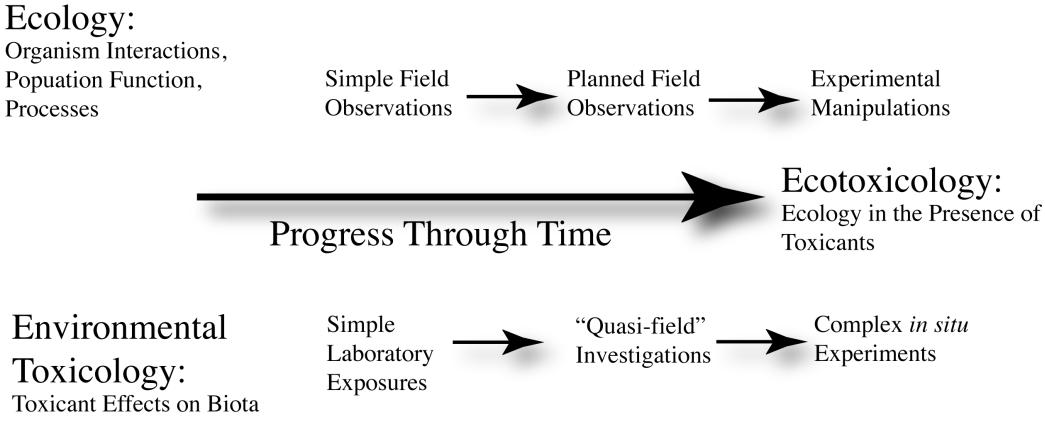


**Fig. 1.1.** The development of ecology, environmental toxicology, and ecotoxicology. Scheme redrawn from Chapman (2002).

As in medical sciences, when considering the developments in pharmacology and toxicology, several lines of reasoning developed in ecotoxicology. Assessment and prediction of effects of contaminants on ecosystems commonly rely on the consideration of chemical exposure, *i.e.*, identifying targets at risk, determining an exposure concentration with respect to the bioavailability of pollutants in a specific environmental milieu and assessing biological responses(Altenburger, 2002).

According to Chapman (2002) there are two key issues specific to ecotoxicology: (1) acute and chronic responses and (2) criteria for species selection. Toxicity test organisms need to be appropriate to the problem being addressed, and the results put into context relative to both reference and baseline comparisons to understand hazard. Use of toxicity tests requires appropriate endpoints and risk hypotheses, considering ecological, not just statistical significance, and recognizing that hazard does not equate to risk. Toxicity should be linked to population and community response to support

decision-making, assessing possible genotypic adaptations that can influence risk estimates, and addressing uncertainty.

Ecotoxicological research is aimed at an understanding of toxicological phenomena in a variety of biota, populations and ecosystems, and diverse aspects such as mechanisms of toxic action and ecological processes in contaminated systems are considered (Fent, 1998). Ecotoxicological effects occur at all levels of biological organization, from the molecular to the ecosystem level. Not only certain organisms may be affected, but the ecosystems as a whole, both terrestrial and aquatic, in its function and structure (Fent, 2004, Fig. 1.2).

Ecotoxicological studies may also focus on ecological and toxicological effects observed in the field in retrospective studies, whereby a causative correlation between effects and chemical residue analysis is often difficult to establish(Fent, 2001).

Ecological investigations such as biomonitoring studies alone do not have sufficient resolving power to identify causative agents. Likewise, chemical analysis of pollutants in ecosystems alone cannot provide evidence for toxicological consequences in biota (Fent, 2003). Only an integrated approach integrating environmental chemical, toxicological and ecological concepts may be suitable for understanding ecotoxicological effects in contaminated ecosystems (Fent, 1996). Each unit of ecological systems functions on the basis of actions and interactions between abiotic factors, which characterize the physico-chemistry of the biotopes, and biotic factors, which relate to the biological component (Kosmehl, 2007).

With respect to the magnitude of assessable factors determining the health status of an ecosystem or the hazard potential of a particular sample, weight-of-evidence (WOE) approaches for assessing contaminations of ecosystems have been advocated (Burton *et al.*, 2002; Keiter *et al.*, 2006; Lowell *et al.*, 2000). According to Chapman (2002), WOE is the process of combining information from multiple lines of evidence to reach a conclusion about an environmental system or stressor. Those lines include *e.g.*, description of ecological risk posed by a hazardous waste site or the determination of effect-levels for chemicals from studies on different species or pathways (Burton *et al.*, 2002).

### 1.2.1 Ecotoxicology and fresh water aquatic sediments

#### Role of sediments in fresh water quality

A river basin is a dynamic system, where interactions between different compartments (atmosphere, river water, sediment, soil, groundwater) greatly

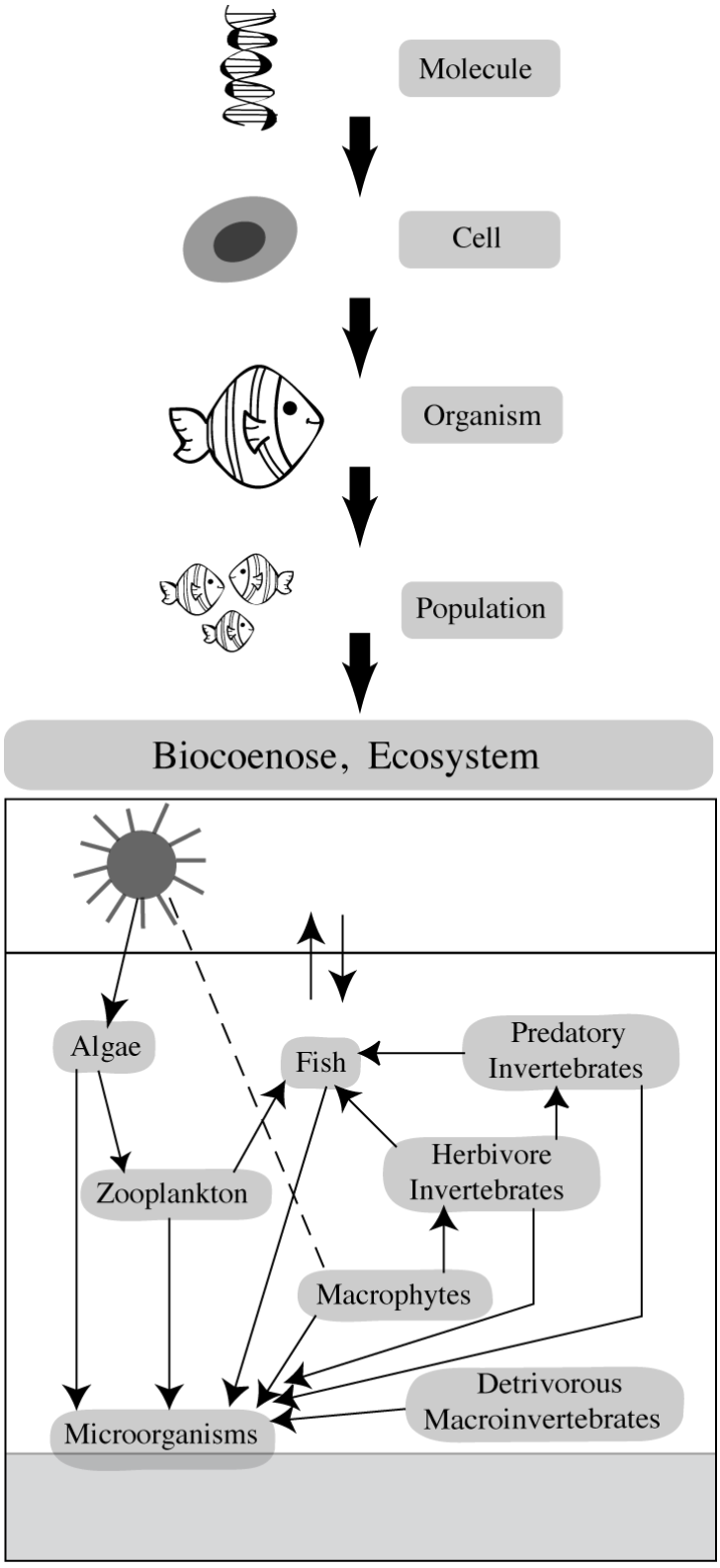


**Fig. 1.2.** Ecotoxicology, an enviromental science focusing on different biological levels. Scheme redrawn from Fent 2001.

influence the ecological quality (Ahlf, 2007). Industrial effluents, agricultural run-off, and municipal wastewaters contain unknown substances and complex mixtures that are released into the environment and can lead to contamination of a river basin. The assessment of water quality in multiple-use water-bodies (rivers, lakes and reservoirs) has gained great importance in recent years, not only because of their ecological, economic and social roles, but also due to the negative impact of a variety of anthropogenic activities (Soares & Mozeto, 2006).

Toxic effects of anthropogenic compounds in biota and ecosystems are regarded in relation to their chemistry and fate in the environment (Fent, 2003). Contaminants at large contaminated sites often share critical properties such as high acute and/or chronic toxicity, high environmental persistence, often high mobility leading to contamination of groundwater, and high lipophilicity leading to bioaccumulation in food webs. Contaminants present at polluted sites occur as mixtures; therefore, interactions between individual compounds are of importance. The bioavailability is a key factor responsible for ecotoxicological effects of contaminants: only the bioavailable fraction induces ecotoxicological effects (Fent, 2003). Once bioavailable for aquatic organisms, xenobiotics may enter, *e.g.*, through the gills, the skin, or the gastrointestinal tract in fish or epidermal cells or root hairs in plants inhabiting chemically polluted aquatic environments (Ohe *et al.*, 2004). Despite the bioavailability of chemicals be an important factor, it is often neglected in ecotoxicological evaluation and hazard assessment. The bioavailable fraction is critical for uptake and, ultimately, for the concentration at the target site in organisms (Fent, 2003, Fig. 1.3).

Sediments in marine and freshwater systems are complex dynamic matrices composed of organic matter in various stages of decomposition, particulate mineral material that varies both in size and chemical composition, and inorganic materials of biogenic origin, *e.g.*, diatom frustules and calcium carbonate (Chen & White, 2004). Humic substances, which represent most of the dissolved organic matter mass in sediments, are highly condensed, polymerized nitrogen- and oxygen-bearing organic complexes (Gagne *et al.*, 1999), and represent an attractive site for binding organic pollutants (Kosmehl, 2007). There is general agreement that sediment-bound substances are of major importance for the fate and effects of trace contaminants as well as water quality in aquatic systems. As a first step during the environmental fate of chemicals, sediment adsorption may lead to a decrease in the presence of persistent pollutants in the water column, which is, in the short term, an improvement of the habitat for pelagic organisms. However, as a result, huge quantities of anthropogenic contaminants are typically associated with aquatic sediments more densely populated areas, making sediment contam-

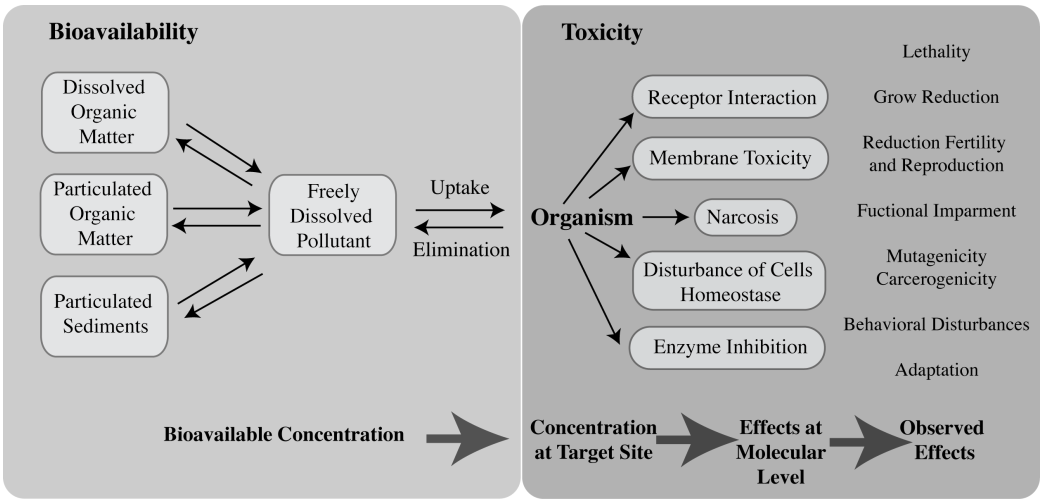


**Fig. 1.3.** Ecotoxicological effects are dependent on the bioavailable fraction of pollutants, and concentrations at the target sites induce molecular effects that propagate to a variety of toxic manifestations in organisms. Scheme adapted from Escher et al. (1997), redrawn from Fent (2003).

ination a serious problem in river sedimentation areas, such as floodplains, lakes, delta areas and estuaries (Thomas *et al.*, 2002; Viganò *et al.*, 2003).

Bioavailability of sediment-bound contaminants, as a prerequisite for exposure, depends on sorption kinetics to the particles, sediment characteristics (capacity controlling properties), and sediment deposition and erosion. Even if sedimentation of particle-bound substances makes sediments a sink of pollutants, they may become a secondary source as well, since contaminated particles can be remobilized to the free water phase via bioturbation (Power & Chapman, 1992) and flood events (Hilscherova *et al.*, 2003; Hollert *et al.*, 2000; Woelz *et al.*, 2008). Relatively unpolluted recent sediment surface layers can cover older contaminants sediments deposited in areas of low flow in river corridors, such as floodplains, river slack-zones and channel beds, reservoirs, and groyne fields. Nevertheless there is an increasing risk of resuspension of old contaminated sediment layer and the transport of the particle-bound pollutants downstream in river systems due to the potential for increasing water discharge associated with both anthropogenic activities (*i.e.*, increased run-off to rivers due to land use changes) and climate change (*i.e.* increased precipitation). Contaminated material can also be introduced to river systems from contaminated soil and other diffuse sources during surface runoff or erosion events (Förstner & Owens, 2007 Fig. 1.4).

Contaminated sediments are known to be able to cause various adverse effects on organisms even when contaminant levels in the overlying water are

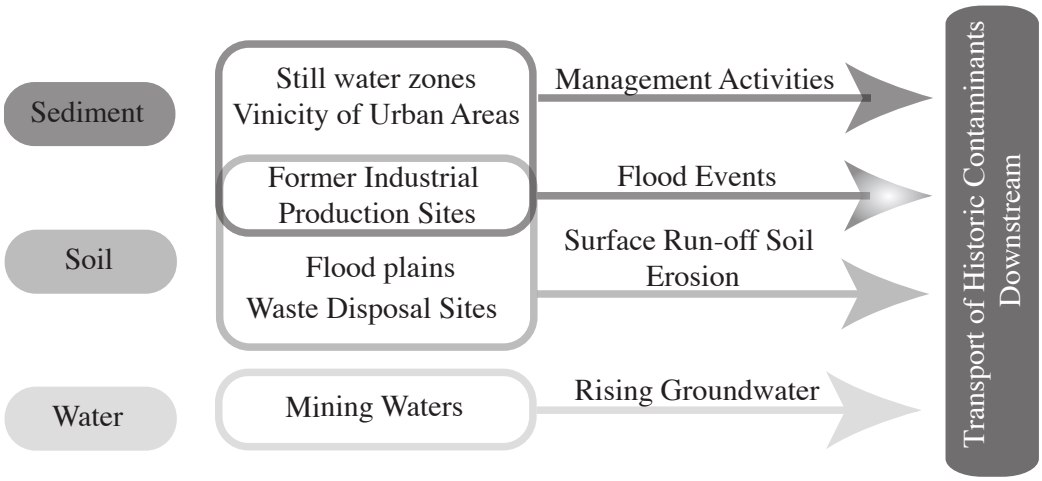


**Fig. 1.4.** Pathways and processes for the transport of historic contaminatin downstream. Scheme redrawed from Heise *et al.* (2004).

low (Chapman, 1989). According to Wang *et al.* (2004), aquatic organisms may be contaminated directly from sediments, from their food, and from overlying water, and the relative importance of these three routes of exposure depends on the contaminants and on the ecology of the particular organism. Moreover, contaminated particulate matter can directly be ingested, *e.g.*, by fish and release their load inside the organism. As a result, there is an urgent need to address sediment-related issues in decision-making processes, since in the field, contaminated sediments may exert effects on the whole ecosystem, including pelagic organisms living in the water column. Even pelagic organisms may be exposed to contaminants which either have diffused from the sediment to the overlying water as a result of chemical partitioning, or are bound to suspended particles (Clement *et al.*, 2004). Thus, from an anthropocentric point of view, a serious problem can arise from sediment pollution, *e.g.*, for people dependant on fish as their major source of animal protein and of course, for the whole aquatic ecosystem (Kosmehl, 2007).

### Evaluation of sediment quality

As stated in section 1.1, to evaluate adverse effects on ecosystems, neither biotests nor chemical-analytic techniques alone are sufficient. Whereas instrumental analyses are useful in identifying the compounds of interest and determining concentrations of specific compounds they provide little information regarding the integrated biological relevance of a complex mixture of compounds associated with environmental samples such as sediments. Therefore, weight-of–evidence studies are recommended.

Formalized use of WOE studies in the environmental sciences is relatively recent. One of the first sediment quality WOE frameworks was the Sediment Quality Triad (SQT) (Chapman, 1990; Chapman *et al.*, 1992; Long & Chapman, 1985; Chapman *et al.*, 2002, Fig. 1.5). The SQT was first based on indices, specifically the development of ratio to reference (RTR) values for each parameter of chemistry, toxicity and benthic community structure (Long and Chapman 1985; Chapman 1990). The SQT has subsequently been refined, deleting the RTR approach, and incorporating generic as well as specific sediment quality values and multivariate analyses (Chapman, 2000).

The SQT approaches evaluate the degree to which contaminants are responsible for the degradation of the sediment health. It is an effects-based technique that involves three components simultaneously: sediments chemistry (a measure of contamination), sediment toxicity testing (a measure of biological effects and bioavailability) and *in situ* community parameters (alterations in the field; Carr *et al.*, 1996).

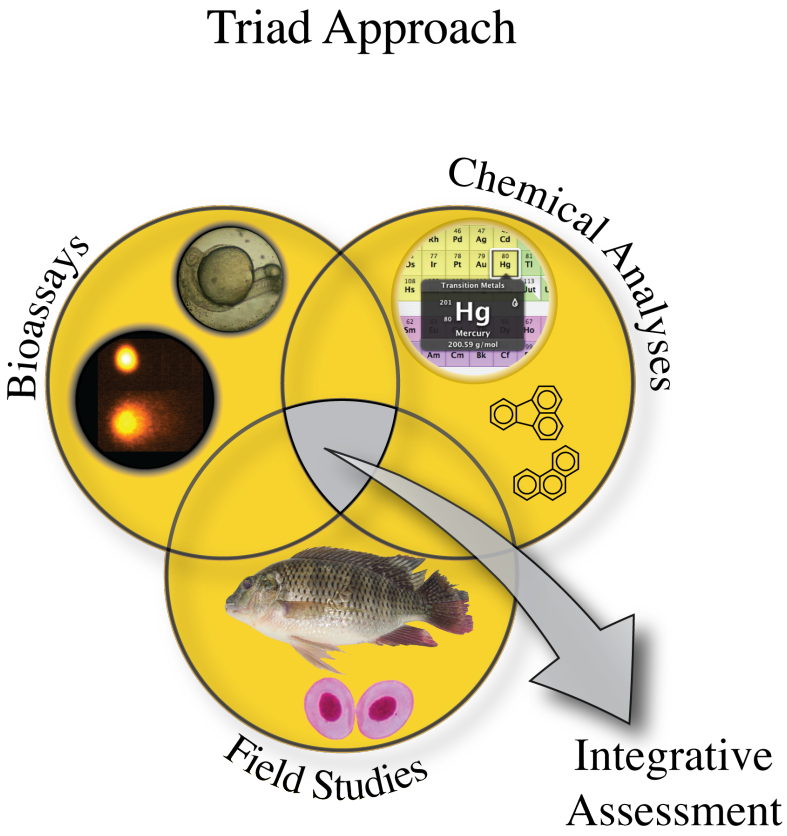


**Fig. 1.5.** Sediment Quality Triad (STQ) according to Chapman *et al.* (1992)

*In situ* community structure and function are powerful and realistic parameters, since they integrate all factors in the field under real exposure conditions over a long time (Schulz, 2001), possibly over many generations (e. g., for some invertebrates species) (Kosmehl, 2007). Structural attributes (such as species numbers, dominant species and abundances) are easiest to visualize and are by far the most commonly used. Functional attributes (such as primary production, community respirations, biomass turnover) require deeper knowledge, but hold the promise of being very broad measures of ecosystem processes that transcend species groups and habitats. However, community functions are much more difficult to measure (Pratt & Suarez, 1990). These criteria often fail to provide sensitive measures of ecosystem health, and the relations to biological status may remain unclear (Cairns *et al.*, 1993). Moreover, due to the complexity of the ecosystem, its intrinsic functions and interactions can only be understood for a very small unit (*e.g.*, two species) (Kosmehl, 2007).

In addition to in-field studies, another line of evidence evaluates the level of environmental contamination and its potential effects by the use of biomarkers to verify the bioavailability and presence of relevant concentrations in biota (Fent, 1996). The response to pollution is reflected as changes in some enzyme activities, especially key enzymes of biotransformation systems of organisms, which can be used as biomarkers that are sensitive to pollution. These biomarkers therefore provide a tool for specific early warning for aquatic pollution (Kammann *et al.*, 2004; Strmac & Braunbeck, 2000). Ecotoxicological studies based on biomarkers allow the determination of the impact of environmental stressors and facilitate following the evolution of the ecosystem towards degradation or restoration (Vasseur & Cossu-Leguile, 2003). In addition to their use as simple indices of exposure and effects to (specific) pollutants, biomarkers can provide insight into ecosystem health. Important advantages of biomarkers are their inherent capacity to detect early biological effects within organisms and to monitor the temporal progression (or regression) of the disturbance of various levels of biological organization (Van der Oost *et al.*, 2003).

As another line of evidence for assessing sediment contaminants, *in vitro* bioassays, can be used as a specific detector for biological effectiveness of complex mixtures, since synergistic and antagonistic effects between all sample components are considered (Fent, 1996). To monitor the sediment quality, ecotoxicological *in vitro* bioassays are first applied to screen if contamination had significant effects on biological functions of the model organisms/systems. Standardized test protocols are available for many species and organisms, ranging from bacteria to vertebrate systems including mammals (Balch *et al.*, 1995; Hollert *et al.*, 2000, 2003; Kinder *et al.*, 2007; Kosmehl,

2007; Kosmehl *et al.*, 2004; Mwase *et al.*, 1998). Whereas acute toxicity was of major concern in the last decades, recently for many river basins there was a change in focus to more specific, chronic (non-lethal) effects (Brack *et al.*, 2005a). While these effects are difficult to assess using *in vivo* tests, they can relatively easily be determined by *in vitro* techniques that allow predicting toxic potentials of complex environmental mixtures (Janosek *et al.*, 2006). The *in vitro* bioassay approach serves as an efficient, fast and cost effective screening for evaluation of the receptor-mediated activities of complex mixtures (Hilscherova *et al.*, 2002). This approach has been successfully used to prioritize contaminated sediment sites (Hilscherova *et al.*, 2003; Hollert *et al.*, 2002; Kammann *et al.*, 2005a; Keiter *et al.*, 2008, 2009; Kosmehl *et al.*, 2004; Metcalfe *et al.*, 1990; Woelz *et al.*, 2009). A further advantage of a bioassay approach is that the combination of different bioanalytical methods allows to investigate multiple endpoints such as genotoxicity or mutagenicity (Kosmehl *et al.*, 2004, 2006), dioxin-like (Brack *et al.*, 2008; Hilscherova *et al.*, 2001; Hollert *et al.*, 2002; Otte *et al.*, 2008; Wong *et al.*, 2005), or various endocrine effects (Hollert *et al.*, 2005; Sumpter & Johnson, 2005) in parallel, *i.e.*, in the same sample. However, since many factors (chemical, physical and biological) can affect environment conditions, the transfer of results obtained by *in vitro* techniques to the field is a complex task. Hence, the establishment of extrapolation parameters is a crucial issue.

Freeze-dried sediments can be used for quality assessment with *in vitro* bioassays using whole-sediment exposure protocols. These protocols represent a most realistic scenario to simulate *in situ* exposure conditions (Burton, 1991; Chapman & Hollert, 2006; Hollert *et al.*, 2003). However, several endpoints (*e.g.*, genotoxicity, mutagenicity) in cell culture-based system can only be tested after transfer of particle-bound substances into the aqueous phase, since exposure to particles is not suitable (Kosmehl, 2007). In ecotoxicological research, cellular effect studies are as important as studies in laboratory species since interactions between chemical contaminants and biological systems take place at the cellular level at the first instance (Fent, 2001; Sanchez-Fortun *et al.*, 2008). The use of *n vitro* cell cultures for ecotoxicological assessment can be a valuable tool for an early and sensitive detection of chemical exposure (Castaño *et al.*, 1996). Fish cell lines, *e.g.*, are useful tools for ecotoxicological evaluation of many chemicals. The use of *in vitro* methods in environmental testing, particularly those employing fish cell cultures, is an area of expanding possibilities in the ecotoxicological evaluation of mixtures, for controlling chemicals, emissions, effluents and hazardous wastes (Castaño *et al.*, 2003; Repetto *et al.*, 2003). Previous studies has been demonstrated that cultured, established fish cell lines can be successfully used for the detection of *in vitro* toxicity screening of aquatic

pollutants (Böttcher *et al.*, in press; Keiter *et al.*, 2006; Kinder *et al.*, 2007; Kosmehl *et al.*, 2004; Seitz *et al.*, 2008; Zurita *et al.*, 2007).

To transfer particle-bound substances from whole sediments to the aqueous phase, and to allow tests using cell cultures, extraction methods are required. A strategy to release organic chemicals from sediments consists of extraction with an organic solvent (*e.g.*, n-hexane, acetone) to dissolve the organic substances and then evaluating the toxicity of the extract (Ho & Quinn, 1993). Acetonic extraction, such as the Soxleht extraction, covers hardly soluble, lipophilic compound in addition to moderately lipophilic substances (Campbell et al 1992,; Ho and Quinn, 1993; True and Heyward, 1990). However, the bioavailability of those compounds may be overestimated compared to the field-like situation. Thus, the relevance and negotiability of results from studies using extracted samples for bioassays studies is somewhat questionable for the ecosystem extrapolation. Even so, sediment extracts are essential for some protocols to transfer particle-bound substances to the liquid phase and to mimic long-time exposure to higher test concentrations of a particular sample of the maximum hazard potential (Kosmehl, 2007).

To extrapolate *in vitro* results to the field a promising approach is the combination of *in vitro* and *in situ* bioassays. *In situ* bioassays approaches are more likely to reflect the real exposure situation in the environment, and, applied in combination with *in vitro* bioassays, the *in situ* assays may be used to verify or falsify the potential toxicity revealed by the *in vitro* bioassays (Fig. 1.6). In fact, the combination of *in vitro* bioassays with *in situ* assays has been applied successfully to detect various ecotoxicological endpoints in several environmental monitoring studies (Böttcher *et al.*, in press; Bolognesi *et al.*, 2004; Buschini *et al.*, 2003; Choueri *et al.*, 2009; Hollert *et al.*, 2002).

**Effect-directed analysis** Integrated approaches such as the sediment quality triad (SQT) provide an evaluation of the ecological relevance of the results of bioassays and chemical analyses for major sites. However, no identification of the harmful substances can be obtained by means of the triad approach. Therefore, reliable hazard and risk assessment require both the detection of adverse effects and the identification of the chemicals causing the effects. This can be done by combining biological testing, physicochemical fractionation and chemical analysis. One approach is the "effect-directed analysis" (Brack, 2003; Brack *et al.*, 2005b). This is done by sequential reduction of the complexity of environmental mixtures eventually to individual toxicants. The sediment extracts are tested for biological effects and subjected to one or several fractionation procedures. After each separation step, the fractions are biotested for selection of active fractions for further

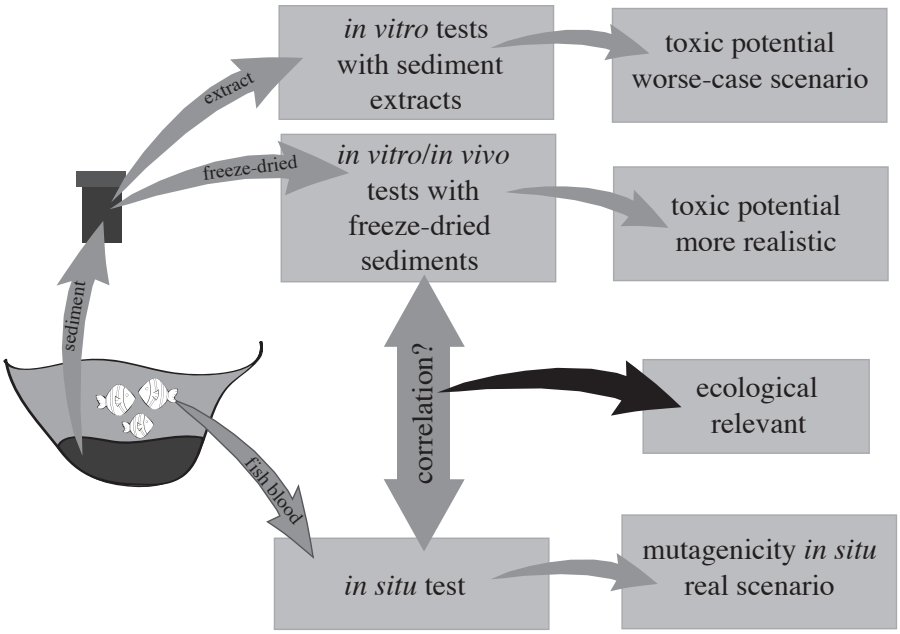


**Fig. 1.6.** Combination of *in vitro* and *in situ* parameters to establish the ecological importance of *in vitro* assays.

investigation. When the complexity of the mixture is reduced to a few individual compounds, the fractions are subjected to chemical identification and quantification. The bioassay-directed fractionation techniques have repeatedly been used to fractionate extracts and examine their toxicity in bioassays, in order to gain insight into the nature of the noxious substances. The combination of specific biomarkers, simple fractionation methods, chemical analyses and *in situ* investigations should prove to be a suitable tool for the assessment of sediments and water.

The integrative assessment (SQT) suggested by Chapman *et al.* (1992) and the principle methodology of effect-directed identification of toxicants in complex environmental samples is presented in Fig. 1.7.

## 1.3 Research questions and outline of this thesis

The objective of this thesis was to determine hazards and provide complex insights into ecotoxicological risks of contaminated sediments from the Tietê River Basin (Brazil) by a combination of ecotoxicological approaches using

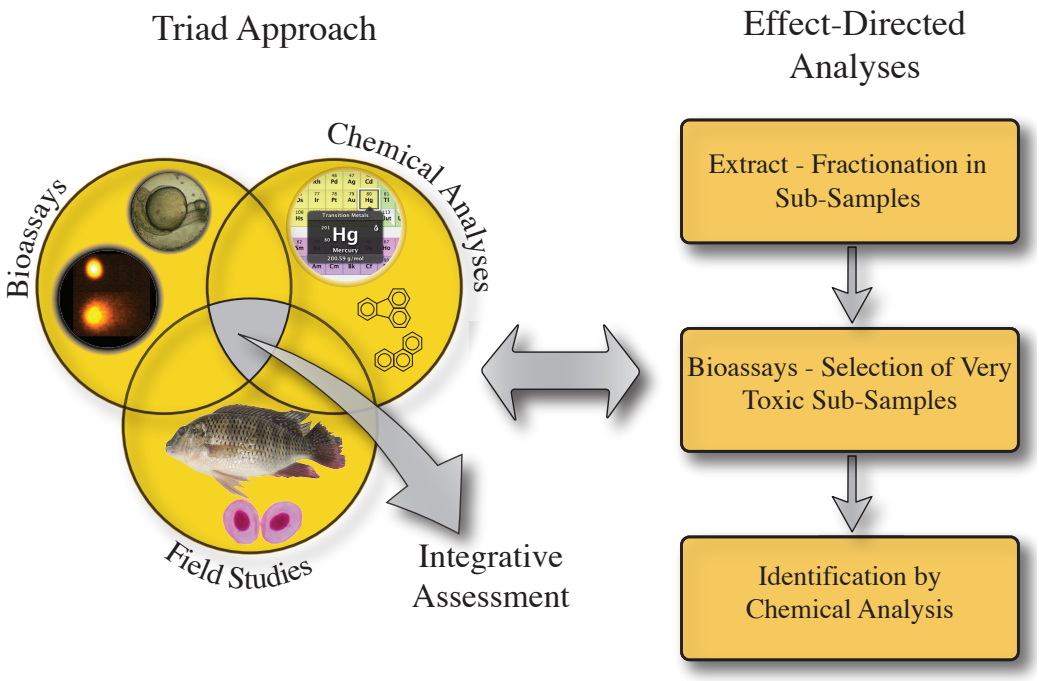


**Fig. 1.7.** The association of the sediment triad approach (Chapman et al., 1992) with effect-directed analyses (Brack, 2003; Brack et al., 2005)

laboratory studies, *in vitro* biological tests as well as *in situ* assessment and chemical analyses. In order to obtain a comprehensive insight into the potential ecotoxicological hazard, acute toxicity and more specific toxicity such as mutagenicity, genotoxicity, teratogenicity and AhR-mediated toxicity were recorded. In order to indentify the unknown pollutants responsible for the toxicity, effect-directed analysis was performed for the most toxic sediment samples, combining an automated on-line multi-step high-performance liquid chromatography (HPLC) fractionation procedure with selected bioassays and chemical analysis.

## 1.4 The studied areas

The Tietê River was selected as an example of a highly polluted river system. This way, surface fresh water sediment samples were collected from eight localities in the Tietê River Basin, São Paulo state, Brazil (Fig. 1.8); these comprised a location at Salesópolis near the Tietê River's spring and the reservoirs Ponte Nova and Billings; Barra Bonita; Bariri and Promissão and Três Irmãos. These localities cover the entire length of the Tietê River

(from its spring to the mouth) and further include 2 important water bodies associated to this river, Pinheiros River and Billings reservoir. The location near the Tietê River spring was selected as reference site.

Sediment samples were collected by means of an Eckman-Birge dredge, with ten replicates at each site (with a distance of 10m from sample to sample). Replicates were homogenized, and 1.5 kg of each sediment sample were frozen immediately, stored at -10°C and transported to Germany. Transfer of the samples to Germany was permitted by the Brazilian National Department of Mineral Production (DNPM). Samples were freeze-dried and kept at 4°C until use in the sediment contact tests. Moreover, extracts were prepared by Soxhlet extraction using acetone p.a. as a solvent and re-dissolved with specific solvents for use in the *in vitro* bioassays and chemical analyses.

As an *in situ* parameter, fish blood from *Oreochromis niloticus* caught at dfferent sites along the Tietê River was collected in order to evaluate *in vivo* mutagenic effects (Chapter 2).

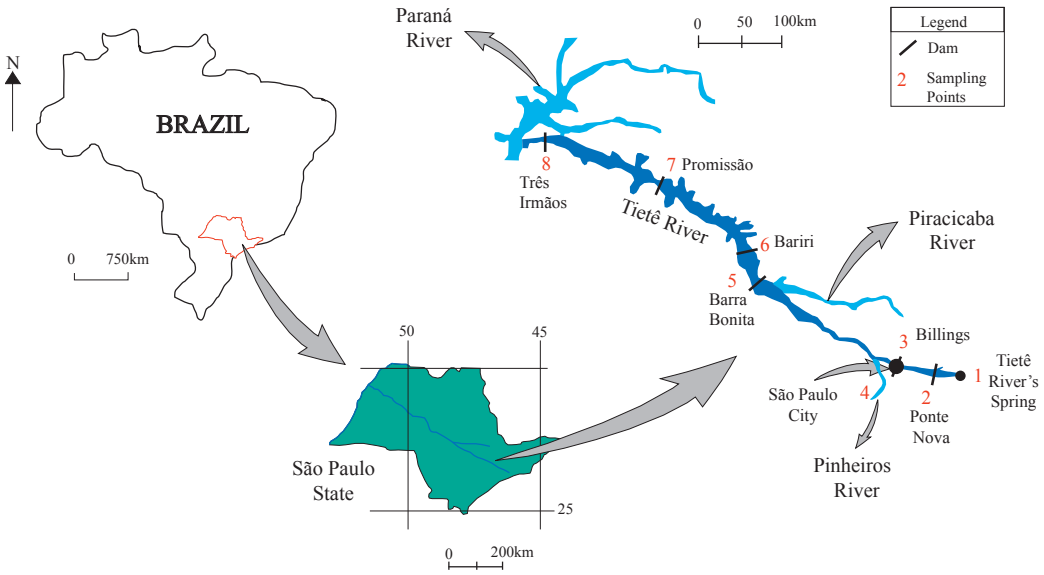


**Fig. 1.8.** Location of the sampling sites in the Tietê River basin, São Paulo state, Brazil.

### 1.4.1 Tietê River Basin

The Tietê River Basin is the largest hydrographical basin of São Paulo state (SMA-SP, 2002). This basin is subdivided into three sub-basins, due to its distinct geomorphologic characteristics: Upper Tietê, Middle Tietê and Lower Tietê. The Upper Tietê River basin corresponds to the drained area of the Tietê River from its spring across the metropolitan area of São Paulo city

characterized by high densities of anthropogenic population and dramatic deterioration subsequent to intensive urbanization. The Superior Middle Tietê River basin is dominated by a high density of urban, industrial and agricultural areas. The Inferior Middle Tietê River basin is characterized predominantly by agricultural areas and the Low Tietê river basin by pastures and sugar cane culture (CETESB, 1997).

### The Tietê River

The Tietê River is inserted in the most important economical center in Brazil, São Paulo state (southeastern Brazil). It practically crosses all of this territory. The Tietê River spring (Fig. 1.9) is located in the municipality of Salesópolis, only 22 km from the ocean, but the geography of the area forces the river to flow towards the center of the continent, from southeast to northwest, till it flows in the Paraná River, in the municipality of Itapura.

**Fig. 1.9.** The Tietê River spring, located in the municipality of Salesópolis (Upper Tietê River Basin), São Paulo state, Brazil.

The total length of the river is 1.150 Km and its level between the mouth and the margins is not more than 860m with a global medium declivity of 74cm/km. The different levels of its route have been utilized for constructing several reservoirs, which were built in response to the growing demand for electric energy in southeastern Brazil, as well as for urban, industrial and agricultural use, including recreation and navigation.

Only few kilometers from its spring, the river passes though the megacity of São Paulo (Fig. 1.10). Especially there (but along its whole course), the river receives a high pollutant load of organic substances, proceeding from domestic sewers, agricultural and industrial residues, and inorganic substances

from industrial sources. Still in the metropolian São Paulo region, the Tietê River receives water from its very polluted tributary, the Pinheiros River (Fig. 1.11).

The São Paulo Metropolitan area (> 19,000,000 inhabitants, Fig. 1.12), comprises 38 cities in addition to the city of São Paulo and occupies an area of 8000 km$^2$, of which 900 km$^2$ is urbanised (Hermann & Braga Jr., 1997).

**Fig. 1.10.** The Tietê River crossing São Paulo city (Upper Tietê River Basin), São Paulo state, Brazil (Source: www.vitruvius.com.br, www.riobranco.org.br).

**Fig. 1.11.** The Pinheiros River crossing São Paulo city (Upper Tietê River Basin), São Paulo state, Brazil (Photo: Robert Portoguá).

**Fig. 1.12.** The Metropolitan area of São Paulo, in São Paulo state, Brazil (Source: www.riobranco.org.br).

### The Tietê River reservoirs

The construction of reservoirs for a variety of purposes represents one of the greatest human experiences in the modification of natural ecosystems. Making use of rivers by building a series of reservoirs is a common practice in large Brazilian river systems (Rodgher *et al.*, 2005). The construction of reservoirs cause changes of social, economic and biological alterations, which can interfere spatially and/or temporally with the ecosystem (Marciano, 2005).

In São Paulo State, this system is commonly adopted for maximum exploitation of hydroelectric energy potentials. Numerous consecutive dams create a group of reservoirs that receive and accumulate organic and inorganic matter from adjacent systems (Rodgher *et al.*, 2005). The Tietê River reservoirs are widely used for providing drinking water, as a water source for agricultural irrigation and as recreation sites. These reservoirs include: Ponte Nova and Billings (Fig. 1.13 and 1.14), in the Upper Tietê River Basin; Barra Bonita (Fig. 1.15), in the superior Middle Tietê River Basin; Bariri and Promissão (also called Álvaro de Souza Lima and Mário Lopes Leão, respectively, Fig. 1.16 and 1.17) in the inferior Middle Tietê River Basin; and Três Irmãos (Fig. 1.18), in the Lower Tietê River Basin. From Barra Bonita to Três Irmãos, the reservoirs were built in cascade arrangements.

As a consequence of the relatively high local population densities and the multiple industrial and agro-industrial activities widespread in the drainage basin, these reservoirs are also used as receptors of domestic and industrial effluents, suffering from poor water quality, mainly in the upper part of the basin (Billings reservoir in Metropolitan São Paulo), as well as its middle reaches (Barra Bonita and Bariri reservoirs; Calijuri, 1999; Conselho Estadual de Recursos Hídricos, 1990). However, the lower Tietê River Basin

**Fig. 1.13.** Ponte Nova reservoir, located in the municipality of Salesópolis (Upper Tietê River Basin), São Paulo state, Brazil.

**Fig. 1.14.** Billings reservoir, located in the Metropolitan region of São Paulo (Upper Tietê River Basin), São Paulo state, Brazil.

(Promissão and Três Irmãos reservoirs) has, in turn, been suffering great transformations, especially in the last decades, firstly with extensive plantations of coffee, cotton, maize and oranges, which have gradually given place to very extensive sugarcane plantations. Since 1975, when the Brazilian alcohol program was launched, the effects of the development of an expanding agro-industrial park producing sugar, alcohol, citrus juices and fabrics must also be considered citepSOARESandMOZETO2006.

**Fig. 1.15.** Barra Bonita reservoir, located in the municipality of Barra Bonita (Middle Tietê River Basin), in São Paulo state, Brazil.

**Fig. 1.16.** Bariri reservoir, located in the municipality of Bariri (Middle Tietê River Basin), São Paulo state, Brazil.

## 1.5 The ecotoxicological assesment of sediments from Tietê River Basin

The Tietê River was selected as a model of a polluted river system. In order to determine hazards and provide complex insights into ecotoxicological risks of contaminated sediments from Tietê River Basin, different ecotoxicological studies were developed. Established test systems were applied to assemble sets of data of toxicological endpoints, and combined with chemical analyses and effect-directed analysis:

**Fig. 1.17.** Promissão reservoir, located in the municipality of Promissão (Lower Tietê River Basin), São Paulo state, Brazil.

**Fig. 1.18.** Três Irmãos reservoir, located between the municipalities of Ilha Solteira and Pereira Barreto (Lower Tietê River Basin), São Paulo state, Brazil.

- In a first approach, *in vitro* bioassays using acetonic sediment extracts with a cell-based monitoring system were combine with *in situ* biomarkers: (1) to find out possible interactive genotoxic and mutagenic effects from multiple contaminants, and (2) to elucidate, by comparison of *in vitro* and *in situ* approaches, the ecological relevance of *in vitro* results and their ability as possible bioindicator systems (Chapter 2).

- Acute cytotoxicity tests and AhR-mediated toxicity tests were applied in combination to chemical analysis of polycyclic aromatic hydrocarbons (PAHs): (3) to assess the ecotoxicological potential of sediments

from the Tietê River reservoirs and the Pinheiros River with respect to cytotoxicity and CYP P450 1A induction potential; (4) to evaluate the contribution of priority PAHs to the EROD induction of these environmental samples (Chapter 3).

- Sediment contact assays, with whole-exposure of fish embryos to solid-phase sediments were applied: (5) to evaluate the embryotoxic and teratogenic effects of sediments from Tietê River Basin by means of the embryo toxicity contact assay with *Danio rerio*, providing a more comprehensive and realistic insight into the bioavailable hazard potential of these sediment samples (Chapter 4).

- Effect-directed analyses were carried out with the most toxic samples in order to (6) identify the unknown pollutants responsible for the toxicity. For this end, a fractionation procedure and selected bioassays were combined (Chapter 5).

## 1.6 Overview of the techniques applied

### 1.6.1 Assessment of genotoxicity and mutagenicity

Growing interest in genotoxicity caused by environmental pollutants has led to the development of various biological tests for detecting and identifying genotoxicants in air, water, sediments and soil (*e.g.*, Chen & White 2004; Claxton *et al.* 2004; Ohe *et al.* 2004; White & Claxton 2004. Genotoxic agents interact with the genetic material forming adducts or inducing DNA alterations or even DNA breakage. In most cases, organisms are capable of repairing such lesions; alternatively, affected cells are eliminated. In case such lesions are permanent and eventually provoke hereditary alterations (mutations), the agent is called mutagen (Mídio & Martins, 2000; Pilot & Dragan, 1996). In addition to a certain rate of spontaneous mutations, most DNA changes are induced by physical, chemical or biological agents, which also humans are naturally exposed to (Matsumoto, 2004). Therefore, the identification and understanding of such agents´ properties allow the evaluation of noxious hereditary alterations or even lethal effects in organisms (Arnaiz, 1995).

According to Kosmehl *et al.* (2004) testing of wastewaters for genotoxicity may become a routine requirement for some industrial wastewater discharge permits, not unlike the more common requirement for routine aquatic toxicity tests. The stimuli for this are concerns that aquatic organisms inhabiting waters impacted by wastewater discharges suffer an increased risk of

genetic damage or cancer. Most importantly, humans utilizing these waters for recreational and drinking water purposes may suffer similar genetic or carcinogenic risks. Wastewater discharges may be one source of genotoxic organic compounds in those impacted areas. With respect to potential human health impacts, there is evidence of increased cancer risk to individual drinking water from surface sources; however, this risk may or may not be related to whether the drinking water source received input of wastewater discharges or known carcinogens.

Since fish represent vertebrates, which are top predators of the food chain, considerable efforts have been undertaken to develop fish-based test systems for the assessment of sediment bound substances (Hollert *et al.*, 2003; Kosmehl *et al.*, 2004). Fish provide a suitable monitoring system within aquatic genotoxicity and wastewater evaluation due to their ability to metabolize xenobiotics and accumulate pollutants. Most importantly, recently a number of laboratory investigations and field studies have documented a correlation between genotoxic pollutants and heritable reproduction effects on individuals as well as a potential link to the declines of fish populations (for reviews see Chen & White, 2004, as well as Keiter *et al.*, 2006).

In the present study, two bioassays were applied to detect the genotoxic potential of the sediments and waters from the Tietê River basin: the *in vitro* version of the comet assay and the *in situ* (*in vivo*) version of the micronucleus assay.

The comet assay is an electrophoresis technique for measuring DNA breaks in individual eukaryotic cells by measuring the migration of DNA from isolated nuclear DNA (Cotelle & Ferard, 1999; Singh *et al.*, 1988). The method is rapid, sensitive and inexpensive (Lee & Steinert, 2003). In various areas, the use of the comet assay has increased, and in recent years the technique has repeatedly been applied in environmental monitoring studies. Due to its versatility and adaptability, the comet assay has, *e.g.*, been successfully used to detect genotoxic activities in different environmental compartments (air, water, soil, sediments) and in a variety of target or test organisms (Kosmehl *et al.*, 2004). In combination with the comet assay, appropriate organisms can be used as biosensors for contamination of the environment by genotoxins. Fish are especially sensitive to pollutants interacting with DNA, as has been shown by several studies (Balch *et al.*, 1995; Bolognesi *et al.*, 2004; Grisolia & Cordeiro, 2000; Metcalfe *et al.*, 1990; Minissi *et al.*, 1996). Lesions may easily result in heritable changes of the DNA with the possibility of negative consequences for future generations, if germ cells are affected. Thus, the measurement of such primary effects of exposure to genotoxicants has promise as an adequate and sensitive tool for examining the actual genotoxic burden of organisms in a given environment. In this study, the comet

assay was carried out with the permanent fish cell line RTL-W1 derived from primary rainbow trout (*Oncorhynchus mykiss*) liver cell cultures (Lee *et al.*, 1993) exposed to acetonic sediment extracts.

The micronucleus assay has frequently been used for the detection of stressor-induced damage to chromosomes or the mitotic apparatus of, *e.g.*, erythroblasts by analyzing erythrocyte samples from peripheral blood cells (Böttcher *et al.*, in press; Grisolia & Cordeiro, 2000; Minissi *et al.*, 1996; Palhares & Grisolia, 2002; Ulupinar & Okumus, 2002). Micronuclei are formed by incorrect condensation of the acentric chromosomal fragments or by whole chromosomes, which are not included into the daughter cells during mitosis (Kato & Shimada, 1975; Hayashi *et al.*, 1990) (Fig. 1.19). A micronucleus is a supernumerary DNA or chromosomal fragment visible in the cytoplasm of a cell under the light microscope (Heddle, 1973; Schmid, 1975). Many types of DNA damage caused by water-borne mutagens may induce alterations in chromosomes; so the measurement of chromosomal aberrations offers an appropriate approach for monitoring mutagenic substances in the field. Moreover, chromosomal aberrations selectively count only the DNA lesions that are not repaired by the machinery of the cell. The selected test fish species should be sensitive for chromosomal aberration and should have a suitable karyotype characterized by a limited number of relatively large chromosomes (Ulupinar & Okumus, 2002).

The micronucleus test in fish erythrocytes has been shown to be a sensitive bioassay for detecting mutagenic pollution in fresh water environments (Minissi *et al.*, 1996). The micronucleus assay was applied with erythrocytes from mature Nile tilapia (*Oreochromis niloticus*), an African fish introduced into Brazil in the 1970s, in order to assess *in vivo* mutagenic effects of waters and sediments in the Tietê River basin *in situ*.

In order to evaluate the ecological relevance of the *in vitro* bioassay results, the results obtained in the *in situ* micronucleus assays were compared with the results obtained by the *in vitro* comet assay.

### 1.6.2 Assessment of acute cytotoxicity and AhR-mediated toxicity

In this study, the acute cytotoxicity was recorded as a basic toxicity indicator. Cytotoxicity assays can be performed at small scale with many replicates, thus allowing simple and rapid measurement of extracts and pore waters in large sample numbers (Hollert *et al.*, 2000). Acute cytotoxicity can be determined by means of the neutral red retention assay (NR assay) (Babich & Borenfreund, 1991b; Borenfreund & Puerner, 1985). The NR assay proce-

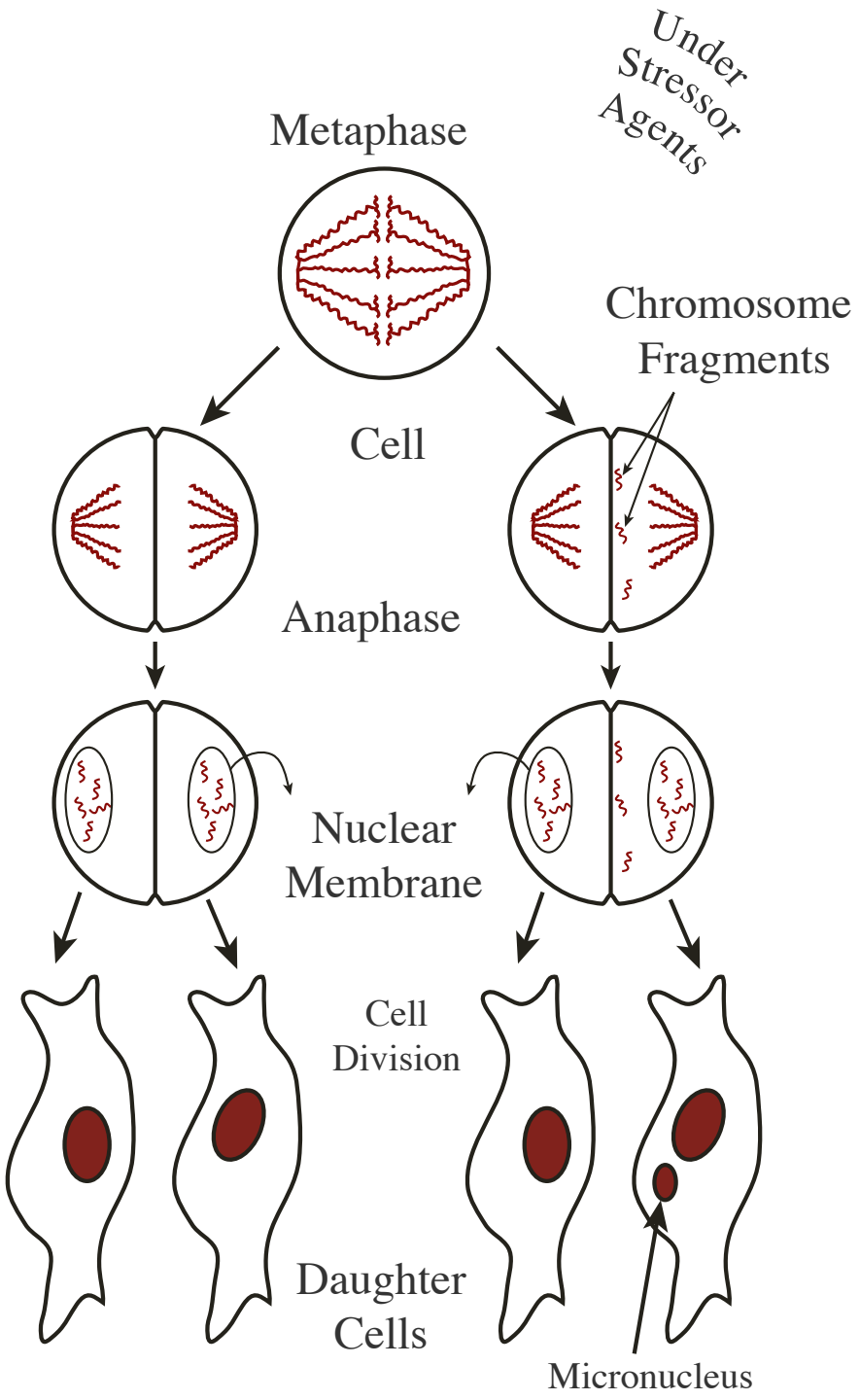


**Fig. 1.19.** A schematic illustration of the mechanism of micronucleus formation in cells, redrawn from Al- Sabti and Metcalfe (1995).

dure is a cell survival/viability technique based on the ability of viable cells to incorporate and bind neutral red, a supravital dye, in the lysosomes after their incubation with toxic substances (Babich & Borenfreund, 1991a). The NR assay is a valuable *in vitro* test since it identifies the cytotoxicity potentials of the samples, determining the maximum concentrations suitable for been used in further tests (meaning maximum concentration that does not induce cytotoxicity). Hence, in an integrative assessment, it is indispensable since the determination of mechanism-specific endpoints is only possible when conflicts with cytotoxic effects are avoided.

As another line of evidence, the *in vitro* detection of cytochrome P4501A (CYP1A) induction was recorded. The induction of CYP1A has been recognized as a sensitive marker of exposure to a number of environmentally relevant toxicants(Behrens *et al.*, 2001; Berbner *et al.*, 1999; Bucheli & Fent, 1995; Engwall *et al.*, 1999). The cytochromes P450 constitute a superfamily of hemoprotein monooxygenases whose members function critically in the metabolism of a wide array of xenobiotic and endogenous substrates. In toxicological studies, the CYP1A has attracted particular attention because of its role in the biotransformation of toxicologically important environmental contaminants (Segner *et al.*, 2000). A variety of environmental pollutants such as dioxin-like compounds are able to induce the biotransformation enzyme CYP1A in fish by ligand binding to the aryl hydrocarbon receptor (AhR; Babin *et al.*, 2005; Hilscherova *et al.*, 2001). Dioxin-like compounds are a class of environmental contaminants that potentially can be found in surface and ground water at contaminated sites (Schirmer *et al.*, 2004). In water, these chemicals are intimately associated with suspended particles and consequently with sediments (Hilscherova *et al.*, 2001; Hollert *et al.*, 2002; Keiter *et al.*, 2009; Seiler *et al.*, 2006; Woelz *et al.*, 2008). In a strict sense, dioxin-like compounds comprise halogenated aromatic hydrocarbons (HAHs), furans (PCDD/Fs) and the polyhalogenated biphenyls (PBs and PBBs), but the attribute "dioxin-like" can also be understood more broadly as being able to act through the AhR. This way, PAHs, which contain many AhR-active compounds can also be referred to as dioxin-like compounds (Schirmer *et al.*, 2004). In fish and other aquatic animals, PAHs have a strong bioconcentration capacity (Connel, 1989) and are readily absorbed during exposure to contaminated food, water and sediment (Conolly & Petersen, 1988; Spacie & Hamelink, 1985; Varanasi *et al.*, 1985). The isoform CYP1A catalyzes the oxidation of many PAHs, yielding highly reactive intermediates (*e.g.*, arene oxides) which can bind to proteins and nucleic acids, resulting in mutagenicity and carcinogenicity in mammals and fish (Varanasi, 1989).

The activity of CYP1A can be determined by measuring 7-ethoxyresorufin-O-deethylase (EROD) activity in several species and test systems (Brun-

ström & Halldin, 1998; Engwall & Hjelm, 2000; Kammann *et al.*, 2005b; Mdegela *et al.*, 2006; Pacheco & Santos, 1998; Segner *et al.*, 2000). In the EROD induction bioassay, cellular induction of CYP1A is measured as specific EROD enzyme activity via the degradation of the artificial substrate 7- ethoxyresorufin to resorufin (Behrens *et al.*, 1998; Fig. 1.20). Compared to other endpoints of the AhR- mediated pathway, the EROD activity is a comparatively sensitive tool and it may even be an indicator for effects at various levels of biological organization (Van der Oost *et al.*, 2003). The permanent cell line RTL-W1 have been widely used for determining EROD activity (Babin *et al.*, 2005; Brack *et al.*, 2000; Billiard *et al.*, 2004; Keiter *et al.*, 2009; Schirmer *et al.*, 2004; Segner *et al.*, 2000) In this study, EROD activity was measured in RTL-W1 cells exposed to the acetonic sediment extracts.

The results obtained from the EROD assay were correlated to chemical analyses of EPA PAHs. The chemical analysis could give an overview of the hazard potential of sediments related to these chemicals. Moreover, in a mass balance calculation, the degree as to which these chemicals account for the EROD-inducing potencies of environmental extracts was calculated.

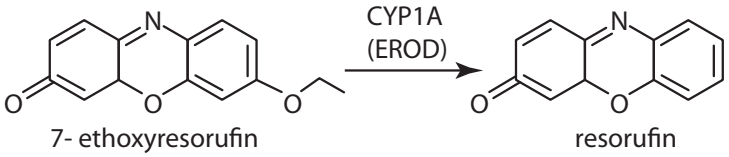


**Fig. 1.20.** Deethylation of 7-ethoxyresorufin to resorufin by the EROD enzyme activity of the cytochrome P450 1A (CYP1A).

### 1.6.3 Whole-fish embryo exposure to solid-phase sediments

For exposure of RTL-W1 cells to the sediment compounds in the comet, cytotoxicity and EROD assays, all samples were extracted using Soxhlet extraction with acetone. Thus, the main interest in these investigations was the total hazard potential of all extractable compounds, an approach that is basically used to screen for pollution hot spots. This, however, neglects field-like exposure parameters like bioavailability; therefore, the ecological relevance is hard to estimate.

Beside the combination of *in vitro* and *in situ* assays, which can be used to confirm or falsify the potential toxicity revealed by the *in vitro* bioassays,

another possibility to obtain ecological relevant results in environmental investigation is to mimic *in situ* exposure conditions. One alternative are contact assays, where a more field-like exposure scenario is used.

It is common sense that fish are an indispensable component of integrated toxicity testing strategies for the aquatic environment (Al-Sabti & Metcalfe, 1995; Balch *et al.*, 1995; Behrens *et al.*, 1998; Braunbeck & Lammer, 2006; Billiard *et al.*, 2004; Lemos *et al.*, 2007). However, in acute tests with their exclusive endpoint of mortality, fish have been hypothesized to suffer severe distress and pain (Braunbeck *et al.*, 2005; Braunbeck & Lammer, 2006; Chandroo *et al.*, 2004; Nagel, 2002), which would be in conflict with current animal welfare legislations (Kosmehl, 2007; Lammer *et al.*, 2009). The fish embryo toxicity test with *Danio rerio* (DIN 38415-6) as well as the use of permanent cell lines has thus become very promising tools to replace the acute fish test (Braunbeck *et al.*, 2005; Nagel, 2002). In this study, based on the protocol of Hollert *et al.* (2003), embryos of *D. rerio* were exposed to freeze-dried sediment samples. The sediment contact fish embryo toxicity assay applied in this study take into account the whole organism exposure to whole sediments and therefore represents a more realistic scenario than exposure to acetonic extracts of the sediments.

There are various advantages of the sediment contact fish embryo assay. First, the test organism *D. rerio* (Fig. 1.21) has been shown to be a suitable test species for early life stage toxicity tests due to several reasons, *e.g.*, its small size, its easy mantenance in different environments, the rapid and synchronous development of the eggs (Hallare *et al.*, 2005). Furthermore, the embryo test with *D. rerio* is a rapid, simple and cost-effective test. Only relatively small amounts of test substance are required, and different toxicological endpoints can be recorded in a short period of time (Nagel, 2002). Moreover, the fish egg assay with solid phase sediments simulates the real exposure of early fish life stages in the field and, consequently, represents a bioassay of highest ecological relevance.

### 1.6.4 Fractionation of selected toxic samples and effect-directed analyses

Polycyclic aromatic compounds (PACs), such as polychlorinated biphenyls (PCBs), naphtalenes (PCNs), PCDD/Fs, and PAHs represent major groups of toxicants in contaminated sediments. The identification of toxic substances in effect-directed analysis is often based on a group-specific fractionation of these PACs (Lübcke-von Varel *et al.*, 2008). In this study, the most toxic samples according to the bioassays results were fractionated with an auto-

**Fig. 1.21.** *Danio rerio*, the species utilized in this study to perform the sediment contact fish embryo toxicity assay (Photo: Erik Leist).

mated on-line multistep HPLC fractionation procedure (Lübcke-von Varel *et al.*, 2008). This procedure allows the class separation of major sediment-associated toxicants, such as PCBs, PCNs, PCDD/Fs, PAHs, hydroxy-, keto- and nitro-PAHs as well as sulphur, oxygen and nitrogen heterocycles. This separation is performed in one run combining three automatically switched normal-phase columns including cyanopropyl (CN), nitrophenyl (NO) and porous graphitized carbon (PGC). Exploiting the potential of each column, compounds are separated mainly according to their polarity, the number of aromatic carbons and their planarity (Lübcke-von Varel *et al.*, 2008).

The fractions obtained were tested individually in the biotests, in order to compare the toxicity of these complex mixtures to the fractions, associate the fraction-related effects. Finally, an attempt was made to identify specific groups of pollutants responsible for most of the toxicity in the samples from the Tietê River Basin.

# Bibliography


Ahlf W (2007) *Sediment Dynamics and Pollutant Mobility in Rivers - An Interdisciplinary Approach*, chap. Sediment Toxicity Data, 379–391. Springer

Al-Sabti K, Metcalfe C (1995) Fish micronuclei for assessing genotoxicity in water. *Mutation Research* 343: 121–135

Altenburger R (2002) Predicting toxic effects of contaminants in ecosystems using single species investigations, habilitationsschrift vorgelegt dem Fachbereich Biologie/Chemie der Universität Bremen

Arnaiz RR (1995) *Las toxinas Ambientales y sus Efectos Genéticos*. 2nd Edition, México

Babich H, Borenfreund E (1991a) Cytotoxicity of T-2 toxin and its metabolites determined with the Neutral red cell viability assay. *Applied and Environmental Microbiology* 57: 2101–2103

Babich H, Borenfreund E (1991b) Cytotoxicity and genotoxicity assays with cultured fish cells: a review. *Toxicology in Vitro* 5: 91–100

Babin M, Casado S, Chana A, Herradon B, Segner H, Tarazona J, Navas J (2005) Cytochrome P4501A induction caused by the imidazole derivative Prochloraz in a rainbow trout cell line. *Toxicology in Vitro* 19: 899–902

Baird DJ, Maltby L, Greig-Smith, W P, Doube PET (eds.) (1996) *ECOtoxicology: Ecological Dimensions*. Chapman and Hall, London

Balch GC, Metcalfe CD, Huestis SY (1995) Identification of potential fish carcinogens in sediment from Hamilton Harbour, Ontario, Canada. *Environmental Toxicology and Chemistry* 14: 79–91

Behrens A, Schirmer K, Bols NC, Segner H (1998) Microassay for rapid measurement of 7-ethoxyresorufin-o-deethylase activity in intact fish hepatocytes. *Marine Environmental Research* 46: 369–373

Behrens A, Schirmer K, Bols NC, Segner H (2001) Polyciclic aromatic hydrocarbons as inducers of cytochrome P4501A enzyme activity in the rainbow trout liver cell line, RTL-W1 and in primary cultures of rainbow trout hepatocytes. *Environmental Toxicology and Chemistry* 20: 632–643

Berbner T, Rahman N, Braunbeck T (1999) Induction of cytochrome P450 1A and DNA damage in isolated rainbow trout (*Oncorhynchus mykiss*) hepatocytes by

2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin. *Biomarkers* 4: 214–228

Billiard S, Bols N, Hodson P (2004) (*In vitro* and (*in vivo* comparisons of fish-specific CYP1A induction relative potency factors for selected polycyclic aromatic hydrocarbons. *Ecotoxicology and Environmental Safety* 59: 292–299

Bolognesi C, Landini E, Perrone E, Roggieri P (2004) Cytogenetic biomonitoring of a floriculturist population in Italy: micronucleus analysis by fluorescence (*in situ* hybridization (FISH) with an all-chromosome centromeric probe. *Mutation Research* 557: 109–17

Borenfreund E, Puerner JA (1985) Tocixity determined *in vitro* b morphological alterations and neutral red absorption. *Toxicological Letters* 24: 119–124

Böttcher M, Grund S, Keiter S, Kosmehl T, Manz W, Seitz N, Rocha PS, Hollert H, Braunbeck T (in press) Comparison of (*in vitro* and (*in situ* genotoxicity in the Danube River by means of the comet assay and the micronucleus test. *Mutation Research*

Brack W (2003) Effect-directed analysis: a promising tool for the identification of organic toxicants in complex mixtures? *Analitical and Bioanalitical Chemistry* 377: 397–407

Brack W, Segner H, Moeder M, Schueuerman G (2000) Fixed-effect-level toxicity equivalents - a suitable parameter for assessing ethoxyresorufin-*O*-deethylase induction potency in complex environmetal samples. *Environmental Toxicology and Chemistry* 19: 2493–2501

Brack W, Bakker J, de Deckere E, Deerenberg C, van Gils J, Hein M, Jurajda P, Kooijiman B, Lamoree M, Lek S, Alda ML, Marcomini A, Munoz I, Rattei S, Segner H, Thomas K, von der Ohe PC, Westrich B, de Zwart D, Schmitt-Jansen M (2005a) Models for assessing and forecasting the impact of environmental key pollutants on freshwater and marine ecosxstems and biodiversity. *Environmental Science and Pollution Research* 12: 252–256

Brack W, Schirmer K, Erdinger L, Hollert H (2005b) Effect-directed analysis of mutagens and ethoxyresorufin-*O*-deethylase inducers in aquatic sediments. *Environmental Toxicology and Chemistry* 24: 2445–2458

Brack W, Blaha JP L ad Giesy, Grote M, Moeder M, Schrader S, Hecker M (2008) Polychlorinated naphthaneles and other dioxin-like compounds in Elbe River sediments. *Environmental Toxicology and Chemistry* 27: 519–528

Braunbeck T, Lammer E (2006) Fish embryo toxicity assays. *German Federal Environment Agency* 1–298

Braunbeck T, Böttcher M, Hollert H, Kosmehl T, Lammer E, Leist E, Rudolf M, Seitz N (2005) Towards an alternative for the acute fish LC (50) test in chemical assessment: the fish embryo toxicity test goes multi-species-an update. *Altex* 22: 87–102

Brunström B, Halldin K (1998) EROD induction by environmental contaminants in avian embryo livers. *Comparative Biochemistry and Physiology* 121: 213–219

Bucheli TD, Fent K (1995) Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical reviews in environmental science and technology* 25: 201–268

Burton GA (1991) Assessing the toxicity of freshwater sediments. *Environmental Toxicology and Chemistry* 10: 1585–1627

Burton GA, Chapman PM, Smith EP (2002) Weight-of-evidence approaches for assessing ecosystem impairment. *Human and Ecological Risk Assessment* 8: 1657–1673

Buschini A, Carboni P, Martino A, Poli P, Rossi C (2003) Effects of temperature on baseline and genotoxicant-induced DNA damage in haemocytes of Dreissena polymorpha. *Mutation Research* 537: 81–92

Cairns J, McCormick PV, Niederlehner BR (1993) A proposed framework for developing indicators of ecosystem health. *Hydrobiologia* 263: 1–44

Calijuri MC (1999) *A comunidade fitoplanctônica em um reservatório tropical (Barra Bonita, SP)*. University of São Paulo, São Carlos, São Paulo, Brazil

Carr RS, Chapman DC, Howard CL, Biedenbach JM (1996) Sediment quality triad assessment survey of the Galveston Bay, Texas system. *Ecotoxicology* 5: 341–364

Castaño A, Cantarino MJ, Castillo P, Tarazona JV (1996) Correlations between the RTG-2 cytotoxicity test EC50 and *in vivo* LC50 rainbow trout bioassay. *Chemosphere* 32: 2141–2157

Castaño A, Segner H, Braunbeck T, Dierickx P, Halder M, Isomaa B, Kawahara K, Lee LEJ, Mothersill C, Part P, Repetto G, Riego J, Rufli H, Smith R, Wood C (2003) The use of fish cells in ecotoxicology. *ECVAM Workshop Report ATLA* 31: 317–351

CETESB (1997) Relatório da Qualidade das Águas Interiores do Estado de São Paulo - 1996. Tech. rep., CETESB

Chandroo KP, Duncan IJH, Moccia RD (2004) Can fish suffer?: perspectives on sentience, pain, fear and stress. *Applied Animal Behaviour Science* 86: 225–250

Chapman P, McDonald B, Lawrence G (2002) Weight-of-evidence issues and frameworks for sediment quality (and other) assessments. *Human and Ecological Risk Assessment* 8: 1489–1515

Chapman PM (1989) Current Approaches to Developing Sediment Quality Criteria. *Environmental Toxicology and Chemistry* 8: 589–599

Chapman PM (1990) The Sediment Quality Triad approach to determining pollution-induced degradation. *Science of the Total Environment* 97: 815–825

Chapman PM (1995) Ecotoxicology and Pollution - Key Issues. *Marine Pollution Bulletin* 31: 167–177

Chapman PM (2000) The Sediment Quality Triad: then, now and tomorrow. *International Journal of Environmental Pollution* 13: 351–356

Chapman PM (2002) Integrating toxicology and ecology: putting the ”eco” into

ecotoxicology. *Marine Pollution Bulletin* 44: 7–15

Chapman PM, Hollert H (2006) Should the sediment quality triad become a tetrad, a pentad, or possibly even a hexad? *Journal of Soils and Sediments* 6: 4–8

Chapman PM, Power EA, Burton Jr GA (1992) *Integrative Assessments in Aquatic Ecosystems*. Boca Raton, USA

Chen G, White PA (2004) The mutagenic hazards of aquatic sediments: a review. *Mutation Research* 567: 151–225

Choueri RB, Cesar A, Torres RJ, Abessa DMS, Morais RD, Pereira CDS, Nascimento MRL, Mozeto AA, Riba I, DelValls TA (2009) Integrated sediment quality assessment in Paranaguá Estuarine System, Southern Brazil. *Ecotoxicology and Environmental Safety* 72: 1824–1831

Claxton LD, Matthews PP, Warren SH (2004) The genotoxicity of ambient outdoor air, a review: Salmonella mutagenicity. *Mutation Research* 567: 347–399

Clement B, Devaux A, Perrodin Y, Danjean M, Ghidini-Fatus M (2004) Assessment of sediment ecotoxicity and genotoxicity in freshwater laboratory microcosms. *Ecotoxicology* 13: 323–333

Conolly JP, Petersen CJ (1988) A thermodynamic-based evaluation of organic chemical accumulation in aquatic organisms. *Environmental Science and Technology* 22: 99–103

Conselho Estadual de Recursos Hídricos (1990) Plano estadual de recursos hídricos. Primeiro plano do Estado de São Paulo: síntese. Tech. rep., DAEE, São Paulo

Cotelle S, Ferard JF (1999) Comet assay in genetic ecotoxicology: a review. *Environmental and Molecular Mutagenesis* 34: 246–255

Engwall M, Hjelm K (2000) Uptake of dioxin-like compounds from sewage sludge into various plant species - assessment of levels using a sensitive bioassay. *Chemosphere* 40: 1189–1195

Engwall M, Brunström B, Näf C, Hjelm K (1999) Levels of dioxin-like compounds in sewage sludge determined with a bioassay based on EROD induction in chicken embryo liver cultures. *Chemosphere* 38: 2327–2343

Fent K (1996) Ecotoxicology of organotin compounds. *Critical Reviews i Toxicology* 26: 1–117

Fent K (1998) *Oekotoxikologie*. Thieme, Stuttgart

Fent K (2001) Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. *Toxicology in Vitro* 15: 477–488

Fent K (2003) Ecotoxicological problems associated with contaminated sites. *Toxicology Letters* 140–141: 353–365

Fent K (2004) Ecotoxicological effects at contaminated sites. *Toxicology* 205: 223–240

Förstner U, Owens PN (2007) *Sediment Dynamics and Pollutant Mobility in Rivers*

- *An Interdisciplinary Approach*, chap. Sediment Quantity and Quality Issues in River Basins, 1–15. Springer

Gagne F, Pardos M, Blaise C, Turcotte P, Quemerais B, A F (1999) Toxicity evaluation of organic sediment extracts resolved by size exclusion chromatography using rainbow trout hepatocytes. *Chemosphere* 39: 1545–1570

Grisolia C, Cordeiro C (2000) Variability in micronucleus induction with different mutagens applied to several species of fish. *Genetics and Molecular Biology* 23: 235–239

Hallare AV, Kosmehl T, Schulze T, Hollert H, Koehler HR, Triebskorn R (2005) Assessing the severity of sediment contamination in Laguna Lake, Philippines using a sediment contact assay with zebrafish (*Danio rerio*) embryos. *Science of the Total Environment* 347: 254–271

Hayashi M, Morita T, Kodama Y, Sofuni T, Ishidate MJ (1990) The Micronucleus Assay with Mouse Peripheral Blood Reticulocytes using Acridine Orange-Coated Slides. *Mutation Research* 245: 245–249

Heddle JA (1973) A Rapid *in vivo* Test for Chromossomal Damage. *Mutation Research* 18: 307–317

Heise S, Förstner U, Westrich B, Jancke T, Karnahl J, Salomons W (2004) *Inventory of historical contaminated sediment in Rhine Basin and its tributaries. On behalf of the Port of Rotterdam*. Report on Behalf of the Port of Rotterdam

Hermann RM, Braga Jr BPF (1997) Case Study VI - The Upper Tietê Basin, Brazil. In: Helmer R, Hespanhol I (eds.) *Water pollution control: a guide to the use of water quality management principles*, E & FN Spon

Hilscherova K, Kannan K, Kang YS, Holoubeck I, Machala M, Masunaga S, Nakanishi J, Giesy JP (2001) Characterization of Dioxin-like activity of sediments from a Czech river basin. *Environmental Toxicology and Chemistry* 20: 2768–2777

Hilscherova K, Kannan K, Holoubeck I, Giesy JP (2002) Characterization of estrogenic activity of riverine sediments from the Czech Republic. *Archives of Environmental Contamination and Toxicology* 43: 175–185

Hilscherova K, Kannan K, Nakata H, Hanari N, Yamashita N, Bradley PW, McCabe JM, Taylor AB, Giesy JP (2003) Polychlorinated dibenzo-p-dioxin and dibenzofuran concentration profiles in sediments and flood-plain soils of the Tittabawassee River, Michigan. *Environmental Science and Technology* 37: 468–474

Ho K, Quinn J (1993) Bioassay-directed fractionation of organic contaminants in an estuarine sediment using the new mutagenic bioassay, Mutatox. *Environmental Toxicology and Chemistry* 12: 823–830

Hollert H, Dürr M, Erdinger L, Braunbeck T (2000) Cytotoxicity of settling particulate matter (SPM) and sediments of the Neckar river (Germany) during a winter flood. *Environmental Toxicology and Chemistry* 19: 528–534

Hollert H, Keiter S, König N, Rudolf M, Ulrich M, Braunbeck T (2003) A New

Sediment Contact Assay to Assess Particle-bound Pollutants Using Zebrafish (*Danio rerio*) Embryos. *Journal of Soils and Sediments* 3: 197–207

Hollert H, Dürr M, Holtey-Weber R, Islinger M, Brack W, Färber H, Erdinger L, Braunbeck T (2005) Endocrine disruption of water and sediment extracts in a non-radioactive dot blot/RNAse protection-assay using isolated hepatocytes of rainbow trout - How explain deficiencies between bioanalytical effectiveness and chemically determined concentrations? *Environmental Science and Pollution Research* 12: 347 – 360

Hollert M Hand Duerr, Olsman H, Halldin K, Bavel Bv, Brack W, Tysklind M, Engwall M, Braunbeck T (2002) Biological and chemical determination of dioxin-like compounds in sediments by means of a sediment triad approach in the catchment area of the Neckar River. *Ecotoxicology* 11: 323–336

Janosek J, Hilscherova K, Blaha L, Holoubek I (2006) Environmental xenobiotics and nuclear receptors—Interactions, effects and *in vitro* assessment. *Toxicology in Vitro* 20: 18–37

Kammann U, Biselli S, Hühnerfuss H, Reineke N, Theobald N, Vobach M, Wosniok W (2004) Genotoxic and teratogenic potential of marine sediment extracts investigated with comet assay and zebrafish test. *Environmental Pollution* 132: 279–287

Kammann U, Biselli S, Reineke N, Wosniok W, Danischewski D, Hühnerfuss H, Kinder A, Sierts-Herrmann A, Theobald N, Vahl H, *et al.* (2005a) Bioassay-directed Fractionation of Organic Extracts of Marine Surface Sediments from the North and Baltic Sea-Part II: Results of the biotest battery and development of a biotest index. *Journal of Soils and Sediments* 5: 225–232

Kammann U, Lang T, Vobach M, Wosniok W (2005b) Ethoxyresorufin-*O*-deethylase (EROD) Activity in Dab (*Limanda limanda*) as Biomarker for Marine Monitoring (6 pp). *Environmental Science and Pollution Research* 12: 140–145

Kato H, Shimada H (1975) Sister Chromatid Exchanges Induced by Mitomycin C: A New Method of Detecting DNA Damage at Chromossomal Level. *Mutation Research* 28: 459–464

Keiter S, Rastall A, Kosmehl T, Erdinger L, Braunbeck T, Hollert H (2006) Ecotoxicological Assessment of Sediment, Suspended Matter and Water Samples in the Upper Danube River. A pilot study in search for the causes for the decline of fish catches. *Environmental Science and Pollution Research* 13: 308–319

Keiter S, Grund S, van Bavel B, Hagberg J, Engwall M, U K, Klempt M, Manz W, Olsman H, Braunbeck T, Hollert H (2008) Activities and identification of aryl hydrocarbon receptor agonists in sediments from the Danube River. *Analytical Bioanalytical Chemistry*

Keiter S, Braunbeck T, Heise S, Pudenz S, Manz W, Hollert H (2009) A fuzzy logic-classification of sediments based on data from *in vitro* biotests. *Journal of Soils and Sediments* 9: 168–179

Kinder A, Sierts-Herrmann A, Biselli S, Heinzel N, Huhnerfuss H, Kammann U, Reineke N, Theobald N, Steinhart H (2007) Expression of heat shock protein 70 in a permanent cell line (EPC) exposed to sediment extracts from the North Sea and the Baltic Sea. *Marine Environmental Research* 63: 506–515

Kosmehl T (2007) *Molecular biomarkes in zebrafish embryos: Towards a more realistic approach in sediment assessment*. Ph.D. thesis, University of Heidelberg, Heidelberg, Germany

Kosmehl T, Krebs W Fand Manz, Erdinger L, Braunbeck H Tand Hollert (2004) Comparative genotoxicity testing of Rhine river sediment extracts using the permanent cell lines RTG-2 and RTL-W1 in the comet assay and Ames assay. *Journal of Soils and Sediments* 4: 84–94

Kosmehl T, Hallare A, Reifferscheid G, Manz W, Braunbeck T, Hollert H (2006) A novel contact A novel contact assay for testing genotoxicity of chemicals and whole sediments in zebrafish embryos. *Environmental Toxicology and Chemistry* 25: 2097–2106

Lammer E, Carr GJ, Wendler K, Rawlings JM, Belanger SE, Braunbeck T (2009) Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? *Comparative Biochemistry and Physiology* 149: 196–209

Lee LE, Clemons JH, Bechtel DG, Caldwell SJ, Han KB, Pasitschniak-Arts M, Mosser D, Bols NC (1993) Development and characterization of a rainbow trout liver cell line expressing cytochrome P450-dependent monooxygenase activity. *Cellular Biology and Toxicology* 9: 279–294

Lee RF, Steinert S (2003) Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. *Mutation Research* 544: 43–64

Lemos CT, Roedel PM, Terra NR, Oliveira NCD, Erdtmann B (2007) River water genotoxicity evaluation using micronucleus assay in fish erythrocytes. *Ecotoxicology and Enviromental Safety* 66: 391–401

Long ER, Chapman PM (1985) A sediment quality triad: Measures of sediment contamination, toxicity and infaunal community composition in Puget Sound. *Marine Pollution Bulletin* 16: 405–415

Lowell R, Culp J, Dubé M (2000) A weight-of-evidence approach for northern river risk assessment: Integrating the effects of multiple stressors. *Environmental Toxicology and Chemistry* 19: 1182–1190

Marciano FT (2005) *Composição, abundância e aspectos reprodutivos das espécies de peixes do reservatório Álvaro de Souza Lima (Bariri, SP) e sua relação com as características ambientais do sistema*. Ph.D. thesis, Universidade de São Paulo, São Carlos, São Paulo, Brazil

Matsumoto ST (2004) *Estudos sobre a influência de efluentes potencialmente genotóxicos, derivados de curtume, na contaminação de recursos hídricos da*

*região de Franca/SP*. Ph.D. thesis, Universidade Estadual Paulista, Franca, São Paulo, Brasil

Mdegela R, Myburgh J, Correia D, Braathen M, Ejobi F, Botha C, Sandvik M, Skaare J (2006) Evaluation of the Gill Filament-Based EROD Assay in African Sharptooth Catfish (*Clarias gariepinus*) as a Monitoring Tool for Waterborne PAH-Type Contaminants. *Ecotoxicology* 15: 51–59

Metcalfe CD, Balch GC, Cairns VW, Fitzsimons JD, Dunn BP (1990) Carcinogenic and genotoxic activity of extracts from contaminated sediments in western Lake Ontario. *Science of the Total Environment* 125–141

Mídio AF, Martins DI (2000) *Toxicologia de alimentos*. Varela, São Paulo, Brasil

Minissi S, Ciccotti E, Rizzoni M (1996) Micronucleus test in erythrocytes of Barbus plebejus (Teleostei, Pisces) from two natural environments: a bioassay for the *in situ* detection of mutagens in freshwater. *Mutation Research* 367: 245–251

Mwase M, Viktor T, Norrgren L (1998) Effects on tropical fish of soil sediments from Kafue River, Zambia. *Bulletin of Environmental Contamination and Toxicology* 61: 96–101

Nagel R (2002) DarT: The embryo test with the Zebrafish *Danio rerio* - a general model in ecotoxicology and toxicology. *Altex* 19: 38–48

Ohe T, Watanabe T, Wakabayashi K (2004) Mutagens in surface waters: a review. *Mutation Research* 567: 109–149

Van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology* 13: 57–149

Otte J, Andersson C, Abrahamson A, Olsman H, Keiter S, Engwall M, Hollert H, Brunstroem B (2008) A bioassay approach to determine the dioxin-like activity in sediment extracts from the Danube River: Ethoxyresorufin-*O*-deethylase induction in gill filaments and liver of three-spined sticklebacks (*Gasterosteus aculeatus* L.). *Environment International* 34: 1176–1184

Pacheco M, Santos MA (1998) Induction of Liver EROD and Erythrocytic Nuclear Abnormalities by Cyclophosphamide and PAHs in *Anguilla anguilla* L. *Ecotoxicology and Environmental Safety* 40: 71–76

Palhares D, Grisolia CK (2002) Comparison between the micronucleus frequencies of kidney and gill erythrocytes in tilapia fish, following mitomycin C treatment. *Genetics and Molecular Biology* 25: 281–284

Pilot H, Dragan Y (1996) *Chemical Carcinogenesis*, 201–267. McGraw Hill, New York, 5 edn.

Power EA, Chapman PM (1992) *Sediment toxicity assessment*, chap. Assessing sediment quality, 1–18. Lewis-Publishers, Boca Raton

Pratt PF, Suarez DL (1990) Irrigation water quality assessment. *American Society of Civil Engineers, Manual and Reports on Engineering Practice* 220–236

Repetto G, del Peso A, Jos A (2003) *In vitro methods in Aquatic Toxicology*, chap. Ecotoxicological characterization of complex mixtures, 295–326. Praxis Publishing Ltd

Rodgher S, Espíndola ELG, Rocha O, Fracácio R, Pereira RHG, Rpdrigues MHS (2005) Limnological and ecotoxicological studies in the cascade of reservoirs in the Tietê River (São Paulo, Brazil). *Brazilian Journal of Biology* 65: 697–710

Sanchez-Fortun S, Llorente MT, Castaño A (2008) Cytotoxic and genotoxic effect in RTG-2 cell line exposed to selected biocides used in the disinfection of cooling towers. *Ecotoxicology* 17: 273–279

Schirmer K, Dayeh V, Bopp S, Russold S, Bols N (2004) Applying whole water samples to cell bioassays for detecting dioxin-like compounds at contaminated sites. *Toxicology* 205: 211–221

Schmid W (1975) The Micronucleus Test. *Mutation Research* 31: 9–15

Schulz R (2001) Comparison of spray drift-and runoff-related input of azinphos-methyl and endosulfan from fruit orchards into the Lourens River, South Africa. *Chemosphere* 45: 543–551

Segner H, Behrens A, Joyce E, Schirmer K, Bols N (2000) Transient induction of 7-ethoxyresorufin-O-deethylase (EROD) activity by medium change in the rainbow trout liver cell line, RTL-W1. *Marine Environmental Research* 50: 489–493

Seiler TB, Rastall AC, Leist E, Erdinger L, Braunbeck T, Hollert H (2006) Membrane dialysis extraction (MDE): A novel approach for extracting toxicologically relevant hydrophobic organic compounds from soils and sediments for assessment in biotests. *Journal of Soils and Sediments* 6: 20–29

Seitz N, Böttcher M, Keiter S, Kosmehl T, Manz W, Hollert H, Braunbeck T (2008) Evaluation of the genotoxic potential of sediment samples from the Danube River using a new expert system and several analyzing methods: How to integrate information from dose-response curves. *Mutation Research* 652: 38–45

Singh N, McCoy M, Tice R, Schneider E (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research* 175: 184–191

SMA-SP (2002) Secretaria do Meio Ambiente do Estado de São Paulo

Soares A, Mozeto AA (2006) Water Quality in the Tietê River Reservoirs (Billings, Barra Bonita, Bariri and Promissão, SP - Brasil) and Nutrient Fluxes across the Sediment-Water Interface (Barra Bonita). *Acta Limnologica Brasiliensia* 18: 247–266

Spacie A, Hamelink JL (1985) *Fundamentals of Aquatic Toxicology*, chap. Bioacummulation. Hemisphere, Washington DC

Strmac M, Braunbeck T (2000) Isolated hepatocytes of rainbow trout (*Oncorhynchus mykiss*) as a tool to discriminate between differently contaminated small river systems. *Toxicology in Vitro* 14: 361–377

Sumpter JP, Johnson AC (2005) Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. *Environmental Science and Technology* 39: 4321–4332

Thomas K, Balaam J, Barnard N, Dyer R, Jones C, Lavender J, McHugh M (2002) Characterisation of potentially genotoxic compounds in sediments collected from United Kingdom estuaries. *Chemosphere* 49: 247–258

Ulupinar, Okumus (2002) Detection of Mutagenic-Carcinogenic Pollutans in Aquatic Systems Using Cytogenetic Methods in Fish. *Turkish Journal of Zoology* 26: 141–148

Varanasi U (1989) *Metabolism of Polycyclic Aromatic Hydrocarbons in the aquatic environment*. CRC, USA

Varanasi U, Reichert WL, Stein JE, Brown DW, Sanborn HR (1985) Bioavailability and biotransformation of aromatic hydrocarbons in benthic organisms exposed to sediment from an urban estuary. *Environmental Science and Technology* 19: 836–841

Lübcke-von Varel U, Streck G, Brack W (2008) Automated fractionation procedure for polycyclic aromatic compounds in sediment extracts on three coupled normal-phase high-performance liquid chromatography columns. *Journal of Chromatography A* 1185: 31–42

Vasseur P, Cossu-Leguile C (2003) Biomarkers and community indices as complementary tools for environmental safety. *Environment International* 28: 711–717

Viganò L, Arillo A, Buffagni A, Camusso M, Ciannarella R, Crosa G, Falugi C, Galassi S, Guzzella L, Lopez A, *et al.* (2003) Quality assessment of bed sediments of the Po River (Italy). *Water research* 37: 501–518

Wang F, Goulet RR, Chapman PM (2004) Testing sediment biological effects with freshwater amphipod *Hyalella azteca*: the gap between laboratory and nature. *Chemosphere* 57: 1713–1724

White PA, Claxton LD (2004) Mutagens in contaminated soil: a review. *Mutation Research* 567: 227–345

Woelz J, Engwall M, Maletz S, Olsman Takner H, van Bavel B, Kammann U, Klempt M, Weber R, Braunbeck T, Hollert H (2008) Changes in toxicity and Ah receptor agonist activity of suspended particulate matter during flood events at the rivers Neckar and Rhine - a mass balance approach using *in vitro* methods and chemical analysis. *Environmental Science and Pollution Research* 15: 536–553

Woelz J, Borck D, Witt G, Hollert H (2009) Ecotoxicological characterization of sediment cores from the western Baltic Sea (Mecklenburg Bight) using GC-MS and *in vitro* biotests. *Journal of Soils and Sediments* DOI 10.1007/s11368-009-0094-9

Wong HL, Giesy JP, Siu WHL, Lam PKS (2005) Estrogenic and Dioxin-like Activities and Cytotoxicity of Sediments and Biota from Hong Kong Mudflats.

*Archives of Environmental Contamination and Toxicology* 48: 575–586

Zurita JL, Jos A, Camean A, Salguero M, Lopez-Artiguez M, Repetto G (2007) Ecotoxicological evaluation of sodium fluoroacetate on aquatic organisms and investigation of the effects on two fish cell lines. *Chemosphere* 67: 1–13

# Chapter 2

# Sediment genotoxicity in the Tietê River (São Paulo, Brazil): *in vitro* comet assay versus *in situ* micronucleus assay studies

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## Abstract

The *in vitro* comet assay with the permanent fish cell line RTL-W1 and the *in situ* micronucleus assay using erythrocytes from indigenous tilapia (*Oreochromis niloticus*) were used to detect genotoxicity in Tietê River sediments (São Paulo, Brazil). Either test was successful in identifying site-specific differences in genotoxicity, with a high correlation between *in situ* and *in vitro* results indicating the relevance of the latter even for environmental studies. Discharges from São Paulo city have major impact on genotoxic effects by sediment-bound contaminants; however, overall genotoxicity decreases downstream. The high genotoxic burden of the Tietê River warrants measures to reduce the input of toxic effluents.

*Keywords*: comet assay, fish erythrocytes, genotoxicity, micronucleus assay, sediments, RTL-W1 cell line, Tietê River, Brazil, effluent

## 2.1 Introduction

Reservoirs are complex aquatic systems mediating between rivers and lakes; they usually reflect multiple impacts generated by a variety of anthropogenic activities (Tundisi *et al.*, 1999). The sediment compartment is the intermediate or final receptor of insoluble (or slightly water soluble) pollutants and can act as a sink for various substances. Sediments accumulate chemicals up to concentrations many times higher than free water column (Ahlf *et al.*, 2002b; Baudo & Muntau, 1990; Burton, 1991; Fracácio *et al.*, 2003; Hollert *et al.*, 2002). As pollutants may be made available under certain environmental conditions (such as dredging or flood events), sediments can also become a source of diffuse contamination to the free water space (Ahlf *et al.*, 2002a; Hollert *et al.*, 2003). Sediment pollutants are not only linked to organisms in aquatic ecosystems, but also to human health via water and fish consumption (Chen & White, 2004; Hollert *et al.*, 2005; Keiter *et al.*, 2006; Maier *et al.*, 2006).

Since fish represent vertebrates, which are top predators in the food web, considerable efforts have been undertaken to develop fish-based test systems for the assessment of sediment-bound substances (Hollert *et al.*, 2003, 2005; Kosmehl *et al.*, 2004). Due to their ability to metabolize xenobiotics and accumulate pollutants, fish represent important monitoring systems within aquatic genotoxicity assessment (Balch *et al.*, 1995; Grisolia & Cordeiro, 2000; Metcalfe *et al.*, 1990; Minissi *et al.*, 1996). Recently a number of laboratory investigations and field studies have documented a correlation between genotoxic pollutants and heritable reproduction effects on individuals as well as a potential link to the declines of fish populations (for reviews, see Chen & White 2004 as well as Keiter *et al.* 2006).

Passing through São Paulo city and São Paulo state in Brazil, the Tietê River is one of the most polluted rivers in the world due to the insufficient effluent treatment and numerous direct industrial sources for a multitude of anthropogenic pollutants. Despite this heavy pollution, little is known about the biological hazard potential of the mixture of chemicals in its waters and sediments. The Tietê River is embedded in the Tietê River Basin, the largest hydrographical basin of São Paulo state. This basin is subdivided into three sub-basins due to distinct geomorphologic characteristics: Upper, Middle and Lower Tietê (Brocanelli, 1998). The river comprises several reservoirs along its course, which are widely used for providing drinking water, as a water source for agricultural irrigation, as receptors of domestic and industrial effluents, and as recreation sites. Given the extent of the pollution and the importance of the river, research into the origin and effects of pollutants and subsequent suggestions for the ecological improvement of the river basin

quality might become an important model for further research and biological risk assessment of highly contaminated river systems not only in Brazil, but throughout the world.

In this study, two bioassays were applied to detect the genotoxic potential of the Tietê River basin: the *in vitro* version of the comet assay with a permanent cell line, and the *in situ* (*in vivo*) version of the micronucleus assay with erythrocytes from fish collected in the field. The *in vitro* approach serves as an efficient, fast and cost-effective screening for the evaluation of the potential biological activities of the complex mixtures (Hilscherova *et al.*, 2002). However, since various factors (chemical, physical and biological) affect environment conditions, the transfer of results obtained by *in vitro* techniques to the field is a complex task, and the establishment of extrapolation parameters is a crucial issue. For this end, a combination of *in vitro* and *in situ* (*in vivo*) bioassays represents a promising approach, since *in situ* bioassays are more likely to reflect the real exposure situation in the environment; applied in combination with *in vitro* bioassays, the *in situ* assays may be used to confirm or falsify the potential toxicity revealed by the *in vitro* bioassays. In fact, the combination of comet and micronucleus assays has been applied successfully to detect genotoxic and mutagenic potentials in several environmental monitoring studies (Böttcher *et al.*, in press; Bolognesi *et al.*, 2004; Buschini *et al.*, 2003).

Thus, the aims of this study were (1) to find out possible interactive genotoxic and mutagenic effects from multiple contaminants, since the Tietê River receives a highly complex load of organic pollutants from domestic sewers, industrial residues, agricultural and agroindustrial activities, as well as numerous inorganic substances of industrial sources, and (2) to elucidate, by comparison of *in vitro* and *in situ* (*in vivo*) approaches, the ecological relevance of *in vitro* results and their ability as possible bioindicator systems. In contrast, this study did not attempt to determine which contaminants were responsible for the genetic damage.

## 2.2 Materials and methods

### 2.2.1 Sediment sampling

The study area comprised a location in Salesópolis near the Tietê River's spring and the reservoirs Ponte Nova and Billings (Upper Tietê); Barra Bonita (Superior Middle Tietê); Bariri and Promissão (Inferior Middle Tietê) and Três Irmãos (Lower Tietê; Fig. 2.1). The Upper Tietê River basin corresponds to the drained area of Tietê River from its spring across the

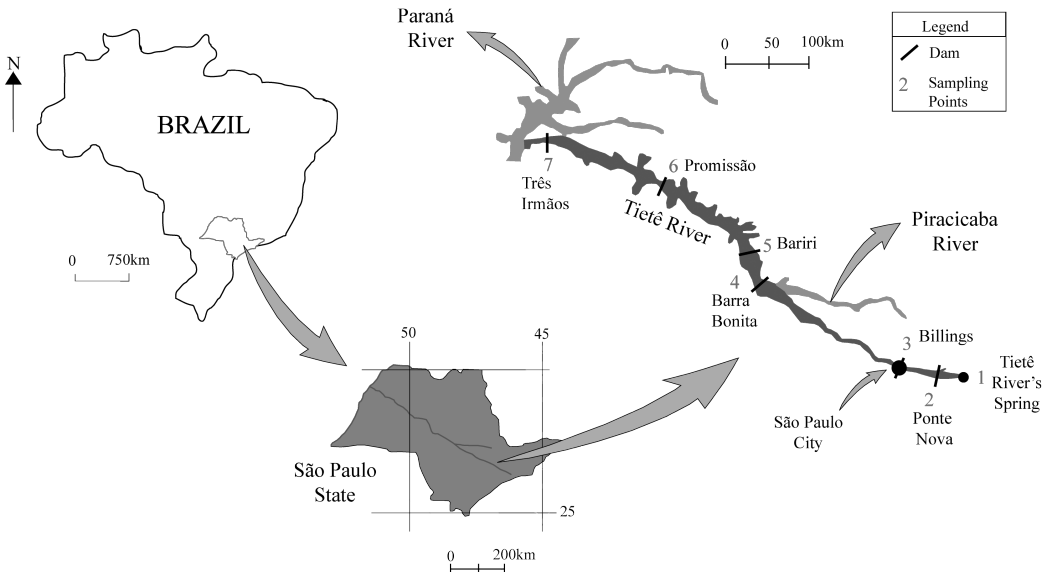


**Fig. 2.1.** Location of the sampling sites in the Tietê River basin, São Paulo, Brazil.

metropolitan area of São Paulo city (> 19,000,000 inhabitants) characterized by high densities of anthropogenic population and dramatic deterioration subsequent to intensive urbanization. The Superior Middle Tietê River basin is dominated by a high density of urban, industrial and agricultural areas. The Inferior Middle Tietê River basin is characterized predominantly by agricultural areas and the Low Tietê River basin by pastures and sugar cane culture (CETESB, 1997).

Surface sediments were collected in May and December 2005 by means of an Eckman-Birge dredge, with ten replicates at each site (with a distance of 10m from sample to sample). Replicates were homogenized, and 1.5 kg of each sediment sample were frozen immediately, stored at -10°C and transported to Germany. Transfer of the samples to Germany was permitted by the Brazilian National Department of Mineral Production (DNPM). Samples were freeze-dried, and extracts were prepared by Soxhlet extraction using acetone p.a. as solvent and re-dissolved with dimethyl sulfoxide (DMSO; Sigma-Aldrich, Deisenhofen, Germany) as described by Hollert *et al.* (2000). The resulting concentration of extracts was 20 g dry sediment-equivalent per 1 ml solvent.

### 2.2.2 Cell culture

The fibroblast-like permanent cell line RTL-W1 (Lee *et al.*, 1993) derived from rainbow trout liver (*Oncorhynchus mykiss*) was used to perform the

comet assay. According toKosmehl *et al.* (2004), RTL-W1 cells are able to detect the genotoxic potential of acetonic sediment extracts. Furthermore, RTL-W1 cells have relatively high biotransformation capacities, if compared to other fish cell lines such as RTG-2 cells Kosmehl *et al.* (2004).

The cells were maintained in 75 cm2 culture flasks (TPP, Trasadingen, Switzerland) in Leibowitz (L15) medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1 % penicillin/streptomycin solution (10,000 U/10,000 µm) in 0.9 % NaCl (Sigma-Aldrich) at 20 °C (Seiler *et al.*, 2006). They were trypsinized using 0.05 % trypsin/ 0.02 % EDTA and washed twice with PBS before being used in experiments Kosmehl *et al.* (2004).

### 2.2.3 Comet assay

The comet assay was performed under alkaline conditions following the procedure of Singh *et al.* (1988) in the modification by Schnurstein & Braunbeck (2001) as well as Kosmehl *et al.* (2004), using acetonic sediment extracts.

In order to determine the highest test concentrations to be used for *in vitro* exposure of RTL-W1 cells, the cytotoxic potential of individual extracts was determined using the neutral red assay as detailed by Babich & Borenfreund (1992) with modifications described by Klee *et al.* (2004). The highest test concentrations were defined as the extract concentrations inducing less than 20 % mortality after 48h of exposure. Cells were exposed to four sequential concentrations of each sediment extract, in serial dilutions from 1:1 to 1:8 of the highest concentrations in supplemented L15 medium (Sigma-Aldrich; Table 2.1).

Each extract was tested in 6-well plates (TTP Renner) after 24h settlement of the cells (Kosmehl *et al.*, 2004) for 48h at 21°C in three independent replicates. Supplemented L15 medium served as a negative control; exposure to UV light at 240 - 280 nm for 5 min was used as a positive control.

After the incubation, cells were rinsed with PBS, trypsinized and embedded in an agarose layer on fully frosted microscope slides (Langenbrink, Emmendingen, Germany) as detailed by Kosmehl *et al.* (2004). Immediately before scoring, the DNA was stained with 75 µl of 20 µM ethidium bromide (Sigma-Aldrich) and covered-slipped. Slides were examined using a fluorescent microscope with 340 x magnification (Axioplan, Zeiss, Germany) equipped with an excitation filter of 518 nm and an image-analysis system (Optilas, Munich, Germany), and cell images were recorded with a high sensitivity CCD camera (Pulnix TM-765E Kinetic; Germany). For each concentration, 100 cells were scored and a computerized image-analysis system (Comet Version 5.5, Kinetic Images, Liverpool, UK) was used to de-

**Table 2.1** Highest test concentrations of sediment extracts used in the comet assay, given in sediment equivalents (mg SEQ) per ml medium.

| Sampling area | (mg SEQ/ml) |
|---|---|
| Spring | 200 |
| Ponte Nova | 22 |
| Billings | 12 |
| Barra Bonita | 152.6 |
| Bariri | 31 |
| Promissão | 19.7 |
| Três Irmãos | 24.5 |

termine DNA tail moments (tail length x fluorescence intensity in the tails). Induction factors were calculated by the comparison of median values of tail moments from exposed cells to median values of tail moments from corresponding negative controls. ANOVA-on-ranks followed by a post-hoc test according to Dunn's ($p < 0.05$) was used to calculate significant statistical differences between groups. After this, maximum induction factors per mg SEQ/ml (IF/(mg SEQ/ml) were computed.

Since the final sediment concentrations (SEQ) differed between samples according to the corresponding LOECs for cytotoxicity, the maximum induction factor induced by the sample was divided by the concentration inducing this effect. Thus, maximum induction factors (IF) per mg SEQ/ml were calculated for each sample in order to allow direct comparison of genotoxic potentials of samples from different sites.

In order to take into account the concentration dependency of the sediment genotoxicity, we also applied the Concentration-dependent induction factor (CDI), developed by Seitz and co-workers (Seitz *et al.*, 2008). The CDI is a simple index that integrates all the important information, providing a basis for a general comparison of the genotoxic potential in the comet assay. The CDI integrates all concentrations and respective induction factors and is calculated according to the following equation:

$$CDI = \sum_{i=1}^{n} \frac{IFi}{ci}$$

where

IFi =induction factor of the concentration i

ci =concentration i

n = n concentrations

### 2.2.4 Fish blood sampling and micronucleus assay

The present study did not involve any animal experiments, since all experimental work was carried out *in vitro* (cell cultures). Sampling at the Tietê River was carried out in compliance with Brazilian conservation regulations. Field fish were sacrificed immediately after catch and never subjected to experimental manipulation.

Fish blood samples were collected from mature Nile tilapia (*Oreochromis niloticus*), an African fish introduced into Brazil in the 1970s. According to Ueng and Ueng (1995), different species of tilapia may serve as biological indicators for environmental pollution partially because the fish survive in highly polluted habitats. This species was selected due to its abundance in the Tietê River and the fact that it has repeatedly been used as a source of cells for the micronucleus test (Bücker & Conceição, 2004; Cavas & Ergene-Gözükara, 2005; Grisolia & Cordeiro, 2000; Palhares & Grisolia, 2002; Ventura *et al.*, 2008). Moreover, tilapia is intensively used for human consumption in Brazil.

At two sites (Spring and Três Irmãos reservoirs), no fish blood could be collected, because it was not possible to catch any *Oreochromis niloticus*. Since no fish blood could be collected from the original reference site, based on previous studies (Mozeto *et al.*, 2004), Promissão reservoir was selected as a complementary reference site in order to allow the comparison between sites.

Peripheral blood samples were obtained with heparinized syringes from the gills of ten fish per site. The blood was immediately smeared onto microscope slides previously cleaned with 99% ethanol. After drying, samples were fixed in methanol for at least 1 minute and subsequently stained with pure Giemsa. For conservation, slides were covered with cover slips by DEPEX (Serva, Heidelberg, Germany).

Two thousand erythrocytes were examined per fish on coded slides (Fig. 2.2). Micronuclei were recorded using a light microscope (Axioplan, Zeiss) equipped with an oil-immersion lens at 1200x magnification. For the identification of micronuclei, the following scoring criteria were used: a) cells with oval appearance and intact cytoplasm, b) oval nuclei with intact nuclear membrane, c) micronuclei less then or equal to one-third the size of the main nuclei, d) micronuclei clearly separated from the main nuclei (Huber *et al.*, 1983; Titenko-Holland *et al.*, 1998).

Results were recorded as percentage of cells containing micronuclei compared to the total number of cells counted. Statistical significances were assessed by using the Chi-square-test with Yates´ correction (ISO/DIS-21427-2, Lovell et al. 1989) using SigmaStat 3.5 (SPSS-Jandel Scientific; Erkrath, FRG).

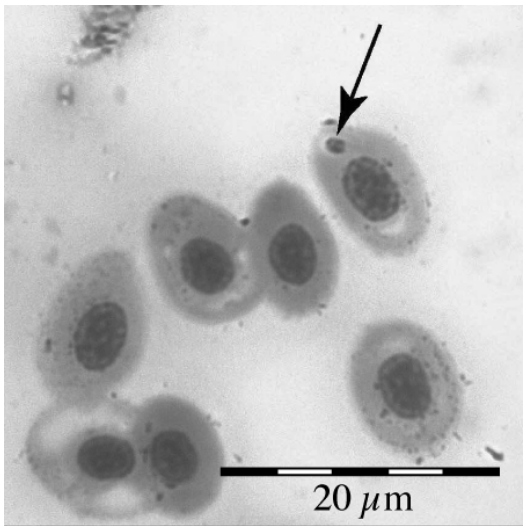


**Fig. 2.2.** Erythrocytes from *Oreochromis niloticus* collected in Billings reservoir, stained with Giemsa. Note micronucleus at arrow.

## 2.3 Results

### 2.3.1 Comet assay

For most sampling locations, the comet assay with RTL-W1 cells documented genotoxic effects with a positive dose-response relationship (Fig. 2.3, showing only data from two sites, near spring and Billings reservoir). Strong genotoxicity was detected in sediments from Billings reservoir near to São Paulo city from concentrations of 1.5 mg SEQ/ml, whereas the reference site near the spring had significant effects only at concentrations $\geq$ 100 mg SEQ/ml. Ponte Nova samples showed significant effects at concentrations of $\geq$ 11mg SEQ/ml. In sediments from Barra Bonita, Promissão and Três Irmãos reservoirs, significant effects were detected in one replicate only, at concentrations $\geq$ 38.2, 2.46 and 24mg SEQ/ml, respectively. Samples from the Bariri reservoir showed significant effects at concentrations of 7.8mg SEQ/ml; however, no effects were seen at any higher concentration.

#### Maximum induction factors per mg SEQ/ml

For most of the samples, the highest genotoxicity was recorded for the 1:4 dilution of the highest test concentration. Therefore, maximum induction factors per mg SEQ/ml were calculated according to these concentrations. The analysis of the induction factors revealed an increase in the genotoxic potential from the spring to Billings reservoir followed by an abrupt decrease

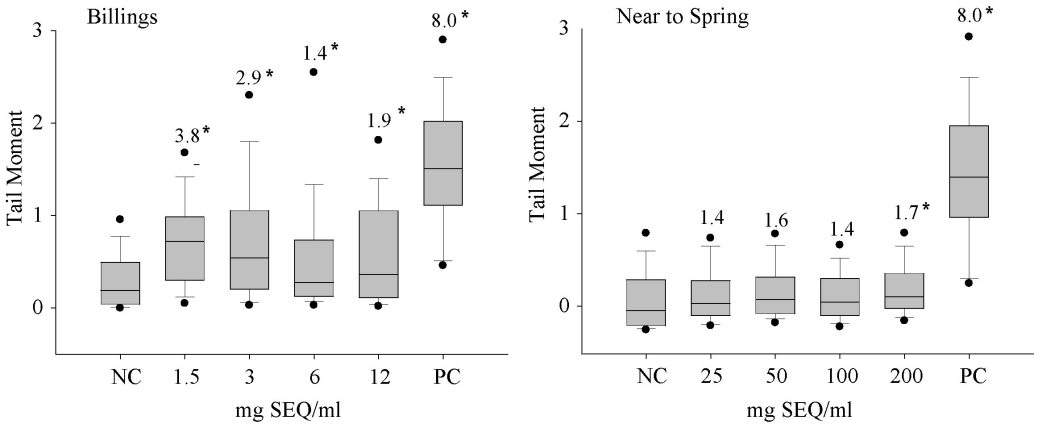


**Fig. 2.3.** Genotoxic effects of acetonic sediment extracts from Billings reservoir (left) and near to the Tietê River spring (right), respectively, in the comet assay using RTL-W1 cells. Each box plot presents the tail moments of four different concentrations of extracts given in sediment equivalent (mg SEQ)/ml medium, as well as negative (NC) and positive controls (PC). Additionally, induction factors are indicated above each box plot. Significant genotoxic effects (post-hoc test according to Dunn; $p < 0.05$) are indicated by asterisks.

in Barra Bonita reservoir. Extracts from Barra Bonita showed the lowest genotoxic effects, comparable to those of the reference site (near spring). The locations downstream Barra Bonita showed an increase in their genotoxic potential again, indicating additional sources of genotoxic acting substances. From Promissão to Três Irmãos reservoir, a minor decrease in the genotoxic potential was recorded (Fig. 2.4).

### 2.3.2 Concentration-dependant induction factor (CDI)

Fig. 2.5 gives a survey on the genotoxicity of the results presented as CDI factors of all locations based on three independent replicates. The CDI confirms that samples from near Tietê River's spring and Barra Bonita reservoir showed very low genotoxic effects (CDI values 0.14 and 0.17, respectively), followed by Três Irmãos and Bariri reservoirs (CDI values 0.64 and 0.72, respectively) and Ponte Nova e Promissão reservoirs (CDI values 1.10 and 1.33, respectively). The genotoxic potential of sediments from Billings reservoir samples becomes even more prominent than if expressed as maximum induction factors (CDI values 4.32, Fig. 2.5). Overall, however, values for CDI and IF closely paralleled each other.

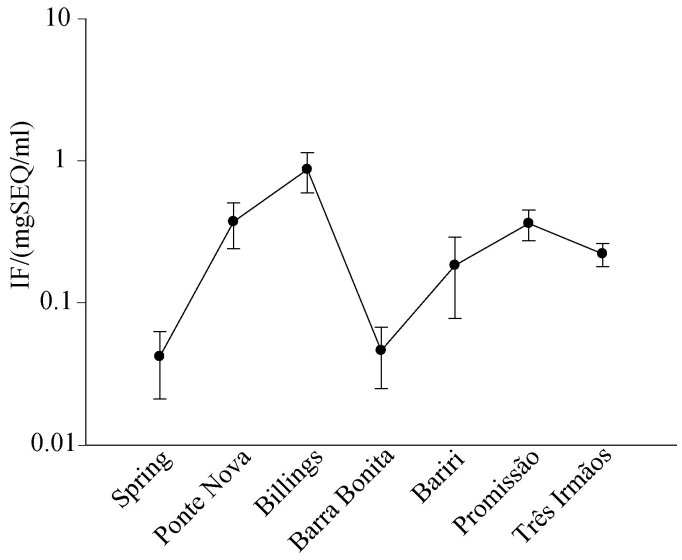


**Fig. 2.4.** Genotoxicity of sediment samples collected at different locations along the Tietê River in the comet assay, given as maximum induction factors in mg SEQ/ml (amounts of extract given in sediment equivalents, SEQs) for the 1:4 dilutions of the highest test concentrations. Highest IF/(mg SEQ/ml) could be observed in cells exposed to Billings reservoir samples, and a decrease of effects was evident in downstream direction. Data are given as means ± SD from three independent experiments.

### 2.3.3 Micronucleus assay

In order to test the mutagenic potentials *in situ* (*in vivo*), the micronucleus test was applied to erythrocytes from Nile tilapia caught in the Tietê River (Fig. 2.6). For statistical analysis (Lemos *et al.*, 2007), 2000 erythrocytes for each fish per location (n = 4 - 9) were analyzed. Due to the influence of the megacity of São Paulo, fish collected from Billings reservoir revealed by far the highest micronucleus frequencies with a median of 6.0 ‰. Clear-cut decreases in micronucleus frequencies were observed downstream from São Paulo. Bariri and Promissão reservoirs had the lowest micronucleus frequencies, with medians of 1.5 and 0.5 respectively.

The micronucleus frequencies significantly differed between individuals caught in the same area: although Ponte Nova and Barra Bonita reservoirs both showed median micronucleus frequencies of 3.5, blood samples from individuals collected at Ponte Nova varied in micronucleus frequency between 0 and 13, whereas fish from Barra Bonita only showed a range between 5 and 9.

According to the Chi-square test, significant differences to Promissão, as a reference location with low contamination, could be observed for Ponte Nova,

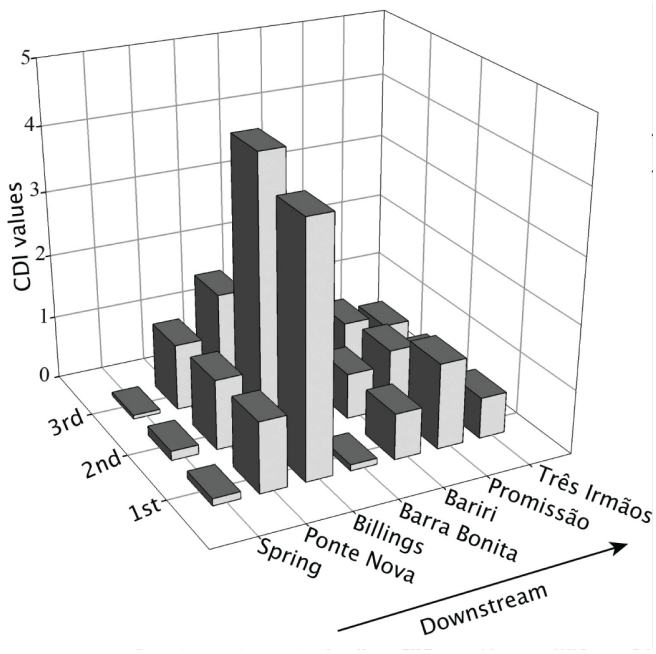


**Fig. 2.5.** Concentration-dependant induction factors (CDI; Seitz *et al.*, 2008) of sediment samples collected from the Tietê River basin. Data are given as means ± SD from three independent determinations. If compared to the maximum induction factors (Fig. 2.4), the differences in genotoxicity between Billings reservoir and the other sampling sites becomes even more prominent.

Billings and Barra Bonita ($p < 0.001$: Fig. 2.6). No significant difference in micronucleus formation was found between Bariri and Promissão.

## 2.4 Discussion

The present study is the first comprehensive investigation into the genotoxic potential of sediments in the catchment area of the Tietê River basin.

In this study, the comet assay results (given as tail moment) documented a strong increase in genotoxicity from Tietê River's spring to Billings (São Paulo city region), and a decrease further downstream. These results were confirmed by the calculation of the IF/(mg SEQ/ml), where only the concentration inducing highest effect was considered and the CDI (taking into account all sediment dilutions) and indicated a strong influence of São Paulo city on the overall genotoxicity of the studied areas.

To appreciate the relative genotoxic potential of Billings and the other reservoirs, however, a comparison to well-known genotoxic water bodies may help: Seitz *et al.* (2008) studied the genotoxic potentials of various locations

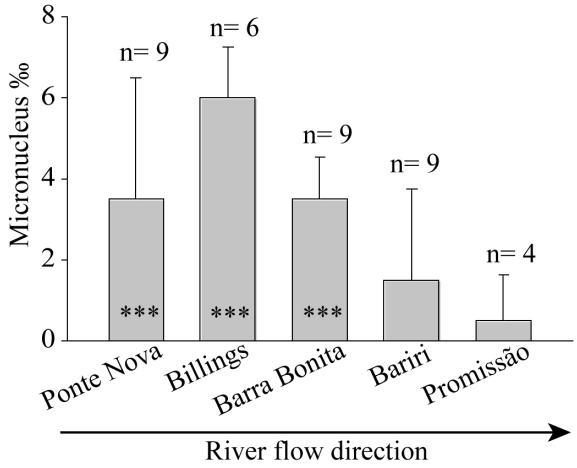


**Fig. 2.6.** Frequency of micronucleated cells (MN) scored from erythrocytes from fish at different sites along the Tietê River. Data are given as median ± SD from 2000 erythrocytes per individual/location. n=number of individuals investigated at each location. *** = significantly different from the control group (Promissão; Chi-square test with Yates´ modification, $p < 0.001$).

along the upper Danube River (Germany) by means of the comet assay, also exposing RTL-W1 cell to sediment extracts. They recorded the highest CDI value of 11.4 from Rottenacker sediment extracts, which is extremely high when compared to the highest value of CDI in the present study (sediment extract from Billings reservoir, with CDI values up to 4.32). However, sediment extracts from the localities Schwarzach and Ehingen, showed values comparable to those from Billings (CDI $\geq 3$). The genotoxic potentials of sediments from Ponte Nova and Promissão are comparable to those from Bad Abbach and Sigmaringen (CDI values $\leq 1.73$), and from Bariri and Três Irmãos are comparable to the Lauchert (CDI 0.66). CDI values from near Tietê River spring and Barra Bonita extracts (CDI 0.17) are considerably lower than all values recorded for upper Danube River sediments. In a fuzzy logic-based classification of sediments from the same locations in upper Danube River, based on data from several *in vitro* biotests, Keiter *et al.* (2009) classified sediments from Schwarzach and Ehingen as strongly toxic, those from Bad Abbach and Sigmaringen as moderately toxic and the samples from the Lauchert as non-toxic. A direct comparison of the results obtained in the present study to results obtained by Keiter *et al.* (2009) is not possible, since their conclusions were based on effects in several *in vitro* biotests. However, taking both studies into account (Seitz *et al.*, 2008; Keiter *et al.*, 2009), it is possible to rate sediments from Billings reservoir as strongly genotoxic,

those from Ponte Nova and Promissão as moderately genotoxic and those from Bariri and Três Irmãos as less genotoxic.

The low genotoxicity by Barra Bonita extracts can be related to significant water input from more than 100 tributaries. However, it may also be related to the potential inefficiency of the sample preparation process for eutrophic sediments such as those from this area (for details, see, *e.g.*, Matsumura-Tundisi & Tundisi 2005; Rodgher *et al.* 2005; Sotero-Santos *et al.* 2006). Interactions between eutrophication and contaminants may occur through many mechanisms: eutrophication may cause dilution of contaminants by increasing the biomass, increased contaminant scavenging by dissolved organic carbon (DOC), increased sedimentation of contaminants and increased uptake in the food chain (Gunnarsson *et al.*, 1995; Taylor *et al.*, 1991; Koelmans *et al.*, 2001). Deposition and recycling of contaminants from bottom sediments may be affected by the eutrophication status of the area (Skei et al. 1996), *e.g.*, eutrophic water bodies contain a great phytoplankton biomass due to the excess of nutrients, causing persistent organic pollutants (POP) retention and greater sedimentation of these pollutants (Larsson *et al.*, 2000). Contaminants and eutrophication factors may also interact to affect bioaccumulation as well as the growth, health and reproduction of benthic organisms (Skei *et al.*, 1996).

Regarding the micronucleus assay data, all Billings fish showed an increase in micronucleus formation over specimens from all other locations. Under aquarium conditions, the micronucleus frequencies for negative controls of *O. niloticus* in previous studies ranged from 0.4 ‰ (Palhares & Grisolia, 2002; Ventura *et al.*, 2008) to 1.72 ‰ (Cavas & Ergene-Gözükara, 2005). In specimens collected in the field, micronucleus frequencies in specimens from Promissão and Bariri reservoirs were in the same range. In in vitro experiments, Cavas & Ergene-Gözükara (2005) exposed *O. niloticus* to cyclophosphamide (as a positive control) at a concentration of 4 mg/L for 3 and 9 days and recorded erythrocyte micronucleus frequencies of 4.12‰ and 5.9 ‰, respectively. Comparing these numbers to those recorded in the present study, the frequency of micronucleated cells in Billings fish (6.0 ‰) was comparable to the cyclophosphamide positive control, thus confirming the strong genotoxic potential in Billings reservoir.

During the last decades, many cases of contamination by heavy metals and organic compounds such as polychlorinated biphenyls (PCBs), organochlorine pesticides and polycyclic aromatic hydrocarbons (PAHs) have been observed in waters in and around São Paulo city, especially in the Billings reservoir (Bainy et al. 1999). In an earlier *in situ* (*in vivo*) study with *O. niloticus* from Billings reservoir, Bainy *et al.* (1996) recorded significantly higher levels of total microsomal cytochromes P450 and *b5* in livers and kid-

neys of specimens from this reservoir compared to specimens from a reference site. These results were confirmed by Bainy *et al.* (1999) and Leitao *et al.* (2000), who, studying the same fish species, observed more than a 20-fold increase in 7-ethoxyresorufine-*O*-deethylase (EROD) in liver microsomes over fish from a non-polluted reservoir. In laboratory studies into the toxicity of Tietê River reservoirs, Almeida and Rocha (2006) applied biotests with *Chironomus xanthus* and *Hyalella azteca* as test organisms and recorded higher mortalities in organisms exposed to Billings reservoir sediments than in organisms exposed to Promissão samples. Assessing the toxicity of sediment samples from Tietê River reservoirs downstream São Paulo city, through chronic-partial toxicity bioassays with *Danio rerio* larvae as test organisms, Fracácio *et al.* (2003) recorded inadequate conditions for the growth of the test organisms when exposed to the sediments of upstream reservoirs and also found an improvement of environmental conditions further down the river system. In an evaluation of the quality of water and sediment samples from the same reservoirs, Rodgher *et al.* (2005) recorded chronic toxicity for *Ceriodaphnia dubia* and acute toxicity for *D. rerio* decreasing by one order of magnitude from Barra Bonita to Três Irmãos, demonstrating an environmental degradation gradient along the reservoirs. According to their findings and in agreement with the findings of the present study, sediment quality of the reservoirs improves with increasing distance from the metropolitan area of São Paulo, indicating the megacity to be the origin of most of the pollutants. However, based on the present findings on genotoxicity, the other locations can also not be considered as being free from anthropogenic impact, since although being less genotoxic than Billings reservoir, significant genotoxic effects were recorded in the other areas.

According to Kosmehl *et al.* (2004), testing of sediments and suspended particulate matters for genotoxicity should become a standard requirement for industrial wastewater discharge permits, as are routine aquatic toxicity and wastewater tests, since aquatic organisms exposed to wastewater discharges and particle-bound substances suffer an increased risk of genetic damage. Aquatic organisms such as fish accumulate pollutants directly from contaminated water or indirectly through the ingestion of contaminated aquatic organisms. Genotoxic pollutants may lead to the contamination not only of the aquatic organisms themselves, but also of the entire ecosystem and, finally, of humans via the food chain (Matsumoto *et al.*, 2006). Thus, humans using water for drinking water purposes may suffer similar genetic or even carcinogenic risks as do fish (Kosmehl *et al.*, 2004, 2008). Moreover, in recent years, there has been increasing concern about the risk for human health following consumption of contaminated fish, since fish are top predators in the food web and can metabolize, concentrate, store and also biomagnify con-

taminants in a way similar to humans. The assessment of sediment quality is, thus, essential for the understanding of processes governing the fate and availability of pollutants in water bodies, which are the final compartment for storage and transformation of most pollutants discharged by anthropogenic activities (Almeida & Rocha, 2006). For this reason, more specific environmental studies are required to elucidate the potential risk for environmental and common welfare, including that of humans.

## 2.5 Conclusions

Differences in genotoxic potentials between different locations could be identified by both the comet assay and the micronucleus test, with a very high correlation between *in vitro* and *in situ* results. The good correlation of these two tests is – in terms of weight-of-evidence approaches – an indication of the high ecological relevance of sediment genotoxicity for the situation in the field (cf. Böttcher *et al.* in press; Chapman & Hollert 2006).

In the present study, strong hazard potentials could especially be detected in samples from Billings reservoir, indicating a major impact of discharges of the megacity São Paulo. Dilution by tributaries further downstream resulted in a significant decrease in genotoxicity of sediment-bound contaminants. Genotoxicity studies thus corroborate conclusions drawn from several studies based on other toxicity parameters (Almeida & Rocha, 2006; Bainy *et al.*, 1996, 1999; Fracácio *et al.*, 2003; Leitao *et al.*, 2000; Rodgher *et al.*, 2005).

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# Bibliography


Ahlf W, Braunbeck T, Heise S, Hollert H (2002a) Sediment and soil quality criteria. *Environmental Monitoring Handbook* 17–11

Ahlf W, Hollert H, Neumann-Hensel H, Ricking M (2002b) A guidance for the assessment and evaluation of sediment quality: A German approach based on ecotoxicological and chemical measurements. *Journal of Soils and Sediments* 2: 37–42

Almeida CA, Rocha O (2006) Estudo Comparativo da Qualidade dos Sedimentos dos Reservatórios do Rio Tietê (SP). *Jornal Brasileiro da Sociedade de Ecotoxicologia* 1: 141–145

Babich H, Borenfreund E (1992) *Neutral red assay for toxicology in vitro. In vitro methods of toxicology*, 238–251. CRC - Press, Boca Raton, Florida

Bainy ACD, Saito E, Carvalho PSM, Junqueira VB (1996) Oxidative stress in gills, erythrocytes, liver and kidney of Nile tilapia (*Oreochromis niloticus*) from a polluted site. *Aquatic Toxicology* 34: 151–162

Bainy ACD, Bruce RW, John JS (1999) Elevated levels of multiple cytochrome P450 forms in tilapia from Billings Reservoir - Sao Paulo, Brazil. *Aquatic Toxicology* 44: 289–305

Balch GC, Metcalfe CD, Huestis SY (1995) Identification of potential fish carcinogens in sediment from Hamilton Harbour, Ontario, Canada. *Environmental Toxicology and Chemistry* 14: 79–91

Baudo R, Muntau H (1990) *Sediments: chemistry and toxicity of in-place pollutants*, chap. Lesser know in-place pollutants and diffuse source problems, 1–14. 1, Boca Raton, Lewis Publishers, Inc.

Bolognesi C, Landini E, Perrone E, Roggieri P (2004) Cytogenetic biomonitoring of a floriculturist population in Italy: micronucleus analysis by fluorescence (*in situ* hybridization (FISH) with an all-chromosome centromeric probe. *Mutation Research* 557: 109–17

Böttcher M, Grund S, Keiter S, Kosmehl T, Manz W, Seitz N, Rocha PS, Hollert H, Braunbeck T (in press) Comparison of (*in vitro* and (*in situ* genotoxicity in the Danube River by means of the comet assay and the micronucleus test. *Mutation Research*

Brocanelli P (1998) *A incorporação da água no meio ambiente da cidade de São Paulo*. Master's thesis, Universidade Presbiteriana Mackenzie, Faculdade de Arquitetura e Urbanismo, São Paulo, São Paulo, Brazil

Bücker, Conceição (2004) Avaliação da mutagênese e genotoxicidade em *Eigenmannia virescens* (Teleostei: Gymnotiformes) expostos ao benzeno. *Acta Amazonica* 36: 357–364

Burton GA (1991) Assessing the toxicity of freshwater sediments. *Environmental Toxicology and Chemistry* 10: 1585–1627

Buschini A, Carboni P, Martino A, Poli P, Rossi C (2003) Effects of temperature on baseline and genotoxicant-induced DNA damage in haemocytes of Dreissena polymorpha. *Mutation Research* 537: 81–92

Cavas T, Ergene-Gözükara S (2005) Induction of micronuclei and nuclear abnormalities in (*Oreochromis niloticus* following exposure to petroleum refinery and chromium processing plant effluents. *Toxicology* 74: 264–271

CETESB (1997) Relatório da Qualidade das Águas Interiores do Estado de São Paulo - 1996. Tech. rep., CETESB

Chapman PM, Hollert H (2006) Should the sediment quality triad become a tetrad, a pentad, or possibly even a hexad? *Journal of Soils and Sediments* 6: 4–8

Chen G, White PA (2004) The mutagenic hazards of aquatic sediments: a review. *Mutation Research* 567: 151–225

Fracácio R, Verani NF, Espíndola ELG, Rocha O, Rigolin-Sá O, Andrade CA (2003) Alterations on growth and gill morphology of *Danio rerio* (Pisces, Ciprinidae) exposed to the toxic sediments. *Brazilian Archives of Biology and Technology* 46: 685–695

Grisolia C, Cordeiro C (2000) Variability in micronucleus induction with different mutagens applied to several species of fish. *Genetics and Molecular Biology* 23: 235–239

Gunnarsson J, Broman D, Jonsson P, Olsson M, Rosenberg R (1995) Interactions between eutrophication and contaminants: Towards a new research concept for the European aquatic environment. *Ambio* 24: 383–385

Hilscherova K, Kannan K, Holoubeck I, Giesy JP (2002) Characterization of estrogenic activity of riverine sediments from the Czech Republic. *Archives of Environmental Contamination and Toxicology* 43: 175–185

Hollert H, Dürr M, Erdinger L, Braunbeck T (2000) Cytotoxicity of settling particulate matter (SPM) and sediments of the Neckar river (Germany) during a winter flood. *Environmental Toxicology and Chemistry* 19: 528–534

Hollert H, Keiter S, König N, Rudolf M, Ulrich M, Braunbeck T (2003) A New Sediment Contact Assay to Assess Particle-bound Pollutants Using Zebrafish (*Danio rerio*) Embryos. *Journal of Soils and Sediments* 3: 197–207

Hollert H, Dürr M, Holtey-Weber R, Islinger M, Brack W, Färber H, Erdinger L,

Braunbeck T (2005) Endocrine disruption of water and sediment extracts in a non-radioactive dot blot/RNAse protection-assay using isolated hepatocytes of rainbow trout - How explain deficiencies between bioanalytical effectiveness and chemically determined concentrations? *Environmental Science and Pollution Research* 12: 347 – 360

Hollert M Hand Duerr, Olsman H, Halldin K, Bavel Bv, Brack W, Tysklind M, Engwall M, Braunbeck T (2002) Biological and chemical determination of dioxin-like compounds in sediments by means of a sediment triad approach in the catchment area of the Neckar River. *Ecotoxicology* 11: 323–336

Huber R, Streng S, Bauchinger M (1983) The suitability of the human lymphocyte micronucleus assay system for biological dosimetry. *Mutation Research* 111: 185–193

Keiter S, Rastall A, Kosmehl T, Erdinger L, Braunbeck T, Hollert H (2006) Ecotoxicological Assessment of Sediment, Suspended Matter and Water Samples in the Upper Danube River. A pilot study in search for the causes for the decline of fish catches. *Environmental Science and Pollution Research* 13: 308–319

Keiter S, Braunbeck T, Heise S, Pudenz S, Manz W, Hollert H (2009) A fuzzy logic-classification of sediments based on data from *in vitro* biotests. *Journal of Soils and Sediments* 9: 168–179

Klee N, Gustavsson LK, Kosmehl T, Engwall M, Erdinger L, Braunbeck T, Hollert H (2004) Changes in toxicity and genotoxicity of industrial sewage sludge samples containing nitro and amino-aromatic compounds following treatment in bioreactors with different oxygen regimes. *Environmental Science and Pollution Research* 11: 313–320

Koelmans AA, Van der Heijde A, Knijff LM, Aalderink RH (2001) Integrated modeling of eutrophication and organic contamination fate and effects in aquatic ecossistems. A review. *Water Research* 35: 3517–3536

Kosmehl T, Krebs W Fand Manz, Erdinger L, Braunbeck H Tand Hollert (2004) Comparative genotoxicity testing of Rhine river sediment extracts using the permanent cell lines RTG-2 and RTL-W1 in the comet assay and Ames assay. *Journal of Soils and Sediments* 4: 84–94

Kosmehl T, Hallare AV, Braunbeck T, Hollert H (2008) DNA damage induced by genotoxicants in zebrafish (*Danio rerio*) embryos after contact exposure to freeze-dried sediment and sediment extracts from Laguna Lake (The Philippines) as measured by the comet assay. *Mutation Research* 650: 1–14

Larsson P, Andersson A, Broman D, Nordbaeck J, Lundberg E (2000) Persistent organic pollutants (POPs) in pelagic systems. *Amio* 29: 202–209

Lee LE, Clemons JH, Bechtel DG, Caldwell SJ, Han KB, Pasitschniak-Arts M, Mosser D, Bols NC (1993) Development and characterization of a rainbow trout liver cell line expressing cytochrome P450-dependent monooxygenase activity. *Cellular Biology and Toxicology* 9: 279–294

Leitao M, Affonso E, Silva M, Meirelles N, Rantin F, Vercesi A, Junqueira V, Degterev I (2000) The liver monooxygenase system of Brazilian freshwater fish. *Comparative Biochemistry and Physiology* 126: 29–38

Lemos CT, Roedel PM, Terra NR, Oliveira NCD, Erdtmann B (2007) River water genotoxicity evaluation using micronucleus assay in fish erythrocytes. *Ecotoxicology and Enviromental Safety* 66: 391–401

Maier M, Kuehlers D, Brauch HJ, Fleig M, Maier D, Jirka GH, Mohrlok E Uand Bethge, Bernhart HH, Lehmann B, Hillebrand G, Woelz J, Hollert H (2006) Flood retention and drinking water supply - Preventing conflicts of interest. *Journal of Soils and Sediments* 6: 113–114

Matsumoto ST, Mantovani MS, Malaguttii MIA, Dias AL, Fonseca IC, Marin-Morales MA (2006) Genotoxicity and mutagenicity of water contaminated with tannery effluents, as evaluated by the micronucleus test and comet assay using the fish *Oreochromis niloticus* and chromosome aberrations in onion root-tips. *Genetics and Molecular Biology* 29: 148–158

Matsumura-Tundisi T, Tundisi JG (2005) Plankton richness in a eutrophic reservoir (Barra Bonita Reservoir, SP, Brazil). *Hydrobiologia* 542: 367–378

Metcalfe CD, Balch GC, Cairns VW, Fitzsimons JD, Dunn BP (1990) Carcinogenic and genotoxic activity of extracts from contaminated sediments in western Lake Ontario. *Science of the Total Environment* 125–141

Minissi S, Ciccotti E, Rizzoni M (1996) Micronucleus test in erythrocytes of Barbus plebejus (Teleostei, Pisces) from two natural environments: a bioassay for the *in situ* detection of mutagens in freshwater. *Mutation Research* 367: 245–251

Mozeto A, Araújo P, Kulmann M, Silvério P, Nascimento M, Almeida F, Umbuzeiro G, Jardim W, Watanabe H, Rodrigues P, Lamparelli M (2004) Interated hierarchical sediment quality assessment program: Qualised project's approach proposal (São Paulo, Brazil). *3rd Workshop Monitoring sediment quality at river basin scale Understanding the behaviour and fate of pollutants SedNet - Lisbon, Portugal*

Palhares D, Grisolia CK (2002) Comparison between the micronucleus frequencies of kidney and gill erythrocytes in tilapia fish, following mitomycin C treatment. *Genetics and Molecular Biology* 25: 281–284

Rodgher S, Espíndola ELG, Rocha O, Fracácio R, Pereira RHG, Rpdrigues MHS (2005) Limnological and ecotoxicological studies in the cascade of reservoirs in the Tietê River (São Paulo, Brazil). *Brazilian Journal of Biology* 65: 697–710

Schnurstein A, Braunbeck T (2001) Tail moment *versus* tail length-application of an *in vitro* version of the comet assay in biomonitoring for genotoxicity in native surface waters using primary hepatocytes and gill cells from zebrafish *(Danio rerio). Ecotoxicology and Enviromental Safety* 49: 187–96

Seiler TB, Rastall AC, Leist E, Erdinger L, Braunbeck T, Hollert H (2006) Membrane dialysis extraction (MDE): A novel approach for extracting toxicologically

relevant hydrophobic organic compounds from soils and sediments for assessment in biotests. *Journal of Soils and Sediments* 6: 20–29

Seitz N, Böttcher M, Keiter S, Kosmehl T, Manz W, Hollert H, Braunbeck T (2008) Evaluation of the genotoxic potential of sediment samples from the Danube River using a new expert system and several analyzing methods: How to integrate information from dose-response curves. *Mutation Research* 652: 38–45

Singh N, McCoy M, Tice R, Schneider E (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research* 175: 184–191

Skei J, Hylland K, Schaanning MT, Berge JA, Gunnarsson JS, Sköld M, Eriksen DO (1996) Interations between eutrophication and contaminants. I. Principles, experimental design and synthesis. *Marine Pollution Bulletin* 33: 64–70

Sotero-Santos RB, Silva CRSES, Verani NF, Nonaka KO, Rocha O (2006) Toxicity of a canobacteria bloom in Barra Bonita Reservoir (Middle Tietê River, São Paulo, Brazil). *Ecotoxicology and Enviromental Safety* 64: 163–170

Taylor WD, Carey JH, Lean DRS, McQueen DJ (1991) Organochlorine concentrations in the plankton of lakes in southern Ontario and their relationship to plankton biomass. *Canadian Journal of Fisheries and Aquatic Sciences* 48: 1960–1966

Titenko-Holland N, Ahlborn T, Lowe X, Shang N, Smith M, Wyrobeck A (1998) Micronuclei and Developmental Abnormalities in 4-Day Mouse Embryo After Paternal Treatment With Acrylamide. *Environmental and Molecular Mutagenesis* 31: 206–217

Tundisi JG, Matsumura-Tundisi T, Rocha O (1999) Theorical Basis for Reservoir Management. In: Tundisi JG, Straskraba M (eds.) *Theorical Reservoir Ecology and Its Application.*, 505–528, São Carlos, SP

Ventura BC, Angelis DF, Marin-Morales MA (2008) Mutagenic and genotoxic effects of the Atrazine herbicide in *Oreochromis niloticus* (Perciformes, Cichlodae) detected by the micronuclei test and the comet assay. *Pesticide Biochemistry and Physiology*

# Chapter 3

# Changes in toxicity and Ah receptor agonist activity of sediments from the Tietê River (São Paulo, Brazil) - a mass balance approach using *in vitro* methods and chemical analysis

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## Abstract

Acute cytotoxicity (neutral red assay), AhR-mediated toxicity (CYP P450 1A induction potential – EROD assay) and concentrations of PAHs were recorded to assess the ecotoxicological potential of sediments from reservoirs along the Tietê River and the Pinheiros River (Brazil). Almost all sediments tested induced cytotoxicity and stimulated cytochrome P450-associated EROD activity. Toxicity increases from Tietê River spring to São Paulo city region and decreases towards downstream. A closer analysis of chemical measurements of PAHs and results from bioassay revealed that the PAHs analyzed could not explain more than 7% of the EROD-inducing potencies. Results confirm that most of the toxicity is due

to the discharges of the metropolitan area of São Paulo. Moreover, they indicate additional sources of pollutants along the river course, which contribute to the degradation of each reservoir.

*Keywords:* acute cytotoxicity; neutral red assay; CYP P450 1A; EROD assay; PAHs, RTL-W1 cell line; sediments; Tietê River; Pinheiros River; Brazil

## 3.1 Introduction

The release of anthropogenic chemicals has led to an increased burden of natural resources. Pollutants such as pesticides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and heavy metals enter freshwater ecosystems from agricultural areas, urban and industrial production sites by drainage, superficial run-off, rainwater and discharges of wastewater, all of which may eventually result in long-term ecotoxicological effects (Strmac & Braunbeck, 2000). The sediment compartment is the intermediate or final receptor of insoluble (or slightly water soluble) pollutants and can act as a sink for various substances. Sediments accumulate chemicals up to concentrations many times higher than free water column concentrations (Ahlf *et al.*, 2002; Baudo & Muntau, 1990; Burton, 1991; Fracácio *et al.*, 2003). Sediments can also become a source of diffuse contamination to the free water column, since pollutants may be made available under certain environmental conditions such as dredging or flood events (Ahlf *et al.*, 2000; Hollert *et al.*, 2003). Sediment pollutants are not only linked to organisms in aquatic ecosystems, but also to human health through drinking water and fish consumption (Chen & White, 2004; Hollert *et al.*, 2005; Keiter *et al.*, 2006; Maier *et al.*, 2006).

Among other effects, the response to pollution is reflected as changes in certain enzyme activities, especially key enzymes of biotransformation systems, which, in turn, can be used as biomarkers of pollution. Such biomarkers therefore serve as early warning signal of aquatic pollution Babin *et al.*, 2005; Hilscherova *et al.*, 2001. Ecotoxicological studies based on biomarkers allow the determination of the impact of environmental stressors and facilitate following the evolution of the ecosystem towards degradation or restoration (Vasseur & Cossu-Leguile, 2003).

The *in vitro* detection of cytochrome P4501A (CYP1A) induction has been recognized as a sensitive marker of exposure to a number of environmentally relevant toxicants (Behrens *et al.*, 2001; Berbner *et al.*, 1999; Bucheli & Fent, 1995; Engwall *et al.*, 1999). In toxicological studies, CYP1A has attracted particular attention because of its role in the biotransformation of toxicologically important environmental contaminants (Segner *et al.*, 2000).

A variety of environmental pollutants, such as dioxin-like compounds, are able to induce the biotransformation enzyme CYP1A in fish, by ligand binding to the aryl hydrocarbon receptor (AhR; Babin *et al.*, 2005; Hilscherova *et al.*, 2001). Dioxin-like compounds are intimately associated with suspended particles and consequently with sediments (Hilscherova *et al.*, 2001; Hollert *et al.*, 2002; Keiter *et al.*, 2009; Seiler *et al.*, 2006; Woelz *et al.*, 2008).

The Tietê River was selected as an example for a contaminated river system. It passes through São Paulo city and São Paulo state (Brazil) and comprises several reservoirs along its course. These reservoirs are intensively used for providing drinking water, as a water source for agricultural irrigation and as recreation sites, but also as receptors of domestic and industrial effluents. Due to the insufficiency of effluent treatment and numerous direct industrial sources for a multitude of anthropogenic pollutants, the Tietê River has thus become one of the most polluted rivers in the world. Despite this heavy pollution, little is know about the biological hazard potential of the mixture of chemicals in waters and sediments in the Tietê River or the substances and sites of concern.

This study is part of an integrative (weight of evidence) assessment of Tietê River sediments, aiming at identifying hazard factors and ecotoxicological risks in the Tietê River Basin (Rocha *et al.*, 2006, 2009). For this end, in the present study, selected ecotoxicological endpoints, such as basic toxicity and aryl hydrocarbon receptor (AhR)-mediated toxicity, were recorded in a cell-based monitoring system using the permanent cell line RTL-W1. Acute cytotoxicity was determined by means of the neutral red retention assay (Babich & Borenfreund, 1991; Borenfreund & Puerner, 1984), and the aryl hydrocarbon receptor (AhR)-mediated toxicity was measured by means of the EROD assay, where the induction of CYP1A in cells is measured as 7-ethoxyresorufin-O-deethylase (EROD) activity via the degradation of the artificial substrate 7-ethoxyresorufin to resorufin (Behrens *et al.*, 1998). Finally, chemical analyses of PAHs were performed to obtain an overview of the hazard potential of these sediments related to these chemicals and to estimate the degree to which these chemicals account for the EROD-inducing potencies of these environmental extracts.

Within the integrated assessment of the Tietê River system, the present study has been designed (1) to assess the ecotoxicological potential of sediments from the Tietê River reservoirs and the Pinheiros River with respect to cytotoxicity and CYP P450 1A induction potential; (2) to evaluate the contribution of priority PAHs to the EROD induction of these environmental samples. To associate chemical analyses with the bioassay results, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) equivalent concentrations of selected PAHs were determined and related to the EROD-inducing potencies of these

environmental extracts.

## 3.2 Material and Methods

### 3.2.1 Sediment sampling

The study area comprised a location in Salesópolis near the Tietê River's spring and the reservoirs Ponte Nova and Billings (Upper Tietê); Barra Bonita (Superior Middle Tietê); Bariri and Promissão (Inferior Middle Tietê) and Três Irmãos (Lower Tietê; Fig. 3.1). The Upper Tietê River basin corresponds to the drained area of the Tietê River from its spring across the metropolitan area of São Paulo city (> 19,000,000 inhabitants) characterized by high densities of anthropogenic population and dramatic deterioration subsequent to intensive urbanization. The Superior Middle Tietê River basin is dominated by urban, industrial and agricultural areas. The Inferior Middle Tietê River basin is characterized predominantly by agricultural areas and the Low Tietê river basin by pastures and sugar cane culture (CETESB, 1997). From Barra Bonita to Três Irmãos, the reservoirs were built in cascade arrangements for the generation of electricity.

Investigation of the Tietê River genotoxic potentials (Rocha *et al.*, 2009) revealed a major impact of São Paulo city discharges on the genotoxic load. This conclusion was based on results obtained for Billings reservoir by means of the comet and micronucleus assays. Since Billings reservoir occasionally also receives water from Pinheiros River system, another highly polluted Tietê River tributary from the São Paulo metropolitan area, samples from the Pinheiros River were also taken as a potential additional source of contamination.

Surface sediments were collected in May and December 2005 in the Tietê River reservoirs, and in December 2006 in the Pinheiros River, by means of an Eckman-Birge dredge, with 10 replicates at each site (with a distance of 10 m from sample to sample). Replicates were homogenized, and 1.5 kg of each sediment sample were frozen immediately, stored at -10°C and transported to Germany. Transfer of the samples was permitted by the Brazilian National Department of Mineral Production (DMPM). Samples were freeze-dried, and extracts were prepared by Soxhlet extraction using acetone p.a. (AppliChem, Darmstadt, Germany) as a solvent and re-dissolved with dimethyl sulfoxide (DMSO; Sigma-Aldrich, Deisenhofen, Germany) for the biossays and hexane p.a. (Riedel-de Haen, Seelze, Germany) for the chemical analyses, as described by Hollert *et al.* (2000). The resulting concentration of the extracts was 20 g dry sediment-equivalent per 1 ml solvent.

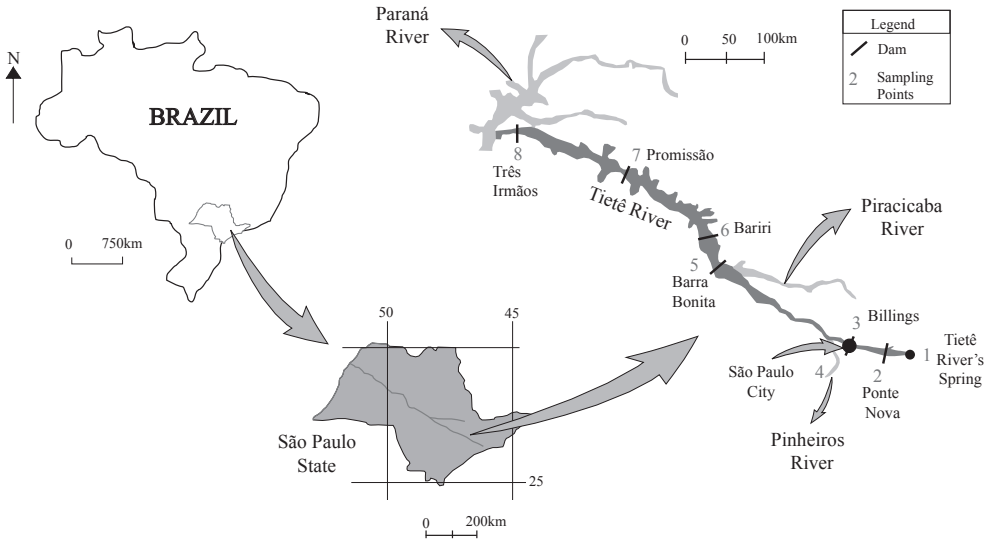


**Fig. 3.1.** Location of the sampling sites in the Tietê River basin, São Paulo, Brazil.

### 3.2.2 Cell culture

The fibroblast-like permanent cell line RTL-W1 (Lee *et al.*, 1993) derived from rainbow trout liver (*Oncorhynchus mykiss*) was used to perform the neutral red and EROD assays. RTL-W1-cells have been successfully used to detect genotoxicity and mutagenic potential of environmental samples (Böttcher *et al.*, in press; Kosmehl *et al.*, 2004; Keiter *et al.*, 2006; Rocha *et al.*, 2009) and EROD activity (Babin *et al.*, 2005; Billiard *et al.*, 2004; Bols *et al.*, 1999; Clemons *et al.*, 1998; Keiter *et al.*, 2009; Lee *et al.*, 1993; Schirmer *et al.*, 2000, 2004; Segner *et al.*, 2000; Woelz *et al.*, 2008). Furthermore, RTL-W1 cells have relatively high biotransformation capacities, if compared to other fish cell lines such as RTG-2 cells (Kosmehl *et al.*, 2004). The cells were maintained in 75 $cm^2$ culture flasks (TPP, Trasadingen, Switzerland) in Leibowitz (L15) medium (Sigma-Aldrich, Deisenhofen, Germany) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin solution (10,000 U/10,000 µg/ml) in 0.9% NaCl (Sigma-Aldrich) at 20°C (Seiler *et al.*, 2006). They were trypsinized using 0.05% trypsin/ 0.02% EDTA and washed twice with PBS before being used in the experiments (Kosmehl *et al.*, 2004).

### 3.2.3 Neutral red (NR) assay

The NR assay was performed to investigate acute cytotoxicity in RTL-W1 cell line exposed to the sediments extracts according to the protocol by Klee *et al.* (2004). Two or more independent assays were run for each sample. Freeze-dried sediment samples (20 g sediment equivalents/ml DMSO) were serially diluted with L15 medium to give a concentration range from 3.1 to 200 mg dry sediment equivalent per ml test medium. The maximum concentration of DMSO in any extract dilution was below its No Observed Effect Concentration (NOEC) for RTL-W1 cells ($< 1\%$; Keiter *et al.*, 2006). Six replicates of each extract dilution were tested by incubation with RTL-W1 cells in 96-well microtiter plates at 20°C for 48 h. 3,5-Dichlorophenol (Riedel de Han, Seelze, Germany) was used as a positive control at a maximum concentration of 80 mg/L medium. Supplemented L15 medium was used as a negative control. After exposure, extract dilutions were discarded, and cells were incubated with neutral red (2-methyl-3-amino-7- dimethylamino-phenanzine) for 3 h. To determine acute cytotoxicity, neutral red retention was measured at 540 nm with a reference wavelength of 690 nm using a GENios plate reader (Tecan, Crailsheim, Germany). The viability of the exposed cells was expressed as a percentage of the controls and the data were plotted as second-order polynomial dose-response curves using Prism 4.0 (GraphPad, San Diego, USA). The cytotoxic potential of individual extracts was calculated as $NR_{50}$ (extract concentrations inducing 50% mortality after 48 h).

### 3.2.4 Ethoxyresofin-O-deethylase (EROD) assay

EROD induction is measured as a specific enzyme activity, with 2,3,7,8-tetra-chlorodibenzo-p-dioxin (TCDD) as a standard, which is known to be one of the most potent inducers of CYP1A activity Okey *et al.*, 1994; Seiler *et al.*, 2006. The EROD assay was carried out according to Behrens *et al.* (1998) with modifications byGustavson *et al.* (2004). This protocol differs from the generally used approach, which includes one separate plate with serially diluted TCDD for each assay, in that the TCDD concentration-response curve was measured on every plate, including the serial dilution TCDD in each sample plate. As described by Seiler *et al.* (2006), cells were pre-incubated in 96-well microtiter plates at 20°C for 72 h. Then cells were exposed for 48h to 1:1 to 1:128 dilutions of the extracts, which had been selected on the basis of the $NR_{85}$ . Due to increased cytotoxicity, the highest test concentration for the Pinheiros River samples was a 1:4 dilution of the $NR_{85}$ . As a positive control, TCDD was serially diluted to give a final concentration range of

3.125 - 100 pM on two separate rows of each plate.

The plates were incubated for 72h at 20°C. Exposure was stopped by removing the medium. For cell lysis, plates were deep-frozen at 80°C for at least 1 h. The plates were then thawed for 10 min. An aliquot of 100 µl of 1.2 µM 7-ethoxyresorufin was added to each well, before deethylation was initiated for 10 min with 0.09 µM NADPH in phosphate buffer. The reaction was stopped by adding 100 µl of 0.54 mM fluorescamine in acetonitrile. After another 15 min, EROD activity was measured fluorometrically at an excitation wavelength of 544 nm and emission at 590 nm using a GENios plate reader. Whole protein was determined fluorometrically using the fluorescamine method (excitation 355 nm, emission 590 nm; Lorenzen & Kennedy, 1993; Brunstroem & Halldin, 1998) with the protocol detailed in Hollert *et al.* (2002). The concentration–response curves for EROD induction in the RTL-W1 bioassay were computed by non-linear regression (GraphPad Prism 4) using the classic sigmoid curve or Boltzmann curve as model equations. The luminescence-inducing potency of the samples was converted to bio-TEQs as described below.

### 3.2.5 Bio-TEQ calculation

Maximal induction rates of the concentration-response curves for the extracts varied in relation to the positive control TCDD. Bioassay-derived TCDD equivalents (bio-TEQs) were calculated by relating biological activities caused by samples to the positive control TCDD. Bio-TEQs for concentration–response curves were calculated following the fixed effect level quantification method, using the EC25 of the maximum response in the TCDD standard curves as the fixed level (Brack *et al.*, 2000; Engwall *et al.*, 1996). The bio-TEQs given in this study are means of n = 2 - 4 independent experiments.

Mean TCDD-$EC_{25}$ values were determined as well as standard deviations (SD) between individual EROD assays and used to calculate bio-TEQs.

The Bio-TEQ concentrations were calculated as:

$$Bio - TEQ = \frac{TCDD\,EC25(pg/ml)}{extract\,EC25TCDD(g/ml)}$$

### 3.2.6 Chemical analysis

Quantification of EPA priority PAHs in the sediment extracts was accomplished by GC-MS analysis. The samples were subjected to clean-up using Florisil® (Merck, Darmstadt, Germany) and, as an eluent, iso-octane:toluene

95:5 (v/v); occasionally, the clean-up was performed twice. Resulting samples were concentrated using a Laborota 4011 digital rotary evaporator (Heidolph, Kelheim, Germany). Final analyses were executed on an Agilent 6890N gas chromatograph (Waldbronn, Germany) coupled to a mass selective Agilent 5973N MSD detector, which was operated in the SIM mode using an Optima-35-MS column (30m x 0.25 mm, film thickness: 0.25 µm; Machery-Nagel, Düren, Germany). The reference compounds (16 EPA Priority Pollutants Mixture) were provided by Promochem (Wesel, Germany) and included naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, 1,2-benzanthracene, chrysene, benzo[b]-fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[ghi]perylene and indeno(1,2,3-cd)pyrene.

### 3.2.7 PAH-TEQ calculation

In order to estimate the degree to which analyzed PAHs account for the EROD-inducing potencies of these sediment extracts, PAH-TEQs were calculated on the basis of PAH potencies relative to TCDD (Bols *et al.*, 1999). The TEQ calculation was done by multiplying the concentration of each PAH in each sample by the corresponding TCDD-related potency given by Bols *et al.* (1999), thus obtaining the relative equivalency potency values (REPs) and summing these values for each sample. To elucidate the percentage of the measured PAHs that is responsible for the induction in the EROD assay in the tested extracts, the PAH-TEQ values were subtracted from the corresponding Bio-TEQ values.

## 3.3 Results

### 3.3.1 Cytotoxicity (neutral red assay)

Fig. 3.2 shows the cytotoxic potentials of individual extracts inducing 50% mortality after 48h ($NR_{50}$). Almost all tested sediments induced cytotoxic effects, with Billings reservoir and Pinheiros River being the most cytotoxic sites ($NR_{50}$ 29 and 35 mg/ml, respectively). The sampling site near the spring (considered as reference site) showed the lowest cytotoxic effect in the study ($NR_{50}$ 225 mg/ml). However, already few kilometers downstream spring, an abrupt increase in cytotoxicity ($NR_{50}$ 44 mg/ml) was recorded for Ponte Nova reservoir. In Barra Bonita reservoir, ca. 270 km downstream São Paulo city, a significant decrease in cytotoxicity was recorded ($NR_{50}$ 176 mg/ml), which, however, was followed by another increase in direction to the

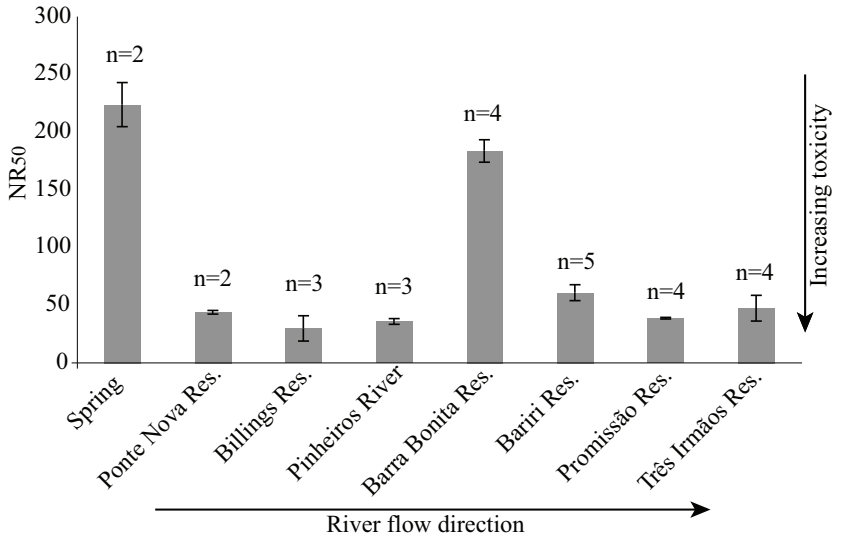


**Fig. 3.2.** Cytotoxicity of extracts from sediment samples collected from the Tietê River basin as revealed in the neutral red assay with RTL-W1 cells: Except for the Barra Bonita Reservoir, sediment extract from all locations induced significant cytotoxic effects when compared to reference site (spring). Data are given as mean $NR_{50}$ (mg/ml; effective concentration for the death of 50% of the cells) values from n independent experiments (6 measurements each).

mouth of the river (Bariri, Promissão and Três Irmãos reservoirs with $NR_{50}$ of 60, 39 and 68 mg/ml, respectively).

The results in the NR assay were used to determine appropriate dilutions for further *in vitro* biotests such as the EROD assay. In order to separate cytotoxic from specific effects, $NR_{85}$ concentrations were selected as the highest concentrations in the EROD assay.

### 3.3.2 Ah receptor agonist activity (EROD assay)

In contrast to the extract of Ponte Nova, which revealed no significant EROD activity, all other sediment extracts investigated clearly induced EROD activity in RTL-W1 cells, each with a positive dose-response relationship (Fig. 3.3). EROD induction potencies are given as toxicity equivalent values relative to TCDD ($EC_{25}$ 6.0 ± 1.4 pg/ml from 27 independent investigations). Highest EROD activities in RTL-W1 cells were induced by extracts from Bariri ($EC_{25TCDD}$ 0.8 - 1.4 mg/ml) and Billings reservoirs ($EC_{25TCDD}$ 1.4 mg/ml).

The $EC_{25TCDD}$ values were used for the calculation of bioassay TCDD-equivalents (Bio-TEQs) in the extracts. The reference site near the spring had the lowest Bio-TEQ value (160 pg/g; Fig. 3.4), whereas Pinheiros River

extracts showed the highest Bio-TEQ value in this study (24170 pg/g). Bariri and Billings extracts also had higher Bio-TEQs (6000 and 5000 pg/g respectively), differing significantly from the other areas. Bio-TEQs for sediments from Barra Bonita and Promissão reservoirs showed fairly low EROD induction (340 and 320 pg/g, respectively), whereas Bio-TEQs for Três Irmãos extract were again higher (1140 pg/g). Since Ponte Nova extract did not induce EROD activity, it was not possible to calculate the Bio-TEQ for this sample. Overall, there were differences by two orders of magnitude between the lowest and the highest Bio-TEQs.

### 3.3.3 Chemical Analyses

Chemical analyses of PAHs revealed by far the highest concentrations for extracted sediment samples from Pinheiros River, Bariri and Billings (3.11, 1.91 and 0.63 µg/g SEQ, respectively). In contrast, PAH concentrations were much lower in sediments from the other areas (Barra Bonita reservoir: 0.37 µg/g SEQ, near spring and Três Irmãos reservoir 0.20 µg/g SEQ, Ponte Nova Reservoir 0.17 µg/g SEQ, and Promissão Reservoir 0.16 µg/g SEQ; Table 3.1). The highest concentrations among individual PAHs were measured for fluoranthene, pyrene and phenanthrene in the samples from Pinheiros River and Bariri reservoir. Concentrations of dibenz[a,h]anthracene and indeno[1,2,3-cd]pyrene, which were found in Pinheiros River sediments only, were also high, when compared to all other PAHs measured.

### 3.3.4 Correlation between Bio-TEQs and chemical analyses (PAH-TEQs)

For most of the extracts, less then 5% of the induction could be explained by known PAHs expressed as PAH-TEQs. For spring and Barra Bonita extracts, approx. 6 and 7 % of the induction could be related to the presence of analyzed PAHs (Table 3.1, Fig. 3.4).

**Fig. 3.3.** ***(following page)*** EROD induction potential of sediment samples from the Tietê River basin in RTL-W1 cells. Data are given as mean $EC_{25TCDD}$ (concentration of each sample which caused 25% of TCDD-induced maximum EROD activity) values from 6 independent measurements. Dashed lines on the diagrams indicate the intersections of EROD activities (in pmol * mg-1 * min -1, y axis) and sediment concentrations in medium (in Log (mg SEQ/ml medium), x axis), corresponding to $EC_{25TCDD}$ values. Figs. 3a to h (left → right, top → down) are displayed according to the flow direction of the river.

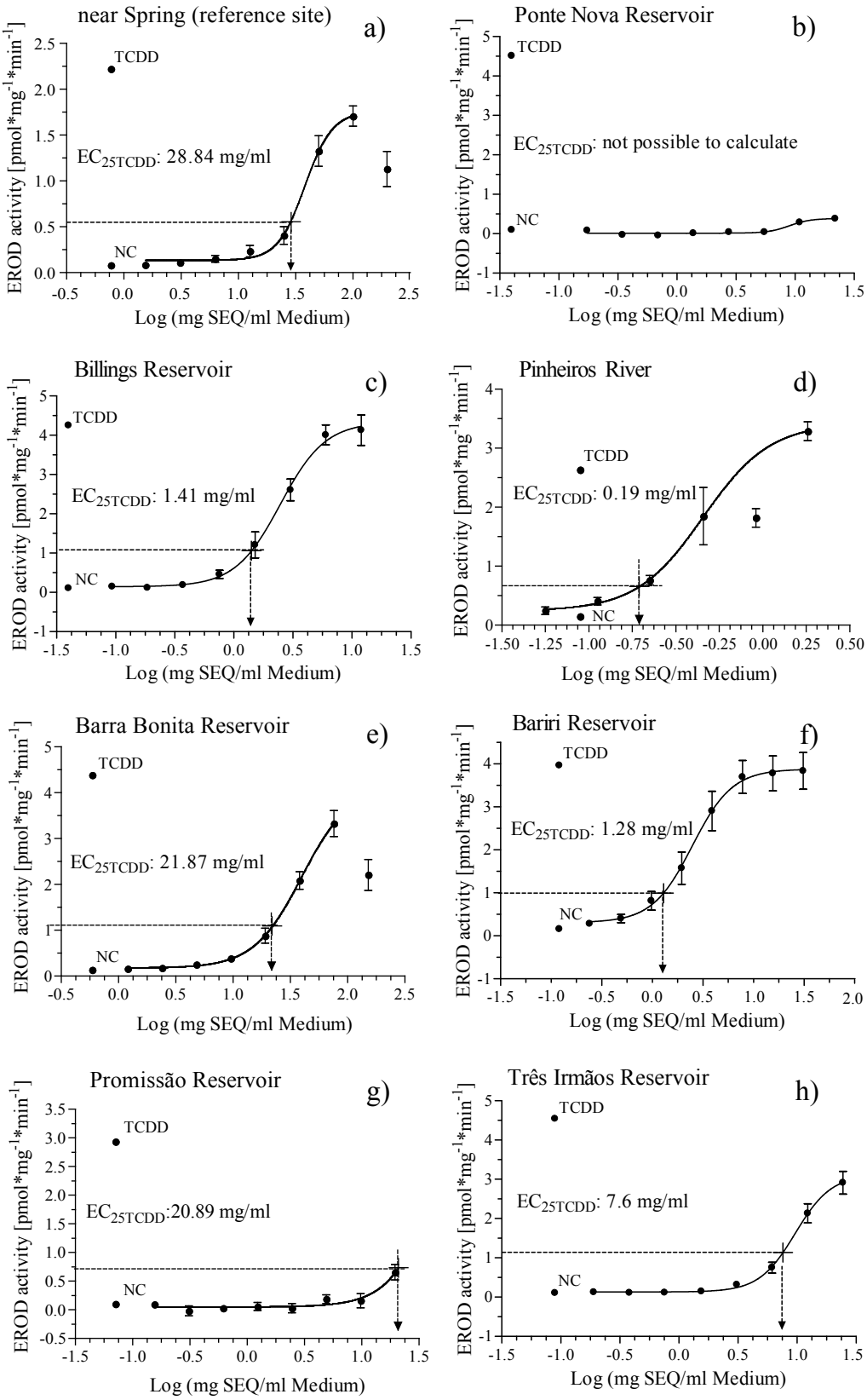
near Spring (reference site)
a)
TCDD
EC25TCDD: 28.84 mg/ml
NC
Ponte Nova Reservoir
b)
TCDD
EC25TCDD: not possible to calculate
NC
Billings Reservoir
c)
TCDD
EC25TCDD: 1.41 mg/ml
NC
Pinheiros River
d)
TCDD
EC25TCDD: 0.19 mg/ml
NC
Barra Bonita Reservoir
e)
TCDD
EC25TCDD: 21.87 mg/ml
NC
Bariri Reservoir
f)
TCDD
EC25TCDD: 1.28 mg/ml
NC
Promissão Reservoir
g)
TCDD
EC25TCDD:20.89 mg/ml
NC
Três Irmãos Reservoir
h)
TCDD
EC25TCDD: 7.6 mg/ml
NC
EROD activity [pmol*mg-1*min-1]
Log (mg SEQ/ml Medium)

**Table 3.1** Chemically analyzed priority EPA-PAHs, REPs, Bio-TEQ and PAH-TEQ values, as well as the calculated contribution of PAHs (percentage) to the EROD induction in each sediment extract. The PAH data are giving in µg/g SEQ.

| PAHs | TEF [a] | Spring | | Ponte Nova Res. | | Billings Res. | | Pinheiros River | | Barra Bonita Res. | | Bariri Res. | | Promissão Res. | | Três Irmãos Res. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PAH (µg/g) | REP (µg/g) | PAH (µg/g) | REP (µg/g) | PAH (µg/g) | REP (µg/g) | PAH (µg/g) | REP (µg/g) | PAH (µg/g) | REP (µg/g) | PAH (µg/g) | REP (µg/g) | PAH (µg/g) | REP (µg/g) | PAH (µg/g) | REP (µg/g) |
| Naphthalene | NI | 0.03550 | - | 0.01065 | - | 0.01233 | - | 0.06444 | - | 0.01503 | - | 0.03133 | - | 0.01267 | - | 0.00778 | - |
| Acenaphthylen | NI | 0.00874 | - | 0.00787 | - | 0.00876 | - | 0.03542 | - | 0.00924 | - | 0.01940 | - | 0.00836 | - | 0.00794 | - |
| Acenaphthene | NI | 0.00427 | - | 0.00827 | - | 0.01027 | - | 0.02139 | - | 0.01185 | - | 0.02764 | - | ND | - | 0.00600 | - |
| Fluorene | NI | 0.00839 | - | 0.00844 | - | 0.01305 | - | 0.03406 | - | 0.00910 | - | 0.04957 | - | 0.00894 | - | 0.00908 | - |
| Phenanthrene | NI | 0.04395 | - | 0.03790 | - | 0.09754 | - | 0.31296 | - | 0.04295 | - | 0.35491 | - | 0.03624 | - | 0.05337 | - |
| Anthracene | NI | 0.01004 | - | 0.00848 | - | 0.01724 | - | 0.06943 | - | 0.00983 | - | 0.09937 | - | 0.00794 | - | 0.00744 | - |
| Fluoranthene | NI | 0.02535 | - | 0.02524 | - | 0.12234 | - | 0.46007 | - | 0.02546 | - | 0.41605 | - | 0.02319 | - | 0.03055 | - |
| Pyrene | NI | 0.01908 | - | 0.02181 | - | 0.10846 | - | 0.43299 | - | 0.02167 | - | 0.32466 | - | 0.01685 | - | 0.02394 | - |
| 1,2-Benzo[a]anthracene | $4*10^{-5}$ | 0.00834 | $3.6*10^{-7}$ | 0.00800 | $3.4*10^{-7}$ | 0.07200 | $3.1*10^{-6}$ | 0.18905 | $8.1*10^{-6}$ | 0.15458 | $6.6*10^{-6}$ | 0.15458 | $6.6*10^{-6}$ | 0.00825 | $3.5*10^{-7}$ | 0.00926 | $4.0*10^{-7}$ |
| Chrysene | $5*10^{-5}$ | 0.00815 | $3.8*10^{-7}$ | 0.00803 | $3.8*10^{-7}$ | 0.05157 | $2.4*10^{-6}$ | 0.23880 | $1.1*10^{-5}$ | 0.01012 | $4.7*10^{-7}$ | 0.13616 | $6.4*10^{-6}$ | 0.00849 | $4.0*10^{-7}$ | 0.01144 | $5.4*10^{-7}$ |
| Benzo[b]fluoranthene | $1.04*10^{-3}$ | ND | - | 0.00822 | $8.5*10^{-6}$ | 0.04680 | $4.9*10^{-5}$ | 0.25155 | $2.6*10^{-4}$ | 0.01030 | $1.1*10^{-5}$ | 0.11088 | $1.1*10^{-4}$ | 0.00886 | $9.2*10^{-6}$ | 0.01046 | $1.1*10^{-5}$ |
| Benzo[k]fluoranthene | 0.00019 | 0.01330 | $2.6*10^{-6}$ | 0.00839 | $1.6*10^{-6}$ | 0.02214 | $4.3*10^{-6}$ | 0.08906 | $1.7*10^{-5}$ | 0.00887 | $1.7*10^{-6}$ | 0.05836 | $1.1*10^{-5}$ | 0.00866 | $1.7*10^{-6}$ | 0.00915 | $1.8*10^{-6}$ |
| Benzo[a]pyrene | 0.00030 | 0.01916 | $5.8*10^{-6}$ | 0.01106 | $3.3*10^{-6}$ | 0.04506 | $1.4*10^{-5}$ | 0.25852 | $7.8*10^{-5}$ | 0.01165 | $3.5*10^{-6}$ | 0.13103 | $3.9*10^{-5}$ | 0.01129 | $3.4*10^{-6}$ | 0.01208 | $3.6*10^{-6}$ |
| Dibenz[a,h]anthracene | 0.00035 | ND | - | ND | - | ND | - | 0.35130 | $1.2*10^{-4}$ | ND | - | ND | - | ND | - | ND | - |
| Benzo[ghi]perylene | NCI | ND | - | ND | - | ND | - | 0.05318 | - | 0.02764 | - | ND | - | ND | - | ND | - |
| Indeno[1,2,3-cd]pyrene | 0.00028 | ND | - | ND | - | ND | - | 0.24502 | $6.8*10^{-5}$ | ND | - | ND | - | ND | - | ND | - |
| Sum | | 0.20427 | $9.1*10^{-6}$ | 0.17236 | $1.4*10^{-5}$ | 0.62756 | $7.2*10^{-5}$ | 3.10727 | $5.7*10^{-4}$ | 0.36829 | $2.3*10^{-5}$ | 1.91394 | $1.8*10^{-4}$ | 0.15975 | $1.5*10^{-5}$ | 0.19848 | $1.7*10^{-5}$ |
| Bio-TEQs (pg/g) | | | 158.79 | | - | | 5077.73 | | 24169.73 | | 336.83 | | 6217.08 | | 318.19 | | 1142.03 |
| PAH-TEQs (pg/g) | | | 9.10 | | 14.22 | | 72.03 | | 567.05 | | 23.05 | | 179.08 | | 15.05 | | 17.21 |
| Percentage | | | 5.73 | | - | | 1.42 | | 2.53 | | 6.84 | | 2.88 | | 4.73 | | 1.51 |

TEFs = toxic equivalency factor (the ability of PAHs to induce EROD activity in relation to TCDD), REP = relative equivalency potencies (product of concentrations of each PAHs in each sample x corresponding TEF); Bio-TEQs = Biological toxicity equivalents; PAH-TEQs = toxicity equivalents of Polycyclic Aromatic Hydrocarbons (sum of REPs); ND = not detectable.
[a] Bols et al. 1999 - NI = no induction (PAHs without EROD induction potency); NCI = no constant induction (PAHs that induce EROD induction inconstantly). For these PAHs TEFs could not be calculated.

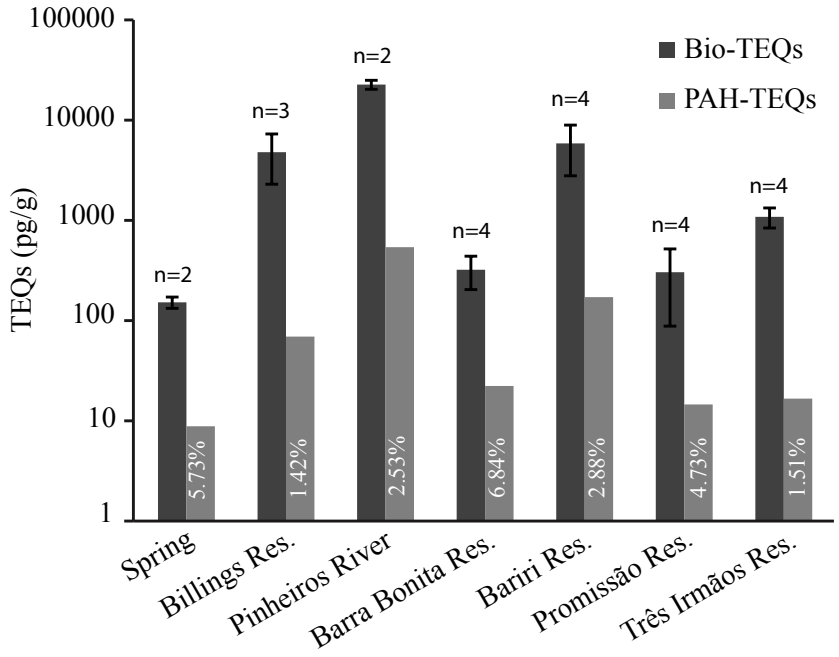


**Fig. 3.4.** Comparison of the total biological response in the EROD assay (Bio-TEQs) and PAH-TEQs (calculated by multiplying compounds concentrations and relative equivalency potencies) and the calculated contribution of these PAHs (in percent) to the EROD induction in each extract. n = number of independent investigations. Error bars represent one sigma standard deviation.

## 3.4 Discussion

The present study is the first comprehensive *in vitro* investigation into the cytotoxic and AhR-mediated toxicity of sediments in the catchment area of Tietê River basin (Brazil), a very large river system. It may thus serve as an example for other river systems. In addition, the study provides an overview of the concentration of priority PAHs and their contribution to the overall EROD induction found in sediment samples.

Findings in the neutral red assay confirm the increase in cytotoxicity from the Tietê River spring (Salesópolis) towards the metropolitan area of São Paulo. Surprisingly, high cytotoxicity was already detected only few kilometers downstream the spring, in the Ponte Nova reservoir, suggesting the existence of pollutant sources already at the beginning of the river course.

Sediment extract from the Pinheiros River (passing through São Paulo city) and Billings reservoir (in São Paulo city region) showed the highest cytotoxicity to exposed cells. From 1952 to 1992, the natural flow of the Pinheiros River had been diverted into Billings reservoir for electricity gen-

eration. After 1992, however, permission for this was withdrawn except for cases of flood control in São Paulo city, in order to prevent the high levels of water pollution in the Billings reservoir (Silva *et al.*, 2002), since the Pinheiros River collected all the sewage from the São Paulo Metropolitan region (CONSEMA, 1993).

In Barra Bonita reservoir, approx. 270 km downstream São Paulo city, a strong decrease in cytotoxicity was recorded followed again by an increase towards the mouth of the river. Comparing the neutral red values of all the samples, it is thus possible to recognize a slight improvement of contamination levels downstream São Paulo. On the other hand, results indicate that most of the toxicity is due to the discharges of the Metropolitan area of São Paulo. The high cytotoxic potentials near the spring and the relative increase towards the mouth of the Tietê River, however, suggest that pollutant sources other than the São Paulo Metropolitan area exist, which also contribute to the overall degradation of each reservoir.

Concerning the Ah receptor induction potential, almost all samples except for Ponte Nova stimulated EROD activity. As for cytotoxicity, the Pinheiros River showed the highest EROD induction rates, with Bio-TEQ values ca. 150 times higher than Bio-TEQs from, *e.g.*, the sample taken near the spring (reference site). Bariri and Billings reservoir samples also showed high inductions, with Bio-TEQs ca. 40 and 30 times higher than the Bio-TEQs from the reference site. Samples from the Três Irmãos, Barra Bonita and Promissão reservoirs were intermediate with Bio-TEQ values 7, 2 and 2.5 times higher than the reference site, respectively. Given the impact of discharges from the São Paulo Metropolitan area, it could be already expected that samples from Pinheiros River and Billings reservoir had higher levels of dioxin-like-toxicity when compared to the other areas. Surprisingly, however, Bariri reservoir, which is located ca. 300 km downstream from São Paulo city, presented values of Bio-TEQs comparable to and even higher than those from Billings reservoir. Bariri reservoir is located in an area characterized predominantly by agriculture activities (especially sugar cane), industries (for sugar and ethanol production) and butchery, however, with only ca. 32800 inhabitants (IBGE, 2008). The reasons for the increased contamination of Bariri reservoir remain, therefore, unknown.

To understand the relative EROD induction potentials of the different locations studied, a comparison to Bio-TEQ values obtained from well-known polluted water bodies may help: Rocha et al. (in preparation) investigating sediment contamination of European fresh water systems, recorded Bio-TEQ values of ca. 4200 pg/g in sediment samples from the significantly polluted Bílina River (Czech Republic) close to the town Jirkov. In an assessment of the potential influence of flood events on the toxicity of suspended particu-

late matter in Neckar River (Germany), Woelz *et al.* (2008) recorded a strong increase of EROD activity during a flood event in correlation with discharge and a maximum Bio-TEQ during the peak of the flood event of 8300 pg/g. Such values are comparable to the Bio-TEQ values recorded for Bariri and Billings sediment extracts, but four to five times lower than values from Pinheiros River. In this context, it should be remembered that in consequence of flood events, runoff and remobilized sediments may cause an increase of ecotoxicologically relevant effects from contaminant reservoirs (Woelz *et al.*, 2008). Carvalho *et al.* (1998) studied the impact of oxidizing effects on metal remobilization from sediments of Billings reservoir by means of a laboratory oxygenation and *Daphnia similis* toxicity test and concluded that oxygenation of highly acid productive field-collected sediments caused increased metal mobilization and toxicity. They suggested that similar responses may occur in the field after dredging activities or natural resuspension of sediments. Taking into account the findings by Carvalho *et al.* (1998) and Woelz *et al.* (2008), it may be concluded that the sediments investigated in the present study, especially those from Pinheiros, Billings and Bariri, most likely represent an important source of diffuse contamination to the entire Tietê River system.

Chemical analyses of PAHs revealed sediments from the Pinheiros River as well as Bariri and Billings reservoirs to carry by far the highest concentrations of these chemicals. The highest concentrations among all identified PAHs were quantified for fluoranthene, pyrene and phenanthrene in the samples from the Pinheiros River and Bariri reservoir. As stated by Bols *et al.* (1999) in investigations of EROD induction by PAHs, these particular substances did not induce EROD activity consistently. The EROD activity related to PAHs in all samples could be associated to 1,2-benzanthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene and benzo[a]pyrene (Bols *et al.*, 1999). Dibenz[a,h]anthracene and indeno[1,2,3-cd]pyrene, which were only found in Pinheiros River sample, could also be related to the EROD activity (Bols *et al.*, 1999).

When associating the Bio-TEQs with the PAH-TEQs and estimating the degree to which analyzed PAHs account for the EROD-inducing potencies of these environmental extracts, less than 7 % of the induction could be explained by the PAHs for all the samples. Results suggest, therefore, that non-analyzed compounds with EROD-inducing potency are also present in these sediments and account for the majority of the EROD-inducing potential; these most likely include both other priority pollutants as well as non-priority pollutants. In any case, given the presence of PAHs in the sediment, they should be considered as relevant contaminants in the Tietê and Pinheiros rivers and should be included in future monitoring and risk assess-

ment programs.

The concentrations of PAHs recorded in samples from Pinheiros River as well as Bariri and Billings reservoirs are comparable to values found in well-known polluted sediments samples from the upper Danube River, in Germany (Keiter *et al.*, 2009). However, when comparing the PAHs concentrations recorded in this study to the threshold values for PAHs stated by Ahlf *et al.* (2002) with classes from I to VI (increasing toxicity), samples from Pinheiros River and Bariri reservoir can be classified as class II, whereas all the other areas can be classified as class I. Based on this classification, these sediments would not be classified as very toxic (Ahlf *et al.*, 2002). Nevertheless, these threshold values were based on the limits for drinking water and living conditions of aquatic organisms only, but did not consider long-time effects or the fact that the NOEC data of many organisms are below targets (Ahlf *et al.*, 2002).

During the last decades, many cases of contamination not only by PAHs, but also by other organic compounds such as PCBs and organochlorine pesticides as well as heavy metals have been observed in waters in and around São Paulo city, especially in the Billings reservoir (Bainy *et al.*, 1999). Silva *et al.* (2002) studied the sediment contamination of reservoirs in the surroundings of the metropolitan area of São Paulo and concluded that the distribution of heavy metals in samples from Billings reservoir indicates recent and intense pollution loads. In the same study, Silva *et al.* (2002) found out that in sediment samples from Barra Bonita reservoir the total heavy metal contents were also high, which could be explained by the transport from upriver areas via fine particles or from diffuse pollution sources. In an assessment of the concentrations of bioavailable metals in the Tietê River cascade reservoirs, Fracácio *et al.* (2003) found that cadmium, lead, cobalt, copper, chromium, iron, magnesium, manganese and zinc were present along all the reservoirs. Although their concentrations decreased downstream, some metal concentrations, *e.g.,* those for cadmium, were also relatively high in the last reservoirs. These results confirmed that, although the evaluated parameters in the sediments showed an improvement of the environmental quality towards Três Irmãos reservoir, punctual and diffuse pollution also occur separately around each water body, thus contributing to the degradation of these environments.

In a recent assessment of genotoxicity of the sediment samples investigated in the present study, Rocha *et al.* (2009) documented a strong increase in genotoxicity from Tietê River's spring to the São Paulo city region (especially in Billings extracts), and a decrease further downstream, again suggesting an improvement of these environments in direction to the mouth of the river. In a comparison with previous studies in the genotoxicity of European fresh water sediments (Rocha *et al.*, 2009), sediments from Billings

reservoir were rated as strongly genotoxic, while those from Ponte Nova and Promissão were classified as moderately genotoxic and those from Bariri and Três Irmãos were recorded as less genotoxic (Rocha *et al.*, 2009).

As exemplified for the Tietê River basin, the bioassays applied in this study in combination with chemical analyses represent suitable tools to function as early warning systems not only for sediment pollution, but also for the entire river system. However, a comprehensive evaluation of the ecotoxicological situation of sediments requires a variety of measured parameters, including more bioassays with other mechanism-specific effects and additional statistical evaluation.

## 3.5 Conclusion

Almost all tested sediments induced cytotoxicity and stimulated cytochrome P450-associated EROD activity. An increase of toxicity was recorded from Tietê River spring to São Paulo city region and a decrease from São Paulo city to downstream direction. Concentrations of PAHs in the sediments were high and comparable to values found in well-known polluted European fresh water sediments. A closer analysis of chemical measurements of PAHs and results from bioassay revealed that these PAHs could not explain more than 7 % of the EROD-inducing potencies, suggesting that EROD activity is related to non-analyzed compounds.

Results of bioassays and chemical analyses suggest that most of the toxicity is due to the discharges of the metropolitan area of São Paulo, but both indicate that pollutants sources occur differently in the whole river course, contributing to the degradation of each reservoir. These results support conclusions obtained from several studies of Tietê River basin based on other toxicity parameters (Bainy *et al.*, 1999; Fracácio *et al.*, 2003; Rocha *et al.*, 2009; Silva *et al.*, 2002).

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# Bibliography


Ahlf W, Braunbeck T, Heise S, Hollert H (2000) *Environmental Monitoring Handbook*, chap. Sediment and soil quality criteria, 1–18. 17, McGraw-Hill, New York

Ahlf W, Hollert H, Neumann-Hensel H, Ricking M (2002) A guidance for the assessment and evaluation of sediment quality: A German approach based on ecotoxicological and chemical measurements. *Journal of Soils and Sediments* 2: 37–42

Babich H, Borenfreund E (1991) Cytotoxicity of T-2 toxin and its metabolites determined with the Neutral red cell viability assay. *Applied and Environmental Microbiology* 57: 2101–2103

Babin M, Casado S, Chana A, Herradon B, Segner H, Tarazona J, Navas J (2005) Cytochrome P4501A induction caused by the imidazole derivative Prochloraz in a rainbow trout cell line. *Toxicology in Vitro* 19: 899–902

Bainy ACD, Bruce RW, John JS (1999) Elevated levels of multiple cytochrome P450 forms in tilapia from Billings Reservoir - Sao Paulo, Brazil. *Aquatic Toxicology* 44: 289–305

Baudo R, Muntau H (1990) *Sediments: chemistry and toxicity of in-place pollutants*, chap. Lesser know in-place pollutants and diffuse source problems, 1–14. 1, Boca Raton, Lewis Publishers, Inc.

Behrens A, Schirmer K, Bols NC, Segner H (1998) Microassay for rapid measurement of 7-ethoxyresorufin-o-deethylase activity in intact fish hepatocytes. *Marine Environmental Research* 46: 369–373

Behrens A, Schirmer K, Bols NC, Segner H (2001) Polyciclic aromatic hydrocarbons as inducers of cytochrome P4501A enzyme activity in the rainbow trout liver cell line, RTL-W1 and in primary cultures of rainbow trout hepatocytes. *Environmental Toxicology and Chemistry* 20: 632–643

Berbner T, Rahman N, Braunbeck T (1999) Induction of cytochrome P450 1A and DNA damage in isolated rainbow trout (*Oncorhynchus mykiss*) hepatocytes by 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin. *Biomarkers* 4: 214–228

Billiard S, Bols N, Hodson P (2004) (*In vitro* and (*in vivo* comparisons of fish-specific CYP1A induction relative potency factors for selected polycyclic aromatic hydrocarbons. *Ecotoxicology and Environmental Safety* 59: 292–299

Bols N, Schirmer K, Joyce E, Dixon D, Greenberg B, Whyte J (1999) Ability of Polycyclic Aromatic Hydrocarbons to Induce 7-Ethoxyresorufin- *O*-deethylase Activity in a trout liver cell line. *Ecotoxicology and Environmental Safety* 44: 118–128

Borenfreund E, Puerner JA (1984) A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90). *Journal of Tissue Culture Methods* 9: 7–9

Böttcher M, Grund S, Keiter S, Kosmehl T, Manz W, Seitz N, Rocha PS, Hollert H, Braunbeck T (in press) Comparison of (*in vitro* and (*in situ* genotoxicity in the Danube River by means of the comet assay and the micronucleus test. *Mutation Research*

Brack W, Segner H, Moeder M, Schueuerman G (2000) Fixed-effect-level toxicity equivalents - a suitable parameter for assessing ethoxyresorufin-*O*-deethylase induction potency in complex environmetal samples. *Environmental Toxicology and Chemistry* 19: 2493–2501

Brunstroem B, Halldin K (1998) EROD induction by environmental contaminants in avian embryo livers. *Comparative Biochemistry and Physiology* 121: 213–219

Bucheli TD, Fent K (1995) Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical reviews in environmental science and technology* 25: 201–268

Burton GA (1991) Assessing the toxicity of freshwater sediments. *Environmental Toxicology and Chemistry* 10: 1585–1627

Carvalho PSM, Zanardi E, Buratini SV, Lamparelli MC, Martins MC (1998) Oxidizing effect on metal remobilization and *Daphnia similis* toxicity from a Brazilian reservoir sediment suspension. *Water Research* 32: 193–199

CETESB (1997) Relatório da Qualidade das Águas Interiores do Estado de São Paulo - 1996. Tech. rep., CETESB

Chen G, White PA (2004) The mutagenic hazards of aquatic sediments: a review. *Mutation Research* 567: 151–225

Clemons J, Myers C, Lee L, Dixon D, Bols N (1998) Induction of cytochrome P4501A by binary mixtures of polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in liver cell lines from rat and trout. *Aquatic Toxicology* 43: 179–194

CONSEMA (1993) CONSEMA: dez anos de atividades. São Paulo: Secretaria do Meio Ambiente. São Paulo, Brasil

Engwall M, Broman D, Ishaq R, Näf C, Zebühr Y, Brunström B (1996) Toxic potencies of lipophilic extracts from sediments and settling particulate matter (SPM) collect in a PCB contaminated river system. *Environmental Toxicology and Chemistry* 15: 213–222

Engwall M, Brunström B, Näf C, Hjelm K (1999) Levels of dioxin-like compounds in

sewage sludge determined with a bioassay based on EROD induction in chicken embryo liver cultures. *Chemosphere* 38: 2327–2343

Fracácio R, Verani NF, Espíndola ELG, Rocha O, Rigolin-Sá O, Andrade CA (2003) Alterations on growth and gill morphology of *Danio rerio* (Pisces, Ciprinidae) exposed to the toxic sediments. *Brazilian Archives of Biology and Technology* 46: 685–695

Gustavson LK, Klee N, Olsman H, Hollert H, Engwall M (2004) Fate of Ah receptor agonistis during biological treatment of an industrial sludge containing explosives and pharmaceutical residues. *Environmental Science and Pollution Research* 11: 379–387

Hilscherova K, Kannan K, Kang YS, Holoubeck I, Machala M, Masunaga S, Nakanishi J, Giesy JP (2001) Characterization of Dioxin-like activity of sediments from a Czech river basin. *Environmental Toxicology and Chemistry* 20: 2768–2777

Hollert H, Dürr M, Erdinger L, Braunbeck T (2000) Cytotoxicity of settling particulate matter (SPM) and sediments of the Neckar river (Germany) during a winter flood. *Environmental Toxicology and Chemistry* 19: 528–534

Hollert H, Keiter S, König N, Rudolf M, Ulrich M, Braunbeck T (2003) A New Sediment Contact Assay to Assess Particle-bound Pollutants Using Zebrafish (*Danio rerio*) Embryos. *Journal of Soils and Sediments* 3: 197–207

Hollert H, Dürr M, Holtey-Weber R, Islinger M, Brack W, Färber H, Erdinger L, Braunbeck T (2005) Endocrine disruption of water and sediment extracts in a non-radioactive dot blot/RNAse protection-assay using isolated hepatocytes of rainbow trout - How explain deficiencies between bioanalytical effectiveness and chemically determined concentrations? *Environmental Science and Pollution Research* 12: 347 – 360

Hollert M Hand Duerr, Olsman H, Halldin K, Bavel Bv, Brack W, Tysklind M, Engwall M, Braunbeck T (2002) Biological and chemical determination of dioxin-like compounds in sediments by means of a sediment triad approach in the catchment area of the Neckar River. *Ecotoxicology* 11: 323–336

IBGE (2008) Instituto Brasileiro de Geografia e Estatística (IBGE) - Estimativas da população para 1º de julho de 2008

Keiter S, Rastall A, Kosmehl T, Erdinger L, Braunbeck T, Hollert H (2006) Ecotoxicological Assessment of Sediment, Suspended Matter and Water Samples in the Upper Danube River. A pilot study in search for the causes for the decline of fish catches. *Environmental Science and Pollution Research* 13: 308–319

Keiter S, Braunbeck T, Heise S, Pudenz S, Manz W, Hollert H (2009) A fuzzy logic-classification of sediments based on data from *in vitro* biotests. *Journal of Soils and Sediments* 9: 168–179

Klee N, Gustavsson LK, Kosmehl T, Engwall M, Erdinger L, Braunbeck T, Hollert H (2004) Changes in toxicity and genotoxicity of industrial sewage sludge samples containing nitro and amino-aromatic compounds following treatment in biore-

actors with different oxygen regimes. *Environmental Science and Pollution Research* 11: 313–320

Kosmehl T, Krebs W Fand Manz, Erdinger L, Braunbeck H Tand Hollert (2004) Comparative genotoxicity testing of Rhine river sediment extracts using the permanent cell lines RTG-2 and RTL-W1 in the comet assay and Ames assay. *Journal of Soils and Sediments* 4: 84–94

Lee LE, Clemons JH, Bechtel DG, Caldwell SJ, Han KB, Pasitschniak-Arts M, Mosser D, Bols NC (1993) Development and characterization of a rainbow trout liver cell line expressing cytochrome P450-dependent monooxygenase activity. *Cellular Biology and Toxicology* 9: 279–294

Lorenzen A, Kennedy S (1993) A fluorescence-based protein assay for use with a microplate reader. *Analytical biochemistry* 214: 346–348

Maier M, Kuehlers D, Brauch HJ, Fleig M, Maier D, Jirka GH, Mohrlok E Uand Bethge, Bernhart HH, Lehmann B, Hillebrand G, Woelz J, Hollert H (2006) Flood retention and drinking water supply - Preventing conflicts of interest. *Journal of Soils and Sediments* 6: 113–114

Okey AB, Riddick DS, Harper PA (1994) The Ah receptor: Mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *Toxicology Letters* 70: 1–22

Rocha PS, Keiter S, Pompêo MLM, Mariani CF, Brandimarte AL, Seiler TB, Kosmehl T, Böttcher M, Wölz J, Braunbeck T, Storch V, Hollert H (2006) Weight-of-Evidence-Studie zur Sedimentbelastung des Tietê River in Brasilien. *Umweltwissenschaften und Schadstoff-Forschung* 18: 70

Rocha PS, Luvizotto GL, Kosmehl T, Böttcher M, Storch V, Braunbeck T, Hollert H (2009) Sediment genotoxicity in the Tietê River (São Paulo, Brazil): *in vitro* comet assay *versus in situ* micronucleus assay studies. *Ecotoxicology and Enviromental Safety* 72: 1842–1848

Schirmer K, Chan AGJ, Bols NC (2000) Transitory metabolic disruption and cytotoxicity elicited by Benzo[*a*]pyrene in two cell lines from rainbow trout liver. *Journal of Biochemical and Molecular Toxicology* 14: 262–276

Schirmer K, Dayeh V, Bopp S, Russold S, Bols N (2004) Applying whole water samples to cell bioassays for detecting dioxin-like compounds at contaminated sites. *Toxicology* 205: 211–221

Segner H, Behrens A, Joyce E, Schirmer K, Bols N (2000) Transient induction of 7-ethoxyresorufin-O-deethylase (EROD) activity by medium change in the rainbow trout liver cell line, RTL-W1. *Marine Environmental Research* 50: 489–493

Seiler TB, Rastall AC, Leist E, Erdinger L, Braunbeck T, Hollert H (2006) Membrane dialysis extraction (MDE): A novel approach for extracting toxicologically relevant hydrophobic organic compounds from soils and sediments for assessment in biotests. *Journal of Soils and Sediments* 6: 20–29

Silva IS, Abate G, Lichtig J, Masini JC (2002) Heavy metal distribution in recent sediments of the Tiete-Pinheiros river system in Sao Paulo state, Brazil. *Applied*

*Geochemistry* 17: 105–116

Strmac M, Braunbeck T (2000) Isolated hepatocytes of rainbow trout (*Oncorhynchus mykiss*) as a tool to discriminate between differently contaminated small river systems. *Toxicology in Vitro* 14: 361–377

Vasseur P, Cossu-Leguile C (2003) Biomarkers and community indices as complementary tools for environmental safety. *Environment International* 28: 711–717

Woelz J, Engwall M, Maletz S, Olsman Takner H, van Bavel B, Kammann U, Klempt M, Weber R, Braunbeck T, Hollert H (2008) Changes in toxicity and Ah receptor agonist activity of suspended particulate matter during flood events at the rivers Neckar and Rhine - a mass balance approach using *in vitro* methods and chemical analysis. *Environmental Science and Pollution Research* 15: 536–553

# Chapter 4

# Sediment-Contact Fish embryo toxicity assay with *Danio rerio* to Assess Particle-bound pollutants in Tietê River basin

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## Abstract

The sediment contact fish embryo test with *Danio rerio* embryos was applied in this study to detect embryotoxicity of sediments from Tietê River Basin (São Paulo state, Brazil). Differences in embryotoxicity could be recorded in the whole river course, and high embryotoxicity could be found in samples near the megacity São Paulo (Billings and Pinheiros samples), but also downstream (Barra Bonita, Promissão and Três Irmãos). Results confirm that most of the toxicity is due to the discharges of the metropolitan area of São Paulo. Moreover, they indicate additional sources of pollutants along the river course, which contribute to the degradation of each area. The sediment contact fish embryo test showed to be powerful tool to detect embryotoxicity in sediments, not only by being a sensitive method, but also for taking into account bioavailability. This test provides an ecological highly realistic and relevant exposure scenario, and should therefore be add in ecotoxicological sediment quality assessments.

## 4.1 Introduction

Contaminated sediments have been recognized not only as a major sink for persistent toxic substances released into the aquatic environment, but also as a potential source, as pollutants may be made available under certain environmental conditions (such as dredging or flood events). These pollutants are not only linked to organisms in aquatic ecosystems, but also to human health through drinking water and fish consumption(Chen & White, 2004; Hollert *et al.*, 2005; Keiter *et al.*, 2006; Maier *et al.*, 2006).

In conventional ecotoxicity testing strategies, fish are an indispensable component of integrated toxicity testing strategies for the aquatic environment (Lammer *et al.*, 2009a). The use of fish as bioindicators of not only water, but also sediment quality assessment provides specific advantages, when compared to other in vivo tests, because fish are especially sensitive to impacts on the aquatic environment and they respond to toxic agents similarly to higher vertebrates including mammals, thus allowing an evaluation of the teratogenic, mutagenic and carcinogenic potentials not only to fish, but also to humans (Lemos *et al.*, 2007).

Fish acute toxicity tests thus play an important role in environmental risk assessment and hazard classification. However, in acute tests with their exclusive endpoint of mortality, fish have been hypothesized to suffer severe distress and pain(Braunbeck *et al.*, 2005; Braunbeck & Lammer, 2006; Chandroo *et al.*, 2004; Nagel, 2002), which would be in conflict with current animal welfare legislations at least in many European countries (Lammer *et al.*, 2009a). Thus, there is an urgent need for the replacement or reduction of *in vivo* tests with adult fish by *in vitro* tests such as cytotoxicity tests, but also tests with early developmental stages of embryos, since these are also not regarded as experimental animals .

Permanent cell lines derived from fish (*e.g.*, RTL-W1 or RTG-2 derived from Oncorhynchus mykiss) have successfully been used in several *in vitro* assays to assess different ecotoxicological endpoints such as cytotoxicity, genotoxicity, dioxin-like activity (Hollert *et al.*, 2000; Kosmehl *et al.*, 2004; Schirmer *et al.*, 2004). Alternatively, fish embryo toxicity tests have also become a promising tool to replace the acute fish test (Braunbeck *et al.*, 2005; Nagel, 2002). Several toxicological studies comparing different life-stages of fish concluded that in most cases long-term toxicity could be extrapolated from results from studies with early life-stages (Chorus, 1987; Mckim, 1977; Woltering, 1984).

In Europe, the use of fish embryos is not regulated by current legislations on animal welfare and is, therefore, considered as a refinement, if not replacement of animal experiments. Fish embryos represent an attractive model for

environmental risk assessment of chemicals, since they offer the possibility to perform small-scale, high-throughput analyses with excellent correlation to conventional in vivo testing with adult fish (Lammer *et al.*, 2009b). Beyond their application for determining the acute toxicity, fish embryos are also excellent models for studies aimed at the understanding of toxic mechanisms and the indication of possible adverse long-term effects (Scholz *et al.*, 2008). Finally, due to its sensitivity, reproducibility and adaptability, the embryo assay test found its way into the laboratories not only for testing chemicals, but also for investigations into environmental samples, *e.g.*, sediments or particulate matters (Hallare *et al.*, 2005b; Hollert *et al.*, 2003; Ulrich *et al.*, 2002).

The present study is part of a weight-of-evidence study aiming at identifying hazard factors and ecotoxicological risks of sediments in the Tietê River Basin(Rocha *et al.*, 2006, 2009b,a).The Tietê River was selected as an example for a contaminated river system. This river and its tributary, the Pinheiros River (São Paulo State, Brazil), are located in the Tietê River Basin and receive a highly complex pollutant load of organic pollutants due to the lack of appropriate treatment of sanitary sewage and industrial effluents in the metropolitan region of São Paulo, as well as numerous inorganic substances from industrial sources. Moreover, along the entire course of the Tietê River, it continues to receive considerable pollutant loads from domestic sewers, agricultural and agroindustrial activities (Calijuri, 1999; CETESB, 1997; Soares & Mozeto, 2006)

The Tietê River comprises several reservoirs in its course, which are widely used for providing drinking water, as a water source for agricultural irrigation purposes and as recreation sites. High mutagenic, genotoxic, as well as aryl hydrocarbon receptor (AhR)-mediated toxicity were recorded in some of these reservoirs with a good correlation between *in situ/in vivo* and *in vitro* assays (Rocha *et al.*, 2009b,a), indicating the high ecological relevance of the *in vitro* assays for these endpoints. However, it is well-know that organic extraction of river sediments usually leads to the transfer of the full spectrum of chemicals adsorbed to the sediment to the dissolved phase and, thus, provide estimations of the total hazard potential, but neglect the bioavailability of sediment contaminants (Fent, 2004; Liß & Ahlf, 1997; Seiler *et al.*, 2006; Wang *et al.*, 2004). Therefore, there is still a need to develop more realistic, field-like exposure scenarios for sediment quality assessment, taking into account both bioavailability and intact organisms in order to improve transferability of results and to better understand fate and behavior of water- and sediment-bound toxicants relevant for toxicity. Thus, in order to simulate *in situ* exposure conditions in a more realistic scenario (Feiler *et al.*, 2005; Triebskorn *et al.*, 1997), a recently developed sediment contact

fish embryo test (Hollert *et al.*, 2003) was applied to sediments collected from locations along the Tietê River, which had proved to be differentially contaminated in previous studies (Rocha *et al.*, 2009a,b).

## 4.2 Materials and methods

### 4.2.1 Sediment sampling

The study area comprised a location in Salesópolis near the Tietê River's spring (considered as a reference site), the reservoirs: Ponte Nova and Billings (Upper Tietê); Barra Bonita (Superior Middle Tietê), Bariri and Promissão (Inferior Middle Tietê), Três Irmãos (Lower Tietê), and the Pinheiros River as a tributary to the Upper Tietê River (Fig. 4.1). The Upper Tietê River basin corresponds to the drained area of the Tietê River from its spring across the metropolitan area of São Paulo city, which is characterized by high densities of anthropogenic agglomerations and dramatic deterioration subsequent to intensive urbanization. The Superior Middle Tietê River basin is dominated by urban, industrial and agricultural areas. The Inferior Middle Tietê River and the Low Tietê basins are characterized predominantly by agricultural areas and pastures and sugar cane culture, respectively (CETESB, 1997). From Barra Bonita to Três Irmãos, the reservoirs were built in cascade arrangements for the generation of electricity.

Surface sediments were collected in May and December 2005 in the Tietê River reservoirs, and in December 2006 in the Pinheiros River, using an Eckman-Birge dredge, with ten replicates at each site (with a distance of 10 m from sample to sample). Replicates were homogenized, and 1.5 kg of each sediment sample were frozen immediately, stored at -10 °C and transported to Germany. Transfer of the samples was permitted by the Brazilian National Department of Mineral Production (DNPM).

Native sediment samples placed each in 500 ml round bottom flasks (Schott, Mainz, Germany) were shock-frozen -30°C over approximately 15 minutes under rotation in an isopropanol bath (N6, C41, Haake, Karlsruhe, Germany) to favor the freeze-drying process. Samples were then freeze-dried in the freeze-drier (Alfa 1-4, Christ, Osterode, Germany) with a negative pressure of approximately -1.4 mbar for 72 hours and sieved with a 1.25 mm mesh sieve (Haver and Boecker, Oelde, Germany) to remove small pieces of vegetation and stones. Samples were then stored at 4°C in brown glass bottles.

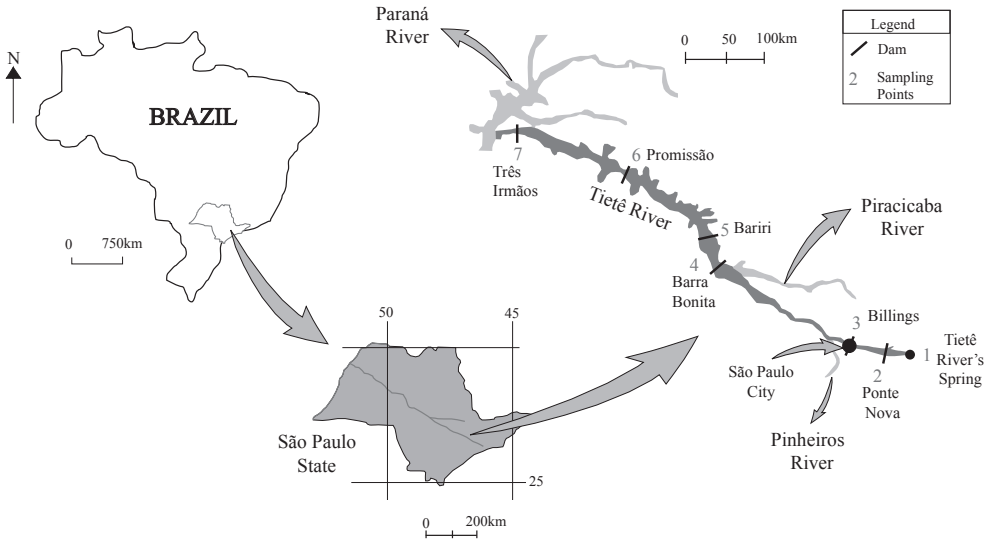



**Fig. 4.1.** Location of the sampling sites in the Tietê River basin, São Paulo, Brazil (modified after Rocha et al 2009a).

### 4.2.2 Oxygen measurement

As a first approach, the oxygen levels were measured in the highest concentrations of selected samples from Promissão, Billings and Barra Bonita to assure that lack of oxygen would not interact with toxic effects in the embryos. Oxygen sensors were delivered from Precision Sensing (Regensburg, Germany). The measuring principle is based on the effect of luminescence quenching by molecular oxygen and consume no oxygen itself in contrast to common sensors (for information on sensor principles see Holst *et al.*, 1997; Klimant & Wolfbeis, 1995; Klimant *et al.*, 1999). The oxygen measurements were performed using the Oxy-4 micro instrument with needle-type sensorsand were carried out immediately (0 to 250 µm) above the respective sediment surface. Mean values of oxygen concentration were calculated from 10 independent measurements with an interval of 2 seconds between measurements at a constant temperature of 25°C.

### 4.2.3 Sediment contact assay with *D. rerio*

Sexually mature D. rerio were obtained from the stocks at at the Department of Zoology, University of Heidelberg, Germany, according to Lammer *et al.* (2009a).

Sediment samples were tested in 6 well plates in serial dilutions of 1:1 to 1:32 in quartz powder (grain size W4; Quartzwerke, Frechen, Germany) and artificial water (ISO7346/3; Table 4.1) according to the protocol of Hollert *et al.* (2003). In brief, the mixtures sediment/quartz were homogenized in a mortar to avoid sediment or quartz powder hot spots. The mixtures were then placed into the well plates, and 5 ml of artificial water, which had been ventilated to oxygen saturation for at least 24 h, was added to each well. As negative controls, 3 g of quartz powder in 5 ml artificial water, as well as only 5 ml of artificial water were used. As positive controls, 3 g of artificial sediment filled up with 5 ml of a 3.7µg/ml 3,4-dichloraniline (3,4-DCA, Fluka, Munich, Germany) solution were used.

**Table 4.1** Concentrations of sediment samples diluted in quartz powder and artificial water.

| Dilution | 1:1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 |
|---|---|---|---|---|---|---|
| Dried sediment (g) | 3 | 1.5 | 0.75 | 0.375 | 0.1875 | 0.09375 |
| Quartz powder (g) | 0 | 1.5 | 2.25 | 2.625 | 2.8125 | 2.90625 |
| Concentrations[a] | 600 | 300 | 150 | 0.75 | 18.75 | 3.125 |

[a] (mg/ml artificial water)

The plates were stored in an incubator at 27°C for 72 h to allow sedimentation and oxygen exchange between sediment samples and water (see sections 4.3.1, 4.2.3 and 4.3.2).

After this period, exposure was started by gentle addition of zebrafish eggs. Three independent assays were run for each sample. Embryo tests were initiated at latest 3h after fertilization of the eggs (ca. 128 cell stage). Five fertilized eggs were placed in each well filled with the samples, giving a total of 10 eggs for each sediment concentration, and incubated at 27 ± 0.5 °C.

To assess the toxic effects, eggs were transfered into another 6-well plates filled with artificial water, and observed under inverted microscope (CK-2 equipped with an SC-35 camera, Olympus, Hamburg, FRG). Toxicological endpoints were recorded after 48 and 96 h, and lethal and non-lethal effects were estimated according to DIN (2001; Table 4.2). Lethal effects were expressed as $LC_{50}$ values (lethal concentration inducing 50% of mortality) and sublethal effects as $EC_{50}$ values (effective concentration inducing 50% effects)

calculated using non-linear regression analyses according to the endpoints after 48 and 96h of exposition, using Prism 4.0 (GraphPad, San Diego, USA).

**Table 4.2** Lethal and sublethal effects used in this study for evaluating the toxicological effects of freeze-dried sediment samples from Tietê River Basin in *Danio rerio* embryos (lethal effects according to DIN, 2001)

| Lethal effects | Sublethal effects |
|---|---|
| Coagulation of the embryo | Lack of blood circulation |
| Lack of somite formation | Edema formation |
| Non-detachment of tail | Developmental retardation |
| Lack of heart function | Malformation[a] |

[a]teratogenic endpoint

**Exposition of embryos to sediments with and without incubation of 72 h**

According to Strecker (2008), one possibility to solve (or at least ease) the problems with oxygen depletion in the sediment contact embryo assay is to incubate the mixture of sediment and artificial water (saturated in oxygen) for 72h prior to exposure of the embryos, in order to allow complete oxygen exchanges between sediment and water. Thus, in order to discriminate between embryotoxic effects by oxygen depletion from effects by chemicals or to the sample itself, the fish embryo test was carried out after 1h and 72h of equilibration of the sediments for four samples (Billings, Pinheiros, Barra Bonita and Promissão.

## 4.3 Results

### 4.3.1 Oxygen measurement

Oxygen levels were measured after 1h and 96h of incubation with sediments. With all sediments, oxygen concentrations were very low after 1h sedimentation (Promissão = 0.02 mg/L, Billings = 0.5 mg/L and Barra Bonita 1.2 mg/L), but increased after 96h (Promissão = 0.9 mg/L, Billings = 1.4 mg/L and Barra Bonita 1.5 mg/L).

### 4.3.2 Sediment contact assay with *Danio rerio*

#### Comparison of effects of sediments with and without pre-incubation

Comparing exposure of the embryos to selected samples with and without sample pre-incubation, effects we could clearly lower or even absent in embryos exposed to the samples pre-incubated for 72h (Fig. 4.2). Mortality rates of embryos exposed to Billings samples, *e.g.*, changed from 100 % in all concentrations without pre-incubation to 100% only in concentrations 300 to 600 mg/ml after pre-incubation for 72 h. A significant decrease in mortality rates could also be observed for the other samples (Pinheiros, Promissão and Barra Bonita). Therefore, all samples from the Tietê River basin were pre-incubated with artificial water for 72h prior to addition of the zebrafish eggs (*cf.* also for Strecker, 2008).

#### Embryotoxicity of the samples

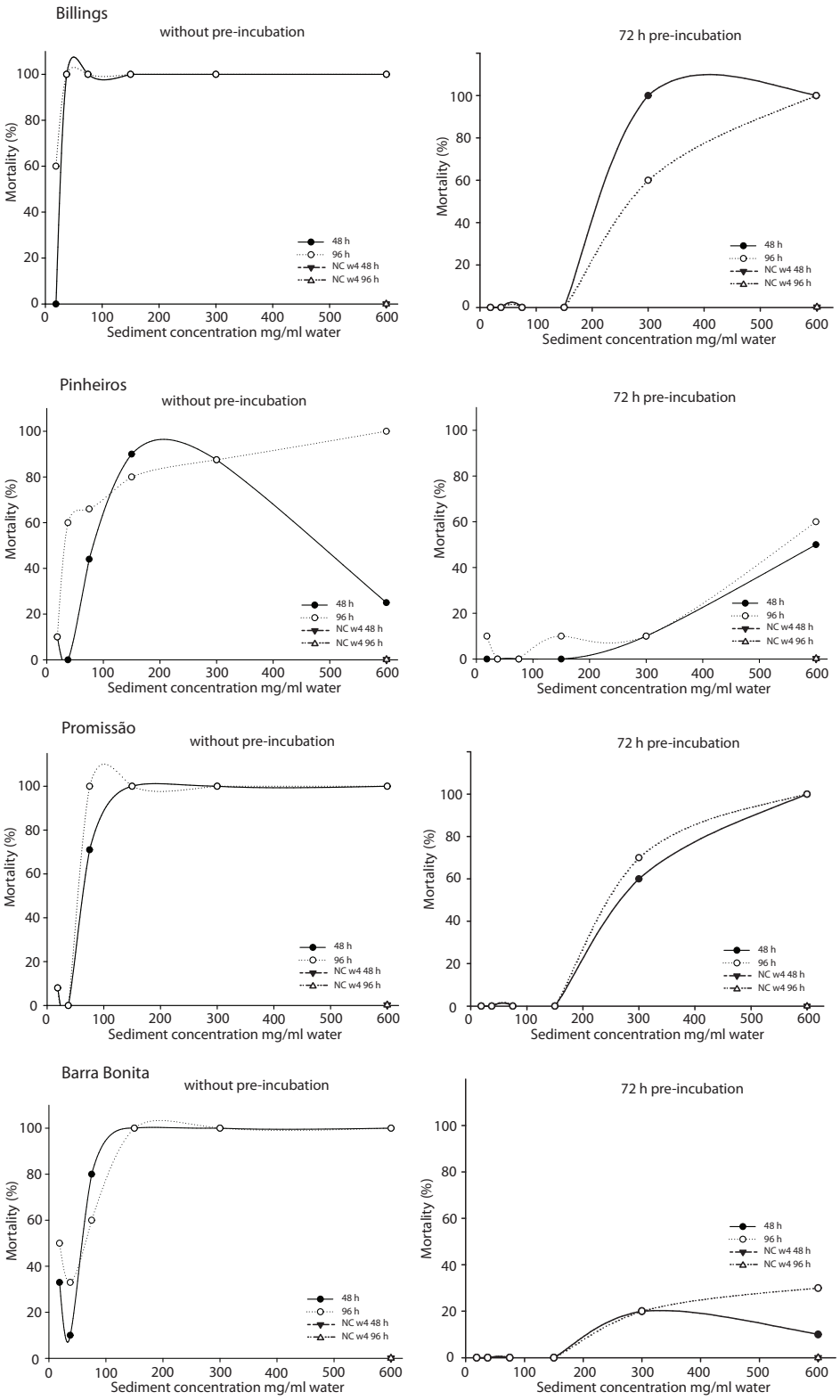
For all native sediment tests, all three independent investigations were considered valid, since mortalities in the negative controls were $< 10$ %, and since the positive controls produced $> 10$ % mortality (according to DIN, 2001 and ISO 15088).

Lethal and non-lethal recorded effects are represented in Fig. 4.3, by mean values of percentage of effects in all three replicates, including $LC_{50}$ and $EC_{50}$ values.

For the sediment samples collected near the spring, very slight edemata were observed in part of the embryos after 48h of exposure to all concentrations except for the lowest one. Since this effect could only be seen in less than 40 % of the embryos, only an effective concentration inducing 25% of effects could be calculated for these samples as $EC_{25} = 57$ mg/ml. In fact, after 96h of exposure, these embryos had recovered, and only a small developmental retardation of less than 2h could be observed, if compared to negative controls. No lethal effects were recorded in embryos exposed to this sample.

After 48h exposure to the sediment samples taken at Ponte Nova, some embryos showed a lack of heart function and blood circulation at all concentrations, but an $LC_{50}$ value could not be calculated, since mortality was $< 50$

**Fig. 4.2.** ***(following page)*** Relative mortality (lethal effects according to DIN, 2001) of *Danio rerio* in the sediment contact assay after exposure for 48 and 96 h to sediments with and without pre-incubation over 72 h. NC w4 = negative control in quartz powder (600 mg/ml artificial water).


Billings
without pre-incubation
72 h pre-incubation
Pinheiros
without pre-incubation
72 h pre-incubation
Promissão
without pre-incubation
72 h pre-incubation
Barra Bonita
without pre-incubation
72 h pre-incubation
Mortality (%)
Sediment concentration mg/ml water
48 h
96 h
NC w4 48 h
NC w4 96 h

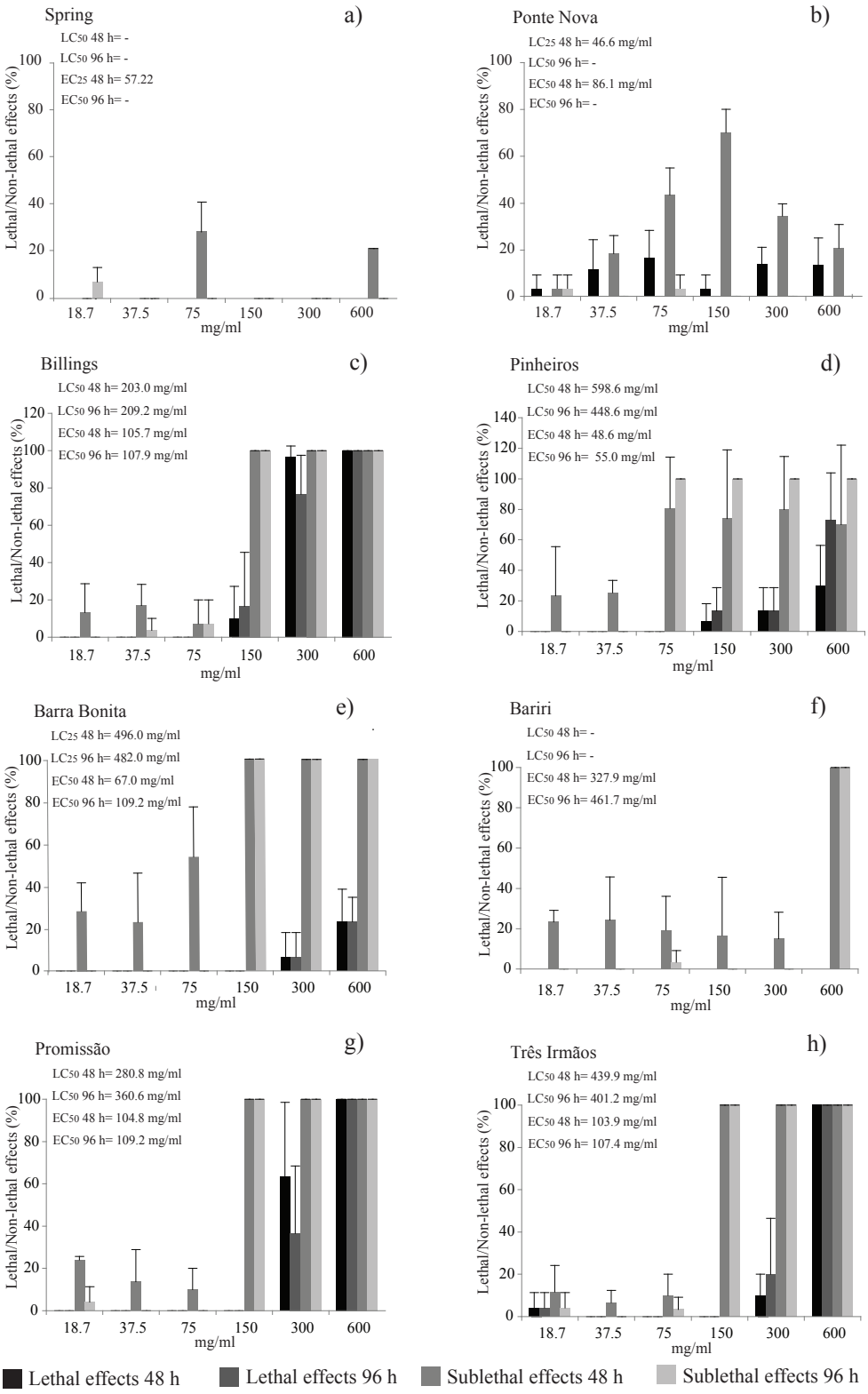
%. Therefore, an $LC_{25}$ as the lethal concentration inducing 25% mortality in the embryos was calculated at 46 mg/ml. In addition, slight edemata were recorded at sediment concentrations between 37.5 mg/ml and 600 mg/ml ($EC_{50}$= 86 mg/ml). No toxic effects were recorded after 96 h.

After 48h exposure to the Billings samples, all eggs were coagulated at the highest sediment concentration. Coagulated eggs were also recorded in concentrations from 75 to 300 mg/ml, but at lower percentages. Exposure to 300 mg/ml also induced a lack of somite formation, non-detachment of the tail, lack of heart function and blood circulation ($LC_{50}$ = 203 mg/ml) as well as developmental retardation, which could be also observed in 150 mg/ml. Edemata were observed at concentrations from 18.75 to 150 mg/ml ($EC_{50}$ = 105 mg/ml). After 96 h of exposure, lethal effects were less prominent even at a concentration of 300 mg/ml, but embryos still showed developmental retardation and lack of blood circulation (Fig. 4.4); almost all of them failed to hatch ($LC_{50}$ = 209 mg/ml, $EC_{50}$ = 110 mg/ml).

The sediments collected at Pinheiros induced coagulation at concentrations of 150 to 600 mg/ml after 48h already. In addition, some embryos lacked somites and heart function or the tail was not detached at the highest concentration ($LC_{50}$ = 600 mg/ml). In all other embryos exposed to > 37.5 mg/ml sediments, development showed retardation between 6 and 35 hours retardation. Some malformation was recorded already at concentrations of 75 mg/ml (Fig. 4.4), and edemata were recorded at all concentrations ($EC_{50}$ = 49 mg/ml). After 96 h, all sublethal effects could still be observed, and developmental retardation could be seen in all surviving embryos between 150 and 600 mg/ml and (at a lower frequency) at 75 mg/ml. In contrast, edemata could only be recorded at a lower percentage at 600 and 150 mg/ml ($EC_{50}$ = 55 mg/ml).

After both 48 and 96h of exposure, Barra Bonita sediment samples only produced low frequencies of lethal effects (coagulation, lack of somites, lack of heart function and non-detached tail) at the two highest concentrations

**Fig. 4.3.** ***(following page)*** Lethal and sublethal effects recorded after 48 and 96h of embryo exposure to freeze-dried sediment samples from Tietê River basin. Percentages of mortality and sublethal effects as well $LC_{50}$ and $EC_{50}$) data for lethal and sublethal effects, respectively, were calculated as means from 3 independent replicates. For samples Ponte Nova and Barra Bonita, $LC_{50}$ values could not be calculated, since effect levels did not reach 50 % of the embryos. For these samples, $LC_{50}$ values were extrapolated. For the sample taken from near the spring, $EC_{25}$ rather than $EC_{50}$ values are given, since sublethal effects were found in less than 50% of the individuals. Figs. 4.3a to 4.3h are arranged from left to right and from top to down according to the flow direction of the river.


Spring
a)
LC50 48 h= -
LC50 96 h= -
EC25 48 h= 57.22
EC50 96 h= -
Ponte Nova
b)
LC25 48 h= 46.6 mg/ml
LC50 96 h= -
EC50 48 h= 86.1 mg/ml
EC50 96 h= -
Billings
c)
LC50 48 h= 203.0 mg/ml
LC50 96 h= 209.2 mg/ml
EC50 48 h= 105.7 mg/ml
EC50 96 h= 107.9 mg/ml
Pinheiros
d)
LC50 48 h= 598.6 mg/ml
LC50 96 h= 448.6 mg/ml
EC50 48 h= 48.6 mg/ml
EC50 96 h= 55.0 mg/ml
Barra Bonita
e)
LC25 48 h= 496.0 mg/ml
LC25 96 h= 482.0 mg/ml
EC50 48 h= 67.0 mg/ml
EC50 96 h= 109.2 mg/ml
Bariri
f)
LC50 48 h= -
LC50 96 h= -
EC50 48 h= 327.9 mg/ml
EC50 96 h= 461.7 mg/ml
Promissão
g)
LC50 48 h= 280.8 mg/ml
LC50 96 h= 360.6 mg/ml
EC50 48 h= 104.8 mg/ml
EC50 96 h= 109.2 mg/ml
Três Irmãos
h)
LC50 48 h= 439.9 mg/ml
LC50 96 h= 401.2 mg/ml
EC50 48 h= 103.9 mg/ml
EC50 96 h= 107.4 mg/ml
Lethal/Non-lethal effects (%)
mg/ml
18.7
37.5
75
150
300
600
Lethal effects 48 h
Lethal effects 96 h
Sublethal effects 48 h
Sublethal effects 96 h

($LC_{25}$ = 496 and 482 mg/ml for 48 and 96h, respectively). After 48 h, developmental retardation and edemata were recorded at all concentrations ($EC_{50}$ = 67 mg/ml); after 96h, these were restricted to the two highest concentrations. With a delay to controls of up to 48 h, developmental retardation was particularly prominent at the highest concentration; most of these embryos did not hatch.

Only after 48 and 96h exposure to the highest concentration of Bariri sediments, embryos showed very minor developmental retardation. Lethal effects were not recorded.

Following exposure to the highest concentration of Promissão sediments, more than half of the eggs coagulated. The surviving embryos failed to develop somites, their tails did not detach, and they lacked functional hearts ($LC_{50}$ = 280 mg/ml). Since lethal effects were also recorded at 300 mg/ml after 48h an $LC_{50}$ of 280 mg/ml could be calculated. All of these embryos suffered from developmental retardation after (48 and 96h exposure to 150 to 600 mg/ml and completely failed to at the two highest concentrations. After 48h exposure, slightly edemata were observed at concentrations between 18.75 and 300 mg/ml ($EC_{50}$ = 105 mg/ml); after 96 h, these were restricted to 150 mg/ml.

Exposure to the Três Irmãos sediments for 48h resulted in $>$ 50 % coagulation of the eggs in highest concentration. All surviving showed lack of somites, non-detachment of the tail and lack of heart function ($LC_{50}$ = 440 mg/ml). Independent of exposure time, developmental retardation was observed in all survivors exposed to 150 to 600 mg/ml, and edema formation was evident at any concentration after 48h ($EC_{50}$ = 104 mg/ml). However, most embryos recovered thereafter, and edemas were recorded after exposure to 300 mg/ml only. At the highest concentration of Três Irmãos sediments, none of the embryos hatched.

## 4.4 Discussion

The aim of the present study was to evaluate the embryotoxic and teratogenic effects of sediments from selected locations in the Tietê River Basin by means of the embryo toxicity contact assay with *Danio rerio* (DIN, 2001; Hollert *et al.*, 2003) in order to provide a comprehensive and realistic into the bioavailable hazard potential of these model sediment samples.

Since this investigation was done with "native" solid-phase sediments, there was a major concern about insufficient levels of oxygen due to degradation processes. There is evidence that oxygen depletion may result in sublethal effects or even lethality for the embryos during the fish embryo test

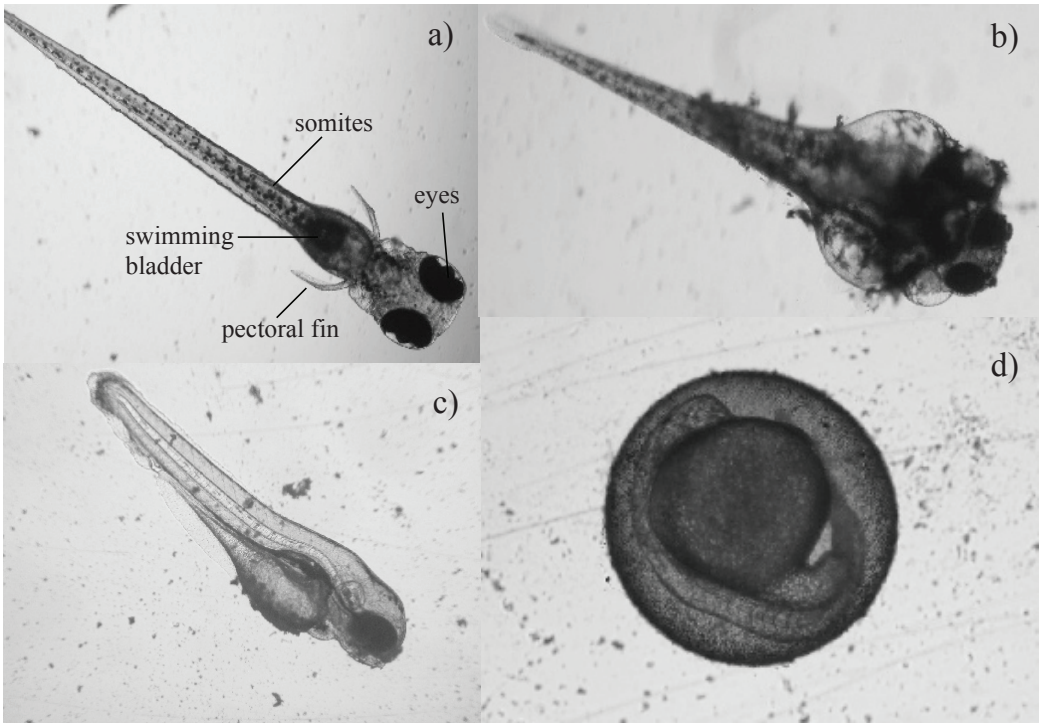



**Fig. 4.4.** Zebrafish embryos after 96 hours post fertilization. Fig. 4.4a: embryo exposed to artificial water and quartz powder (negative control), showing normal development; Fig. 4.4b: embryo exposed to 3,4-dichloraniline (3.7 µg/ml, positive control), showing edematas; Fig. 4.4c: embryo exposed to sediments from Pinheiros River (75 mg/ml), showing malformation; Fig. 4.4d: embryo exposed to sediments from Billings reservoir (300 mg/ml) showing developmental retardation.

(Kuster & Altenburger, 2008; Padilla & Roth, 2001). Although zebrafish is capable of adapting to low oxygen tensions (Braunbeck & Lammer, 2006) and can survive the first 24h post-fertilization under completely anoxic conditions, extended exposure to such conditions is likely to be lethal (Padilla & Roth, 2001). In order to make sure that toxic effects would not be caused by oxygen depletion, oxygen levels in the test plates were controlled after 1 and 96 h.

Moreover, particular attention was given to an equilibration period of 72 h, in which the sediment-water interface was allowed to stabilize. As pointed out by Strecker (2008), a 72h pre-test incubation of the sediments in the wells can be used to establish a minimum level of oxygen concentrations above the sediment surface. In fish embryo contact tests with native sediments from Lake Skadar (Montenegro), Strecker (2008) demonstrated that oxygen concentrations rapidly decreased upon addition of the sediments to the sediments to levels as low as 0.8 mg/L, but recovered to 2.5 and 3.5

mg/L after 72 and 144 h, respectively. Apparently during the initial mixing procedure, dissolved oxygen is rapidly used up by inorganic and organic redox processes; upon stabilization of the sediment surface, however, albeit low, but sufficient oxygen conditions are re-established with time. Therefore, particular care was taken to saturate the medium with oxygen before use and to guarantee sufficient access of oxygen to the wells in the test systems during the equilibration phase of 72 h.

After 1h incubation at 27°C, observed oxygen concentrations were generally very low. For Promissão sediments, the medium above the sediment surface was anoxic (0.02 mg/L). Oxygen concentrations of Billings and Barra Bonita were higher, but still critical for a normal development (0.49 and 1.20 mg/L, respectively). After 96 h, oxygen concentrations had increased considerably, and measurements above Promissão sediments revealed oxygen levels 50 times higher than at the beginning of the incubation (0.9 mg/L); conditions above Billings and Barra Bonita sediments had also improved to 1.4 mg/L. Thus, after 96h of incubation, oxygen levels had recovered significantly, but were still critical. However, Braunbeck & Lammer (2006) stated that even at oxygen concentrations as low as 2 mg/L, which should be expected to be lethal to adults of most other cyprinid fish species, *D. rerio* embryos did not show any symptom of malformation or even growth retardation. Thus, based on these studies, it could be expected that oxygen levels should not have caused adverse effects in the zebrafish embryos. In fact, in a comparison of results from fish embryo tests performed with samples with and without pre-incubation of 72 h, embryos exposed to pre-incubated sediments displayed much less effects than embryos exposed to non-pre-incubated samples. Since, Hollert *et al.* (2003) reported *D. rerio* embryos to survive at oxygen levels of 0.5 mg/ml, toxic effects recorded in this study for experiments with sediments pre-incubated for 72h can be assumed to be a consequence of exposure to sediment-bound contaminants rather than oxygen depletion.

Based on previous studies conducted by Rocha *et al.* (2009b,a) and the fact that, *e.g.*, the Pinheiros River and Billings reservoir are both located in the São Paulo metropolitan region, embryo toxicity by sediments from these areas could be expected to be considerable. The Pinheiros River collects all the sewage from the São Paulo Metropolitan region (CONSEMA, 1993) and has been reported to be free of fish, due to its high pollution load in conjunction with anoxic conditions. These high levels of water pollution directly influence Billings reservoir, since from 1952 to 1992, the natural flow of this river had been diverted into Billings reservoir for electricity generation, and only after 1992 permission for this was withdrawn except for cases of flood control in São Paulo city (Silva *et al.*, 2002).

As expected, sediments from Billings reservoir turned out to be most toxic with lethal effect levels ($LC_{50}$ values) of 200 - 210 mg/ml after 48 and 96h of exposure, respectively. However, in a ranking, this was not followed by Pinheiros River, but by Promissão and Três Irmãos reservoirs ($LC_{50}$ from 280 mg/ml to 440 mg/ml), which are located downstream by more than 400 and 600 km from São Paulo city, respectively). The Pinheiros River only followed on position 5 with $LC_{50}$ values of 600 and 450 mg/ml after 48 and 96h exposure.

In contrast, sediment samples from Ponte Nova (located only few kilometers from the Tietê River spring) as well as Barra Bonita reservoir (ca. 270 km downstream São Paulo city) induced low embryo mortalities ($LC_{50}$ values not calculable); sediments from the reference sites (near Tietê River's spring and Bariri reservoir) did not induce any toxic effects in zebrafish embryos.

With respect to sublethal effects, however, Pinheiros River sediments proved to be more effective than the other sampling areas with $EC_{50}$ (48 h) approx. 50 mg/ml. Likewise, sediments from Barra Bonita reservoir, which induced low acute mortality, also induced sublethal effects with $EC_{50}$ values of 70 to 110 mg/ml after 48 and 96 h of exposure. Sublethal toxicities of sediments from Billings, Promissão, Três Irmãos and Ponte Nova were comparable $EC_{50}$ between 90 and 110 mg/ml, and only Bariri sediments proved less effective even for sublethal changes ($EC_{50}$= 330 and 460 mg/ml for 48 and 96 h, respectively. Most importantly, as for acute toxicity, the reference sites were free of sublethal effects.

Fracácio *et al.* (2003) investigated the toxicity of sediments from Barra Bonita, Bariri, Promissão and Três Irmãos in D. rerio larvae at a concentration of 250 mg fresh sediment/ml artificial water over 7 days from hatching and recorded mortalities of 93.3, 33.3, 43.3 and 10 %, respectively. For samples from Promissão and Três Irmãos, results of the present study perfectly correlate with those by Fracácio *et al.* (2003). In contrast, sediments from Barra Bonita reservoir showed the highest mortality in the study by Fracácio *et al.* (2003), but did not even induce 50 % mortality at the highest concentration of 600 mg/ml in the present study. This is most likely due to different exposure scenarios: Whereas in the present study embryos were exposed for at maximum 96 h post-fertilization, Fracácio *et al.* (2003) exposed larvae for 7 days from the point of hatch. Most interestingly, however, if sublethal effects were included in the comparison, effect levels were not only similar, but also comparable to those for Pinheiros.

Keiter *et al.* (2006) applied the sediment contact fish embryo assay with *D. rerio* to samples collected in 2003 from well-known polluted areas along the upper Danube River. With $LC_{50}$ values of 5.5 to 23 mg/ml, mortality rates were, thus, considerably lower than for sediment samples from Tietê

River basin. However, in 2004, Seitz (2005) collected sediment samples from the same locations along the Danube River and obtained $EC_{50}$ values comparable to those of Pinheiros and Barra Bonita samples (45 to 70 mg/ml) as well as of Billings, Promissão and Três Irmãos (ca. 100 mg/ml). This comparison illustrates that the embryo toxicity of sediments not only depends on the exact sampling location, but also on sampling time.

In previous investigations within this weight-of-evidence study, Rocha *et al.* (2009a) documented acute cytotoxicity (RTL-W1 cells) for almost all extracts from the Tietê River Basin, with Billings reservoir and Pinheiros River being the most cytotoxic sites ($NR_{50}$ approx. 30 mg/ml). As for embryo toxicity, the reference site spring showed the lowest cytotoxic potential in the study ($NR_{50} > 220$ mg/ml). However, only few kilometers downstream spring, in the Ponte Nova reservoir, an abrupt increase in cytotoxicity was recorded ($NR_{50}$ aprox. 50 mg/ml), suggesting the existence of pollutant sources already in the beginning of the river course. In Barra Bonita reservoir, ca. 270 km downstream São Paulo city, a strong decrease in cytotoxicity was recorded ($NR_{50} > 170$ mg/ml) followed again by an increase towards the mouth of the river ($NR_{50}$ between 40 and 60 mg/ml for Bariri, Promissão and Três Irmãos reservoirs).

With respect to genotoxicity, sediments from the Pinheiros River and Billings Rocha *et al.* (2009b) showed a higher genotoxic potential than the other areas. The concentration-dependant induction factors (CDI, Seitz *et al.*, 2008) recorded for Billings sediment extracts were more than 3 times higher than those from Ponte Nova and Promissão, almost 7 times higher than those from Bariri and Três Irmãos, and more than 30 times higher than those from Barra Bonita and the reference site near the Tietê River spring. On the other hand, the genotoxicity of Pinheiros River sediments was twice as high than that Billings samples.

In order to evaluate the mutagenicity *in situ* (*in vivo*), Rocha *et al.* (2009b) applied the micronucleus test to erythrocytes from *Oreochromis niloticus* caught in these reservoirs. Fish collected from Billings reservoir revealed by far the highest micronucleus frequencies with a median of 6.0 ‰. Fish from Ponte Nova and Barra Bonita reservoirs presented frequencies of 3.5 ‰, and Bariri and Promissão reservoirs had the lowest micronucleus frequencies (1.5 and 0.5 ‰, respectively).

For the dioxin-like activity (EROD activity in RTL-W1 cells), Rocha *et al.* (2009a) found the following ranking: Pinheiros River $\gg$ Bariri reservoir $>$ Billings reservoir $\gg$ Três Irmãos $\gg$ Barra Bonita reservoir $\approx$ Promissão reservoir $\gg$ Ponte Nova = reference site (spring).

Comparing all these results (Rocha *et al.*, 2009b,a) with the results obtained with the sediment fish contact assay, sediments from Billings reservoir

and Pinheiros River have to be classified by far as the most toxic ones. Overall, Billings has to be rated more toxic than Pinheiros River, since sediments collected at Pinheiros induced mainly sublethal effects in the embryos, but were less effective with respect to acute toxicity. The lower acute mortality recorded for native Pinheiros samples might be due to reduced bioavailability of specific compounds, which could well have been made available in acetonic extracts, which were used for the other bioassays. However, bioavailability itself is a poorly understood phenomenon (Strmac *et al.*, 2002), rather variable and subject to many undefined variables (biological, physical, and chemical), and thus, difficult to quantify (Hallare *et al.*, 2005a). Likewise, native sediment samples from Barra Bonita, Promissão and Três Irmãos reservoirs proved to be quite toxic to the embryos, whereas organic extracts were less effective in the other bioassays. The fact that acute toxicity data (*e.g.*, cytotoxicity and fish embryo toxicity) for the various locations along the Tietê River do not necessarily parallel specific effects such as genotoxicity, mutagenicity and dioxin-like activity, corroborates the view that acute toxicity data are insufficient to extrapolate to specific toxic mechanisms and that a thorough assessment of the contamination level of natural ecosystems like a river system definitely require a battery of different endpoints. Whole sediment exposure scenarios with whole-organism mimic normal bioavailability of toxicants (Kosmehl *et al.*, 2006), and have therefore high ecological relevance (Feiler *et al.*, 2005; Hollert *et al.*, 2003; Kosmehl *et al.*, 2006). This is of high relevance especially in cases, when results are to be extrapolated to field conditions in rivers and lakes (Kosmehl *et al.*, 2006). Even if the sensitivity to non-bioavailable fractions could be low (which could be the case for Pinheiros River sample), the addition of a contact assay in ecotoxicological sediment quality assessment is essential to improve the understanding of fate and behavior of relevant toxicants in the sediment and water.

Since the megacity São Paulo is situated close to the Tietê river spring, there is an impact along the entire course of the Tietê River. The solid-phase embryo test with the whole (native) sediments, simulating *in situ* exposure conditions, confirms that surface sediments of the Tietê River basin, especially from Billings reservoir (considering lethal and sublethal effects) and Pinheiros (considering sublethal effects) have very high toxic hazard potentials due to the discharges of the metropolitan area of São Paulo. However, high embryo toxicity recorded in locations far downstream São Paulo such as Barra Bonita, Promissão and Três Irmãos indicate the existence of additional sources of pollutants along the Tietê River course.

## 4.5 Conclusions

The sediment contact fish embryo test uses native sediments and is, thus, a powerful tool to detect toxic effects of sediments not only very sensitively, but also with high ecological relevance, since it takes bioavailability into account. Significant differences in embryo toxicity could be recorded along Tietê River course, with high embryo toxicity in samples from the megacity of São Paulo (Billings and Pinheiros River), but also downstream (Barra Bonita, Promissão and Três Irmãos). Especially for the latter reservoirs, the toxicity of whole (native) sediments was more prominent than expected from results from experiments with sediment extracts, which usually are interpreted as "worse-case scenarios". Whereas embryo toxicity and cytotoxicity in permanent cell lines show parallels with respect to acute toxicity, there is no way to extrapolate from acute toxicity to specific toxic effects with different modes of action. Therefore, for a comprehensive estimation of sediment pollution, the use of a test battery covering not only acute toxicity, but also sublethal endpoints, is indispensible.

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# Bibliography


Braunbeck T, Lammer E (2006) Fish embryo toxicity assays. *German Federal Environment Agency* 1–298

Braunbeck T, Böttcher M, Hollert H, Kosmehl T, Lammer E, Leist E, Rudolf M, Seitz N (2005) Towards an alternative for the acute fish LC (50) test in chemical assessment: the fish embryo toxicity test goes multi-species-an update. *Altex* 22: 87–102

Calijuri MC (1999) *A comunidade fitoplanctônica em um reservatório tropical (Barra Bonita, SP)*. University of São Paulo, São Carlos, São Paulo, Brazil

CETESB (1997) Relatório da Qualidade das Águas Interiores do Estado de São Paulo - 1996. Tech. rep., CETESB

Chandroo KP, Duncan IJH, Moccia RD (2004) Can fish suffer?: perspectives on sentience, pain, fear and stress. *Applied Animal Behaviour Science* 86: 225–250

Chen G, White PA (2004) The mutagenic hazards of aquatic sediments: a review. *Mutation Research* 567: 151–225

Chorus D (1987) Literaturrecherche und Auswertung zur Notwendigkeit chronischer Tests - insbesondere des Reproduktionstests–am Fisch f
"ur die Stufe II nach dem Chemikaliengesetz. *UBA I* 4: 316

CONSEMA (1993) CONSEMA: dez anos de atividades. São Paulo: Secretaria do Meio Ambiente. São Paulo, Brasil

DIN (2001) German standard methods for the examination of water, waste and sludge - Subanimal testing (group T) - Part 6: Toxicity to fish. Determination of the non-acute-poisonous effect of wate water to fish eggs by dilution limits (T6). *DIN 38415 - 6; German Standardization Organization*

Feiler U, Ahlf W, Höss S, Hollert H, Neumann-Hensel H, Meller M, Weber J, Heininger P (2005) The SeKT Joint Research Project: Definition of reference conditions, control sediments and toxicity thresholds for limnic sediment contact tests. *Environmental Science and Pollution Research* 12: 257–258

Fent K (2004) Ecotoxicological effects at contaminated sites. *Toxicology* 205: 223–240

Fracácio R, Verani NF, Espíndola ELG, Rocha O, Rigolin-Sá O, Andrade CA (2003) Alterations on growth and gill morphology of *Danio rerio* (Pisces,

Ciprinidae) exposed to the toxic sediments. *Brazilian Archives of Biology and Technology* 46: 685–695

Hallare A, Schirling M, Luckenbach T, Koehler H, Triebskorn R (2005a) Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos. *Journal of Thermal Biology* 30: 7–17

Hallare AV, Kosmehl T, Schulze T, Hollert H, Koehler HR, Triebskorn R (2005b) Assessing the severity of sediment contamination in Laguna Lake, Philippines using a sediment contact assay with zebrafish (*Danio rerio*) embryos. *Science of the Total Environment* 347: 254–271

Hollert H, Dürr M, Erdinger L, Braunbeck T (2000) Cytotoxicity of settling particulate matter (SPM) and sediments of the Neckar river (Germany) during a winter flood. *Environmental Toxicology and Chemistry* 19: 528–534

Hollert H, Keiter S, König N, Rudolf M, Ulrich M, Braunbeck T (2003) A New Sediment Contact Assay to Assess Particle-bound Pollutants Using Zebrafish (*Danio rerio*) Embryos. *Journal of Soils and Sediments* 3: 197–207

Hollert H, Dürr M, Holtey-Weber R, Islinger M, Brack W, Färber H, Erdinger L, Braunbeck T (2005) Endocrine disruption of water and sediment extracts in a non-radioactive dot blot/RNAse protection-assay using isolated hepatocytes of rainbow trout - How explain deficiencies between bioanalytical effectiveness and chemically determined concentrations? *Environmental Science and Pollution Research* 12: 347 – 360

Holst G, Glud RN, K
"uhl M, Klimant I (1997) A microoptode array for fine-scale measurement of oxygen distribution. *Sensors & Actuators: B Chemical* 38: 122–129

ISO7346/3 (1996) *Water quality - Determination of the acute lethal toxicity of substances to a freshwater fish (Brachydanio rerio Hamilton-Buchanan (Teleostei, Cyprinidae)). - Part 3: Flow-through method*

Keiter S, Rastall A, Kosmehl T, Erdinger L, Braunbeck T, Hollert H (2006) Ecotoxicological Assessment of Sediment, Suspended Matter and Water Samples in the Upper Danube River. A pilot study in search for the causes for the decline of fish catches. *Environmental Science and Pollution Research* 13: 308–319

Klimant I, Wolfbeis O (1995) Oxygen-sensitive luminescent materials based on silicone-soluble ruthenium diimine complexes. *Analytical Chemistry* 67: 3160–3166

Klimant I, Ruckruh F, Liebsch G, Stangelmayer A, Wolfbeis OS (1999) Fast response oxygen micro-optodes based on novel soluble ormosil glasses. *Microchimica Acta* 131: 35–46

Kosmehl T, Krebs W Fand Manz, Erdinger L, Braunbeck H Tand Hollert (2004) Comparative genotoxicity testing of Rhine river sediment extracts using the permanent cell lines RTG-2 and RTL-W1 in the comet assay and Ames assay. *Jour-*

*nal of Soils and Sediments* 4: 84–94

Kosmehl T, Hallare A, Reifferscheid G, Manz W, Braunbeck T, Hollert H (2006) A novel contact A novel contact assay for testing genotoxicity of chemicals and whole sediments in zebrafish embryos. *Environmental Toxicology and Chemistry* 25: 2097–2106

Kuster E, Altenburger R (2008) Oxygen decline in biotesting of environmental samples-Is there a need for consideration in the acute zebrafish embryo assay? *Environmental Toxicology* 23: 745–750

Lammer E, Carr GJ, Wendler K, Rawlings JM, Belanger SE, Braunbeck T (2009a) Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? *Comparative Biochemistry and Physiology* 149: 196–209

Lammer E, Kamp HG, Hisgen V, Koch M, Reinhard D, Salinas ER, Wendler K, Zok S, Braunbeck T (2009b) Development of a flow-through system for the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*). *Toxicology in Vitro* 23: 1436–1442

Lemos CT, Roedel PM, Terra NR, Oliveira NCD, Erdtmann B (2007) River water genotoxicity evaluation using micronucleus assay in fish erythrocytes. *Ecotoxicology and Enviromental Safety* 66: 391–401

Liß W, Ahlf W (1997) Evidence from whole-sediment, porewater, and elutriate testing in toxicity assessment of contaminated sediments. *Ecotoxicology and Environmental Safety* 36: 140–147

Maier M, Kuehlers D, Brauch HJ, Fleig M, Maier D, Jirka GH, Mohrlok E Uand Bethge, Bernhart HH, Lehmann B, Hillebrand G, Woelz J, Hollert H (2006) Flood retention and drinking water supply - Preventing conflicts of interest. *Journal of Soils and Sediments* 6: 113–114

Mckim JM (1977) Evaluation of tests with early life stages of fish for predicting long-term toxicity. *Journal of the Fisheries Research Board of Canada* 34: 1148—1154

Nagel R (2002) DarT: The embryo test with the Zebrafish *Danio rerio* - a general model in ecotoxicology and toxicology. *Altex* 19: 38–48

Padilla P, Roth M (2001) Oxygen deprivation causes suspended animation in the zebrafish embryo. *Proceedings of the National Academy of Sciences* 98: 7331

Rocha PS, Keiter S, Pompêo MLM, Mariani CF, Brandimarte AL, Seiler TB, Kosmehl T, Böttcher M, Wölz J, Braunbeck T, Storch V, Hollert H (2006) Weight-of-Evidence-Studie zur Sedimentbelastung des Tietê River in Brasilien. *Umweltwissenschaften und Schadstoff-Forschung* 18: 70

Rocha PS, Azab E, Schmidt B, Storch V, Hollert H, Braunbeck T (2009a) Changes in toxicity and Ah receptor agonist activity of sediments from the Tietê River (São Paulo, Brazil) - a mass balance approach using *in vitro* methods and chemical analysis. *Ecotoxicology and Environmental Safety*

Rocha PS, Luvizotto GL, Kosmehl T, Böttcher M, Storch V, Braunbeck T, Hollert H (2009b) Sediment genotoxicity in the Tietê River (São Paulo, Brazil): *in vitro* comet assay *versus in situ* micronucleus assay studies. *Ecotoxicology and Enviromental Safety* 72: 1842–1848

Schirmer K, Dayeh V, Bopp S, Russold S, Bols N (2004) Applying whole water samples to cell bioassays for detecting dioxin-like compounds at contaminated sites. *Toxicology* 205: 211–221

Scholz S, Fischer S, Gündel U, Küster E, Luckenbach T, Voelker D (2008) The zebrafish embryo model in environmental risk assessment—applications beyond acute toxicity testing. *Environmental Science and Pollution Research* 15: 394–404

Seiler TB, Rastall AC, Leist E, Erdinger L, Braunbeck T, Hollert H (2006) Membrane dialysis extraction (MDE): A novel approach for extracting toxicologically relevant hydrophobic organic compounds from soils and sediments for assessment in biotests. *Journal of Soils and Sediments* 6: 20–29

Seitz N (2005) *Der ökologische Zustand der oberen Donau – eine integrierte Bewertung auf Grundlage von Makrozoobenthos und Sedimentkontakttests mit Danio rerio.* Master's thesis, University of Heidelberg

Seitz N, Böttcher M, Keiter S, Kosmehl T, Manz W, Hollert H, Braunbeck T (2008) Evaluation of the genotoxic potential of sediment samples from the Danube River using a new expert system and several analyzing methods: How to integrate information from dose-response curves. *Mutation Research* 652: 38–45

Silva IS, Abate G, Lichtig J, Masini JC (2002) Heavy metal distribution in recent sediments of the Tiete-Pinheiros river system in Sao Paulo state, Brazil. *Applied Geochemistry* 17: 105–116

Soares A, Mozeto AA (2006) Water Quality in the Tietê River Reservoirs (Billings, Barra Bonita, Bariri and Promissão, SP - Brasil) and Nutrient Fluxes across the Sediment-Water Interface (Barra Bonita). *Acta Limnologica Brasiliensia* 18: 247–266

Strecker R (2008) *Untersuchen zum Sauerstoffbedarf des Zebrabärblings (Danio rerio) im Sedimentkontakttest.* Master's thesis, University of Heidelberg, Germany

Strmac M, Oberemm A, Braunbeck T (2002) Effects of sediment eluates and extracts from differently polluted small rivers on zebrafish embryos and larvae. *Journal of Fish Biology* 61: 24–38

Triebskorn R, Köhler H, Honnen W, Schramm M, Adams S, Müller E (1997) Induction of heat shock proteins, changes in liver ultrastructure, and alterations of fish behavior: Are these biomarkers related and are they useful to reflect the state of pollution in the field? *Journal of Aquatic Ecosystem Stress and Recovery* 6: 57–73

Ulrich M, Schulze T, Leist E, Glaß B, Maier M, Maier D, Braunbeck T, Hollert H (2002) Ökotoxikologische Untersuchung von Sedimenten und Schwebstof-

fen: Abschätzung des Gefährdungspotenzials für Trinkwasser und Korrelation verschiedener Expositionspfade (Acetonischer Extrakt, Natives Sediment) im Bakterienkontakttest und Fischeitest. *Umweltwissenschaften und Schadstoff-Forschung* 14: 132–137

Wang F, Goulet RR, Chapman PM (2004) Testing sediment biological effects with freshwater amphipod *Hyalella azteca*: the gap between laboratory and nature. *Chemosphere* 57: 1713–1724

Woltering D (1984) The growth response in fish chronic and early life stage toxicity tests: A critical review. *Aquatic toxicology* 5: 1–21

# Chapter 5

# Effect-directed analysis (EDA) of genotoxicants and Ethoxyresorufin-O-deethylase inducers in Brazilian sediments

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## Abstract

Pinheiros River and Billings reservoir are polluted sites of major concern in the Tietê River Basin, in Brazil. Sediment extracts from these localities were investigated combining a new fractionation method with the *in vitro* assays comet and EROD to chemical analysis, in order to identify the unknown pollutants responsible for the toxicity. Major genotoxicity and EROD induction potency were detected in different fractions, indicating different sets of toxicants inducing genotoxicity and metabolic activation. Overall, results obtained from the investigation of the Pinheiros and Billings fractions suggested that genotoxicity could be mostly related to Alkanes, polychlorinated biphenyls (PCBs), naphtalenes (PCNs) and medium polar to polar polycyclic aromatic compounds (PACs), while AhR-mediated toxicity could be mostly related to polycyclic aromatic hydrocarbons (PAHs). A closer

analysis of chemical measurements of EPA PAHs and results from EROD assay revealed that the analyzed PAHs could not explain more than 3% of the EROD-inducing potencies, suggesting that major determined EROD activity could be related to non-priority PAHs. The fractionation procedure applied in this study allowed the identification of specific groups of pollutants responsible for the toxicity, and prioritize individual fractions for subsequent effect-directed analysis.

## 5.1 Introduction

Sediments are a major issue for many scientists and authorities due to their close linkage to the aquatic food web and their potential to accumulate pollutants (Chen & White, 2004; Keiter *et al.*, 2006; Kosmehl, 2007; Maier *et al.*, 2006). As pollutants may be made available under certain environmental conditions (such as dredging or flood events), sediments can also become a source of diffuse contamination to the free water space (Ahlf *et al.*, 2002; Hollert *et al.*, 2003). Their associated load of pollutants can be characterized particularly by its high complexity, which is hardly to characterize based on chemical-analytic methods only. Integrated approaches such as the sediment quality triad (SQT) (Chapman, 2000) provide an evaluation of the ecological relevance of the results of bioassays and chemical analyses for major sites. However, no identification of the harmful substances can be obtained by means of the triad approach. Therefore, reliable hazard and risk assessment require both the detection of adverse effects and the identification of the chemicals causing the effects. This can be done by combining biological testing, physicochemical fractionation and chemical analysis. One approach is the "Effect-directed analysis" (Brack, 2003; Brack *et al.*, 2005). This approach includes sequential reduction of the complexity of environmental mixtures aiming to identify chemical groups and /or individual toxicants. Therefore, the sediment extracts are tested for biological effects and subjected to one or several fractionation procedures. After each separation step the fractions are biotested for selection of active fractions for further investigation.

Polychlorinated biphenyls (PCBs), naphtalenes (PCNs), dibenzo-p-dioxins and furans (PCDD/Fs), as well as polyaromatic hydrocarbons (PAHs), hydroxy-, keto- and nitro-PAHs and sulphur, oxygen and nitrogen heterocycles represent major group of toxicants in contaminated sediments. Several studies demostrated that these lipophilic pollutants are known to induce cytochrome P450 1A (CYP1A) by ligand-activation of the aryl hydrocarbon receptor (AhR), as well as genotoxicity and mutagenicity, and are often present in contaminated sediments (Brack *et al.*, 2007; Engwall *et al.*, 1999; Keiter *et al.*, 2008; Woelz *et al.*, 2008; Rocha *et al.*, 2009a). The identification of

toxic substances in effect-directed analysis is often based on a group-specific fractionation of these polycyclic aromatic compounds (PACs) (Lübcke-von Varel *et al.*, 2008). The bioassay-directed fractionation techniques have repeatedly been used to fractionate sediment extracts and examine their toxicity in bioassays, in order to gain insight into the nature of the noxious substances (Biselli *et al.*, 2005; Brack *et al.*, 2007; Ho & Quinn, 1993; Kammann *et al.*, 2005; Lübcke-von Varel *et al.*, 2008; Marvin *et al.*, 1999; Schwab & Brack, 2007).

This study is part of an integrative (weigh-of-evidence) assessment of ecotoxicological risks in the Tietê River basin sediments (Brazil). Tietê River was selected as an example for a contaminated river system, and several of its reservoirs and its tributary Pinheiros River have being investigated (Rocha *et al.*, 2006, 2009b,a, in prep.). The Tietê River and Pinheiros River, both passing through the metropolitan area of São Paulo ($>$ 19,000,000 inhabitants), are known as highly polluted rivers due to the insufficiency of effluent treatment and numerous direct industrial sources for a multitude of anthropogenic pollutants. From 1952 to 1992, the natural flow of the Pinheiros River had been diverted into Billings reservoir for electricity generation. After 1992, permission for this was withdrawn except for cases of flood control in São Paulo city, in order to prevent the high levels of water pollution in the Billings reservoir (Silva *et al.*, 2002), since the Pinheiros River collected all the sewage from the São Paulo Metropolitan region (CONSEMA, 1993). However, the dumping of domestic sewage and the deforestation of green areas, due to the irregular growing and unorganized settlements around the reservoir (with aproximately 700 thousand inhabitants) continue to contribute to the pollution of this area.

In previous studies, Rocha and co-workers recorded extremely high toxicity in sediment samples from Billings reservoir (the largest water body in the Metropolitan São Paulo region) and Pinheiros River (acute-toxicity, geno- and mutagenicity, EROD activity, embryotoxicity) (Rocha *et al.*, 2009b,a, in prep.). Genotoxic and embryo toxic effects recorded in sediment extracts from Billings reservoir were comparable to those recorded in strongly toxic sediment extracts from the Upper Danube River (Keiter *et al.*, 2009; Seitz, 2005; Seitz *et al.*, 2008). When comparing Bio-TEQs values determined by the EROD assay, the values obtained for Billings reservoir were comparable to those obtained from significantly polluted sediment extracts from Bílina River (Czech Republic) (Rocha et al, in prep.), while Bio-TEQs values from Pinheiros samples were more than 5 times higher than those.

In order to identify key pollutants causing the high toxicity of sediments samples from Pinheiros River and Billings reservoir, a recently establish fractionation procedure was applied in sediment extracts from these two sites,

and the obtained fractions were tested by comet and EROD assays. Moreover the most toxic fractions were chemically analyzed.

The automated on-line multi-step high-performance liquid chromatography (HPLC) fractionantion procedure (Lübcke-von Varel *et al.*, 2008) used in this study allows the class separation of major sediment-associated toxicants in one run combining three automatically switched normal-phase columns. The system was designed to provide 18 fractions co-eluting with major halogenated aromatic compounds such as PCBs, PCNs and PCDD/Fs with increasing planarity and degree of chlorination, PAHs with increasing numbers of aromatic carbon atoms, and several more polar compounds including nitro-PAHs, azaarenes and PAH-quinones (Lübcke-von Varel *et al.*, 2008).

## 5.2 Material and Methods

### 5.2.1 Sediment sampling

Surface sediment samples were collected from two sites, Pinheiros River (in the Traicão elevatory dam), and Billings Reservoir, both located in São Paulo city region (Fig. 5.1), in may and december 2006, using an Eckman-Birge dredge. The Traicão elevatory dam was contructed to revert Pinheiros River course and elevate its waters to the Billings reservoir level.

Ten replicates at each site, with a distance of 10m from sample to sample, were collected. Replicates were homogenized, and 1.5kg of each sediment sample were frozen immediately, stored at -10°C and tranported to Germany. Transfer of the samples to Germany was permitted by the Brazilian National Department of Mineral Production (DNPM).

Samples were then freeze-dried and extracts were prepare with Soxhlet extraction using dichloromethane p.a. (DCM; Merck, Darmstadt, Germany) and acetone p.a. (AppliChem, Darmstadt, Germany) as solvent (3:1, v/v, 400mL). After extraction, the extracts were concentrated first by a rotary evaporator and then by a gentle stream of nitrogen. Residues from each sample were then re-dissolved in 1mL hexane (Hx; p.a.; Merck) and acetone (7:3, v/v) toluene (Fluka) for following clean up procedure. Differing from Billings samples, genotoxicity was not recorded for Pinheiros River in previous studies. For this reason, to obtain results for comet assay with raw sediment sample from Pinheiros River, the respective sediment extract was re-dissolved in 1 mL with dimethyl sulfoxide (DMSO; Sigma-Aldrich, Deisenhofen, Germany), after being evaporated in nitrogen stream, as described by Hollert *et al.* (2000).

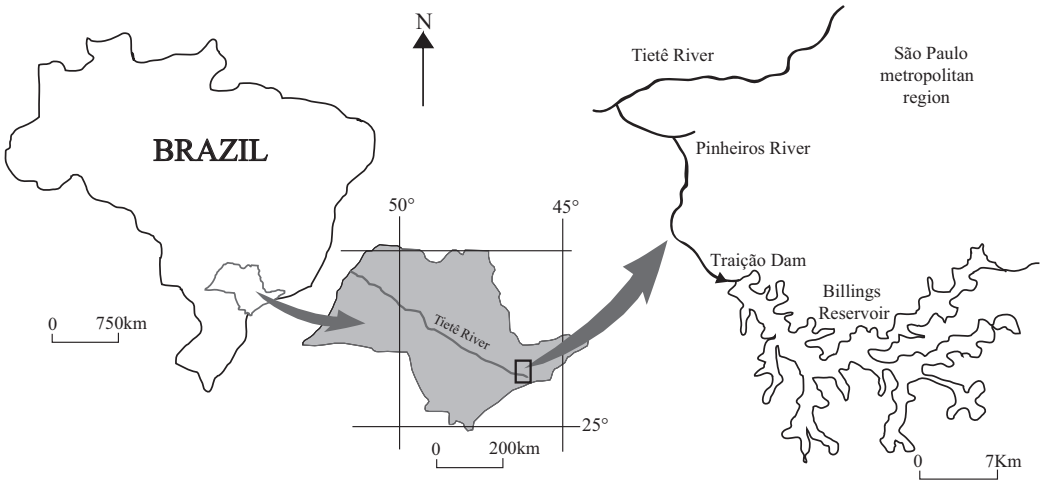



**Fig. 5.1.** Location of sampling areas, in São Paulo, Brazil. Detail on Pinheiros River and Billings Reservoir, located in São Paulo metropolitan area.

### 5.2.2 Accelerated membrane-assisted Clean-up of sediment extracts

An accelerated membrane-assisted clean-up (AMAC) technique was used to purify the complex matrix of the sediment extracts according to an optimized protocol described by Streck *et al.* (2008). This is a new technique to purify lipid-rich extracts of biota samples. The required solvents were acetone, Hx, toluene, methanol and DCM (Suprasolv® or LiChrosolv® grade - Merck, Darmstadt, Germany). Briefly, 40g of freeze-dried sediment (40g sediment equivalents - SEQ) were transferred to dialysis membranes (high density polyethylene tubes, 50 µm membrane thickness; Polymer-Synthese-Werk GmbH, Rheinberg, Germany) and dialyzed using an ASE 200 device (Dionex, Sunnyvale, CA). The temperature, pressure, number and duration of cycles were chosen as described by Lübcke-von Varel *et al.* (2008). Dialysis extracts were collected in glass ASE vials and capped with a PTFE-coated screw cap. After evaporating the extracts to dryness, the residue was redissolved in Hx:DCM (9:1, v/v) and an equivalent of 25g SEQ/ml was then used for fractionation procedure.

### 5.2.3 Fractionation procedure

The fractionantion method used in this study was the novel automated online multi-step HPLC fractionantion procedure. It was developed using couple and automatically connected columns, including cyanopropyl- and

nitrophenylpropyl-bonded silica and porous graphitised carbon stationary phases. In this method, the compounds are separated mainly according to their polarity, number of aromatic carbons and planarity (Table 5.1, Lübcke-von Varel *et al.*, 2008). The sediment fractionantion procedure followed Lübcke-von Varel *et al.* (2008). All solvents used in this procedure were Suprasolv® or LiChrosolv® grade. Briefly, in the first step medium polar and polar compounds are trapped on cyanopropyl silica (CN) with Hx as mobile phase, while non-polar substances are flushed to the nitrophenylpropyl silica (NO) and porous graphitized carbon (PGC) stationary phases. To separate PAHs with more than two aromatic rings from the more polar PACs such as nitro- and keto-PACs, the CN-column is switched off-line. Flushing of the NO and PGC phases with Hx continues and the remaining chlorinated diaromatic compounds elute from NO to the PGC column. Then a sequential fractionated elution from each of the columns begins, starting with the separation of chlorinated diaromatic compounds on PGC in forward and back-flush mode using Hx and toluene as mobile phases. The NO phase is then eluted with Hx:DCM (95:5). At last, the CN column is eluted with Hx, DCM and acetonitrile. After passing through the detector, the eluent is collected by the fraction collector into 18 glass bottles (fractions 1 to 18). After fractionation, the fractions of each sample were evaporated to dryness and the residue was re-dissolved in DMSO for the biotests and in hexane p.a. (Riedel-de Haen, Seelze, Germany) for the chemical analyses, with a final concentration of 20 mg SEQ/ml solvent.

### 5.2.4 Cell culture

The fibroblast-like permanent cell line RTL-W1 (Lee *et al.*, 1993) derived from rainbow trout liver (*Oncorhynchus mykiss*) was used to perform the biotests. The cells were maintained in 75 $cm^2$ culture flasks (TPP, Trasadingen, Switzerland) in Leibowitz (L15) medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1 % penicillin/streptomycin solution (10,000 U/10,000 µm) in 0.9 % NaCl (Sigma-Aldrich) at 20°C. They were trypsinized using 0.05 % trypsin/ 0.02 % EDTA and washed twice with PBS before being used in experiments (Kosmehl *et al.*, 2004).

**Table 5.1** Summary of solvent compositions, applied columns and eluting compounds depending on the respective fraction according to Lübcke-von Varel *et al.* (2008).

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

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

### 5.2.5 Comet assay

The comet assay was performed under alkaline conditions following the procedure of Singh *et al.* (1988) in the modification by Schnurstein & Braunbeck (2001) as well as Kosmehl *et al.* (2004). Two or more independent assays were run for each sample.

Cells were exposed to 100mg SEQ/ml supplemented L15 medium (Sigma-Aldrich, Deisenhofen, Germany), corresponding to 0.5% DMSO. The fractions were tested in 6-well plates (TTP Renner) after 24h settlement of the

cells (Kosmehl et al. 2004) for 48h at 21°C in two independent experiments. Supplemented L15 medium served as a negative control; exposure to UV light at 240 - 280 nm for 5 min was used as a positive control.

After the incubation, cells were rinsed with PBS, trypsinized and embedded in an agarose layer on fully frosted microscope slides (Langenbrink, Emmendingen, Germany) as detailed by Kosmehl et al. (2004). Immediately before scoring, the DNA was stained with 75 µl of 20 µM ethidium bromide (Sigma-Aldrich, Deisenhofen, Germany) and covered-slipped. Slides were examined using a fluorescent microscope with 340 x magnification (Axioplan, Zeiss, Germany) equipped with an excitation filter of 518 nm and an image analysis system (Optilas, Munich, Germany), and cell images were recorded with a high sensitivity CCD camera (Pulnix TM-765E Kinetic; Germany). For each fraction, 100 cells were scored and a computerized image-analysis system (Comet Version 5.5, Kinetic Images, Liverpool, UK) was used to determine DNA tail moments (tail length x fluorescence intensity in the tails). Induction factors were calculated by the comparison of median values of tail moments from exposed cells to median values of tail moments from corresponding negative controls. ANOVA-on-ranks followed by a post-hoc test according to Dunn's ($p < 0.05$) was used to calculate significant statistical differences between groups. As a first approach, only one concentration was tested for each fraction (100mg SEQ/ml medium). Then, for the most toxic fractions, cells were exposed to serial extracts dilutions from 1:1 to 1:8, with 100mg SEQ/ml medium as highest concentration, to record dose-response curves.

Results of genotoxic potencial of fractions were compared to genotoxic potential of Billings (Rocha *et al.*, 2009b) and Pinheiros raw sediment extractss, in order to compare the genotoxicity of complex mixtures and fractions. Comet assay with Pinheiros raw sample was performed exactly as describe in Rocha *et al.* (2009b), with serial dilutions of 1:1 to 1:8, with highest concentration of 5.9 mg SEQ/ml medium (based on $NR_{85}$, were less than 15% mortalily of cells could be recorded in the cytotoxicity test).

### 5.2.6 Concentration-dependant Induction factor (CDI)

In order to take into account the concentration dependency of the sediment genotoxicity, the Concentration-dependent induction factor (CDI), developed by Seitz and co-workers (Seitz *et al.*, 2008) was calculated. The CDI is a simple index that integrates all the important information, providing a basis for a general comparison of the genotoxic potential in the comet assay. The CDI integrates all concentrations and respective induction factors and is calculated according to the following equation:

$$CDI = \sum_{i=1}^{n} \frac{IFi}{ci}$$

where

IFi =induction factor of the concentration i

ci =concentration i

n = n concentrations

### 5.2.7 Ethoxyresorufin-*O*-deethylase (EROD) assay

As EROD induction is measured as specific enzyme activity, it has to be compared to a standard for evaluation of the toxic potential of tested samples (Seiler *et al.*, 2006). This standard is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which is known to be one of the most potent inducer of CYP1A activity (Okey *et al.*, 1994).

The EROD assay was carried out according to Behrens *et al.* (1998), with modifications by Gustavson *et al.* (2004). This protocol differs from the generally used approach, which includes one separate plate with serially diluted TCDD for each assay, in that the TCDD concentration-response curve was measured on every plate, including the serial dilution TCDD in each sample plate. Two or more independent assays were run for each sample.

As described by Seiler *et al.* (2006) cells were incubated at 20°C in 96-well microtiter plate for 72 h. Subsequently, cells were exposed for 48h to the the fractions, in a serial dilution from 1:1 to 1:128, with highest concentration of 100 mg SEQ/ml supplemented L15 medium, corresponding to 0.5% DMSO. As a positive control, TCDD was serially diluted to give a final concentration range of 3.125 - 100 pM on two separate rows of each plate. The plates were incubated for 72h at 20°C. Exposure was stopped by removing the medium. For cell lysis, plates were deep-frozen at 80°C for at least 1 h. The plates were then thawed for 10 min. An aliquot of 100 µl of 1.2 µM 7-ethoxyresorufin were added to each well, before deethylation was initiated for 10 min with 0.09 µM NADPH in phosphate buffer. The reaction was stopped by adding 100 µl of 0.54 mM fluorescamine in acetonitrile. After another 15 min, EROD activity was measured fluorometrically at an excitation wavelength of 544 nm and emission at 590 nm using a GENios plate reader. Whole protein was determined fluorometrically using the fluorescamine method (excitation 355 nm, emission 590 nm; (Brunstroem & Halldin, 1998; Lorenzen & Kennedy, 1993; Hollert *et al.*, 2002)) with the protocol detailed in Hollert *et al.* (2002). The concentration–response curves for EROD induction in the RTL-W1 bioassay were computed by nonlinear regression (GraphPad Prism 4) using the classic

sigmoid curve or Boltzmann curve as model equations. The luminescence-inducing potency of the samples was converted to bio-TEQs as described below.

Results of EROD activity potencies of the fractions were compared to EROD potencies of raw samples(Rocha *et al.*, 2009a) in order to compare the AhR-mediated toxicity of complex mixtures and fractions.

### 5.2.8 Bio-TEQ calculation

Maximal induction rates of the concentration-response curves for the extracts varied in relation to the positive control, 2,3,7,8-TCDD. Bioassay-derived TCDD equivalents (bio-TEQs) were calculated by relating biological activities caused by samples to the positive control 2,3,7,8-TCDD. Bio-TEQs for concentration–response curves were calculated following the fixed effect level quantification method, using the $EC_{25}$ of the maximum response in the TCDD standard curves as the fixed level (Brack *et al.*, 2000; Engwall & Hjelm, 2000). The bio-TEQs given in this study are means of n=2-3 independent experiments. Mean TCDD-$EC_{25}$ values were determined between individual EROD assays and used to calculate bio-TEQs. The Bio-TEQ concentrations were calculated as:

$$Bio - TEQ = \frac{TCDD\,EC25(pg/ml)}{extract\,EC25TCDD(g/ml)}$$

### 5.2.9 Chemical analysis

Chemical analysis were perform with the most toxic fractions. Sediment extract fractions were analyzed by gas chromatography-mass spectrometry (GC-MS) for PAH identification and quantification. An 6890N gas chromatograph system coupled with a G2589A-5973N mass selective detector (MSD) and a 7683 automatic sample injector (all instruments from Agilent, CA, USA) was equipped with an Optima 35 MS capillary column (30.0 m × 0.25 mm i.d. and 0.25 µm film thickness (Macherey and Nagel, Düren, Germany) for chromatographic separation. Helium was used as carrier gas at a constant flow rate of 1.1 mL min-1. The system operated at the following conditions: injector temperature 280°C, injection volume 1 µl in splitless mode; GC-MS transfer line temperature 320°C; ionization by electron impact at 70 eV; oven temperature program: 50°C (5 min) ramped up at 10°C min-1 to 280°C and held for 5 min, then ramped up at 10°C min-1 to 280°C and held for 6 min. The MSD was operated in scan mode. Data acquisition and processing

was performed with the Agilent Technologies MS ChemStation data analysis software and the NIST MS Search Program.

The target PAHs were identified by two criteria: retention time and library search using a spectral library (NIST 08) to identify each PAH according to its mass spectrum. Quantification was executed by external calibration using standard solutions (DE-PROM 16, LGC Standards, Wesel, Germany) with known amounts of the PAHs.

### 5.2.10 PAH-TEQs calculation

In order to estimate the degree to which analyzed PAHs account for the EROD-inducing potencies of these sediment extract fractions, PAH-TEQs were calculated on the basis of PAH potencies relative to TCDD (Bols *et al.*, 1999). The TEQ calculation was done by multiplying the concentration of each PAH in each sample by the corresponding TCDD-related potency (toxic equivalent factors related to TCDD - TEF) given by Bols *et al.* (1999), thus obtaining the relative equivalency potency values (REPs) and summing these values for each sample. To elucidate the percentage of the measured PAHs that is responsible for the induction in the EROD assay in the tested extracts, the PAH-TEQ values were subtracted from the corresponding Bio-TEQ values.

## 5.3 Results

### 5.3.1 Comet assay

Strong genotoxicity was detected in sediments from Pinheiros raw samples. Significant genotoxic effects were recorded even in concentrations of 0.64 mg SEQ/ml (Fig. 5.2). For Billings raw samples, significant genotoxic effects were recorded even in concentrations of 1.5 mg SEQ/ml (Rocha *et al.*, 2009b).

Regarding fractions of sediment extracts from both areas, Billings and Pinheiros, the most toxic potential was found in fractions 2 (related to PCBs, eluting in order of chlorination in ortho-position and chlorination degree, and PCNs with 3 Cl) and 16 (related to (Hydroxy-)quinones, keto-, dinitro-, hydroxy-PAHs, and N-Heterocycles with rising polarity). For these two fractions, the DNA damage in 100 m SEQ/ml was extremely high, and tail moments could not even be recorded. For this reason, the selected fractions were tested in serial extracts dilutions (1:1 to 1:8 of highest concentration), to obtain dose-response curves. Genotoxic effects were documented in RTL-W1 cells, with a positive dose-response relationship for both Pinheiros and

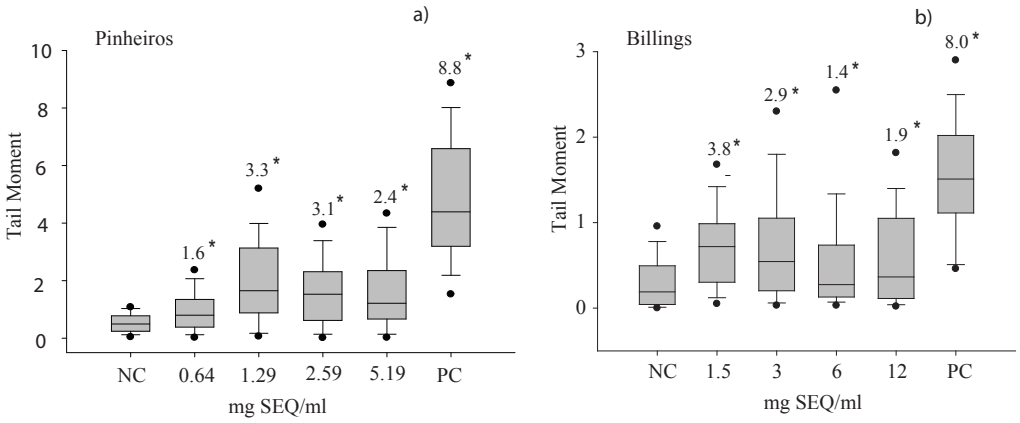



**Fig. 5.2.** Genotoxic effects of raw sediment extracts from Pinheiros River (Fig. 5.2a, this study), and Billings reservoir (Fig. 5.2b, data obtaind from Rocha *et al.*, 2009b) in the comet assay using RTL-W1 cells. Each box plot presents the tail moments of four different concentrations of extracts given in sediment equivalent (mg SEQ)/ml medium, as well as negative (NC) and positive controls (PC). Additionally, induction factors are indicated above each box plot. Significant genotoxic effects (post-hoc test according to Dunn; $p < 0.05$) are indicated by asterisks.

Billings selected fractions, and significant genotoxic effects were recorded even in lowest concentration (12.5 mg SEQ/ml, Fig. 5.3c and 5.3d).

Pinheiros fractions 9, 10, 11, 13, 14, 15 and 17 and Billings fractions 7, 12, 15, 17 and 18 also showed significant genotoxic effects. For fractions 1, 3-8 and 12 from Pinheiros samples and 1, 3-5, 8-11, 13 and 14 from Billings samples no genotoxicity was recorded (Fig. 5.3a and 5.3b).

The CDI values calculated for Pinheiros raw sediment extrat was 6.18, and from fractions 2 and 16 from Pinheiros were 0.84 and 0.36, respectively. Billings raw sediment extract (according to Rocha *et al.*, 2009b) presented CDI value of 3.3, and Billings fractions 2 and 16 of 0.36 and 0.32, respectively (Fig. 5.4).

### 5.3.2 Ethoxyresofin-*O*-deethylase (EROD) assay

The raw samples Pinheiros and Billings revealed a high EROD potency, with $EC_{25TCDD}$ values of ca. 0.19 and 1.4 mgSEQ/ml, respectively, and Bio-TEQs of ca. 22402 and 5078 pg/g, respectively (Rocha *et al.*, 2009a). According to EROD assay results and calculated BioTEQs in the present study, for Pinheiros fractions highest toxic potentials were found in fractions 8, 9 and 10, with with $EC_{25TCDD}$ values ca. 0.79, 1.07 and 0.72 mgSEQ/ml, respectively (Fig. 5.5 a, b and c), and BioTEQs ca. 600, 605 and 810 pg/g SEQ, respectively. For fractions 11, 13, 14 and 15 BioTEQs values ranged from

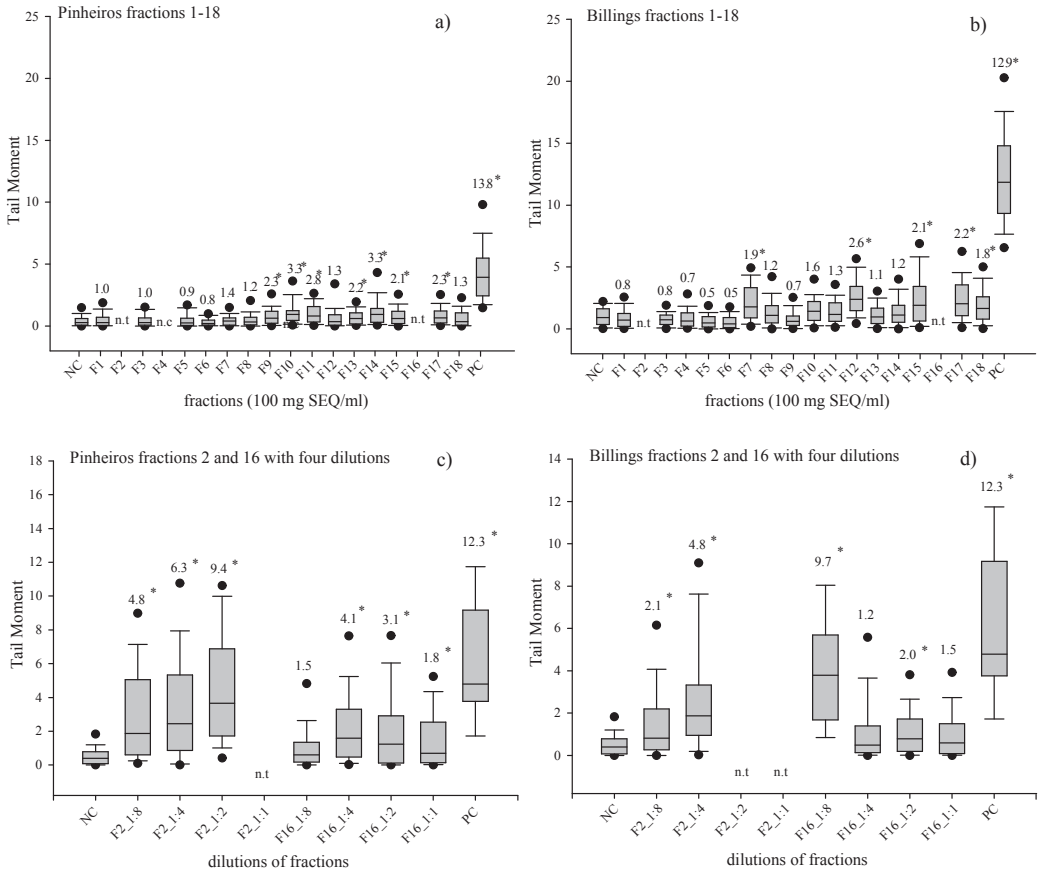



**Fig. 5.3.** Genotoxic effects of fractions from Pinheiros and Billings extracts, in the comet assay using RTL-W1 cells. Each box plot represents tail moments of 100 mg SEQ/ml medium of fractions 1 to 18 of each sample (Fig. 5.3a and b) and in four different concentrations of the most toxic fractions 2 and 16 (100, 50, 25 and 12.5 mg SEQ/ml medium, Fig. 5.3c and d). Negative and positive controls are represented as NC and PC, respectively. Additionally, induction factors are indicated above each box plot. Significant genotoxic effects (post-hoc test according to Dunn; $p<0.05$ are indicated by asterisks. F= fraction, nt=not possible to calculate tail moments (probably due to extremely high genotoxicity), nc=no cells in the slides (probably due to cytotoxicity).

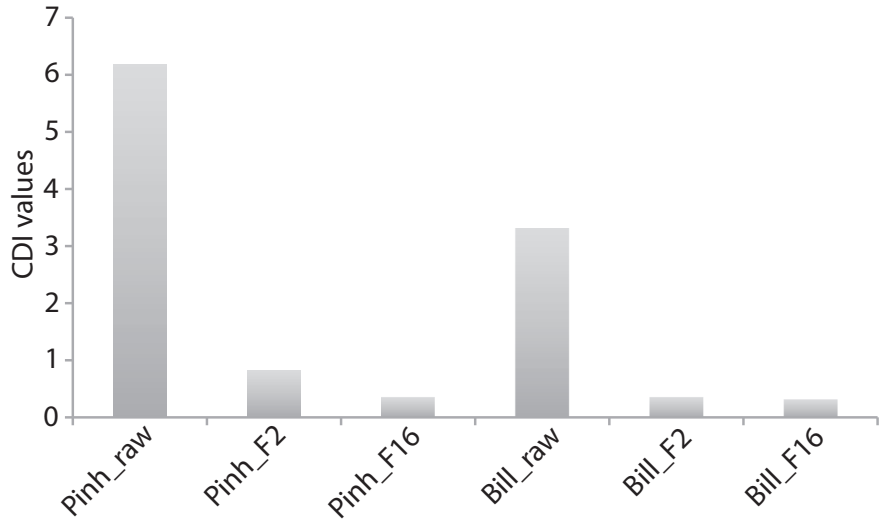
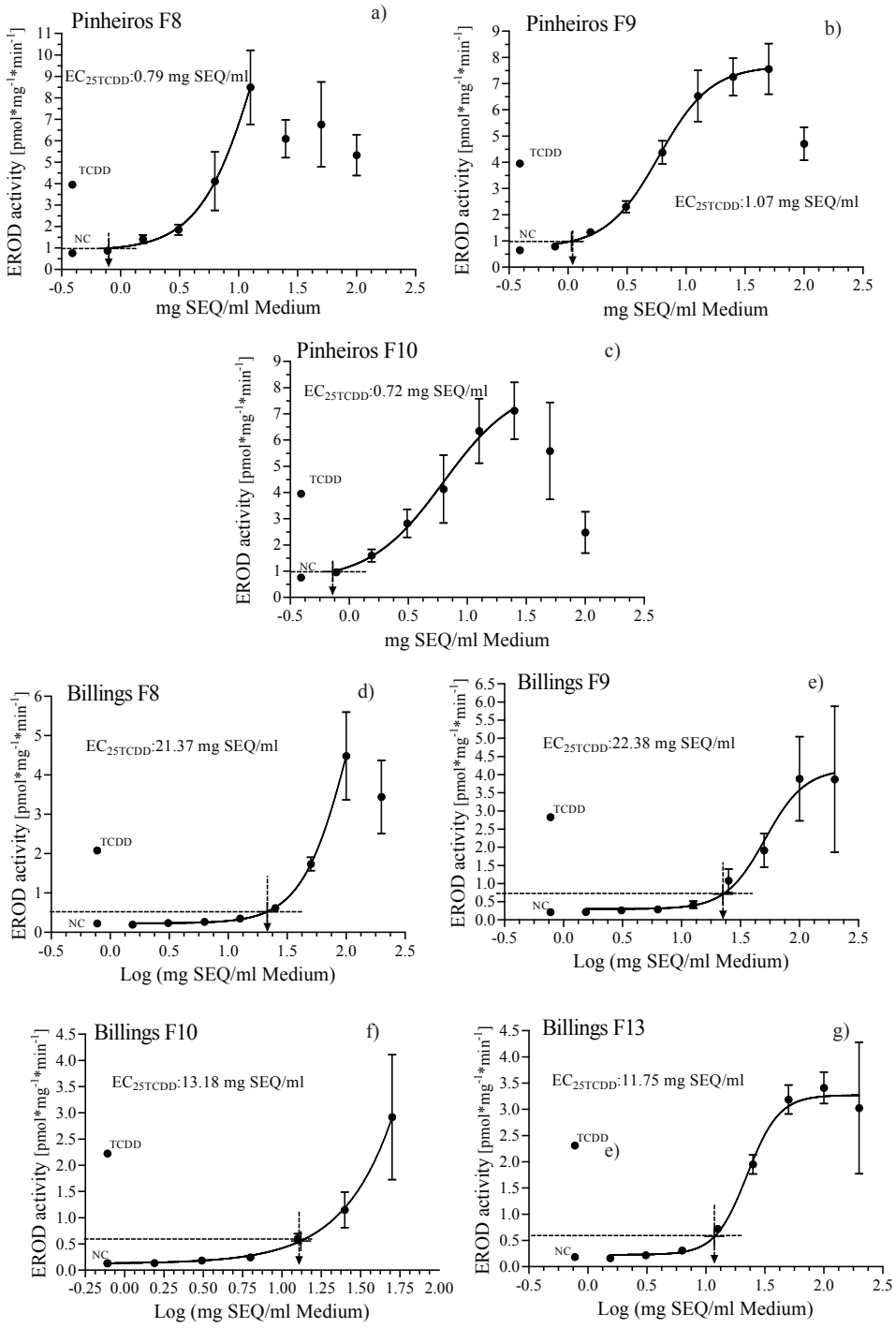



**Fig. 5.4.** Concentration-dependant induction factors (CDI; Seitz et al. 2008) of sediment raw extracts (raw) and fractions 2 (F2) and 16 (F16) from Pinheiros River and Billings reservoir. CDI value of Billings raw extract was obtained from Rocha *et al.* (2009b).

250 to 400 pg/g. Fraction 7, 16 and 17 showed BioTEQs between 150 and 200 pg/g, while fractions 4, 5, 6 and 18 showed the lowest values, between 20 and 60 pg/g (Fig. 5.6). For fractions 1 to 3 no EROD activity was recorded.

For Billings fractions, the most toxic potential was found in fractions 8, 9, 10 and 13, with $EC_{25TCDD}$ values ca. 21.37, 22.38, 13.18 and 11.75 mgSEQ/ml, respectively (Fig. 5.5 d, e and f) , and with BioTEQs ca. 170, 150 , 200 and 185 pg/g, respectively. Fractions 7, 11, 12, 14, 15, 16 and 17 showed BioTEQs values between 50 and 100 pg/g. The lower BioTEQ value was recorded in fraction 18 ($>$ 25 pg/g) and fractions 1 to 6 showed no EROD activity (Fig. 5.6).

**Fig. 5.5.** ***(following page)*** EROD induction potential of most toxic fractions from sediment extracts from Pinheiros River (Fig. 5.5a, b and c) and Billings reservoir (Fig. 5.5d, e, f, and g) in RTL-W1 cells. Data are given as mean $EC_{25TCDD}$ (concentration of each sample which caused 25% of TCDD-induced maximum EROD activity) values from 6 independent measurements. Dashed lines on the diagrams indicate the intersections of EROD activities (in pmol * mg-1 * min -1, y axis) and sediment concentrations in medium (in mg SEQ/ml for Fig. 5.5a, b and c, and Log (mg SEQ/ml medium) for Fig. 5.5d, e, f, and g, x axis), corresponding to $EC_{25TCDD}$ values. F= fraction.


a)
Pinheiros F8
EC25TCDD:0.79 mg SEQ/ml
TCDD
NC
EROD activity [pmol*mg-1*min-1]
mg SEQ/ml Medium
b)
Pinheiros F9
EC25TCDD:1.07 mg SEQ/ml
TCDD
NC
EROD activity [pmol*mg-1*min-1]
mg SEQ/ml Medium
c)
Pinheiros F10
EC25TCDD:0.72 mg SEQ/ml
TCDD
NC
EROD activity [pmol*mg-1*min-1]
mg SEQ/ml Medium
d)
Billings F8
EC25TCDD:21.37 mg SEQ/ml
TCDD
NC
EROD activity [pmol*mg-1*min-1]
Log (mg SEQ/ml Medium)
e)
Billings F9
EC25TCDD:22.38 mg SEQ/ml
TCDD
NC
EROD activity [pmol*mg-1*min-1]
Log (mg SEQ/ml Medium)
f)
Billings F10
EC25TCDD:13.18 mg SEQ/ml
TCDD
NC
EROD activity [pmol*mg-1*min-1]
Log (mg SEQ/ml Medium)
g)
Billings F13
EC25TCDD:11.75 mg SEQ/ml
TCDD
e)
NC
EROD activity [pmol*mg-1*min-1]
Log (mg SEQ/ml Medium)

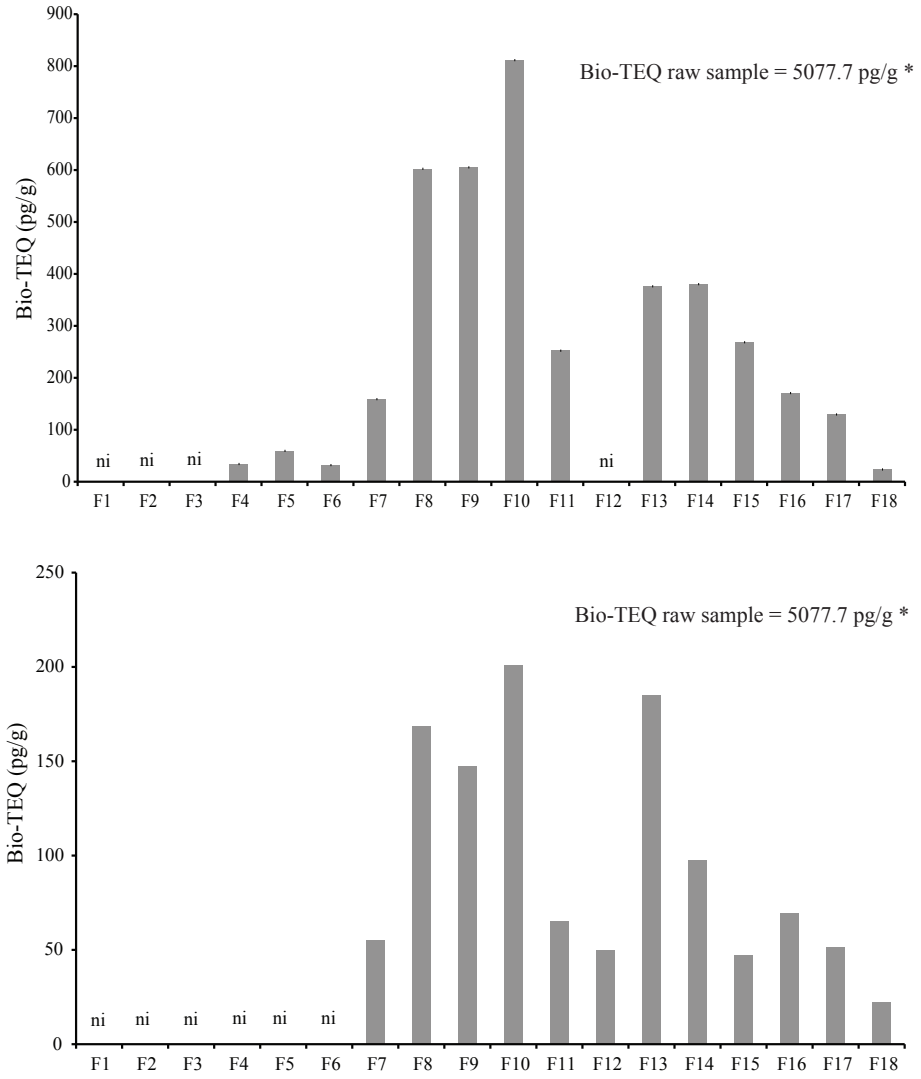



**Fig. 5.6.** BioTEQ values for selected fractions of sediment extracts from Pinheiros River (Fig. 5.6a) and Billings reservoir (Fig. 5.6b) sediment extract fractions. Bio-TEQ values of raw Pinheiros and Billings sediment extracts from Rocha *et al.* (2009a), represented with asterisks. F=fraction, ni= no EROD induction.

### 5.3.3 Chemical Analysis

Results from both applied bioassays suggested that most of the toxicity is related to PCBs, PCNs and PAHs. As standards were available only for PAHs, chemical analyses of these compounds where performed with the most toxic fractions.

For Billings, fractions 8, 9, 10 and 13 were analysed, and for Pinheiros, fractions 8, 9 and 10. Results showed that for both, Billings and Pinheiros fractions, fraction 8 was related to fluoranthene and pyrene, while fraction 9 to 1,2 benzo[a]nthracene and chrysene and fraction 10 to benzo[b]fluoranthene, benzo[k]fluoranthene and benzo[a]pyrene. For Billings' fraction 13 no compounds could be determined (Table 5.2).

### 5.3.4 Correlation between Bio-TEQs and chemical analyses (PAH-TEQs)

PAH-TEQs for the fractions could be calculated only for the PAHs capable of inducing EROD activity (according to Bols *et al.*, 1999, Table 5.2). According to PAH-TEQ calculation, only 1% of the EROD activity could be explained by the PAHs recorded in fractions 9 and 10, from both Pinheiros and Billings samples.

## 5.4 Discussion

The present study is part of an integrative (weight of evidence) assessment of Tietê River sediments, aiming to identify hazard factors and ecotoxicological risks in the Tietê River Basin. Based on results obtained in previous studies, sediment samples from Pinheiros River and Billings reservoir were considered the most toxic from all selected areas in the Tietê River basin (Rocha *et al.*, 2009b,a, in prep.) . In order to identify key pollutants causing the high toxicity of these sediments samples, a recently establish fractionation procedure was applied in sediment extracts from these two areas, and the obtained fractions were tested by comet and EROD assays. Such detailed investigation has never been carried out in these areas before.

Raw samples from Pinheiros and Billings extracts showed a high genotoxic potential, as demostrated by Rocha *et al.* (2009b) and this study. Billings raw extract presented CDI value of ca. 3.3, while Pinheiros value was almost two times higher (CDI = 6.18). According to the threshold values proposed by Keiter *et al.* (2009), sediments with CDI values $> 0.65$ are already considered

**Table 5.2** Chemically analyzed priority EPA-PAHs, REPs, Bio-TEQ and PAH-TEQ values, as well as the calculated contribution of PAHs (percentage) to the EROD induction in the very toxic fractions (according to EROD assay results). The PAH data are giving in µg/g SEQ.

| EPA PAHs | TEF[a] | Pinheiros conc. (µg/g SEQ) | | | | | | Billings conc. (µg/g SEQ) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | F8 | REP | F9 | REP | F10 | REP | F8 | REP | F9 | REP | F10 | REP | F13[b] |
| Naphthalene | NI | ND | - | ND | - | ND | - | ND | - | ND | - | ND | - | ND |
| Acenaphtylene | NI | ND | - | ND | - | ND | - | ND | - | ND | - | ND | - | ND |
| Acenaphtene | NI | ND | - | ND | - | ND | - | ND | - | ND | - | ND | - | ND |
| Fluorene | NI | ND | - | ND | - | ND | - | ND | - | ND | - | ND | - | ND |
| Phenanthrene | NI | ND | - | ND | - | ND | - | ND | - | ND | - | ND | - | ND |
| Anthracene | NI | ND | - | ND | - | ND | - | ND | - | ND | - | ND | - | ND |
| Fluoranthene | NI | 0.3661 | - | ND | - | ND | - | 0.9334 | - | ND | - | ND | - | ND |
| Pyrene | NI | 0.3632 | - | ND | - | ND | - | 0.0747 | - | ND | - | ND | - | ND |
| Benzo[a]anthracene | $4*10^{-5}$ | ND | - | 0.2160 | $9.3*10^{-6}$ | ND | - | ND | - | 0.1297 | $5.57*10^{-6}$ | ND | - | ND |
| Chrysene | $5*10^{-5}$ | ND | - | 0.3267 | $1.5*10^{-5}$ | ND | - | ND | - | 0.0935 | $4.39*10^{-6}$ | ND | - | ND |
| Benzo[b]fluoranthene | $1.03*10^{-3}$ | ND | - | ND | - | 0.1463 | 0.00015 | ND | - | ND | - | 0.0303 | $3.15*10^{-5}$ | ND |
| Benzo[k]fluoranthene | 0.00019 | ND | - | ND | - | 0.0966 | $1.86*10^{-5}$ | ND | - | ND | - | 0.0377 | $7.27*10^{-6}$ | ND |
| Benzo[a]pyrene | 0.00030 | ND | - | ND | - | 0.2058 | $6.21*10^{-5}$ | ND | - | ND | - | 0.0247 | $7.45*10^{-6}$ | ND |
| Indeno[1,2,3 - c, d]anthracene | 0.00028 | ND | - | ND | - | ND | - | ND | - | ND | - | ND | - | ND |
| Dibenz[a,b]anthracene | 0.00035 | ND | - | ND | - | ND | - | ND | - | ND | - | ND | - | ND |
| Benzo[g,h,i]perylene | NCI | ND | - | ND | - | ND | - | ND | - | ND | - | ND | - | ND |
| sum PAH conc. ((µg/g SEQ) | | 0.7293 | | 0.5426 | | 0.4486 | | 0.1680 | | 0.2231 | | 0.0927 | | - |
| Bio_TEQs (pg/g) | | | 600 | | 605 | | 810 | | 170 | | 150 | | 200 | 185 |
| PAh-TEQs (pg/g) | | | - | | 2.46 | | 23.27 | | - | | 0.99 | | 4.62 | - |
| Percentage | | | - | | 0.41 | | 2.9 | | - | | 0.66 | | 2.3 | - |

TEFs = toxic equivalency factor (the ability of PAHs to induce EROD activity in relation to TCDD), REP = relative equivalency potencies (product of concentrations of each PAHs in each sample x corresponding TEF); Bio-TEQs = Biological toxicity equivalents; PAH-TEQs = toxicity equivalents of Polycyclic Aromatic Hydrocarbons (sum of REPs); ND = not detectable. F = fraction.

[a] Bols et al. 1999 - NI = no induction (PAHs without EROD induction potency); NCI = no constant induction (PAHs that induce EROD induction inconstantly). For these PAHs TEFs could not be calculated.

[b] For fraction 13, concentrations of all PAHs were bellow detection limit (ND). Therefore, REP could be not calculated.

strongly genotoxic. Evaluation of Pinheiros and Billings fractions showed that for both samples, higher genotoxic effects were obtained from fractions 2 and 16. According to the fractionation method developed by Lübcke-von Varel *et al.* (2008), these two fractions can be related to Alkanes, PCBs (with 2 or 4 chlorines in ortho-position) and PCNs with 3 chlorines atoms (fraction 2) and medim polar to polar PACs such as (Hydroxy-)quinones, keto-, dinitro-, hydroxy-PAHs, and N-Heterocycles (fraction 16). Genotoxic effects of these fractions were recorded in RTL-W1 cells even at the lowest tested concentration of 12.5 mg SEQ/ml. Moreover, in the highest concentrations of these two fractions DNA damage was extremely high, and tail moments were not possible to calculate due to the high fluorescence intensity. CDI values calculated for the most genotoxic Pinheiros fractions were 0.84 (fraction 2) and 0.36 (fraction 16), representing 14% and 6% of CDI of raw sample. For Billings fractions, CDI values of most genotoxic fractions were 0.36 and 0.32, representing 11% and 10% of CDI of raw sample.

Significant genotoxic effects of Pinheiros sediment samples were also related to fractions 8, 9 and 10 (PAHs with four to six aromatic rings, respectively), as well as to fraction 13 (mononitro-PAHs) and fractions 14, 15 and 17 (medim polar to polar PACs, like for fraction 16). According to the chemical analyses of EPA priority PAHs, performed in the present study, fraction 8 presented Fluoranthene and Pyrene. Fractions 9 presented Benzo[a]anthracene and Chrysene and fraction 10 Benzo[b]fluoranthene, Benzo[k]fluoranthene and Benzo[a]pyrene, which are very well known carcinogenic inducers (Harvey, 1991; Neff, 1979). In a study of toxicity of European fresh water sediments Luebcke-von Varel *et al.* (in prep.) combined the automated multistep fractionation procedure with *in vitro* mutagenic biotest, and recorded mutagenic effects in fractions 9, 10 and 11, as well as in 14 and 15.

With respect to Billings' fractions, significant genotoxic effects were also related to fractions 7 and 12 (PAHs with three and seven aromatic rings, respectively), as well as to fractions 15, 17 and 18. Differing from Pinheiros, no significant genotoxic effect were recorded for Billings fractions 8, 9 and 10. The concentration of analysed PAHs in Pinheiros fractions were almost 4 times higher than in Billings fractions.

According to EROD assay results, PAHs play an important role in the Ah-R mediated toxicity of both areas. The most toxic fractions from Pinheiros River and Billings reservoir extracts were related to fractions 8, 9 and 10 (PAHs with four to six aromatic rings, respectively), while for Billings were also be related to fraction 13 (mono-nitro PAHs). Bio-TEQs of the most Ah-mediated toxic fractions from Pinheiros were 600 , 605, and 810 pg/g (fractions 8, 9 and 10 , respectively) and from Billings 170, 150, 200 and 185 pg/g (fractions 8, 9, 10 and 13 respectively). The highest Bio-TEQ

value of fractions from both areas were determined for fraction 10. However, Pinheiros' fraction 10 presented a Bio-TEQ more than four times higher then the same fraction of Billings (810 and 200 pg/g, respectively). The same was observed for Bio-TEQ values of the sediment raw extracts. Pinheiros and Billings raw sediment extracts presented extremely high Bio-TEQ values of 22403 and 5078 pg/g, respectively (Rocha *et al.*, 2009a). Comparing Bio-TEQs of raw sediment extracts to the respective fractions, for both areas, Bio-TEQ values of raw samples were almost 30 times higher than the highest Bio-TEQ recorded in the fractions.

Bols *et al.* (1999) investigated the ability of PAHs to induce EROD activity and stated that fluoranthene and pyrene (found in fraction 8) are not able to induce EROD activity consistently, while 1,2-benzanthracene and chrysene (found in fraction 9) and benzo[b]fluoranthene, benzo[k]fluoranthene and benzo[a]pyrene (found in fraction 10) induce EROD activity consistently. The calculation of PAH-TEQs was done based on these results obtained by Bols *et al.* (1999). When associating the Bio-TEQs with the PAH-TEQs calculated in this study and estimating the degree to which analyzed PAHs account for the EROD-inducing potencies of these fractions, only ca. 3% of the induction could be explained by the PAHs for the fractions in each sample. These results are in accordance to results obtained by Rocha *et al.* (2009a), where, for the raw Pinheiros and Billings sediment extracts, only 2.5% and 1.4% of EROD activity induction could be explained by priority PAHs. Therefore, results confirm that non-analysed compounds with EROD-inducing potency account for the majority of the determined EROD-inducing potential; for the fractions, these most likely include non-priority PAHs, as most toxic fractions were related to these compounds; for the raw sediment extracts other priority pollutants as well as non-priority pollutants should also be consider, as the fractionation procedure was based on PACs and did not take into account other classes of pollutants. In fact, in contrast to the Pinheiros fractions 1 to 3 and 12, and Billings fractions 1 to 6, which do not induce any EROD activity, all the other fractions investigated are clearly EROD activity inducers in RTL-W1 cells, each with a positive dose-response relationship. For instance, fractions 13 and 14 for both samples (related to mainly mononitro PAHs) were also responsable for high EROD activity induction, being Billings fraction 13 one of the most potent inducer from the these sample fractions.

Results obtained in this study suggested that genotoxicity could be moslty related to Alkanes, PCBs, PCNs and medim polar to polar PACs, while AhR-mediated could be mostly related to PAHs. Many cases of contamination by PAHs, PCBs and organochlorine pesticides as well as heavy metals have been observed in waters in and around São Paulo city, especially in the Billings

reservoir(Bainy *et al.*, 1999). Rocha *et al.* (2009a) recorded high PAH concentrations in Billings and Pinheiros sediment samples, with values comparable to those obtained in well-known polluted European fresh water sediments (Keiter *et al.*, 2008). According to Leitão (1999), sediment PCB levels in Billings reservoir ranged from 7 to 101 µg/kg. Bainy *et al.* (1996) observed an unusually high level of total microsomal cytochrome P450 in liver and kidney of *Oreochromis niloticus* caught at this reservoir, which were associated with the PCBs detected in these organisms, and may also have been related to oxidative stress observed in several tissues of these animals (Bainy *et al.*, 1996). According to the São Paulo State Environmental Agency (CETESB, 1993), fish from Billings Reservoir were chronically exposed not only to high levels of PCBs but also compounds, but also to lindane, hexachlorobenzene, pentachlorophenol and dichlorodiphenyldichloroethylene (DDE), some of which are common carcinogenic and inducers of CYP1A. Moreover, Silva *et al.* (2002) recorded high levels of heavy metals in Billings reservoir and in the surrondings of the metropolitan area of São Paulo city.

Aquatic organisms such as fish accumulate pollutants directly from contaminated water or indirectly through the ingestion of contaminated aquatic organisms. In recent years, there has been increasing concern about the risk for human health following consumption of contaminated fish, since fish are top predators in the food web and can metabolize, concentrate, store and also biomagnify contaminants in a way similar to humans. Dioxin-like compounds display a wide variety of toxic effects in mammals, birds and fishes, including immunotoxicity, carcinogenicity, metabolic changes, endocrine disruption and even death (Van den Berg *et al.*, 1998) and are intimately associated to sediments. Aquatic organisms inhabiting waters impacted by carcinogens, *e.g.*, may suffer an increased risk of genetic damage or cancer. Humans utilizing these waters for recreational and drinking water purposes may suffer similar genetic or carcinogenic risks (Kosmehl *et al.*, 2004). Hence, the assessment of sediment quality is essential for the understanding of processes governing the fate and availability of these contaminants in water bodies.

The combination of *in vitro* bioassays with fractionation techniques helps to associate effects to groups of contaminants with similar physico-chemical properties and thus to prioritize individual fractions for subsequent effect-directed analysis. The on-line fractionation procedure applyed in this study (Lübcke-von Varel *et al.*, 2008) in combination to comet and EROD assays, allows the understanding of the toxicity of these sediment extracts for fraction-specific adverse effects, associating these effects to specific groups of pollutants.

## 5.5 Conclusion

Major genotoxicity and EROD induction potency were detected in different fractions of sediment extracts from Pinheiros River and Billings reservoir, indicating different sets of toxicants inducing genotoxicity and metabolic activation. Overall, results obtained from the investigation of the Pinheiros and Billings fractions suggested that genotoxicity could be moslty related to Alkanes, PCBs, PCNs and medim polar to polar PACs, while AhR-mediated toxicity could be mostly related to PAHs.

Genotoxicity and AhR-mediated toxicity recorded in Pinheiros fractions were four times higher than in Billings fractions.

The correlation of measured EPA PAHs in the most EROD inducers fractions and results from EROD assay revealed that these PAHs could explain only ca. 3% of the EROD-inducing potencies, suggesting that EROD activity in the fractions is related to non-priority PAHs. The fractionation method applied in this study only retains and fractionate PACs. Hence, it has to be consider that toxicity of raw sediment extracts can also be related to other priority and non-priority pollutants.

The fractionation procedure applyed in this study allowed the identification of specific groups of pollutants responsible for the toxicity, and prioritize individual fractions for subsequent effect-directed analysis, with more specific sequential reduction of the complexity of these environmental mixtures, eventually to individual toxicants.

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# Bibliography


Ahlf W, Braunbeck T, Heise S, Hollert H (2002) Sediment and soil quality criteria. *Environmental Monitoring Handbook* 17–11

Bainy ACD, Saito E, Carvalho PSM, Junqueira VB (1996) Oxidative stress in gills, erythrocytes, liver and kidney of Nile tilapia (*Oreochromis niloticus*) from a polluted site. *Aquatic Toxicology* 34: 151–162

Bainy ACD, Bruce RW, John JS (1999) Elevated levels of multiple cytochrome P450 forms in tilapia from Billings Reservoir - Sao Paulo, Brazil. *Aquatic Toxicology* 44: 289–305

Behrens A, Schirmer K, Bols NC, Segner H (1998) Microassay for rapid measurement of 7-ethoxyresorufin-o-deethylase activity in intact fish hepatocytes. *Marine Environmental Research* 46: 369–373

Van den Berg M, Birnbaum L, Bosveld A, Brunstr
"om B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, *et al.* (1998) Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environmental Health Perspectives* 106: 775–792

Biselli S, Reineke N, Heinzel N, Kammann U, Franke S, Huhnerfuss H, Theobald N (2005) Bioassay-directed fractionation of organic extracts of marine surface sediments from the North and Baltic Sea. Part I: Determination and identification of organic pollutants. *Journal of Soils and Sediments* 5: 171–181

Bols N, Schirmer K, Joyce E, Dixon D, Greenberg B, Whyte J (1999) Ability of Polycyclic Aromatic Hydrocarbons to Induce 7-Ethoxyresorufin- *O*-deethylase Activity in a trout liver cell line. *Ecotoxicology and Environmental Safety* 44: 118–128

Brack W (2003) Effect-directed analysis: a promising tool for the identification of organic toxicants in complex mixtures? *Analitical and Bioanalitical Chemistry* 377: 397–407

Brack W, Segner H, Moeder M, Schueuerman G (2000) Fixed-effect-level toxicity equivalents - a suitable parameter for assessing ethoxyresorufin-*O*-deethylase induction potency in complex environmetal samples. *Environmental Toxicology and Chemistry* 19: 2493–2501

Brack W, Schirmer K, Erdinger L, Hollert H (2005) Effect-directed analysis of

mutagens and ethoxyresorufin-*O*-deethylase inducers in aquatic sediments. *Environmental Toxicology and Chemistry* 24: 2445–2458

Brack W, Klamer JCH, Alda ML, Barceló D (2007) Effect-Directed Analysis of Key Toxicants in European River Basins: A Review. *Environmental Science and Pollution Research* 14: 30–38

Brunstroem B, Halldin K (1998) EROD induction by environmental contaminants in avian embryo livers. *Comparative Biochemistry and Physiology* 121: 213–219

CETESB (1993) Relatório da Qualidade das Águas Interiores do Estado de São Paulo - 1992. Tech. rep., CETESB

Chapman PM (2000) The Sediment Quality Triad: then, now and tomorrow. *International Journal of Environmental Pollution* 13: 351–356

Chen G, White PA (2004) The mutagenic hazards of aquatic sediments: a review. *Mutation Research* 567: 151–225

CONSEMA (1993) CONSEMA: dez anos de atividades. São Paulo: Secretaria do Meio Ambiente. São Paulo, Brasil

Engwall M, Hjelm K (2000) Uptake of dioxin-like compounds from sewage sludge into various plant species - assessment of levels using a sensitive bioassay. *Chemosphere* 40: 1189–1195

Engwall M, Brunström B, Näf C, Hjelm K (1999) Levels of dioxin-like compounds in sewage sludge determined with a bioassay based on EROD induction in chicken embryo liver cultures. *Chemosphere* 38: 2327–2343

Gustavson LK, Klee N, Olsman H, Hollert H, Engwall M (2004) Fate of Ah receptor agonistis during biological treatment of an industrial sludge containing explosives and pharmaceutical residues. *Environmental Science and Pollution Research* 11: 379–387

Harvey R (1991) *Polycyclic aromatic hydrocarbons: chemistry and carcinogenicity.* Cambridge University Press

Ho K, Quinn J (1993) Bioassay-directed fractionation of organic contaminants in an estuarine sediment using the new mutagenic bioassay, Mutatox. *Environmental Toxicology and Chemistry* 12: 823–830

Hollert H, Dürr M, Erdinger L, Braunbeck T (2000) Cytotoxicity of settling particulate matter (SPM) and sediments of the Neckar river (Germany) during a winter flood. *Environmental Toxicology and Chemistry* 19: 528–534

Hollert H, Keiter S, König N, Rudolf M, Ulrich M, Braunbeck T (2003) A New Sediment Contact Assay to Assess Particle-bound Pollutants Using Zebrafish (*Danio rerio*) Embryos. *Journal of Soils and Sediments* 3: 197–207

Hollert M Hand Duerr, Olsman H, Halldin K, Bavel Bv, Brack W, Tysklind M, Engwall M, Braunbeck T (2002) Biological and chemical determination of dioxin-like compounds in sediments by means of a sediment triad approach in the catchment area of the Neckar River. *Ecotoxicology* 11: 323–336

Kammann U, Biselli S, Reineke N, Wosniok W, Danischewski D, Hühnerfuss H, Kinder A, Sierts-Herrmann A, Theobald N, Vahl H, *et al.* (2005) Bioassay-directed Fractionation of Organic Extracts of Marine Surface Sediments from the North and Baltic Sea-Part II: Results of the biotest battery and development of a biotest index. *Journal of Soils and Sediments* 5: 225–232

Keiter S, Rastall A, Kosmehl T, Erdinger L, Braunbeck T, Hollert H (2006) Ecotoxicological Assessment of Sediment, Suspended Matter and Water Samples in the Upper Danube River. A pilot study in search for the causes for the decline of fish catches. *Environmental Science and Pollution Research* 13: 308–319

Keiter S, Grund S, van Bavel B, Hagberg J, Engwall M, U K, Klempt M, Manz W, Olsman H, Braunbeck T, Hollert H (2008) Activities and identification of aryl hydrocarbon receptor agonists in sediments from the Danube River. *Analytical Bioanalytical Chemistry*

Keiter S, Braunbeck T, Heise S, Pudenz S, Manz W, Hollert H (2009) A fuzzy logic-classification of sediments based on data from *in vitro* biotests. *Journal of Soils and Sediments* 9: 168–179

Kosmehl T (2007) *Molecular biomarkes in zebrafish embryos: Towards a more realistic approach in sediment assessment.* Ph.D. thesis, University of Heidelberg, Heidelberg, Germany

Kosmehl T, Krebs W Fand Manz, Erdinger L, Braunbeck H Tand Hollert (2004) Comparative genotoxicity testing of Rhine river sediment extracts using the permanent cell lines RTG-2 and RTL-W1 in the comet assay and Ames assay. *Journal of Soils and Sediments* 4: 84–94

Lee LE, Clemons JH, Bechtel DG, Caldwell SJ, Han KB, Pasitschniak-Arts M, Mosser D, Bols NC (1993) Development and characterization of a rainbow trout liver cell line expressing cytochrome P450-dependent monooxygenase activity. *Cellular Biology and Toxicology* 9: 279–294

Leitão MAS (1999) *Estudo sazonal de enzimas biomarcadoras de poluição aquática, em tilápias do nilo (Oreochromis niloticus) da represa Billings - SP.* Ph.D. thesis, Universidade de São Paulo

Lorenzen A, Kennedy S (1993) A fluorescence-based protein assay for use with a microplate reader. *Analytical biochemistry* 214: 346–348

Maier M, Kuehlers D, Brauch HJ, Fleig M, Maier D, Jirka GH, Mohrlok E Uand Bethge, Bernhart HH, Lehmann B, Hillebrand G, Woelz J, Hollert H (2006) Flood retention and drinking water supply - Preventing conflicts of interest. *Journal of Soils and Sediments* 6: 113–114

Marvin C, McCarry B, Lundrigan J, Roberts K, Bryant D (1999) Bioassay-directed fractionation of PAH of molecular mass 302 in coal tar-contaminated sediment. *Science of the Total Environment* 231: 135–144

Neff JM (1979) *Polycyclic aromatic hydrocarbons in the aquatic environment. Sources, fates and biological effects.* Applied Science Publishers (London)

Okey AB, Riddick DS, Harper PA (1994) The Ah receptor: Mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *Toxicology Letters* 70: 1–22

Rocha PS, Keiter S, Pompêo MLM, Mariani CF, Brandimarte AL, Seiler TB, Kosmehl T, Böttcher M, Wölz J, Braunbeck T, Storch V, Hollert H (2006) Weight-of-Evidence-Studie zur Sedimentbelastung des Tietê River in Brasilien. *Umweltwissenschaften und Schadstoff-Forschung* 18: 70

Rocha PS, Azab E, Schmidt B, Storch V, Hollert H, Braunbeck T (2009a) Changes in toxicity and Ah receptor agonist activity of sediments from the Tietê River (São Paulo, Brazil) - a mass balance approach using *in vitro* methods and chemical analysis. *Ecotoxicology and Environmental Safety*

Rocha PS, Luvizotto GL, Kosmehl T, Böttcher M, Storch V, Braunbeck T, Hollert H (2009b) Sediment genotoxicity in the Tietê River (São Paulo, Brazil): *in vitro* comet assay *versus in situ* micronucleus assay studies. *Ecotoxicology and Enviromental Safety* 72: 1842–1848

Rocha PS, Bernecker C, Strecker R, Storch V, Hollert H, Braunbeck T (in prep.) Sediment contact fish embryo test with *Danio rerio* to assess particle-bound pollutants in Tietê River basin

Schnurstein A, Braunbeck T (2001) Tail moment *versus* tail length-application of an *in vitro* version of the comet assay in biomonitoring for genotoxicity in native surface waters using primary hepatocytes and gill cells from zebrafish *(Danio rerio). Ecotoxicology and Enviromental Safety* 49: 187–96

Schwab K, Brack W (2007) Large volume TENAX® Extraction of the bioaccessible fraction of sediment-associated organic compounds for a subsequent effect-directed analysis. *Journal of Soils and Sediments* 7: 178–186

Seiler TB, Rastall AC, Leist E, Erdinger L, Braunbeck T, Hollert H (2006) Membrane dialysis extraction (MDE): A novel approach for extracting toxicologically relevant hydrophobic organic compounds from soils and sediments for assessment in biotests. *Journal of Soils and Sediments* 6: 20–29

Seitz N (2005) *Der ökologische Zustand der oberen Donau – eine integrierte Bewertung auf Grundlage von Makrozoobenthos und Sedimentkontakttests mit Danio rerio.* Master's thesis, University of Heidelberg

Seitz N, Böttcher M, Keiter S, Kosmehl T, Manz W, Hollert H, Braunbeck T (2008) Evaluation of the genotoxic potential of sediment samples from the Danube River using a new expert system and several analyzing methods: How to integrate information from dose-response curves. *Mutation Research* 652: 38–45

Silva IS, Abate G, Lichtig J, Masini JC (2002) Heavy metal distribution in recent sediments of the Tiete-Pinheiros river system in Sao Paulo state, Brazil. *Applied Geochemistry* 17: 105–116

Singh N, McCoy M, Tice R, Schneider E (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research* 175: 184–191

Streck H, Schulze T, Brack W (2008) Accelerated membrane-assisted clean-up as a tool for the clean-up of extracts from biological tissues. *Journal of Chromatography A* 1196: 33–40

Lübcke-von Varel U, Streck G, Brack W (2008) Automated fractionation procedure for polycyclic aromatic compounds in sediment extracts on three coupled normal-phase high-performance liquid chromatography columns. *Journal of Chromatography A* 1185: 31–42

Luebcke-von Varel U, Loeffler I, Streck G, Machala M, Ciganek M, Neca J, Pecikova K, Vykopalova L, Vondracek J, Reifferscheid G, Flueckiger-Isler S, Weiss JW, Lamoree M, Brack W (in prep.) Toxicity patterns of three contaminated sediments. Combining an automated multistep fractionation procedure and a battery of *in vitro* assays

Woelz J, Engwall M, Maletz S, Olsman Takner H, van Bavel B, Kammann U, Klempt M, Weber R, Braunbeck T, Hollert H (2008) Changes in toxicity and Ah receptor agonist activity of suspended particulate matter during flood events at the rivers Neckar and Rhine - a mass balance approach using *in vitro* methods and chemical analysis. *Environmental Science and Pollution Research* 15: 536–553

# Chapter 6

# Final discussion and conclusions

The problematics of polluted sediments of rivers have attracted world-wide attention and become a serious ecotoxicological and regulatory issue (Burton, 1991; Chapman, 2000; Hilscherova *et al.*, 2003; Viganò *et al.*, 2003). Sediment problematics are of course not restricted to rivers, since they also transport their pollution loads to lakes, estuaries and the marine environment, where polluted sediments pose a threat to any form of organism (Hallare *et al.*, 2005; Marvin *et al.*, 2000). Thus, research into the origin and effects of pollutants and subsequent suggestions for the ecological improvement of the Tietê River basin quality might become a model for further research and biological risk assessment of highly contaminated river systems worldwide.

The Tietê River, selected in this study as an example for a highly contaminated river system, is located in the most important economical center in Brazil, São Paulo state. The reservoirs constructed along its course are widely used for providing drinking water, as water sources for agricultural irrigation and as recreation sites.

The studied areas (Tietê River's spring, the reservoirs Ponte Nova, Barra Bonita, Bariri, Promissão and Três Irmãos), cover the entire length of the Tietê River from its spring to the mouth and also include two important water bodies associated to this river, Pinheiros River and Billings reservoir.

Several approaches involving different test organisms, several endpoints and different sediment phases (liquid as extract and solid as freeze-dried samples) were followed under laboratory conditions. Results document that sediment samples from various sites along the Tietê River Basin are differentially polluted with contaminants which cause not only acute cytotoxicity, but also genotoxicity and AhR-mediated toxicity in fish cells, as well as embryo toxicity. Moreover, mutagenicity was recorded *in situ* in fish caught from the field.

Differences in cytotoxicity, genotoxicity and AhR-mediated toxic poten-

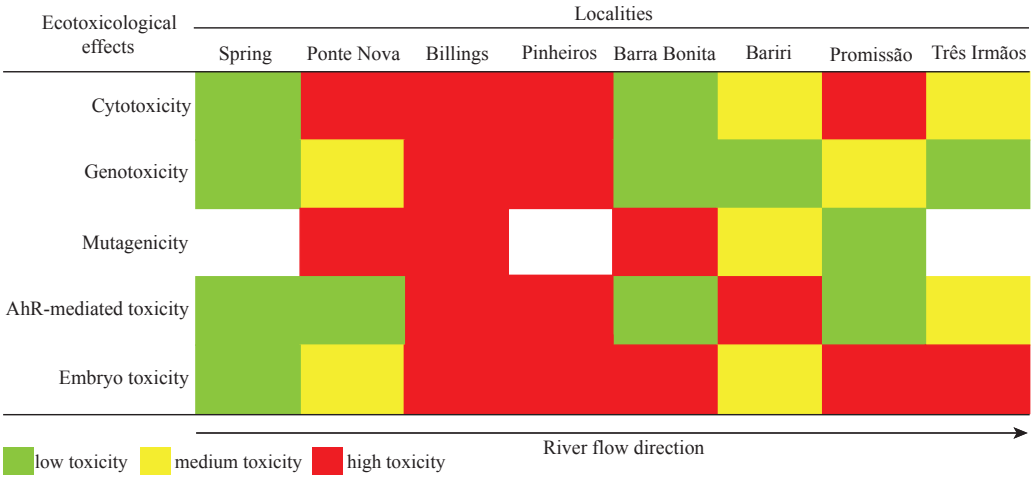


**Fig. 6.1.** Ecotoxicological effects recorded in the different locations along the Tietê River course. Relative intensities of effects represented with the traffic light colors, with low toxicity in green, medium toxicity in yellow and high toxicity in red (blank: not measured). Locations are arranged from left to right in the flow direction of the river. Classification is based on toxicity ranking for each ecotoxicological effect.

tials between different locations could be identified by the neutral red, comet and EROD assays, respectively, with RTL-W1 cells exposed to acetonic sediment extracts. A high correlation between genotoxicity *in vitro* and mutagenicity *in situ*/*in vivo* (micronucleus assay with fish blood erythrocytes) was obtained, underlining the high ecological relevance of sediment genotoxicity for the situation in the field. However, since organic extracts provide estimations of the total hazard potential, but neglect the bioavailability of sediment contaminants, there was still a need to develop more field-like exposure scenario for sediment quality assessment, taking into account both bioavailability and intact organisms in order to improve transferability of results and to better understand fate and behavior of water- and sediment-bound toxicants relevant for toxicity. Thus, in order to simulate *in situ* exposure conditions in a more realistic scenario, a sediment-contact fish embryo test was applied and changes in embryotoxicity were recorded in the whole river course. The fish embryo assay with solid phase sediments simulate the real exposure of early fish life stages in the field and, consequently, represents a bioassay of highest ecological relevance. In fact, the fish embryo assays revealed significant effects even for Barra Bonita, Promissão and Três Irmãos; thus, there is evidence that even the extended list of specific endpoints used in the present weight-of-evidence approach does not necessarily cover all aspects of toxicity.

Chemical analyses of sediment extracts revealed high PAH concentrations in Billings and Pinheiros sediment samples, as well as in samples from

Bariri reservoir, with values comparable to those obtained in well-known polluted European fresh water sediments (Keiter *et al.*, 2008). However, a closer analysis of chemical measurements of PAHs and results from EROD bioassay revealed that these PAHs could not explain more than 7 % of AhR-mediated toxicity, suggesting that EROD induction could be also related to non-analyzed compounds.

Overall, results suggest that most of the toxicity is due to the discharges of the metropolitan area of São Paulo, but indicate that pollutants sources occur differently along the whole river course (Fig. 6.1), contributing to the degradation of each reservoir. These results support conclusions obtained from several studies of Tietê River basin based on other toxicity parameters (Bainy *et al.*, 1999; Fracácio *et al.*, 2003; Rocha *et al.*, 2009; Silva *et al.*, 2002).

Fig. 6.2 presents a ranking of the ecotoxicological effects recorded in the different locations along the Tietê River course, with respect to results obtained by the different bioassays.

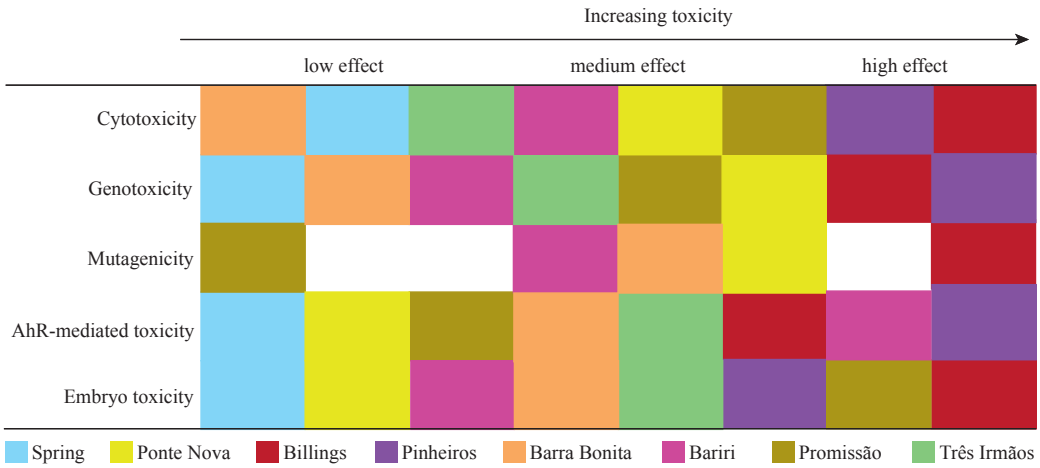


**Fig. 6.2.** Ranking of ecotoxicological effects recorded in the different locations along the Tietê River course. Increasing severity of toxicity is represented from left to right. Each location is represented by a specific color. Colors do not represent toxicity intensities.

Since major toxicities were associated to samples from the metropolitan region, sediment samples from Pinheiros River and Billings reservoir were submitted to a fractionation procedure, in order to identify key pollutants causing the high toxicity of sediments. In an effect-directed analysis, genotoxicity and EROD induction potency were detected in fractions of Billings and Pinheiros sediment extracts, indicating different sets of toxicants inducing genotoxicity and metabolic activation. Overall, results obtained from the investigation of the Pinheiros and Billings fractions suggested that genotoxicity

could be mostly related to alkanes, polychlorinated biphenyls (PCBs), naphthalenes (PCNs) as well as medium polar to polar polycyclic aromatic compounds (PACs), whereas AhR-mediated toxicity could mostly be attributed to polycyclic aromatic hydrocarbons (PAHs). The correlation of EPA-PAHs measured in the fractions which were strongest inducers of EROD activity and results from EROD assay revealed that these PAHs could explain only ca. 3% of the EROD-inducing potencies, suggesting that EROD activity in the fractions is related to non-priority PAHs. The fractionation method applied in this study only takes into account polycyclic aromatic compounds (PACs). Hence, it has to be considered that toxicity of raw sediment extracts can also be related to other priority and non-priority pollutants.

In fact, many cases of contamination not only by PAHs, but also by PCBs and organochlorine pesticides as well as heavy metals have been observed in waters in the Tietê River Basin (Bainy *et al.*, 1999): According to Leitão (1999), sediment from Billings reservoir showed high values of PCBs. Bainy *et al.* (1996) observed an unusual high level of total microsomal cytochrome P450 in liver and kidney of *O. niloticus* caught at this reservoir, which were associated with the PCBs detected in these organisms, and may also have been related to oxidative stress observed in several tissues of these animals (Bainy *et al.*, 1996). Fish from Billings reservoir were chronically exposed not only to high levels of PCBs but also to lindane, hexachlorobenzene, pentachlorophenol and dichlorodiphenyldichloroethylene (DDE), some of which are common inducers of CYP1A (CETESB, 1993). Silva *et al.* (2002) recorded high heavy metal concentrations in samples from Billings and Barra Bonita reservoirs. In an assessment of the concentrations of bioavailable metals, Fracácio *et al.* (2003) found that cadmium, lead, cobalt, copper, chromium, iron, magnesium, manganese and zinc were present along all Tietê River cascade reservoirs. Although their concentrations decreased downstream, some metal concentrations, *e.g.,* those for cadmium, were also relatively high in Três Irmãos, the last reservoir of the cascade.

In the present study, the combination of several test systems with different biological endpoints and chemical data allowed the determination of hazards and could give insights into ecotoxicological risks associated with contaminated sediments from Tietê River basin, and can therefore, be used as a model for further research and biological risk assessment of other highly contaminated river systems in the world. However, in an assessment of potential contamination of environmental samples, such as sediments, several points should be considered, among them: problems and possibilities for the interpretation of the results with respect to possible effects to the aquatic ecosystem; extrapolation of effects recorded in the test organisms to human health; scientifically well-based recommendations for the ecological improve-

ment of the environment studied; appropriate consideration of the costs for the assays, in case there is a need to cut them down.

In the present study, effectiveness analyses taking into consideration both ecological relevance and costs of the bioassays resulted in the conclusion, that specifically the sediment-contact fish embryo test proved to be a powerful tool for the determination of toxic effects, since whole sediment exposure scenarios with whole-organism realistically mimic the true bioavailability of toxicants and have, therefore, high ecological relevance, especially in cases when results are to be extrapolated to field conditions in rivers and lakes. Moreover, the costs are relative low, and the necessary materials to apply the tests are relatively easy to be obtained. No fancy laboratory equipment, *e.g.*, sterile benches, is necessary.

On the other hand, many times (and as exemplified here by Tietê River sediments) acute toxicity data (such as fish embryo toxicity) do not necessarily parallel specific effects such as genotoxicity, mutagenicity and dioxin-like activity. This corroborates the view that acute toxicity data are insufficient to extrapolate to specific toxic mechanisms and that a thorough assessment of the specific contamination levels of natural ecosystems like a river system definitely require a battery of different endpoints.

The ecotoxicological investigation of sediment samples from Tietê River basin revealed specific effects like genotoxicity and mutagenicity, which are of major concern in environmental evaluation of contaminants. In contrast to acute toxicity, which is most problematic for the present generation of target organisms, genotoxicity and mutagenicity can generally be correlated with the reproductive effects such as gamete loss due to cell death, embryonic mortality, and heritable mutations in a range of model animals including polychaete worms, nematodes, sea urchins, amphibians, and fish (Anderson & Wild, 1994; Chen & White, 2004). Aquatic organisms exposed to wastewater discharges and particle-bound substances suffer an increased risk of genetic damage. Fish, *e.g.*, accumulate pollutants directly from contaminated water or indirectly through the ingestion of contaminated aquatic organisms. Genotoxic pollutants may lead to the contamination not only of the aquatic organisms themselves, but also of the entire ecosystem and, finally, of humans via the food web (Matsumoto *et al.*, 2006). Thus, humans using water for drinking water purposes may suffer similar genetic or even carcinogenic risks as do fish (Kosmehl *et al.*, 2004, 2008). Moreover, in recent years, there has been increasing concern about the risk for human health following consumption of contaminated fish, since fish are top predators in the food web and can metabolize, concentrate, store and also biomagnify contaminants in a way similar to humans. The *in vitro* comet assay with permanent cells derived from fish, proved to be an effective tool for the detection of geno-

toxicity in the case of sediments from the Tietê River basin. However, *in vitro* assays using sediment extracts usually do not sufficiently reflect the actual hazard potential of sediments, since they seem to overestimate the bioavailability of pollutants. Moreover, when high costs are a problem to be considered, this assay (as applied here), has disadvantages since it requires sophisticated laboratory equipments, including, *e.g.*, sterile benches, extraction devices to transfer particle-bound substances from whole-sediment to the aqueous phase, specific computer programs, which altogether increase the costs of the test. On the other hand, the application of the micronucleus test with erythrocytes from fish collected in the field, is a suitable alternative and an affordable method to record mutagenicity *in vivo*, with the advantage that *in situ* assays are more likely to reflect the real exposure situation in the environment.

However, even with the disadvantage of representing "worst-case scenarios" and being "more expensive" than tests such as the micronucleus *in situ* assay with fish blood erythrocytes, *in vitro* bioassay approaches serve as efficient and fast screening tools for the evaluation of receptor-mediated activities of the complex mixtures (Hilscherova *et al.*, 2002), and the use of cell cultures for ecotoxicological assessment can be a valuable tool for an early and sensitive detection of chemical exposure (Castaño *et al.*, 1996). Since interactions between chemical contaminants and biological systems take place at the molecular and cellular levels at the first instance, cellular effect studies should be considered just as important as studies in laboratory species (Fent, 2001; Sanchez-Fortun *et al.*, 2008). Moreover, several endpoints (*e.g.*, genotoxicity, AhR-mediated toxicity) in cell culture-based system can only be tested after transfer of particle-bound substances into the aqueous phase, since particle-exposure is not (yet) suitable for these endpoints. For instance, in this study, AhR-mediated toxicity was detected based on induction of EROD activity in permanent cells derived from fish. The *in vitro* detection of cytochrome P4501A (CYP1A) induction has been recognized as a sensitive marker of exposure to a number of environmentally relevant toxicants (Behrens *et al.*, 2001; Berbner *et al.*, 1999; Bucheli & Fent, 1995; Engwall *et al.*, 1999). A variety of environmental pollutants, such as dioxin-like compounds, are able to induce the biotransformation enzyme CYP1A in fish, by ligand binding to the aryl hydrocarbon receptor (AhR; Babin *et al.*, 2005; Hilscherova *et al.*, 2001). The EROD activity is a sensitive tool, and it may even be an indicator for effects at various levels of biological organization (Van der Oost *et al.*, 2003). The EROD activities recorded in sediments from Pinheiros River, Billings and Bariri reservoirs, *e.g.*, are comparable to those recorded by Woelz *et al.* (2008) during a flood event at the Neckar, a river with well-documented pollution in Germany. In this context, it should be remembered that in

consequence of flood events, runoff and remobilized sediments may cause an increase of ecotoxicologically relevant effects from contaminant reservoirs. Hence, based on the results obtained from the present assay, it is possible to confirm that Pinheiros, Billings and Bariri, most likely represent important sources of diffuse contamination to the entire Tietê River system. Particular atention should be taken for Pinheiros and Billings, since the metropolitan region of São Paulo constantly suffers with flood events.

To conclude, to determine the health status of an ecosystem or hazard potential of a particulate sample, even when taking into account all the possible problems and possibilities of interpretation of results, weight-of-evidence approaches have to be applied. Only an integrated approach combining environmental chemical, toxicological and ecological concepts have a realistic chance of interpreting and understanding ecotoxicogical effects in contaminated ecosystems (Fent, 1996).

In the case of aquatic systems, the assessment of sediment quality is an indispensible component for the understanding of processes governing the fate and availability of pollutants since sediments are the final compartment for storage and transformation of most pollutants discharged by anthropogenic activities (Almeida & Rocha, 2006). A comprehensive evaluation of the ecotoxicological situation of sediments requires different approaches and broad knowledge for interpreting results. As exemplified for the Tietê River basin, a battery of bioassays applied in combination with chemical analyses and effect-directed analysis represent suitable tools to function as early warning systems not only for sediment pollution, but also for hazards for the entire river system. Fractionation of the most toxic samples allowed the identification of specific groups of pollutants responsible for the toxicity, and allowed to prioritize individual fractions for subsequent effect-directed analysis. Fractionation should allow sequential reduction of the complexity of these environmental mixtures to specific-classes of toxicants and eventually to individual toxicants. Finally, additional lines of evidence, *e.g.,* considering sediment dynamics, should be applied in future research, to further improve the assessment of the risk and hazard in natural ecosystems.

# Bibliography


Almeida CA, Rocha O (2006) Estudo Comparativo da Qualidade dos Sedimentos dos Reservatórios do Rio Tietê (SP). *Jornal Brasileiro da Sociedade de Ecotoxicologia* 1: 141–145

Anderson S, Wild GC (1994) Linking genotoxic responses to reproductive success in ecotoxicology. *Environmental Health Perspectives* 102: 9–12

Babin M, Casado S, Chana A, Herradon B, Segner H, Tarazona J, Navas J (2005) Cytochrome P4501A induction caused by the imidazole derivative Prochloraz in a rainbow trout cell line. *Toxicology in Vitro* 19: 899–902

Bainy ACD, Saito E, Carvalho PSM, Junqueira VB (1996) Oxidative stress in gills, erythrocytes, liver and kidney of Nile tilapia (*Oreochromis niloticus*) from a polluted site. *Aquatic Toxicology* 34: 151–162

Bainy ACD, Bruce RW, John JS (1999) Elevated levels of multiple cytochrome P450 forms in tilapia from Billings Reservoir - Sao Paulo, Brazil. *Aquatic Toxicology* 44: 289–305

Behrens A, Schirmer K, Bols NC, Segner H (2001) Polyciclic aromatic hydrocarbons as inducers of cytochrome P4501A enzyme activity in the rainbow trout liver cell line, RTL-W1 and in primary cultures of rainbow trout hepatocytes. *Environmental Toxicology and Chemistry* 20: 632–643

Berbner T, Rahman N, Braunbeck T (1999) Induction of cytochrome P450 1A and DNA damage in isolated rainbow trout (*Oncorhynchus mykiss*) hepatocytes by 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin. *Biomarkers* 4: 214–228

Bucheli TD, Fent K (1995) Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical reviews in environmental science and technology* 25: 201–268

Burton GA (1991) Assessing the toxicity of freshwater sediments. *Environmental Toxicology and Chemistry* 10: 1585–1627

Castaño A, Cantarino MJ, Castillo P, Tarazona JV (1996) Correlations between the RTG-2 cytotoxicity test EC50 and *in vivo* LC50 rainbow trout bioassay. *Chemosphere* 32: 2141–2157

CETESB (1993) Relatório da Qualidade das Águas Interiores do Estado de São Paulo - 1992. Tech. rep., CETESB

Chapman PM (2000) The Sediment Quality Triad: then, now and tomorrow. *International Journal of Environmental Pollution* 13: 351–356

Chen G, White PA (2004) The mutagenic hazards of aquatic sediments: a review. *Mutation Research* 567: 151–225

Engwall M, Brunström B, Näf C, Hjelm K (1999) Levels of dioxin-like compounds in sewage sludge determined with a bioassay based on EROD induction in chicken embryo liver cultures. *Chemosphere* 38: 2327–2343

Fent K (1996) Ecotoxicology of organotin compounds. *Critical Reviews i Toxicology* 26: 1–117

Fent K (2001) Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. *Toxicology in Vitro* 15: 477–488

Fracácio R, Verani NF, Espíndola ELG, Rocha O, Rigolin-Sá O, Andrade CA (2003) Alterations on growth and gill morphology of *Danio rerio* (Pisces, Ciprinidae) exposed to the toxic sediments. *Brazilian Archives of Biology and Technology* 46: 685–695

Hallare AV, Kosmehl T, Schulze T, Hollert H, Koehler HR, Triebskorn R (2005) Assessing the severity of sediment contamination in Laguna Lake, Philippines using a sediment contact assay with zebrafish (*Danio rerio*) embryos. *Science of the Total Environment* 347: 254–271

Hilscherova K, Kannan K, Kang YS, Holoubeck I, Machala M, Masunaga S, Nakanishi J, Giesy JP (2001) Characterization of Dioxin-like activity of sediments from a Czech river basin. *Environmental Toxicology and Chemistry* 20: 2768–2777

Hilscherova K, Kannan K, Holoubeck I, Giesy JP (2002) Characterization of estrogenic activity of riverine sediments from the Czech Republic. *Archives of Environmental Contamination and Toxicology* 43: 175–185

Hilscherova K, Kannan K, Nakata H, Hanari N, Yamashita N, Bradley PW, McCabe JM, Taylor AB, Giesy JP (2003) Polychlorinated dibenzo-p-dioxin and dibenzofuran concentration profiles in sediments and flood-plain soils of the Tittabawassee River, Michigan. *Environmental Science and Technology* 37: 468–474

Keiter S, Grund S, van Bavel B, Hagberg J, Engwall M, U K, Klempt M, Manz W, Olsman H, Braunbeck T, Hollert H (2008) Activities and identification of aryl hydrocarbon receptor agonists in sediments from the Danube River. *Analytical Bioanalytical Chemistry*

Kosmehl T, Krebs W Fand Manz, Erdinger L, Braunbeck H Tand Hollert (2004) Comparative genotoxicity testing of Rhine river sediment extracts using the permanent cell lines RTG-2 and RTL-W1 in the comet assay and Ames assay. *Journal of Soils and Sediments* 4: 84–94

Kosmehl T, Hallare AV, Braunbeck T, Hollert H (2008) DNA damage induced by genotoxicants in zebrafish (*Danio rerio*) embryos after contact exposure to freeze-dried sediment and sediment extracts from Laguna Lake (The Philippines)

as measured by the comet assay. *Mutation Research* 650: 1–14

Leitão MAS (1999) *Estudo sazonal de enzimas biomarcadoras de poluição aquática, em tilápias do nilo (Oreochromis niloticus) da represa Billings - SP*. Ph.D. thesis, Universidade de São Paulo

Marvin CH, McCarry BE, Villella J, Allan LM, Bryant DW (2000) Chemical and biological profiles of sediments as indicators of sources of genotoxic contamination in Hamilton Harbour. Part I: Analysis of polycyclic aromatic hydrocarbons and thia-arene compounds. *Chemosphere* 41: 979–988

Matsumoto ST, Mantovani MS, Malaguttii MIA, Dias AL, Fonseca IC, Marin-Morales MA (2006) Genotoxicity and mutagenicity of water contaminated with tannery effluents, as evaluated by the micronucleus test and comet assay using the fish *Oreochromis niloticus* and chromosome aberrations in onion root-tips. *Genetics and Molecular Biology* 29: 148–158

Van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology* 13: 57–149

Rocha PS, Luvizotto GL, Kosmehl T, Böttcher M, Storch V, Braunbeck T, Hollert H (2009) Sediment genotoxicity in the Tietê River (São Paulo, Brazil): *in vitro* comet assay *versus in situ* micronucleus assay studies. *Ecotoxicology and Enviromental Safety* 72: 1842–1848

Sanchez-Fortun S, Llorente MT, Castaño A (2008) Cytotoxic and genotoxic effect in RTG-2 cell line exposed to selected biocides used in the disinfection of cooling towers. *Ecotoxicology* 17: 273–279

Silva IS, Abate G, Lichtig J, Masini JC (2002) Heavy metal distribution in recent sediments of the Tiete-Pinheiros river system in Sao Paulo state, Brazil. *Applied Geochemistry* 17: 105–116

Viganò L, Arillo A, Buffagni A, Camusso M, Ciannarella R, Crosa G, Falugi C, Galassi S, Guzzella L, Lopez A, *et al.* (2003) Quality assessment of bed sediments of the Po River (Italy). *Water research* 37: 501–518

Woelz J, Engwall M, Maletz S, Olsman Takner H, van Bavel B, Kammann U, Klempt M, Weber R, Braunbeck T, Hollert H (2008) Changes in toxicity and Ah receptor agonist activity of suspended particulate matter during flood events at the rivers Neckar and Rhine - a mass balance approach using *in vitro* methods and chemical analysis. *Environmental Science and Pollution Research* 15: 536–553

# Appendices

# Appendix A

# Weight-of-Evidence-Studie zur Sedimentbelastung des Tietê River in Brasilien

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## Projektvorstellung

# Weight-of-Evidence-Studie zur Sedimentbelastung des Tietê River in Brasilien

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Der Fluss Tietê im südlichen Brasilien hat eine Gesamtlänge von 1150 km und durchquert den gesamten Bundesstaat Sao Paulo. Der Höhenunterschied zwischen Quelle und Mündung beträgt etwa 860 m und wird mittels einer Reihe von Stauhaltungen zur Stromerzeugung genutzt. Sowohl hinsichtlich seiner organischen als auch der anorganischen Schadstofffrachten gilt der Tietê als stark belastet. Bereits fünf Kilometer nach der Quelle beginnt die Verunreinigung des Tietê und erreicht ihren Höhenpunkt in São Paulo. Dort gleicht der Tietê einem vollständig verschmutzten und stinkenden Abwasserkanal, in dem selbst Autos und Möbelstücke umher treiben. Nach weiteren 300 km, nahe der Kleinstadt Barra Bonita, ist die Wasserqualität bereits deutlich verbessert.

Die Bewertung von Sedimenten nimmt in diesem Weight-of-evidence-Projekt einen hohen Stellenwert ein, da aus verschiedenen anderen Studien bekannt ist, dass viele Schadstoffeinträge eine hohe Affinität zu Schwebstoffen besitzen, an diesen adsorbieren und dadurch der Wassersäule entzogen werden. Die Sedimente stellen nach Sedimentation der Schwebstoffe eine Senke für diese Schadstoffe dar; sie können in staureguliertenFlusssystemen insbesondere durch Hochwasserereignisse, Verklappungen und Spülungen von Stauhaltungen wieder remobilisiert werden. Sedimentgebundene Schadstoffe sind aus zahlreichen Studien für ihre nachteilige Wirkung insbesondere gegenüber benthischen Fischen bekannt und können bei Verzehr von kontaminierten Fischen ein Gesundheitsrisiko für den Menschen darstellen. Auch die Sedimentation kontaminierter Schwebstoffe auf landwirtschaftlich genutzten Überflutungsflächen und eine mögliche Beeinträchtigung des Trinkwassers können problematisch sein.

Ziel dieses Projektes ist es, eine umfassende Weight-of-evidence-Studie durchzuführen, bei der nicht nur die Sedimentqualität des Tietê sondern auch das Ausmaß der ökotoxikologischen Belastung *in situ* bewertet werden sollen, um eine Risikoabschätzung und ggf. eine Toxicity Reduction Evaluation zu ermöglichen.

Hierzu werden an sieben verschiedenen Standorten entlang des Tietê Sedimentproben für ökotoxikologische Wirktests und chemische Analysen entnommen sowie Fische für Mikrokerntests *in situ* gefangen (**Abb. 1**): Während die Quelle des Tietê und die Stauhaltung Ponte nova als Referenzstandorte oberhalb von São Paulo dienen, repräsentiert die Stauhaltung Billings einen Flussabschnitt, der von der Metropole ökotoxikologisch stark beeinträchtigt wird. Für die stromabwärts gelegenen Stau-

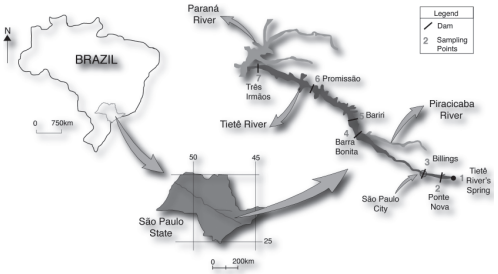


**Abb. 1:** Standorte der Sedimentprobennahmen entlang des Tietê

haltungen Bairi und Bara Bonita ist aus chemischen Untersuchungen bereits eine Abnahme der partikelgebunden Schadstoffkonzentrationen bekannt. Promissao und Três Irmaos gelten aus vorhergehenden Untersuchungen als relativ gering belastet.

Für eine umfassende ökotoxikologische Risikobewertung des Tietê werden in diesem Projekt sowohl akut toxische als auch spezifische Endpunkte (mutagene, gentoxische, teratogene, östrogene und dioxinähnliche Wirksamkeit) eingesetzt. Die akute Cytotoxizität wird im Neutralrottest mit der Zelllinie RTL-W1 aus der Regenbogenforelle (*Oncorhynchus mykiss*) ermittelt. Unter Verwendung des Sedimentkontakttests mit *Danio rerio* und des Bakterienkontakttests mit *Arthrobacter globiformis* werden native Sedimentproben hinsichtlich ihrer bioverfügbaren Toxizität untersucht. Die mutagene, gentoxische und dioxinähnliche Wirksamkeit von Sedimentextrakten wird mit dem Ames-Test, dem Comet- und EROD-Assay mit der Zelllinie RTL-W1 ermittelt. Der Mikronukleustest mit Fischzellen stellt eine geeignete Methode dar, um sowohl gentoxische *In vivo*- als auch *In situ*-Untersuchungen durchzuführen und die Gewässerqualität zu überprüfen. In verschiedenen Freiland- und Laboruntersuchungen konnte gezeigt werden, dass in Fischerythrocyten nach Exposition mit unterschiedlichen Schadstoffen die Anzahl der Mikronuklei deutlich zunimmt. Daher wird der Mikronukleustest mit Erythrocyten von Fischen aus dem Tietê in dieser Studie eingesetzt, um die Relevanz der gewonnen *In vitro*-Biotests für die aquatische Gemeinschaft *in situ* zu überprüfen. In dem Projekt sollen weiterhin die Konzentrationen von organischen und anorganischen Schadstoffen erfasst werden.

# Appendix B

# Perfluorooctane sulfonate is increasing the genotoxicity of cyclophosphamide in the micronucleus assay with V79 cells

## Further proof of alterations in cell membrane properties caused by PFOS

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## Abstract

Perfluorooctane sulfonate (PFOS, $C_8F_{17}SO_3^-$) is a fully fluorinated organic compound which has been manufactured for decades and was used widely in industrial and commercial products. The recent toxicological knowledge of PFOS mainly concerns mono-substance exposures of PFOS to biological systems, leaving the potential interactive effects of PFOS with other compounds as an area where un-

derstanding is significantly lacking. However, a recent study, reported the potential of PFOS to enhance the toxicity of two compounds by increasing cell membrane permeability. This is of particular concern since PFOS has been reported to be widely distributed in the environment where contaminants are known to occur in complex mixtures. In this study, PFOS was evaluated alone and in combination with cyclophosphamide (CPP) to investigate whether a presence of PFOS leads to an increased genotoxic potential of CPP towards hamster lung V79 cells. Genotoxicity was investigated using the micronucleus (MN) assay according to the recent draft ISO/DIS 21427-2 method. PFOS alone demonstrated no genotoxicity up to a concentration of 12.5 µg/ml. However, PFOS combined with two different concentrations of CPP, with metabolic activation, caused a significant increase in the number of micronucleated cells compared to treatments with CPP alone. These results provide a first indication that PFOS has the potential to enhance the genotoxic action of CPP towards V79 cells, suggesting, together with the alterations in cell membrane properties shown previously, that genotoxicity of complex mixtures may be increased significantly by changes in chemical uptake. Together with an earlier study performed by the own working group, it can be concluded that PFOS alone is not genotoxic in this bioassay using V79 cells up to 12.5 µg/ml, but that further investigations are needed to assess the potential interaction between PFOS and other substances, in particular regarding the impact of membrane alterations on the uptake of toxic substances.

*Keywords*: Alterations in cell membrane properties; genotoxicity; micronucleus assay; perfluorooctane sulfonate; PFOS

## B.1 Introduction

Perfluorooctane sulfonate (PFOS, $C_8F_{17}SO_3^-$) belongs to a group of fully or 'perfluorinated' compounds which has recently received increasing attention based on its occurrence in the environment and toxicological effects. PFOS has been synthetically produced for more than 50 years and, due to its unique surface-active properties, is widespread in industrial and commercial products, *e.g.*, fire fighting foams and coatings for textiles and paper products approved for food contact. The chemical structure of PFOS is characterized by an alkyl chain with fluorine substitutions forming strong carbon-fluorine bonds (C-F). Due to these high-energy bonds, PFOS demonstrates great resistance to hydrolysis, photolysis, microbial degradation and metabolism by vertebrates. PFOS is therefore considered to be persistent in the environment (Giesy & Kannan, 2002). PFOS is among the most commonly detected perfluorinated chemical in the environment (Martin *et al.*, 2004; Tseng *et al.*, 2006) and has been identified in arctic mammals (Giesy & Kannan, 2001; Bossi *et al.*, 2005; Smithwick *et al.*, 2006) as well as in human blood

and serum samples (Yeung *et al.*, 2006; Karrman *et al.*, 40). Recently, another perfluorinated chemical, perfluorooctanoic acid (PFOA), was detected at concentrations of 3,640 ng/L and 519 ng/L in German surface water and drinking water, respectively (Skutlarek *et al.*, 2006a,b). These findings are of concern and demonstrate the need for a comprehensive understanding of the toxic potential of these chemicals. Another issue that needs to be addressed in the context of the environmental toxicology of PFOS is the fact that contaminants in the environment almost always occur in complex mixtures. This leads to exposure situations where contaminants can interact and cause synergistic or antagonistic effects and, thus, gives rise to unexpected consequences compared to the individual toxicity of the contaminants. PFOS has been reported to be non-genotoxic in a number of microbial and mammalian assays (Chemical Committee and Working Party on Chemicals Pesticides and Biotchenology, 2002). The aim of the present study was to investigate the genotoxicity of PFOS as well as to evaluate whether PFOS possess the potential to increase the toxic action of a standard genotoxic substance, cyclophosphamide (CPP), towards hamster lung V79 cells. Genotoxicity was investigated using the micronucleus (MN) assay.

## B.2 Method

The MN assay was performed according to the ISO Draft International Standard ( ISO/DIS21427-2, Reifferscheid *et al.*, 2007). V79 cells were seeded at a density of 5 x 104 cells/ml onto slides in culture dishes and incubated at 37$^{o}$C for 6 h. V79 cells with metabolic activation (rat liver S9 mix) were treated with 12.5 µg/ml PFOS both alone and in combination with 1.25 µg/ml or 2.5 µg/ml CPP for 4 h. Control cells were treated both with and without DMSO (1%). After fixation and air-drying preparations, slides were stained with Giemsa for 20 min. Two independent experiments were performed with two replicates for each treatment. Per treatment replicate, a total of 1000 cells were scored for the evaluation of the frequency of MN. In addition, the concentration of PFOS was determined using LC-MS/MS (Skutlarek *et al.*, 2006b). This was performed in order to assess differences in nominal and real concentrations. The chemical analysis indicated a good recovery of PFOS in the bioassay (100–110% of the nominal concentration).

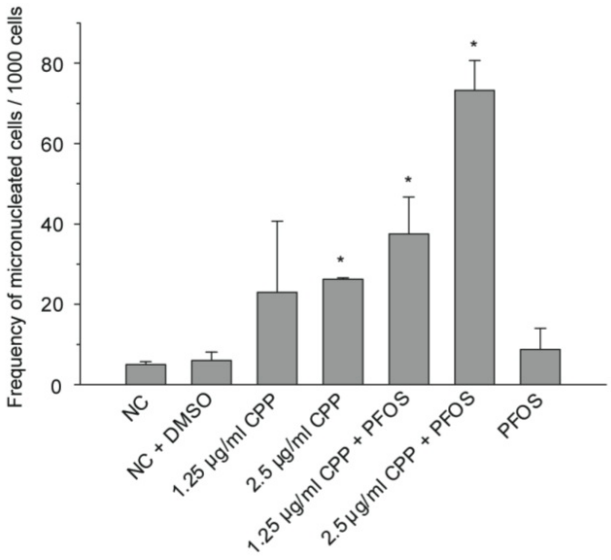


**Fig. B.1.** Frequency of micronucleated V79 cells exposed to PFOS (12.5 mg/L) combined with CPP (1.25 µg/ml and 2.5 µg/ml) with metabolic activation. The frequency of micronucleated cells is showing a clear increase in treatments with both substances compared with the single-substance treatments. Minimum Essential Medium (MEM) with DMSO (1 %) served as a negative control (NC). Data are means ± SD of two independent experiments with 2 replicates each. 1000 cells were assessed within each replicate.

## B.3 Results

PFOS alone exhibited no increase in the frequency of micronucleated cells relative to the control (Fig. B.1).

These results are in agreement with an earlier MN assay performed by our working group where PFOS (12.5 µg/ml) was evaluated towards V79 cells both with and without metabolic activation (data not shown). Co-exposure of cells to PFOS and CPP caused a significantly greater incidence of MN when compared to both controls. PFOS combined with CPP (2.5 µg/ml) resulted in a clear increase in micronucleated cells compared with the same concentrations of CPP and PFOS alone. A similar tendency was observed for PFOS combined with CPP (1.25 µg/ml), although cautious interpretation is required due to high variations in the treatment with CPP (1.25 µg/ml). Addition of PFOS to CPP (1.25 µg/ml) caused a greater frequency of micronucleated cells than the greater concentration of CPP (2.5 µg/ml) alone.

## B.4 Discussion and conclusion

The amphiphilic properties of PFOS suggest that cell membranes could be affected, potentially leading to an increase of the cellular 'accessibility' of other substances and a loss in homeostasis. Considering the global distribution of PFOS in wildlife and humans, the interactive effects of PFOS with other compounds could represent a cause for potential human and environmental health risks. This study offers the first indication of the potential of PFOS to increase the genotoxic action of CPP towards V79 cells. These results demonstrate that the frequency of micronucleated cells is greater in the combined treatments with metabolic activation compared to treatments with CPP and PFOS alone. PFOS along with metabolic activation showed no genotoxic potential towards V79 cells. Co-exposure of cells to PFOS and CPP (2.5 µg/ml) induced approximately three and eight times the amount of micronucleated cells compared to treatments with either CPP (2.5 µg/ml) or PFOS, respectively. Due to the high variation in treatments with 1.25 µg CPP/ml, no clear conclusions can be drawn concerning the same concentration combined with PFOS. PFOS combined with 1.25 µg CPP/ml, however, did reveal a slightly greater genotoxicity than 2.5 µg CPP/ml alone, *i.e.*, the twofold increased concentration. It therefore seems reasonable to suggest that, while PFOS itself is inactive at micronuclei induction, the combination with CPP increases the mutagenic action of CPP by some undefined mechanism. Co-exposure of the two chemicals could suggest an additive effect with respect to the significant increase of micronucleated cells. The combination of PFOS and the higher concentration of CPP caused a larger increase of MN than would have been expected based on an additive toxicity assumption. Therefore, it appears that the greater genotoxicity of CPP in the presence of PFOS is caused by a potentiation. A possible explanation for this observation might be due to increased permeability of cell membranes. PFOS was found to affect membrane fluidity and mitochondrial membrane potential in fish leukocytes (Hu *et al.*, 2003) and to inhibit gap junctional, intercellular communication in both rat liver cells and dolphin kidney cells (Hu *et al.*, 2002). These reports provide strong indications of the membrane alteration abilities of PFOS. Furthermore, PFOS was reported to increase the permeability of cell membranes to 2,3,7,8-tetrachlorodibenzo-p-dioxin and estradiol (Hu *et al.*, 2003). However, while PFOS (0.1 µg/ml) significantly increased responses to TCDD and E2, the increases observed were only approximately 40%. In the current study the potentiation of genotoxicity was greater. This observation may indicate that PFOS differentially increases the membrane permeability of structurally different compounds. The results suggest that the permeability of polar compounds, resulting from metabolic activation,

may be more greatly enhanced than that of the previously studied, relatively non-polar compounds. From these observations, along with the present study, it seems possible that alterations in cellular membrane properties caused by PFOS could have considerable impact on the availability of CPP to V79 cells, leading to the enhanced genotoxic action of CPP. We conclude that these results indicate a need for further and continued research activity to comprehensively assess the interactive effects of PFOS with other compounds in complex environmental mixtures, in particular with respect to the impact of membrane alterations on the uptake and effects of toxic substances. Because PFOS binds tightly to proteins (Jones *et al.*, 2003) and the *in vitro* system studied here can not completely mimic the pharmacokinetics in an *in vivo* system, the combination studies need to be repeated *in vivo* with exposures that are environmentally relevant in both dose and dose rate.

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# Bibliography


Bossi R, Riget FF, Dietz R (2005) Temporal and spatial trends of perfluorinated compounds in ringed seal (*Phoca hispida*) from Greenland. *Environmental Science and Technology* 39: 7416–7422

Chemical Committee and Working Party on Chemicals Pesticides and Biotchenology (2002) Hazard assessment of perfluorooctane sulfonate (PFOS) and its salts. *ENV/JM/RD 17/Final, OECD*

Giesy JP, Kannan K (2001) Global distribution of perfluorooctane sulfonate in wildlife. *Environmental Science and Technology* 35: 1339–1342

Giesy JP, Kannan K (2002) Perfluorochemical surfactants in the environment. *Environmental Science and Technology* 36: 146A–152A

Hu W, Jones PD, Upham BL, Trosko JE, Lau C, Giesy JP (2002) Inhibition of gap junctional intercellular communication by perfluorinated compounds in rat liver and dlphin kidney epithelial cell lines *in vitro* and Sprague-Dawley rats *in vivo*. *Toxicological Sciences* 429–436

Hu W, Jones PD, DeCoen W, King L, Fraker P, Newsted J, Giesy JP (2003) Alterations on cell membrane properties caused by perfluorinated compounds. *Comparative Biochemistry and Physiology* 135: 77–88

ISO/DIS21427-2 (2006) *Water quality - Evaluation of genotoxicity by measurement of the induction of micronuclei - Part 2; "Mixed population" method using the cell line V79*

Jones PD, Hu W, DeCoen W, Newsted JL, Giesy JP (2003) Binding of perfluorinated fatty acids to serum proteins. *Environmental Toxicology and Chemistry* 22: 2639–2649

Karrman A, Mueller JF, van Bavel B, Harden F, Toms LML, Lindstrom G (40) Levels of 12 perfluorinated chemicals in pooled Australian serum, collected 2002-2003, in relation to age, gender and region. *Environmental Science and Technology* 12: 3742–3748

Martin JW, Smithwick MM, Braune BM, Hockstra PF, Muir DCG, Mabury SA (2004) Identification of long-chain perfluorinated acids in biota from the Canadian artic. *Environmental Science and Technology* 38: 373–380

Reifferscheid G, Dill F, Fieblinger D, Gminski R, Grummt HJ, Hafner C, Hollert

H, Kunz S, Rodrigo G, Stopper C H Ziemann, Selke D (2007) Untersuchung von Abwasserproben auf Genotoxizitaet Ergebnisse eines Ringversuchs mit dem *in vitro* - Mikrokerntest im Rahmen der Standardisierung nach ISO. *Umweltwissenschaften und Schadstoff-Forschung* 19: 7–16

Skutlarek D, Exner M, Faerber H (2006a) Perfluoriniert Tenside (PFT) in der aquatischen Umwelt und im Trinkwasser. *UWSF - Z Umwelchem Oekotox* 18: 151–154

Skutlarek D, Exner M, Faerber H (2006b) Perfluorinated surfactants in surface and drinking waters. *Environmental Science and Pollution Research* 13: 299–307

Smithwick M, Norstrom RJ, Mabury SA, Solomon K, Evans TJ, Stirling I, Taylor MK, Muir DCG (2006) Temporal trends of perfluoroalkyl contaminants in polar bears (*Ursus maritimus*) from two locations in the North American artic, 1972-2002. *Environmental Science and Technology* 40: 1139–1143

Tseng CL, Liu LL, Chen CM, Ding WH (2006) Analysis of perfluorooctanesulfonate and related fluorochemicals in water and biological tissue samples by liquid chromatography-ion trap mass spectometry. *Journal of Chromatography A* 1105: 119–126

Yeung LWY, So MK, Jiang G, Taniyasu S, Yamashita N, Song M, Wu Y, Li J, Giesy JP, Guruge KS, Lam PKS (2006) Perfluorooctanesulfonate and related fluorochemicals in human blood samples from China. *Environmental Science and Technology* 40: 715–720

# Appendix C

# Comparison of *in vitro* and *in situ* genotoxicity in the Danube River by means of the comet assay and the micronucleus test

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## Abstract

Genotoxicity can be correlated with adverse reproductive effects or may even result in elevated extinction risk. It may thus be a valuable tool for screening of pollution and potential environmental harm. Since many genotoxicants tend to adsorb to particulate matter, sediments and suspended materials are of particular interest for genotoxicity screening under field conditions. In order to relate the genotoxic potential of sediments to genetic damage of fish, RTL-W1 cells were exposed *in vitro* to acetonic sediments extracts collected at 10 selected sites along the upper Danube River and analyzed in the comet and micronucleus assays, and these *in vitro* results were compared to micronucleus formation in erythrocytes of European barbel (*Barbus barbus*) caught in the field. The two *in vitro* bioassays showed excellent correlation indicating comparability of genotoxic potentials

*in vitro*. Sampling sites could be clearly differentiated with respect to severity of effects, with Rottenacker as the most heavily contaminated site, with Ehingen and Schwarzach as moderately genotoxic, and with least effects in the tributary Lauchert. All other sediment extracts showed intermediate genotoxic or mutagenic effects. *In situ*, micronucleus formation in barbel erythrocytes indicated severe genotoxicity at Rottenacker, moderate effects at Ehingen, but minor contamination at Riedlingen and Sigmaringen. *In situ* observations thus showed excellent correlation with corresponding *in vitro* tests and document the ecological relevance of *in vitro* studies with sediment extracts. With respect to the ecological status of the Danube River, results overall indicate a moderate to severe genotoxic potential with a highly differential localization.

*Keywords*: Genotoxicity, micronucleus assay, comet assay, *in situ*, *in vitro*

# C.1 Introduction

Records of the upper Danube River document improvements of water quality over the last three decades. The reasons for the improvement of major water quality parameters are more restrictive waste water legislation, implementation of water protection programs as well as modernization of sewage plants (WWA, 2004). This improvement, however, is in contradiction to only minor recoveries or even further declines of fish stocks in the Danube River (Keiter *et al.*, 2006); similar trends have been observed for many other streams in Europe, USA and Canada since the mid 1980s (Burkhardt-Holm *et al.*, 2005; Cook *et al.*, 2003; Faller *et al.*, 2003; Lafontaine *et al.*, 2002). It may be concluded that common health and quality parameters apparently fail in particular river systems at least with respect to fish populations. As a consequence, more subtle parameters are required for assessing the status of aquatic ecosystems.

Biotests, for example, give signals of pollution and potential damage in the environment (Huschek & Hansen, 2005). Among other parameters, genotoxicity is of special interest, since it may directly be correlated with adverse reproductive effects (Anderson & Wild, 1994) or even lead to an elevated risk of extinction (Diekmann *et al.*, 2004b). Previous studies on fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*) and fern (*Onoclea sensibilis*) have shown that exposure to mutagens in water may well enhance the frequency of heritable recessive lethal mutations (Diekmann *et al.*, 2004a; Schoen *et al.*, 2002; White *et al.*, 1999), and the accumulation of such deleterious mutations can directly contribute to the decline of small populations via a phenomenon known as mutational meltdown (Lynch *et al.*, 1995).

Genotoxic activity of organic compounds in various types of industrial

effluents (*e.g.*, metal refining and founding) or runoff is predominantly associated with particulate materials (White *et al.*, 1996), and, consequently, with sediments. Sediments therefore represent reservoirs of genotoxic and mutagenic hazard, which can continually be reintroduced into the water column via resuspension or trophic transfer. Sediments may thus contribute substantially to the exposure of benthic biota as well as subsequent bioaccumulation (Chen & White, 2004; Van den Berg *et al.*, 1998).

For the screening of sediment genotoxicity, *in vitro* bioassays proved to be valuable tools in aquatic ecotoxicology to determine the total biological potential of a mixture of chemicals or environmental extracts as well as individual substances (Chen & White, 2004). In the present study, the comet assay was selected as an *in vitro* genotoxicity assay, since it represents a rapid, sensitive and inexpensive method for measuring genotoxic effects in individual cells (Fairbairn *et al.*, 1995; Lee & Steinert, 2003). It allows the detection of DNA strand breaks and alkalilabile sites by measuring the migration of DNA fragments from immobilized nuclear DNA (Singh *et al.*, 1988). Additionally, it can be conducted on virtually any eukaryotic cell type, *in vivo* as well as *in vitro* (Lee & Steinert, 2003). In this study, the comet assay was performed *in vitro* following exposure of the cell line RTL-W1 (Lee *et al.*, 1993) to organic sediment extracts from the upper Danube River.

In contrast, the micronucleus assay is based on the loss of chromosomes or chromosome fragments during meiosis, which are not reincorporated into the nucleus after cell division and, therefore, are transformed into a smaller nucleus or micronucleus (Grisolia, 2002; Jenssen & Ramel, 1980). The micronucleus test was also used for RTL-W1 cells previously exposed to the organic sediment extracts. Surprisingly, although this fish-derived permanent cell line has repeatedly proved to be a useful tool for the detection of genotoxic pollutants in sediments, it has not been used previously in the micronucleus assay.

However, even though organic extracts of sediments have frequently been used to assess the potential ecotoxicological hazard of sediments (Chen & White, 2004; Hollert *et al.*, 2000; Schwab & Brack, 2007; Seiler *et al.*, 2006), the relevance of such studies for the field situation is difficult to assess (Reid & Semple, 2000). In contrast, bioassays with animals collected in the field represent a more realistic exposure scenario, which better reflects ecosystem health status and the fate of introduced contaminants (Chapman & Hollert, 2006). Therefore, European barbel was selected for assessing the genotoxic potential *in situ* by means of the micronucleus test in erythrocytes. Since fish occupy a top position in the food web, they are eligible bioindicators. Especially European barbel (*Barbus barbus*), as benthic fish, may accumulate toxicants from sediments and should, thus, be particularly more at risk to en-

vironmental pollution (Vindimian *et al.*, 1991). Furthermore, in common fish zonation along river systems (Huet, 1949), a 'barbel zone' has been defined with the barbel as the dominant species. The ecological significance of this species thus further corroborates its value as an indicator species, and the wide zoogeographic distribution of Barbus sp. in Europe allows the future comparison between different rivers within *in situ* monitoring programs.

For decades, researchers have tried to identify a causal link between chemical contamination and genotoxicity in fish populations (Macek, 1980). Nonetheless, to the best of our knowledge, studies comparing results of *in situ* assays, reflecting real exposure conditions (actual ecological status), and results from *in vitro* approaches (*e.g.*, using samples such as sediments) from the same ecosystem for exposure are scant. This is even more surprising, since, in order to elucidate the comparability and adequacy of *in vitro* tests for replacing field studies, combination and correlation of both approaches are indispensable.

Thus, the objectives of this study were (1) to compile data from different *in vitro* test systems to identify the total hazard potential of sediments from the upper Danube River, and (2) to compare this to the *in situ* genotoxic potential in order to elucidate the ecological relevance of *in vitro* results and their suitability as possible bioassay systems.

## C.2 Material and Methods

### C.2.1 Samples

In May, July and October 2004, near-surface sediment samples were taken by means of a van Veengripper or a stainless steal shovel at eight locations along the upper Danube River (Sigmaringen, Riedlingen, Rottenacker, Ehingen, Oepfingen, Ingolstadt, Bad Abbach, Jochenstein, all Germany) as well as at two tributaries (Lauchert and Schwarzach; Fig. C.1 ). Cooled samples were transferred to the laboratory, freeze-dried and sieved at 2 mm. Sub-samples were used for preparation of organic extracts in a Soxhlet apparatus (for further details, see Keiter *et al.*, 2006). The resulting extract concentration was adjusted to an equivalent of 20 g sediment equivalents dry weight per ml DMSO (Serva, Heidelberg, Germany). Extract concentrations used in the comet assay with RTL-W1 cells are given in mg sediment equivalent per ml (SEQ/ml).

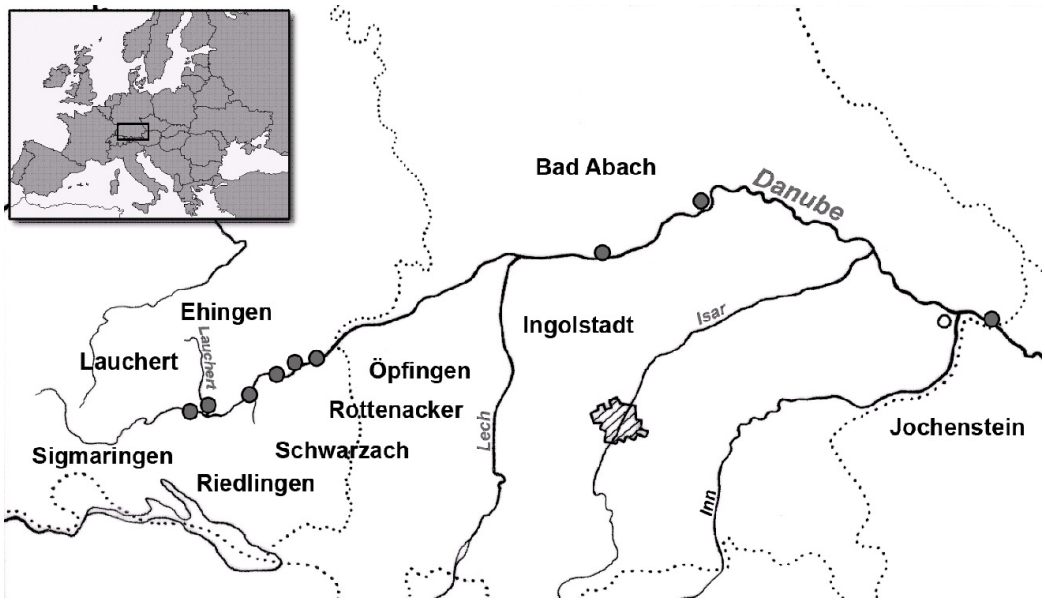


**Fig. C.1.** Study area and sampling locations.

## C.2.2 *In vitro* tests

### Cell culture conditions

RTL-W1 cells derived from rainbow trout liver (*Oncorhynchus mykiss*; were cultured in Leibovitz L15 medium (Sigma-Aldrich, Deisenhofen, Germany) according to Klee *et al.* (2004). Prior to use in the *in vitro* assays, RTL-W1 cells were washed twice with phosphate-buffered saline (PBS; Sigma-Aldrich) and trypsinized for 2 min according to Kosmehl *et al.* (2004), using 0.05 % trypsin (Sigma-Aldrich) and 0.02 % ethylene diamine tetra acetic acid (EDTA; Sigma-Aldrich).

### Negative controls

For the *in vitro* tests, three different types of negative control were used: (1) a medium control (test medium only), (2) a solvent control with a concentration range between 2.5 and 40 mg DMSO/ml in the test medium, and (3) a process control for Soxhlet extraction with empty extraction thimbles. The resulting Soxhlet control extract was conce(Borenfreund & Puerner, 1984)ntrated and re-solved in DMSO for testing. Since no significant differences could be observed between all three controls, they will collectively be referred to as 'negative controls'.

### Comet assay

The comet assay was performed under alkaline conditions following the procedure of Singh *et al.* (1988) with modifications according to Schnurstein & Braunbeck (2001) as well as Kosmehl *et al.* (2004) using acetonic sediment extracts and RTL-W1 cells.

UV light (240 - 280 nm for 5 min) was used as a positive control. For exposure to sediment extracts, cells were transferred to 6-well plates (TTP Renner, Dannstadt, Germany) and incu-bated for 12h in pure medium to allow for complete cell attachment. Afterwards the medium was changed to different extract concentrations, which, in previous acute toxicity tests, had been shown to not induce more than 20 % lethality in the neutral red assay . DMSO was used as solvent and never exceeded a concentration of 1 %. Exposure was carried out for 24h at 20°C. After incubation, cells were washed with PBS, trypsinized and processed for the comet assay.

In order to guarantee for optimal adhesion, fully frosted slides (Langenbrink, Emmendingen, Germany) were used. After cleaning in 99 % ethanol, slides were coated with 1 % normal melting agarose (NMA; SeaKem, FMC Bioproducts, Rockland, USA), which was allowed to dry for 5 min at 37°C and scraped off afterwards. This procedure increases the adhesion of the following 0.5% NMA layer. Cells were embedded in 0.7 % low melting agarose (LMA; SeaKem, FMC Bioproducts) layers on the pre-coated slide and again coated with an additional layer of 0.7 % NMA. Slides were cooled on ice for 3 min and dried at 37 °C for 5 min, followed by lysis in 100 mM EDTA, 2.5 M NaCl, 1% Triton X-100 and 10 % DMSO (pH 13.0) in the dark at 4°C for 1.5 h. After electrophoresis in the same buffer at 25 V and 310 mA for 20 min, samples were neutralised by incubation in 400 mM Tris at pH 7,4 for 2 min. Slides where either analyzed directly or stored in a humid box (PBS) for at maximum 8 days at 4°C. Immediately before scoring, the DNA was stained with 75 µl of 20 µM ethidium bromide (Sigma) and coated with a cover slip.

All slides were examined at a magnification of 320x using a fluorescent microscope (Aristoplan, Leica, FRG) equipped with an excitation filter of 518 nm and an image analysis system (Optilas, Munich, Germany) with a greyscale CCD camera (JAI Pulnix TM-765E Kinetic, Glostrup, DK) and the Comet 3.0 software (Kinetic Images, Liverpool, UK). For each concentration, the tail moments of 100 randomly selected cells were analyzed.

For statistical analysis, data were analyzed with the H-test according to Kruskal and Wallis (SigmaStat 3.5; SPSS-Jandel, Erkrath, Germany). In cases of significant differences, a post-hoc test according to Dunn was used to identify groups differing significantly. The induction factor (IF) was

calculated by dividing the median of each concentration by the median of the corresponding control group.

To simplify the comparisons, data were converted into the "concentration-dependant induction factor" CDI according to Seitz *et al.* (2008). The CDI is a simple index value that integrates all important information, providing a basis for a general comparison of the genotoxic potential in the comet assay. The CDI is calculated as follows:

$$CDI = \sum_{i=1}^{n} \frac{IFi}{ci}$$

IFi = induction factor of the concentration i
ci = concentration i
n = n concentrations

### Micronucleus test

For the *in vitro* version of the micronucleus test, RTL-W1 cells were exposed to sediment extracts and to nitroquinoline-N-oxide (NQO, Sigma-Aldrich). For exposure, cells were transferred to 6-well plates with ethanol-cleaned cover slips (Assistent, Sondheim, Germany) and incubated for 12h in pure medium to allow for complete cell attachment. Afterwards, exposure and fixation were conducted as described by Schnurstein & Braunbeck (2001). Subsequently to fixation, the slides were stained for one minute with undiluted Giemsa (Gurr, BDH Labaratory Supplies, Poole, UK) and covered with DePeX (Serva, Heidelberg, Germany). After 24 hours of solidification, 2000 cells per slide were scored.

Micronuclei were counted under the light microscope equipped with an oil immersion lens at 1200x magnification. Criteria for micronuclei in RTL-W1 cells were set as follows: (a) maximum size of micronuclei must not exceed 30 % of the main nucleus; (b) micronuclei and nuclei should stain similarly; (c) micronuclei should be clearly separated from the nucleus; (d) only cells with good cytoplasmic outlines are used for reading (ISO21427-2, 2003).

Results were recorded as percentage of cells containing micronuclei compared to the total number of counted cells. The induction factor for each site was calculated by computing the percentage of micronuclei from exposed cells to the percentage of micronuclei in the negative controls. Statistical significances were assessed by using the Chi-square-test with Yates correction (ISO21427-2, 2003; Lovell *et al.*, 1989). Therefore, for each concentration and sediment extract, the number of cells with micronuclei was compared with those without micronuclei and with the corresponding negative control

(SigmaStat 3.5). Values differing significantly from the negative control were marked in the graph with a probability value from $p \leq 0.05$ with one asterisk (*), $p \leq 0.01$ with two asterisks (**) and for $p \leq 0.001$ with three asterisks (***).

To further evaluate the induction assessed in the micronucleus test, genotoxicity was related to NQO mutagenicity by calculating NQO equivalents. Since NQO is a strong, well-known mutagenic substance, it has widely been used as a reference substance in various biotests (Diekmann *et al.*, 2004b,a; Homme *et al.*, 2000; Nunoshiba & Demple, 1993; Yang *et al.*, 1991). For this purpose, an NQO calibration curve was generated (Fig. C.2). Cells were exposed to NQO at concentrations of 11.9 to 190 µg/L. By relating the induction factors of sediment extracts to an NQO calibration curve, an equivalent to the NQO concentration was obtained. The value of the corresponding NQO concentration will be expressed as induction per gram sediment (see formula). This conversion of mutagenic potentials from sediment extracts allows a direct comparison of the sampling sites under consideration of each tested concentration.

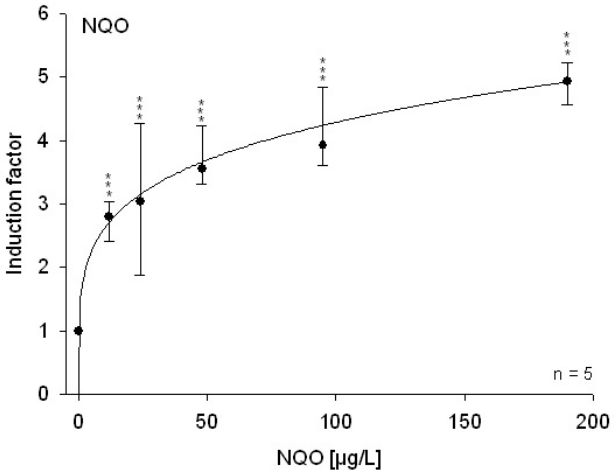


**Fig. C.2.** Induction of genotoxicity by NQO in the micronucleus assay using RTL-W1 cells. Asterisks (***): Values differ significantly from the negative control (Chi-square test with Yates correction; $p \leq 0.01$).

NQO concentrations obtained from the calibration curve were translated by the following formula:

$$NEQ\,[\mu g\,NQO\,equivalent/g\,sediment] = \frac{NQO\,[\mu g/L]}{extract\,concentration[\mu g/L]}$$

The average NQO equivalents ($NEQ_{average}$) for all sites were calculated from the NQO equivalents of each tested concentration.

## C.2.3 Field studies

### Blood samples

Blood samples were collected from wild European barbel (*Barbus barbus*) immediately after capture at the sites Riedlingen, Sigmaringen, Rottenacker and Ehingen.

At each sampling site, five mature fish were collected by electro fishing, anesthetized with a saturated benzocaine (Sigma) solution and opened ventrally. Cardiovascular blood samples were obtained by puncture of the heart with heparinized syringes (Hawksley and Sons Limited, Lancing, UK). The blood was smeared immediately at ethanol-cleaned slides. After drying, the slides were fixed in methanol for at least 1 min and stained with undiluted Giemsa (Gurr). The slides were then covered with DePeX (Serva). Sex of the fish was not determined, the length ranged between 12 and 33 cm, the weight between 14.5 and 41 g.

As a reference, fish from Riedlingen were caught and maintained in Heidelberg University facilities for 60 days under flow-through conditions (4 L/h) in a 400 L basin, with continuous dechlorinated tap water and aeration. The photoperiod was adjusted to a 12h light/12h dark cycle, water temperature averaged 14 ± 1°C. Once a week, barbel were fed with blood-worms, otherwise with trout flake food (Trouvit pro aqua, Milkivit, Burgheim, Germany)

### Micronucleus test

The frequency of micronuclei in erythrocytes was assessed from fish sampled along the River Danube. Per slide, 2000 erythrocytes were examined. Since the micronucleus frequency of at least four individuals (8000 erythrocytes) per sampling site was assessed, a statistical analysis could be carried out (Lemos *et al.*, 2007). For evaluation and scoring criteria, see section 2.2.4.

An all pairwise comparison according to Dunnett´s test ($p \leq 0.05$) was used to identify groups that differed significantly.

# C.3 Results

## C.3.1 Comet assay in RTL cells exposed to sediment extracts *in vitro*

Acetonic sediment extracts from the Danube River were tested in the comet assay with RTL-W1 cells. 8 of 10 sediment extracts displayed genotoxic activity in terms of DNA fragmentation (tail moment), revealing clear dose-response relationships. Sediment extracts from Rottenacker, Oepfingen and Schwarzach differed significantly from the negative control at all concentrations tested. In order to demonstrate the relation between sediment test concentra-tion and the resulting tail moment and induction factor, two examples are illustrated in detail (Fig. C.3). For the sediment extract from the Lauchert tributary, only one concentration in each of the two replicates showed significant genotoxicity compared to the negative control (5 mg SEQ/ml and 40 mg SEQ/ml, respectively). In contrast, for sediment extracts from Oepfingen, all extract concentrations in either replicate displayed elevated genotoxic effects compared to the negative controls. Furthermore, the induction factor increased with concentration (Fig. C.3).

Results for the comet assay with RTL-W1 cells were computed as concentration-dependent induction factors (CDIs) to enable a better comparison of the results from different sampling sites (Fig. C.4). The sediment extract from Rottenacker induced the highest CDI of 11.4. The CDIs for the extracts from Ehingen, Schwarzach and Oepfingen ranged between 2 and 4, the sediment extracts from Bad Abbach, Jochenstein, Ingolstadt and Sigmaringen caused CDIs between 1 and 1.6. The CDIs for the other extracts from Riedlingen and the tributary Lauchert were below 1.

## C.3.2 Micronucleus test in RTL-W1 cells exposed *in vitro*

### NQO in the micronucleus test with RTL-W1 cells

4-Nitroquinolin-N-oxid was tested in 5 independent replicates (Fig. C.4). The induction of micronuclei in RTL-W1 cells increased with concentration, finally reaching a maximum induc-tion factor of 5. All concentrations tested differed significantly from the control ($p \leq 0.01$). Based on the curve progression of each replicate (single data/graphs not shown), it is unlikely that the maximum induction factor of 5 will be exceeded by higher NQO concentrations.

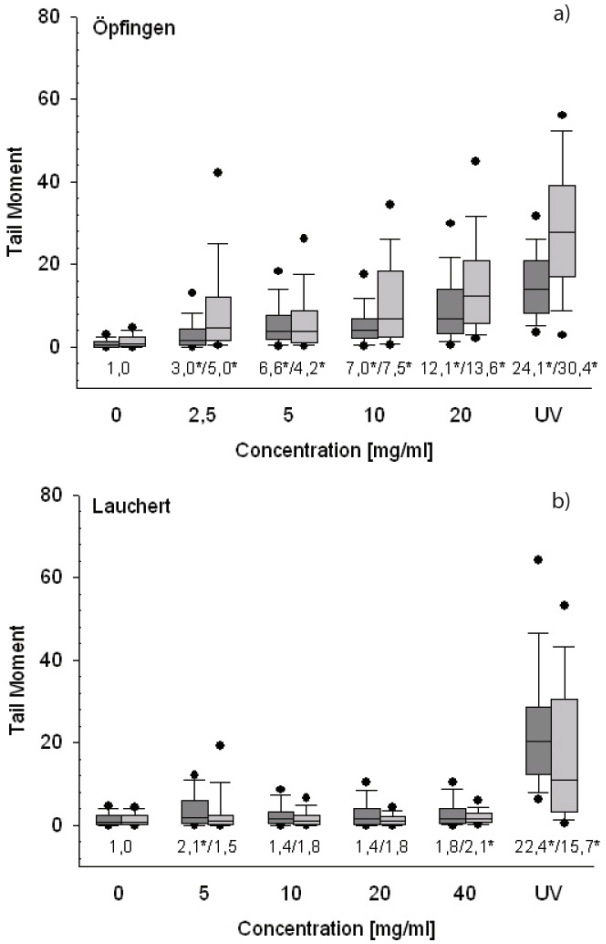


**Fig. C.3.** a and b - Genotoxic effects of acetonic sediment extracts from Lauchert and Oepfingen respectively, in the comet assay. Numbers below the box plots identify the induction factor for each concentration. Asterisks (*): Values differ significantly from the negative control (post hoc test according to Dunn's; $p < 0.05$).

### Induction of micronuclei in RTL-W1 cells by sediment extracts

All tested sediment samples produced a significant induction of micronuclei, if compared to the negative control. The extracts from Schwarzach, Rottenacker and Ehingen showed a strong increase of the induction factor. Doserelated effects of the extracts showed scarce similarities: At low concentrations, micronucleus induction for all extracts increased with concentration. However, this initial increase stagnated for higher concentrations of sediments from Schwarzach, Rottenacker and Ehingen, induction (Ehingen: Fig. C.5a) or even decreased to negative control levels (Sigmaringen, Schwarzach and Rottenacker; Fig. C.5b). The micronucleus rate at the highest concentration

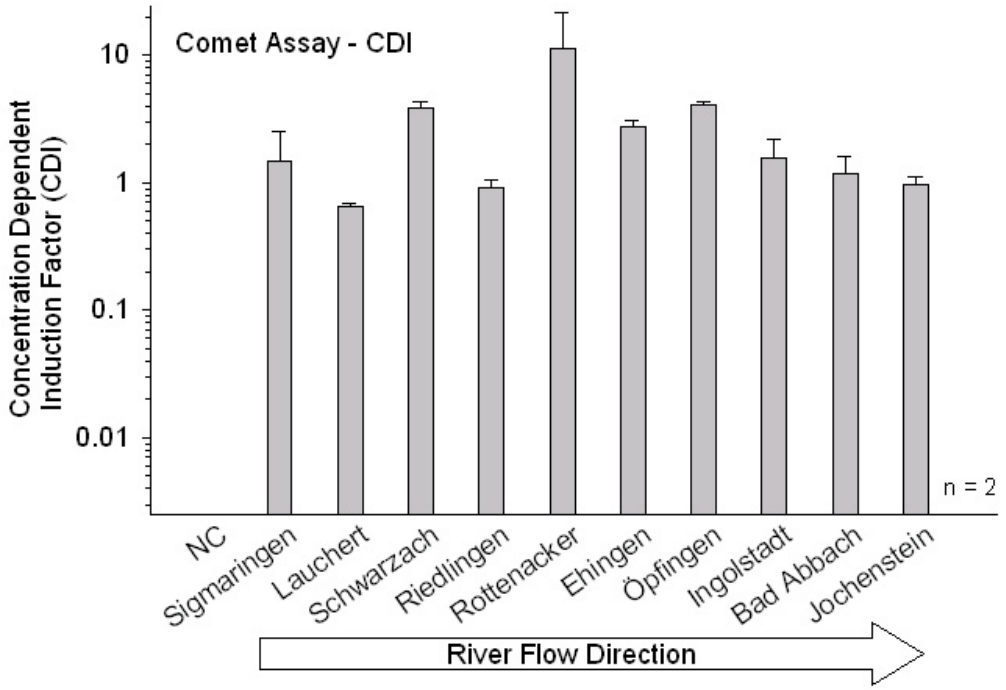


**Fig. C.4.** Genotoxic activity of sediment extracts in the comet assay with RTL-W1 cells from two independent series of experiments in river flow direction, expressed as concentration-dependent induction factor (CDI; Seitz et al. 2007). NC = negative control.

tested in this assay (40 mg SEQ/ml) could not be assessed due to cytotoxic effects of the sites Rottenacker, Ehingen, Schwarzach, Ingolstadt, Jochenstadt and Bad Abbach. In contrast, the induction rate of sediment extracts from Lauchert (Fig. C.5c), Riedlingen and Oepfingen showed a linear dose-response relationship.

The results of the micronucleus test with RTL-W1 cells were analyzed by means of NQO equivalents (NEQ; Fig. C.6). The genotoxic effects in RTL-W1 cells of 1 g sediment from Rottenacker were identical to those caused by 3.1 µg/L NQO. Elevated NEQs were also calculated for sediment extracts from Ehingen (1.3 µg/L) and the Schwarzach tributary (1.4 µg/L). In contrast, NEQs from the Lauchert tributary was not elevated (NEQ = 0.1 µg/L).

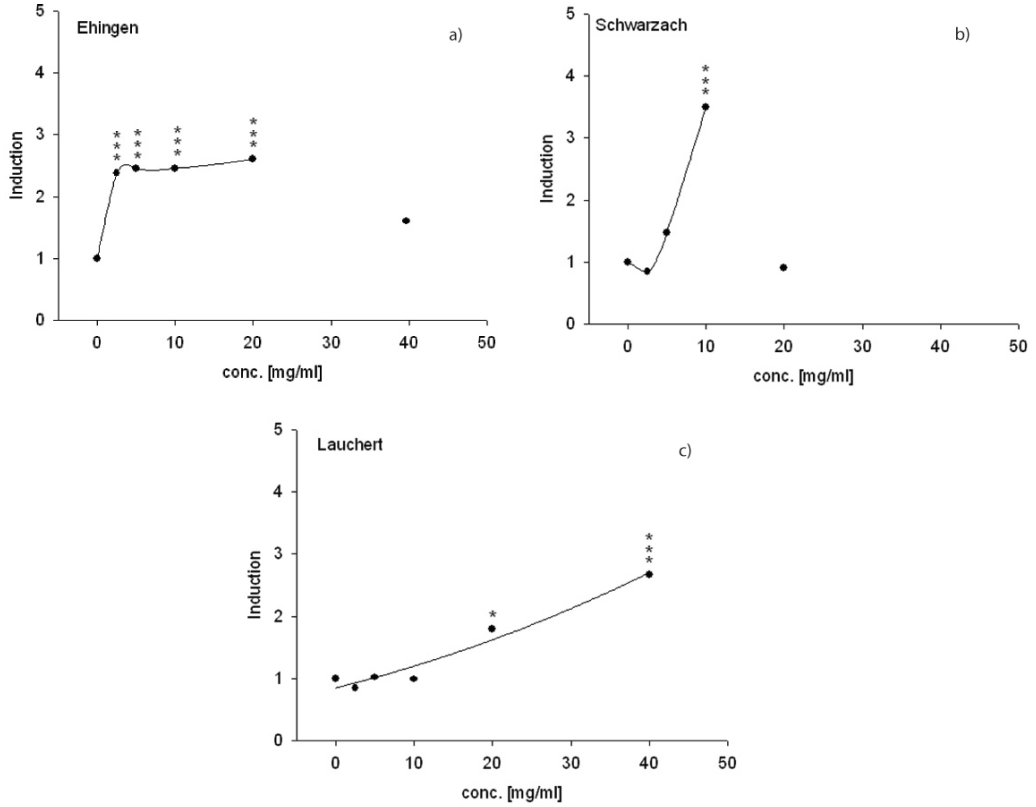


**Fig. C.5.** a,b e c - Mutagenic activity of acetonic sediment extracts in the micronucleus assay with RTL-W1 cells, displayed as induction factors. Asterisks: Values differ significantly from the negative control (Chi-square test with Yates correction; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

## Micronucleus test in erythrocytes from barbel after *in situ* exposure in the Danube River

The frequency of micronuclei in erythrocytes was assessed from barbel sampled along the River Danube (Sigmaringen, Riedlingen, Rottenacker and Ehingen; Fig. C.7); the micronucleus frequency is given as percentage (MN[%]). The highest micronucleus rate was determined for Rottenacker (MN[%] 0.31); however, only slightly lower rates were recorded for Ehingen (MN[%] 0.27) and Riedlingen (MN[%] 0.24). The micronucleus frequencies for these three sites were significantly higher, if compared to negative controls (MN[%] 0.06). Only the micronucleus rates from erythrocytes from barbel caught at Sigmaringen (MN[%] 0.14) were not significantly elevated.

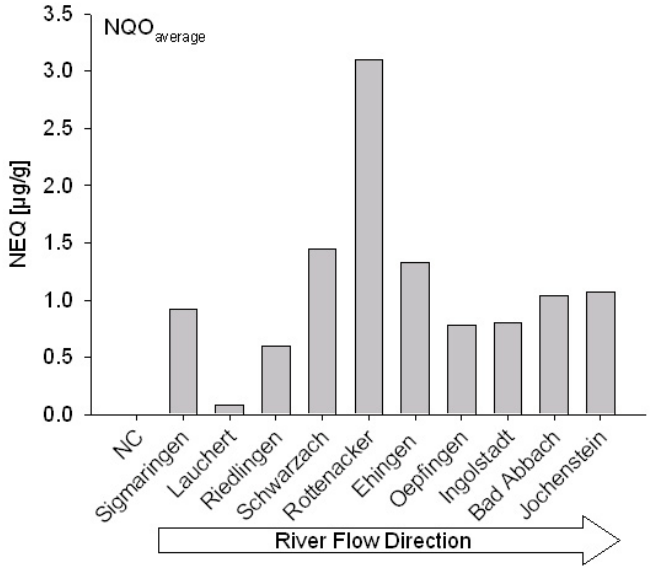


**Fig. C.6.** Mutagenic activity of sediment extracts from the Danube River, tested in the micronucleus assay with RTL-W1 cells. Mutagenicity is displayed via the NEQ value in µg/g. Extracts are arranged in river flow direction.

# C.4 Discussion

## C.4.1 *In vitro* test systems: comet assay *versus* micronucleus test

The comet assay detects a broad range of interactions of various genotoxicants with the DNA, however without information about their eventual importance for long-term effects. The micronucleus assay, in contrast, only documents established genotoxic deletions, but fails to detect transient (reparable) DNA alterations. Since, as applied in the present study, the micronucleus assay does not detect DNA lesions in germ cells, the established DNA damage reported will not necessarily be translated to the next generation. However, the likelihood of mutations in germ cells strongly increases with the number of micronuclei in the somatic cells examined (erythrocytes, RTL-W1). The correlation between germ and somatic cells strongly depends on the chemical nature of the pollutants in sediments. Apart from this, in case a significant number of individuals were affected, even transient primary changes in the DNA structure might result in carcinogenic processes and, eventually, in implications at the population level. As a consequence, for risk assessment purposes, a combination of both assays seems appropriate to avoid false negatives in genotoxicity evaluation.

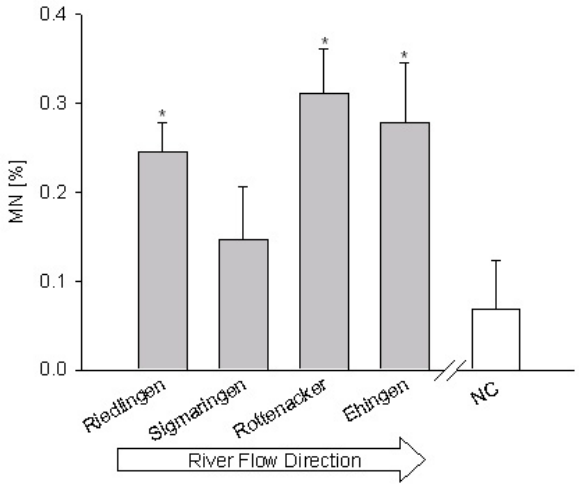


**Fig. C.7.** Micronucleus frequency (MN [%]) in barbel (*Barbus barbus*) erythrocytes from selected sites along the Danube River. Each box contains data from five barbels with 2000 erythrocytes assessed per barbel. NC: Negative control; Asterisk (*): Values differ significantly from the negative control (post hoc test according to Dunnett; $p < 0.05$).

The sediment extract from Rottenacker showed the highest genotoxicity with a CDI of 11.4. The CDIs for the extracts from Ehingen, Schwarzach and Oepfingen were also elevated (values ranged between 2 and 4) and, thus, rated moderately genotoxic. Minor genotoxic activity could be observed for sediment extracts from Bad Abbach, Jochenstein, Ingolstadt and Sigmaringen. The CDIs for the remaining extracts from Riedlingen and the tributary Lauchert were below 1, which is considered as non-genotoxic.

Rocha *et al.* (2009) exposed RTL-W1 cells to sediment extracts from the Tiête River in Sao Paulo State (Brazil) and analyzed these using the comet assay. The upper part of this river includes the origin as well as the metropolitan region of Sao Paulo City. The CDI for sediments from the spring was below 1 (0.37) and, thus, comparable to sediment extracts from the Danube tributary Lauchert (0.5). Approaching Sao Paul City, the CDI increased, finally reaching a maximum of 3.3 for sediment extracts from Billings's reservoir located in Sao Paulo City. The sediment extract from Rottenacker exceeds this value by a factor of 3.5. The sediment extracts from Ehingen, Schwarzach and Oepfingen are comparable with those from Billings. Downstream Sao Paulo City the CDIs decreased below 1 again. Preliminary studies revealed Billings's reservoir as a water body extremely polluted by domestic sewage as well as by industrial effluents (Bainy *et al.*, 1999; Leitao *et al.*, 2000). As a consequence, the results obtained for sediment extracts from the Danube Rive are even more striking.

The sediments studied in the present investigation have also been tested in the sediment-contact version of the comet assay with zebrafish (*Danio rerio*) embryos, *i.e.*, in an assay that applies the comet assay to cells from full organisms with intact cell-to-cell interactions (Jarvis & Knowles, 2003). The results obtained by Seiler *et al.* (2006) provided clear evidence of genotoxic contamination of sediments from the upper Danube River. At some localities, there was significantly increased genotoxicity in native sediments even at dilutions of up to 32-fold (Rottenacker). Again, sediments from the Lauchert tributary were free of genotoxicity. A correlation analysis confirmed a high correlation between comet assays with Danio rerio exposed to native sediments (Seitz *et al.*, 2008) and comet assays with RTL-W1 cells exposed to acetonic sediment extracts (this study; $r_{Pearson} = 0.75$ with $p < 0.05$; $R^2 = 0.56$).

The results of the micronucleus test with RTL-W1 cells were compared by means of NEQs (nitroquinoline-N-oxide (NQO) equivalents; Fig. C.7). As indicated by the comet assay, sediment extracts from Rottenacker displayed the highest genotoxic potential. One gram sediment equivalent exhibits the same mutagenic activity to RTL-W1 cells as 3.1 µg/L NQO. Slightly lower NEQs were calculated for sediment extracts from Ehingen (1.3 µg/L) and the contaminated Schwarzach tributary (1.4 µg/L). Little to no mutagenic activity could be measured after exposure to extracts from the Lauchert tributary (NEQ = 0.1 µg/L). The other sediment extracts induced minor genotoxic effects with NEQs ranging from 0.6 – 1.1 µg/L.

Diekmann *et al.* (2004a) showed that NQO at concentrations as low as 0.1 µg/L may significantly affect egg production in zebrafish. Since NQO only causes genotoxicity and mutagenicity, but no general and cellular toxicity, effects reported by Diekmann *et al.* (2004b) can be related to DNA damage or DNA loss. Even though the genetic damage was assessed in a somatic cell line, in cannot be excluded that similar genotoxic effects might as well appear in the somatic cells and consequently might be transmitted to future generations. Therefore, an NEQ from Rottenacker of 3.1 µg/L might well have consequences on fish populations. Furthermore, an NQO concentration of 14.6 µg/L proved to be lethal in a full life-cycle test with *Danio rerio*; unfortunately concentrations between 2.9 µg/L und 14.6 µg/L had not been tested (Diekmann *et al.*, 2004a). The sediment extract from Rottenacker would reach this lethal NEQ concentration with approximately 5 g sediment equivalent/L water, the extracts from Schwarzach and Ehingen with approximately 10 g sediment equivalent/L water. The fact that during a flood event one litre of water from the Elbe River can contain up to one gram suspended matter (Wilken *et al.*, 1991), this potential for genetic damage is of more concern.

By comparing the CDIs and NEQs along the river flow direction, 'hot spots', *i.e.*, sampling sites with evaluated genotoxic potential could be determined. In both tests, the highest genotoxicity was induced by sediment extracts from Rottenacker. The genotoxic activity measured in the comet assay for sediment extracts from Ehingen, Schwarzach and Oepfingen could be classified as also genotoxic in the micronucleus test with exception of Oepfingen, which only caused minor genotoxicity.

In conclusion, based on these two *in vitro* tests, sediment extracts from the sampling site Rottenacker would be categorized as severely genotoxic, those from Ehingen and Schwarzach as moderately genotoxic. Little to no genotoxic activity was shown by the tributary Lauchert. The remaining sediment extracts induced low genotoxic effects.

A correlation coefficient of 0.97 ($p < 0.001$; $R^2 = 0.81$), was calculated for the tested sediment extracts in the two *in vitro* test systems indicating genotoxic potential of analyzed sediment extracts were highly correlated.

### C.4.2 *In situ* genotoxicity - the micronucleus test in Danube River fish

For the assessment of genotoxicity *in situ*, sampling sites were selected based on their results in the *in vitro* assays. Rottenacker was ranked as a severely genotoxic/mutagenic site, Ehingen as a moderately contaminated site, and Riedlingen as well as Sigmaringen as mildly to minimally polluted sites. Consistent with the previous *in vitro* test results, the mutagenic potentials of the sampling sites did not change with river flow direction.

Minissi *et al.* (1996) conducted the micronucleus rates in European barbel sampled from an uncontaminated river in Italy (Mignone; low to mild contamination) with those from a contaminated river in Italy (Tiber; heavily contaminated). Barbel erythrocytes from the Mignone River showed a micronucleus induction rate of 0.015 %, those from Tiber a rate of 0.032 %. The control fish induced micronucleus formation in 0.005 % of the cells.

Rocha *et al.* (2009) also determined the micronucleus frequency in erythrocytes from fish (Oreochromis niloticus) collected in the Tiête River in Sao Paulo State, Brazil. The average micronucleus induction rates for the two reference sites (Bariri and Promissao) were 0.1 %. In comparison, along the highly contaminated river sections around Sao Paulo City (Billings's reservoir; MN[%] 0.6 %) there is a 6-fold higher induction of micronuclei in erythro-cytes. Comparing results from the present study with those from Minissi *et al.* (1996) and Rocha *et al.* (2009), micronuclei in erythrocytes from barbel caught at Rottenacker (induction factor = 4.6) were induced

almost as strong as for the Tiber (induction factor = 6.4) and for the Tiête River in Sao Paulo City (induction factor = 6.0). Therefore, the mutagenic contamination of the Danube River in Rottenacker is comparable to two other highly contaminated rivers in Italy and Brazil. Similar induction rates of micronuclei were found for barbel from Ehingen (induction factor = 4.1). In contrast, micronuclei induction from barbel of the Danube River near Sigmaringen (induction factor = 2.1) is comparable to the induction of micronuclei in Mignone, Italy (induction factor = 3), and, thus, the mutagenic potential corresponds to a non-contaminated river in Italy.

### C.4.3 Correlation between findings in the *in vitro* and *in situ* tests

Since only four sites along the Danube River were sampled for *in situ* micronucleus tests, the correlation analysis is restricted to these four sites and the negative controls. To the best of our knowledge, this is the first study comparing results of the *in situ* micronucleus test with those of *in vitro* micronucleus tests. Correlation analysis between these two test systems revealed a correlation coefficient of $r_{Spearman} = 0.90$ (with $p < 0.1$; $R^2 = 0.61$), indicating high correlation. Likewise, the same correlation coefficient was calculated for the analysis between the comet assay and the micronucleus assay with barbel erythrocytes ($r_{Spearman=0.90}$ with $p < 0.1$; $R^2 = 0.61$). Results of the *in situ* tests confirm Rottenacker and Ehingen as 'hot spots' for genotoxic potency. In the present study, findings obtained in *in vitro* test correlate well with field studies, and are, therefore, consistent. Similar studies conducted by Rocha and colleagues (Rocha *et al.*, 2009) in the Tiête River with the micronucleus test *in situ* and the comet assay *in vitro* with RTL-W1 cells, also confirmed a high correlation between these two test systems.L

Furthermore, studies applied on four sites (*i.e.*, Black Rock Harbour, Connecticut; Puget Sound, Washington; Hamilton Harbour, Lake Ontario, Canada; the Black River, Ohio, USA), *i.e.*, highly contaminated with known mutagens (*e.g.*, PAHs), high levels of sediment genotoxicity, and an abnormally high incidence of genotoxic effects, neoplasms and preneoplastic lesions in indigenous biota, demonstrated a causal relationship between sediment genotoxicity and *in situ* effects (*e.g.*, cancer, DNA damage, etc.) in selected species of aquatic biota (Chen & White, 2004). These tests, based on bacterial mutations (Balch *et al.*, 1995; Gardner *et al.*, 1987; Ho *et al.*, 1994; Ho & Quinn, 1993; Marvin *et al.*, 1993, 1999, 2000; Metcalfe *et al.*, 1990)or aberrations in fish and mouse cell lines (Metcalfe *et al.*, 1990; Kocan & Powell, 1985), corroborate the proposed link between genotoxicity *in vitro* and

*in situ*.

## C.5 Conclusion

Conventional water quality criteria do not include assessments of important parameters such as genotoxicity and, thus, fail to account for effects by genotoxic compounds. The present study documents that fish collected from certain localities in the Danube River display marked genotoxicity, whereas fish sampled at other sites do not. This differential distribution of the genotoxic potential could be confirmed by *in vitro* studies into the effects of organic sediment extracts in permanent fish cell cultures. However, the good correlation between *in vitro* and *in situ* genotoxicity bioassays found for the upper Danube River needs to be confirmed in additional studies.

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# Bibliography


Anderson S, Wild GC (1994) Linking genotoxic responses to reproductive success in ecotoxicology. *Environmental Health Perspectives* 102: 9–12

Bainy ACD, Bruce RW, John JS (1999) Elevated levels of multiple cytochrome P450 forms in tilapia from Billings Reservoir - Sao Paulo, Brazil. *Aquatic Toxicology* 44: 289–305

Balch GC, Metcalfe CD, Huestis SY (1995) Identification of potential fish carcinogens in sediment from Hamilton Harbour, Ontario, Canada. *Environmental Toxicology and Chemistry* 14: 79–91

Van den Berg M, Birnbaum L, Bosveld A, Brunstrom B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy S, Kubiak T, Larsen J, Leeuwen FX, van, Liem AK, Nolt C, Peterson RE, Poellinger L, Safe S, Schrenk D, Tillitt D, Tysklind M, Younes M, Waern F, Zacharewski T (1998) Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife, EROD. *Environ Health Perspect* 106: 775–792

Borenfreund E, Puerner JA (1984) A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90). *Journal of Tissue Culture Methods* 9: 7–9

Burkhardt-Holm P, Giger W, Guttinger H, Ochsenbein U, Peter A, Scheurer K, Segner H, Staub E, Suter MJ (2005) Where have all the fish gone? The reasons why fish catches in Swiss rivers are declining. *Environmental Science and Technology* 39: 441A–447A

Chapman PM, Hollert H (2006) Should the sediment quality triad become a tetrad, a pentad, or possibly even a hexad? *Journal of Soils and Sediments* 6: 4–8

Chen G, White PA (2004) The mutagenic hazards of aquatic sediments: a review. *Mutation Research* 567: 151–225

Cook P, Robbins J, Endicott D, Lodge K, Guiney PD, Walkero M, Zabelo E, Peterson R (2003) Effects of aryl hydrocarbon receptor-mediated early life stage toxicity on lake trout populations in Lake Ontario during the 20th Century. *Environmental Science and Technology* 37: 3864–3877

Diekmann M, Hultsch V, Nagel R (2004a) On the relevance of genotoxicity for fish populations I: effects of a model genotoxicant on zebrafish (*Danio rerio*) in a

complete life-cycle test. *Aquatic Toxicology* 68: 13–26

Diekmann M, Waldmann P, Schnurstein A, Grummt T, Braunbeck T, Nagel R (2004b) On the relevance of genotoxicity for fish populations II: genotoxic effects in zebrafish (*Danio rerio*) exposed to 4-nitroquinoline-1-oxide in a complete life-cycle test. *Aquatic Toxicology* 68: 27–37

Fairbairn DW, Olive PL, O'Neil KL (1995) The comet assay: a comprehensive review. *Mutation Research* 339: 37–59

Faller P, Kobler B, Peter A, Sumpter J, Burkhardt-Holm P (2003) Stress status of gugeon (*Gobio gobio*) from rivers in switzerland with and without input of sewage treatment plant influent. *Environmental Toxicology and Chemistry* 22: 2063–2072

Gardner G, Yevich P, Malcolm A, Pruell R (1987) Carcinogenic effects of Black Rock Harbor sediment on american oysters and winter flounder. *US Environmental Protection Agency and The National Cancer Institute, Narragensett*

Grisolia C (2002) A comparison between mouse and fish micronucleus test using cyclophosphamide, mitomycin C and various pesticides. *Mutation Research* 518: 145–150

Ho K, Quinn J (1993) Bioassay-directed fractionation of organic contaminants in an estuarine sediment using the new mutagenic bioassay, Mutatox. *Environmental Toxicology and Chemistry* 12: 823–830

Ho K, Mills L, Mueller C, Anderson S, Malcolm A (1994) The influence of sediment extract fractionation methods on bioassay results. *Environmental Toxicology and Water Quality* 9: 145–154

Hollert H, Dürr M, Erdinger L, Braunbeck T (2000) Cytotoxicity of settling particulate matter (SPM) and sediments of the Neckar river (Germany) during a winter flood. *Environmental Toxicology and Chemistry* 19: 528–534

Homme M, Jacobi H, Juhl-Strauss U, Witte I (2000) Synergistic DNA damaging effects of 4-nitroquinoline-1-oxide and non-effective concentrations of methyl methanesulfonate in human fibroblasts. *Mutation Research* 461: 211–219

Huet M (1949) Apercu des relation entre la pente et les population piscicoles dans les eaux courantes. *Revue Suisse d' Hydrologie* 11: 332–351

Huschek G, Hansen PD (2005) Ecotoxicological classification of the Berlin river system using bioassays in respect to the European Water Framework Directive. *Environmental Monitoring and Assessment* 121: 15–31

ISO21427-2 (2003) *Water quality - Evaluation of gentoxicity by measurement of the induction of micronuclei - Part 1: "Mixed population" method using the cell line V 79*

Jarvis R, Knowles JF (2003) DNA damage in zebrafish larvae induced by exposure to low-dose rate gamma-radiation: detection by the alkaline comet assay. *Mutation Research* 541: 63–69

Jenssen D, Ramel C (1980) The micronucleus test as part of a short-term mutagenicity test program for the prediction of carcinogenicity evaluated by 143 agents tested. *Mutation Research* 75: 191–202

Keiter S, Rastall A, Kosmehl T, Erdinger L, Braunbeck T, Hollert H (2006) Ecotoxicological Assessment of Sediment, Suspended Matter and Water Samples in the Upper Danube River. A pilot study in search for the causes for the decline of fish catches. *Environmental Science and Pollution Research* 13: 308–319

Klee N, Gustavsson LK, Kosmehl T, Engwall M, Erdinger L, Braunbeck T, Hollert H (2004) Changes in toxicity and genotoxicity of industrial sewage sludge samples containing nitro and amino-aromatic compounds following treatment in bioreactors with different oxygen regimes. *Environmental Science and Pollution Research* 11: 313–320

Kocan R, Powell D (1985) *Short-Term Bioassays of Complex Environmental Mixtures IV*, chap. Anaphase aberrations: An *in vitro* test for assessing the genotoxicity of individual chemicals and complex mixtures. Plenum Press, New York

Kosmehl T, Krebs W Fand Manz, Erdinger L, Braunbeck H Tand Hollert (2004) Comparative genotoxicity testing of Rhine river sediment extracts using the permanent cell lines RTG-2 and RTL-W1 in the comet assay and Ames assay. *Journal of Soils and Sediments* 4: 84–94

Lafontaine Y, Gilbert N, Dumouchel F, Brochub C, Moore S, Pelletierc E, Dumont P, Branchaude A (2002) Is chemical contamination responsible for the decline of the copper redhorse (*Moxostoma hubbsi*), an endangered fish species, in Canada? *Science of the Total Environment* 298: 25–44

Lee LE, Clemons JH, Bechtel DG, Caldwell SJ, Han KB, Pasitschniak-Arts M, Mosser D, Bols NC (1993) Development and characterization of a rainbow trout liver cell line expressing cytochrome P450-dependent monooxygenase activity. *Cellular Biology and Toxicology* 9: 279–294

Lee RF, Steinert S (2003) Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. *Mutation Research* 544: 43–64

Leitao M, Affonso E, Silva M, Meirelles N, Rantin F, Vercesi A, Junqueira V, Degterev I (2000) The liver monooxygenase system of Brazilian freshwater fish. *Comparative Biochemistry and Physiology* 126: 29–38

Lemos CT, Roedel PM, Terra NR, Oliveira NCD, Erdtmann B (2007) River water genotoxicity evaluation using micronucleus assay in fish erythrocytes. *Ecotoxicology and Enviromental Safety* 66: 391–401

Lovell DP, Anderson D, Albanese R, Amphlett G, Clare G, Ferguson R, Richold M, Papworth D, Savage J (1989) *Statistical evaluation of mutagenicity test data : UKEMS Sub-Committee on Guidelines for Mutagenicity Testing, report, part III*, chap. Statistical analysis of *in vivo* cytogenetic assays, 184–232. Cambridge University Press, Cambridge (England); New York

Lynch M, Conery J, Burger R (1995) Mutational Meltdowns in Sexual Populations. *Evolution* 49: 1067–1080

Macek KJ (1980) Aquatic toxicology: fact or fiction? *Environmental Health Perspectives* 34: 159–162

Marvin C, McCarry B, Lundrigan J, Roberts K, Bryant D (1999) Bioassay-directed fractionation of PAH of molecular mass 302 in coal tar-contaminated sediment. *Science of the Total Environment* 231: 135–144

Marvin CH, Allan L, McCarry BE, Bryant DW (1993) Chemico/biological investigation of contaminated sediment from the Hamilton Harbour area of western Lake Ontario. *Environmental and Molecular Mutagenesis* 22: 61–70

Marvin CH, McCarry BE, Villella J, Allan LM, Bryant DW (2000) Chemical and biological profiles of sediments as indicators of sources of genotoxic contamination in Hamilton Harbour. Part I: Analysis of polycyclic aromatic hydrocarbons and thia-arene compounds. *Chemosphere* 41: 979–988

Metcalfe CD, Balch GC, Cairns VW, Fitzsimons JD, Dunn BP (1990) Carcinogenic and genotoxic activity of extracts from contaminated sediments in western Lake Ontario. *Science of the Total Environment* 125–141

Minissi S, Ciccotti E, Rizzoni M (1996) Micronucleus test in erythrocytes of Barbus plebejus (Teleostei, Pisces) from two natural environments: a bioassay for the *in situ* detection of mutagens in freshwater. *Mutation Research* 367: 245–251

Nunoshiba T, Demple B (1993) Potent intracellular oxidative stress exerted by the carcinogen 4-nitroquinoline-N-oxide. *Cancer Research* 53: 3250–3252

Reid KC B J Jones, Semple KT (2000) Bioavailability of persistent organic pollutants in soils and sediments - a perspective on mechanisms, consequences and assessment. *Environmental Pollution* 108: 103–112

Rocha PS, Luvizotto GL, Kosmehl T, Böttcher M, Storch V, Braunbeck T, Hollert H (2009) Sediment genotoxicity in the Tietê River (São Paulo, Brazil): *in vitro* comet assay *versus in situ* micronucleus assay studies. *Ecotoxicology and Enviromental Safety* 72: 1842–1848

Schnurstein A, Braunbeck T (2001) Tail moment *versus* tail length-application of an *in vitro* version of the comet assay in biomonitoring for genotoxicity in native surface waters using primary hepatocytes and gill cells from zebrafish *(Danio rerio). Ecotoxicology and Enviromental Safety* 49: 187–96

Schoen DJ, Brownstein J, White PA (2002) Mutation load in natural populations of the sensitive fern *Onoclea sensibilis* exposed to soil mutagens. *Ecological Applications* 12: 124–137

Schwab K, Brack W (2007) Large volume TENAX® Extraction of the bioaccessible fraction of sediment-associated organic compounds for a subsequent effect-directed analysis. *Journal of Soils and Sediments* 7: 178–186

Seiler TB, Rastall AC, Leist E, Erdinger L, Braunbeck T, Hollert H (2006) Mem-

brane dialysis extraction (MDE): A novel approach for extracting toxicologically relevant hydrophobic organic compounds from soils and sediments for assessment in biotests. *Journal of Soils and Sediments* 6: 20–29

Seitz N, Böttcher M, Keiter S, Kosmehl T, Manz W, Hollert H, Braunbeck T (2008) Evaluation of the genotoxic potential of sediment samples from the Danube River using a new expert system and several analyzing methods: How to integrate information from dose-response curves. *Mutation Research* 652: 38–45

Singh N, McCoy M, Tice R, Schneider E (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research* 175: 184–191

Vindimian E, Namour P, Migeon B, Garric J (1991) *In situ* pollution induced cytochrome P450 activity of freshwater fish: Barbel (*Barbus barbus*), chub (*Leuciscus cephalus*) and nase (*Chondrostoma nasus*). *Aquatic Toxicology* 255–266

White P, Rasmussen J, Blaise C (1996) Sorption of organic genotoxins to particulate matter in industrial effluents. *Environmental and Molecular Mutagenesis* 27: 140–151

White P, Robitaille S, Rasmussen J (1999) Heritable reproductive effects of benzo[a]pyrene on the fatheas minnow (Pimephales promelas). *Environmental Toxicology and Chemistry* 1843–1847

Wilken R, Christiansen H, Fanger H, Greiser N, Haar S, Puls W, Reinecke H, Spott D, Vollmer M (1991) Fakten und Hypothesen zum Schwebstoff- und Schadstofftransport in der Elbe. *Vom Wasser* 167–189

WWA (2004) Wasserwirtschaftsamt Ingolstadt - Geschaeftsbereich des Bayer. *Staatsministeriums fuer Umwelt, Gesundheit und Verbraucherschutz, Ingolstadt*

Yang A, Zdzienicka M, Simons J, Waters R (1991) The repair of 4-nitroquinoline-1-oxide induced DNA adducts in hypersensitive Chinese hamster mutants: lack of repair of UV induced (6-4) photoproduct correlates with reduced repair of adducts at the N2 of guanosine. *Mutagenesis* 449–453

# Appendix D

# Publications related to this thesis

**Publications in international peer-reviewed journals**

2009 Rocha, P. S., Azab, E., Schmidt, B., Storch, V., Hollert, H., Braunbeck, T. Changes in toxicity and Ah receptor agonist activity of sediments from the Tietê River (São Paulo, Brazil) - a mass balance approach using *in vitro* methods and chemical analysis. *Submitted to* Ecotoxicology and Environmental Safety

2009 Böttcher, M., Grund, S., Keiter, S., Kosmehl, T., Manz, W., Seitz, N., Rocha, P. S., Hollert, H., Braunbeck, T. Comparison of *in vitro* and *in situ* genotoxicity in the Danube River by means of the comet assay and the micronucleus test. *Submitted to* Mutation Research

2009 Rocha, P. S., Luvizotto, G. L., Kosmehl, K., Böttcher, M., Storch, V., Braunbeck, T., Hollert, H. Sediment genotoxicity in the Tietê River (São Paulo, Brazil): *in vitro* versus *in situ* studies. *In press in* Ecotoxicology and Environmental Safety. 72:1842–1848

2007 Jernbro, S., Rocha, P. S., Keiter, S., Skutlarek, D., Färber, H., Jones, P. D., Giesy, J. P., Hollert, H., Engwall, M. Perfluorooctane Sulfonate Increases the Genotoxicity of Cyclophosphamide in the Micronucleus Assay with V79 Cells – Further Proof of Alterations in Cell Membrane Properties Caused by PFOS. Environmental Science and Pollution Research International. 14:85-7

**Projectvorstellung - Concept of Project**

2006 Rocha, P. S., Keiter, S., Pompêo, M., Mariani, C., Brandimarte, A. L., Seiler, T-B., Kosmehl, T., Böttcher, M., Wölz, J., Braunbeck, T., Storch, V., Hollert, H. Weight-of-Evidence-Studie zur Sedimentbelastung des Tietê River in Brasilien, UWSF – Umweltwissenschaften und Schadstoffforschung, 18: page 70.

**Oral Presentations**

2007 Rocha, P. S., Brack, W., Erdinger, L., Braunbeck, T., Storch, V., Hollert, H. Sediment ecotoxicology: Identification of hazard factors and ecotoxicological risks in the Tietê River. Oral presentation in the Annual meeting "SETAC – GLB 2007", in Leipzig, Germany.

**Poster proceedings without ISSN number**

2009 Mariani, C., Rocha, P. S., Pompêo, M. M., Zimmer, H., Erdinger, L., Zielke, H., Wölz, J., Hollert, H. Integrated Assessment of Sediment from a shallow polymitic reservoir: preliminary results from biotests. Oral presentation in the Annual meeting "SETAC North America 2009", in New Orleans, United States of America.

2008 Rocha, P. S., Brack, W., Grund, S., Braunbeck, T., Storch, V., Hollert, H. Characterization of hazard factors and ecotoxicological risks of contaminated freshwater sediments in Brazil, South America. Poster presentation in the Annual meeting "SETAC Europe 2008" in Warsaw, Poland.

2008 Otte, J. C., Rocha, P. S., Brinkmann, M., Faßbender, C., Higley, E. B., Wahrendorf, D-S., Manz, W., Keiter, S., Giesy, J. P., Braunbeck, T., Hecker, M., Hollert, H. Ah receptor mediated activities of cotaminants in sediment samles from the Elbe River, Germany. Poster presentation in the Annual meeting "SETAC Europe 2008" in Warsaw, Poland.

2008 Mariane, C., Pompêo, M. M., Rocha, P. S. , Zimmer, H., Zielke, H., Wölz, J., Erdinger, L., Hollert, H. Integrated assessment of sediments from Rio Grande resrvoir: Biotests preliminary results. Poster presentation in the Annual meeting "SETAC Europe 2008" in Warsaw, Poland.

2008 Mariane, C., Pompêo, M. M., Rocha, P. S., Zimmer, H., Zielke, H., Wölz, J., Erdinger, L., Hollert, H. Towards and integrated assessment of sediment from Rio Grande reservoir: preliminary results from bioassays. Poster presentation in the "X Congresso Brasileiro de Ecotoxicologia, Ecotox 2008", in Bento Gonçalves, Rio Grande do Sul, Brazil.

2007 Rocha, P. S., Brack, W., Jurajda, P., Ondracková, M., Wölz, J., Seiler, T-B., Kosmehl, T., Braunbeck, T., Storch, V., Hollert, H. Assessment of ecotoxicological risks and hazard factors of contaminated sediments from European freshwater ecosystems. Poster presentation in the conference "Risk Assessment in European River Basins – State of the Art and Future Challenges", in Leipzig, Germany.

2007 Rocha, P. S., Luvizotto, G. L., Kosmehl, T., Keiter, S., Böttcher, M., Wölfle, S., Storch, V., Braunbeck, T., Hollert, H. Assessment of genotoxicity of sediments from Tietê River basin (Sao Paulo, Brazil) combining the comet assay *in vitro* and the micronucleus assay *in situ* using fish blood cells. Poster presentation in the Annual meeting "SETAC – GLB 2007", in Leipzig, Germany.

2007 Rocha, P. S., Brack, W., Jurajda, P., Ondracková, M., Wölz, J., Seiler, T-B., Kosmehl, T., Braunbeck, T., Storch, V., Hollert, H. Assessment of ecotoxicological risks and hazard factors of contaminated sediments from European freshwater ecosystems. Poster presentation in the Annual meeting "SETAC – GLB 2007", in Leipzig, Germany.

2007 Jernbro, S., Rocha, P. S., Keiter, S., Skutlarek, D., Färber, H., Giesy, J., Jones, P., Hollert, H., Engwall, M. Teratogenetic and genotoxic evaluation of several perfluorinated chemicals (PFCs). Poster presentation in the Annual meeting "SETAC – GLB 2007", in Leipzig, Germany.

2007 Wahrendorf, D-S., Wetzel, M., Hollert, H., Reifferscheid, G., Rocha, P. S., Koop, J., Manz, W. Wie gut werden durch ökotoxikologische Sedimentuntersuchungen Beeinträchtigungen von benthischen Organismengemeinschaften abgebildet? Poster presentation in the Annual meeting "SETAC – GLB 2007", in Leipzig, Germany.

2007 Rocha, P. S., Luvizotto, G. L., Kosmehl, T., Keiter, S., Böttcher, M., Wölfle, S., Storch, V., Braunbeck, T., Hollert, H. Assessment of genotoxicity of sediments from Tietê River basin (Sao Paulo, Brazil) combining the comet assay *in vitro* and the micronucleus assay *in situ* using fish blood cells. Poster presentation in the Annual meeting "SETAC Europe 2007", in Porto, Portugal.

2006 Rocha, P. S,, Keiter, S,, Seiler, T-B,, Kosmehl, T,, Böttcher, M,, Wölz, J,, Pompêo, M,, Brandimarte, A. L., Mariani, C., Braunbeck, T., Storch, V., Hollert, H. Integrated assessment of sediment contamination in Tietê River, Brazil: first results. Poster presentation in the "IX Congresso Brasileiro de Ecotoxicologia, Ecotox 2006", in São Pedro, São Paulo, Brazil.

2006 Rocha, P. S., Keiter, S., Seiler, T-B., Wölz, J., Kosmehl, T., Braunbeck, T., Storch, V., Hollert, H. Weight-of-evidence study to assess sediment contamination in the Tietê River, Brazil. Poster presentation in the Annual meeting "SETAC – GLB 2006", in Landau, Germany.

2006 Jernbro, S., Rocha, P. S., Keiter, S., Skutlarek, D., Färber, H., Giesy, J., Jones, P., Hollert, H., Engwall, M. Teratogenetic and genotoxic evaluation of several perfluorinated chemicals (PFCs). Poster presentation in the Annual meeting "SETAC – GLB 2006", in Landau, Germany.

2006 Rocha, P. S., Storch, V., Braunbeck, T., Pompêo, M., Brandimarte, A .L., Mariani, C., Seiler, T-B., Kosmehl, T., Keiter, S., Böttcher, M., Hollert, H. Integrated assessment of sediment contamination in Tietê River, Brazil: first results. Poster presentation in the Annual meeting "SETAC Europe 2006", in The Hague, Netherlands.

2005 Rocha, P. S., Storch, V., Braunbeck, T., Pompêo, M., Brandimarte, A. L., Mariani, C., Seiler, T-B., Kosmehl, T., Keiter, S., Böttcher, M., Hollert, H. Integrated assessment of sediment contamination in Tietê River, Brazil. Poster presentation in the Annual meeting "SETAC-GLB 2005", in Basel, Germany.

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# Eidesstattliche Erklärung

Hiermit erkläre ich, Paula Suares Rocha, geboren am 27.10.1976 in Andradina - SP, Brasilien, an Eides statt, dass ich die vorliegende Dissertation selbst verfasst und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Ich, Paula Suares Rocha, geboren am 27.10.1976 in Andradina - SP, Brasilien, erkläre zudem an Eides statt, dass ich an keiner anderen Stelle ein Prüfungsverfahren beantragt habe, dass ich die Dissertation nicht in dieser oder anderer Form bereits anderweitig als Prüfungsarbeit verwendet habe und dass ich sie an keiner anderen Fakultät als Dissertation vorgelegt habe.

Heidelberg, October 12, 2009

Paula Suares Rocha