Carolina Fiorillo Mariani

Variação temporal do teor de SVA/ MES e avaliação integrada do sedimento do Braço do Rio Grande (Complexo Billings – SP)

São Paulo

2010

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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor em Ciências, na Área de Ecologia.

Orientador: Marcelo L. M. Pompêo
Colaborador Externo: Henner Hollert

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Mariani, Carolina Fiorillo
Variação temporal do teor de SVA/ MES e avaliação integrada do sedimento do Braço do Rio Grande (Complexo Billings – SP)
143 páginas.
Tese (Doutorado) - Instituto de Biociências da Universidade de São Paulo. Departamento de Ecologia.
1. Sedimento 2. Bioensaios 3. Ecotoxicidade I. Universidade de São Paulo. Instituto de Biociências. Departamento de Ecologia.

Comissão Julgadora:

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Prof. Dr.(a)
Orientador

Ao meu pai.

DA ANÁLISE

Eis um problema! E cada sábio nele aplica
As suas lentes abismais
Mas quem com isso ganha é o problema, que fica
Sempre com um *x* a mais...

Mario Quintana
Prosa & Verso

Agradecimentos

Gostaria de agradecer ao meu orientador Prof. Dr. Marcelo Pompêo, que desde o mestrado confiou no meu trabalho e me deu liberdade para explorar a ecotoxicologia, sem contudo me deixar esquecer a limnologia.

Thanks to my german supervisor, Prof. Dr. Henner Hollert, for the opportunity, for taking me as part of his research group and for all the support during my stay in Germany.

Thanks to Dr. Holger Zimmer and Dr. Lothar Erdinger for the possibility of using UV-light apparatus and ICP/MS for digesting and measuring metals in fish eggs.

Agradeço a Paula S. Rocha pela co-orientação, amizade de pensamentos positivos!

Aos meus queridos amigos Daniele Gallindo, Leonardo Silva, Fabiano de Bona, Pedro Aleixo, Vicente Sampaio, Kim Egerton, Maira Toledo, Daniel Baló, Particia Carvalho, Sheila C. da Silva, Juliana Braidotti, Paula Y. Nishimura, Paula R. Padial, Fabio Peixoto, Silvio Tambara, José Pedro N. Ribeiro.

Aos meus amigos que se multiplicam! Os Tanaka, os Pompéia, os D'Addio-Iwashita, os Osima, os Kaneko.

Sem vocês, amigos, tudo seria mais difícil.

Thanks to the German work group and friends, in Aachen and Heidelberg: Jan Wölz, Markus Brinkmann, Marit Ernst, Andrea Gerstner, Katja Großhans, Nina Best, Thomas-Benjamin Seiler, Hanno Zielke, Stephanie Grund, Rubens Strecker, Lisa Baumann, Eva Lammer, Kerstin Schaudt, Steffen Keiter. Viellen Danke für die Schöne Zeit in Deutschland.

Obrigada à Capes, à FAPESP e ao DAAD pelas bolsas de doutorado e doutorado sanduiche.

Obrigada aos funcionários da Universidade de São Paulo e das Universidades de Aachen e Heidelberg.

Obrigada à Conestoga-Rovers e Associados, amigos e colegas.

Agradeço aos meu pais Edson e Maria Auxiliadora, aos meus irmãos Sarah e Thiago, aos meus avós, e a minha família, por todo o amor e carinho.

Agradeço também a minha família Bernardini, Ungar e DeSimone.

Obrigada àquelas pessoas que, mesmo sem o nome nesta página, colaboraram para este trabalho.

Ao meu marido e meu amor, Alexandre Ungar, por estar ao meu lado (mesmo que de longe às vezes) me apoiando durante todo este tempo.

Lista de Abreviações

AVS	Acid Volatile Sulfide
Bio-TEQ	Biological Tocicity Equivalent
CCME	Canadian Concil of Ministers of the Environment
CETEB	Companhia de Tecnologia de Saneamento Ambiental
DMSO	dimethylsulfoxide
DO	Dissolved Oxygen
EqP	Equilibrium Partitioning
EROD	Ethoxyresorufin-*O*-deethylase
ICP/MS	Induced Coupled Plasma – Mass Spectrometer
ISQG	Interim Sediment Quality Guideline
OC	Organic Carbon
OM	Organic Matter
PAH	Polycyclic Aromatic Hydrocarbon
PAH-TEQ	PAH Tocicity Equivalent
PEL	Probable Effect Level
RBV	Regional Background Value
REP	Relative Equivalency Potency
RTL-W1	Rainbow trout liver – Waterloo 1 cell line
SEM	Simultaneously Extracted Metals
SEQ	Sediment Equivalent
SQG	Sediment Quality Guidelines
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEL	Threshold Effect Level
TEF	Toxic Equivalency Factor
US EPA	United States Environmental Protection Agency

Apresentação

O presente trabalho de doutorado é uma continuação do meu trabalho de mestrado, quando eu também optei pelo reservatório Rio Grande como local de estudo. Durante o mestrado, eu e meu orientador Marcelo conversamos muito sobre a estabilidade do sedimento daquele reservatório, e da possível relação com a dinâmica de estratificação/desestratificação da coluna d'água, pois afinal também somos limnólogos. Além disso, um dos nossos maiores desafios foi discutir os resultados então obtidos de teores de metais no sedimento na medida em que os parâmetros comparativos existentes eram (e ainda são) produzidos fora do Brasil, em especial nos EUA, Canadá e Europa. Nós nos preocupávamos com a dinâmica desses metais encontrados em grande quantidade no sedimento: será que os sedimentos são tão estáveis a ponto de os metais ficarem lá para sempre? Será que não vão para a água?

Após a conclusão do mestrado, tivemos a oportunidade de ser indagados pela mídia quanto ao risco a que seres humanos estavam expostos, quando tomavam água ou comiam peixe oriundo do Rio Grande. Nessa ocasião tivemos que "traduzir" minha dissertação e pensar melhor sobre a questão prática dos resultados no cotidiano das pessoas. E qual não foi a minha surpresa nessa época ouvir de um especialista em água/sedimento, ocupante de um cargo importante em um órgão público, que as pessoas não precisavam se preocupar com o teor exacerbado de metais no sedimento do Rio Grande, porque o sedimento não tinha nada haver com a água. No fim, todas essas questões foram muito importantes para amadurecer a idéia e para escrevermos um artigo para Ciência Hoje, justamente com o objetivo de divulgação científica.

Nossa hipótese passou a girar em torno do fato de as avaliações que tangem o sedimento serem feitas muito espaçadas no tempo e no espaço. A questão do espaço foi a abordagem do mestrado, faltava a abordagem temporal. Queríamos também saber qual o potencial tóxico desse sedimento medido com parâmetros biológicos, e não somente valores comparativos importados, já que em última análise o objetivo é contribuir para avaliação de risco ecológico da biota exposta a esse sedimento.

O reservatório Rio Grande é um dos braços do Complexo Billings, que é o maior reservatório de água da Região Metropolitana de São Paulo. Dele (Complexo Billings) é retirada água diretamente para abastecer o ABC, e ainda indiretamente (através da transposição para a Guarapiranga) parte da cidade de São Paulo. Desta forma, muitas vezes, fazemos menção a essa importância, que para nós um dos pontos-chave deste trabalho. No entanto, como nosso objetivo também é publicação em revistas científicas internacionais, precisamos muitas vezes tirar o peso da região e o apelo local, para que as questões de interesse mais global pudessem ser ressaltadas. Desta forma, organizamos este trabalho de modo que contribuísse ao máximo com a escala local (referências sobre a importância dos resultados para o ecossistema local, inclusive as pessoas) e global (artigos científicos). Outra preocupação nossa foi referente à língua, posto que tivemos colaboradores estrangeiros, em virtude do estágio de doutorado realizado na Alemanha.

Durante essa experiência no exterior, foi muito importante o contato com diversas metodologias de avaliação ecotoxicológia, algumas delas pouco conhecidas (ou aplicadas) no Brasil, e sobre as quais eu escrevi nos Relatórios enviados para a FAPESP e para a CAPES (agências concessoras das bolsas de estudo).

Como essas descrições não cabiam nos capítulos com formato de artigos, nós criamos um capítulo de metodologias para aproveitar esse material.

O **Capítulo 1**, bem como o **Capítulo 7** foram redigidos em português e trazem uma introdução geral sobre a ecotoxicologia e um fechamento sobre as conclusões do trabalho. O **Capítulo 2** apresenta as metodologias utilizadas em detalhes. Os Capítulos do meio (**Capítulos 3, 4, 5 e 6**) trazem o trabalho técnico em si, na forma de artigos científicos a serem submetidos a revistas científicas internacionais, e estão em Inglês.

Por últimos, o **Capítulo 6** foi idealizado para dar jus ao título deste trabalho, ou seja, avaliação integrada. Nele nós sumarizamos as informações de uma forma mais visual, e utilizamos cores semafóricas para avaliar os dados todos juntos. Parte da idéia da criação visual deste capítulo nasceu da apresentação de parte dos resultados no congresso norte-americano da Sociedade de Toxicologia e Química Ambientais (SETAC – Society of Environmental Toxicology and Chemistry) e também do meu trabalho como consultora ambiental.

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Abstract

Assessment of complex mixture sediments is a great challenge, especially for reservoir located near urban areas where a multitude of pollution sources is present, such as Rio Grande reservoir. Aiming an integrated assessment, we performed a battery of bioassays on sediment from Rio Grande reservoir, using permanent cell line RTL-W1 and bacteria exposed sediment acetonic extracts (cytotoxicity test, EROD assay, comet assay, micronucleus assay and Ames Fluctuation test) and *Danio rerio* embryos (whole sediment contact). After exposure, we measured metal (ICP-MS) in dechorionated non-coagulated fish embryos (digestion: H_2O_2 and HNO_3 under UV light radiation). PAH was also measured in extracts and metal dynamics was evaluated as temporal heterogeneity and remobilization to water column. Sediment extract was highly cytotoxic (NR_{50} = 10.3 mg sediment equivalent /mL; n=4) and whole sediment showed high toxic potential to fish embryos (EC_{50} 48h=12 mg sediment/ mL; n=4). No metal could be detected in the non-coagulated fish eggs, although metals were found in high concentrations with potential to cause negative effect. EROD assay showed dioxin-like activity, with $EC_{25\ TCDD}$ = 0.82 mg of SEQ/mL, and Bio-TEQ as high as 1884 pg/g (n=6). Sum of target PAH in sediment extract was 763.6 µg/kg; though 4.64 % of Bio-TEQ could be explained by PAH-TEQ. Ames Fluctuation test and comet assay reveled high potentials for mutageno- and genotoxicity. Studied metals appear not to be the cause of primary acute effect observed toxicity tests, nor seam target PAHs. Yet, we observed high concentration of metal in sediment, short-time temporal heterogeneity of metals coupled with front cold passage and possible remobilization into the water column, what raises the need for better understanding of metal dynamics in such aquatic environment (tropical polymitic) and careful planning for sediment sampling designs (considering temporal and spatial scales and the whole water column above the sediment). Integrated assessment through Weight-of-Evidence (WOE) approach indicates the need of management actions regarding sediment from Rio Grande reservoir. However, characterization of the risk in within an Ecological Risk Assessment (ERA) framework should precede action, in order to better address the problem. Further studies should prioritize chronic exposure of organisms to sediment samples from Rio Grande; PCBs and dioxins should be considered as possible EROD activity inducer; chemical analysis should be expanded and consider other organic chemicals; sediment fractionation technique should also be applied in order to provide insights on causality from organic compounds.

Resumo

A avaliação de sedimentos contendo de mistura complexa de substâncias contaminantes é um grande desafio, especialmente em reservatórios localizados próximos a áreas urbanas, onde existe um grande número de fontes de poluição, como é o caso do reservatório Rio Grande. Com o objetivo de proceder uma análise integrada, nós realizamos uma bateria de biotestes usando amostras de sedimento do reservatório Rio Grande e células permanentes da linhagem RTL-W1 e bactérias expostas a extrato acetônico (teste de citotoxicidade, teste de EROD, teste cometa, teste do micronúcleo e teste de flutuação de Ames) e também embriões de *Danio rerio* (teste de contato com o sedimento). Após a exposição dos embriões, nós retiramos o córion e realizamos medição de metais (ICP-MS) em embriões não coagulados (digestão: H_2O_2 e HNO_3 sob radiação UV). Nós realizamos análises químicas de HPAs alvos no extrato e de dinâmica de metais, esta última avaliada como heterogeneidade temporal e remobilização para a coluna d'água. O extrato do sedimento se mostrou citotóxico (NR_{50} = 10,3 mg sedimento equivalente /mL; n=4) e o sedimento se mostrou com elevado potencial tóxico para embriões de peixe (EC_{50} 48h=12 mg sedimento/ mL; n=4). Não foram detectados metais no tecido de embriões não coagulados. Teste de EROD revelou alta atividade enzimática, com $EC_{25\ TCDD}$ = 0,82 mg of SEQ/mL, e Bio-TEQ = 1884 pg/g (n=6). A concentração total de HPAs analisados no extrato foi de 763,6 µg/kg; apesar disso, apenas 4.64 % do Bio-TEQ pôde ser explicado pelo PAH-TEQ. O teste de flutuação de Ames e o teste cometa revelaram altos potenciais para mutageno e genotóxicos. Os metais estudados aparentemente não são a causa primária para o efeito agudo observado, assim como os HPAs analisados. Porém, nós encontramos uma alta concentração de metais no sedimento, além de uma heterogeneidade temporal de curto tempo coincidente com a passagem de uma frente fria, e uma possível remobilização de metais para a coluna d'água, o que levanta a necessidade de um melhor entendimento da dinâmica dos metais em um ambiente aquático como o estudado (tropical e polimítico), além da necessidade de um planejamento cuidadoso de desenhos experimentais de estudos de sedimentos (considerando as escalas temporal e espacial e a massa d'água como um todo). Análise Integrada por meio de "ponderação das evidências" indicou a necessidade de ações de manejo para o sedimento do reservatório Rio Grande. No entanto, deve ser realizada a caracterização do risco, no contexto de uma Análise de Risco Ecológica, antes que se decida qual ação tomar, de forma a melhor dirigir o problema. Estudos complementares devem priorizar testes com organismos com exposição crônica a amostras do sedimento do Rio Grande; PCBs e dioxinas devem ser considerados como possíveis indutores de atividade de EROD; análises químicas do

sedimento devem ser expandidas e considerar outros compostos orgânicos; técnicas de fracionamento de sedimento devem ser empregadas visando o estabelecimento de causalidade em respeito aos compostos orgânicos.

Cap.1 Introdução

Todos os dias, novos compostos químicos são descobertos ou sintetizados para usos diversos, incluindo agricultura, como lubrificantes, como remédios, comonentes de produtos de beleza, etc. De acordo com levantamento realizado pela União Européia, em 1981 havia 100.000 substâncias conhecidas no mercado. A partir de então, outras 3800 foram registradas, sendo que após 25 anos de regulamentação ainda existem lacunas de informação referentes a 30.000 dessas substâncias (Environment Directorate General 2007).

Atualmente, produtos tóxicos são uma questão global e por décadas, têm sido objeto de negociação internacional, de modo que existem mais de 50 tratados e acordos regionais e internacionais relacionados ao tema. Alguns dos tratados globais mais importante são:

- Convenção sobre Prevenção da Poluição Marinha por Alijamento de Resíduos e Outras Matérias, concluída em Londres, a 29 de dezembro de 1972 e promulgada pelo Brasil em 1982;
- Protocolo de Montreal sobre Substancias que Agridem a Camada de Ozônio, redigido a partir de reuniões realizadas em Londres (1990), Copenhagen (1992), Montreal (1997) e Beijing (1999); promulgada pelo Brasil em 1990. Todas as emendas e alterações quanto a aspectos técnicos realizados no texto do Protocolo foram ratificadas pelo Brasil;
- Convenção da Basiléia sobre Movimento Transfronteiriços de Resíduos Perigosos e seu Depósito, redigida em 1989 e ratificada pelo Brasil em 1992;
- Convenção de Roterdã sobre Procedimento para Consentimento Prévio Informado para Comércio Internacional de Certos Químicos e Pesticidas Perigosos (PIC), redigida em 1998 e ratificada pelo Brasil em 2004;
- Convenção de Estocolmo sobre Poluentes Orgânicos Persistentes (POPS), redigida em 2001 e ratificada pelo Brasil em 2004;
- Enfoque Estratégico para o Gerenciamento Internacional de Produtos – SAICM, redigida em 2006.

Apesar da vasta gama de compostos previstos nesses tratados e dos esforços internacionais direcionados ao controle dessas substâncias com respeito ao registro, aplicações e conhecimento da

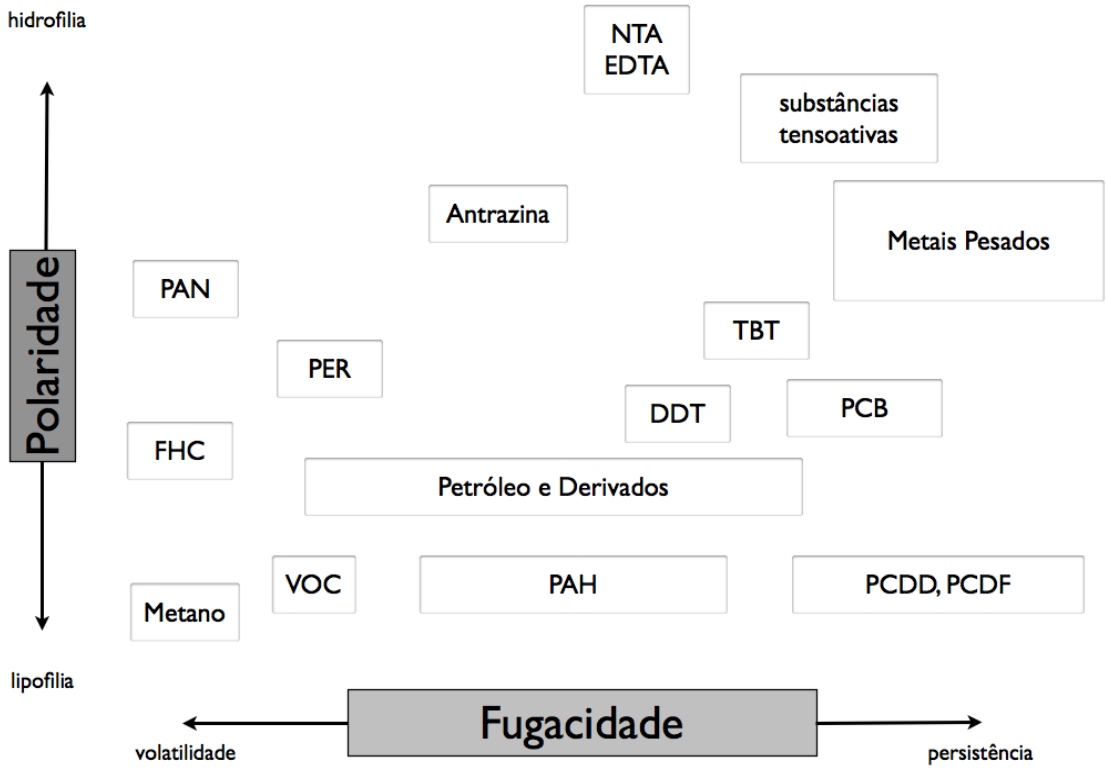
interação com o meio ambiente, todos os dias novos produtos são inventados. As chamadas substâncias emergentes (“Emerging Substances”) podem ser divididas em 4 categorias: éteres difenil polibromados (PBDEs) e outros poluentes orgânicos persistentes (POPs), produtos farmacêuticos e de cuidado pessoal, desruptores endócrinos e produtos de nanotecnologia (Chapman 2006).

Nesse contexto, o estudo das substâncias contaminantes no ambiente e os riscos oferecidos por elas aos organismos é uma tarefa grande e urgente, que deve ter como o objetivo final o melhor gerenciamento dos riscos à saúde do meio ambiente e dos seres humanos.

1 Substâncias contaminantes no ambiente

A Organização Mundial da Saúde elegeu 10 poluentes de interesse prioritário para a saúde humana, a saber, poluentes do ar, arsênio, asbestos, benzeno, cádmio, dioxinas e substâncias semelhantes (incluindo PCBs), concentrações inadequadas ou excessivas de fluoreto, chumbo, mercúrio, pesticidas altamente perigosos (WHO 2010).

Algumas dessas sustâncias foram eleitas por fazerem parte dos chamados PBTs (Persistent, Bioaccumulative, and Toxic compounds), por possuírem, como o próprio nome já sugere, as seguintes características que elevam grau de criticidade para avaliação ecotoxicológica de uma substância: alta toxicidade, persistência no ambiente e alto potencial de bioacumulação/ biomagnificação. A Figura 1 apresenta graficamente algumas classes de contaminantes quanto à persistência (em função da fugacidade) e a polaridade. Desta forma, as substâncias mais críticas estão localizadas à direita na figura.



PCDD = policloro dibenzo-1,4-dioxina
PCDF = policloro dibenzo furano
PAN = Peroxiacetil nitrato
PER = tetracloroeteno (percloroetileno)
EDTA = ácido etilenodiamino tetra-acético
DDT = Dicloro-Difenil-Tricloroetano

NTA = Ácido Nitrilo Triacético
FHC = Hidrocarbonetos fluorados
TBT = Tributil-estanho
VOC = Compostos Orgânicos Voláteis
PCB = Bifenilas Policloradas
PAH = Hidrocarbonetos Policíclicos Aromáticos

Adaptado de Fent (2007)

Figura 1. Representação de classes de contaminantes quanto à persistência (função da fugacidade) e da polaridade.

Uma vez no ambiente, o destino dos compostos químicos poluentes dependem de processos de transporte, de transferência e de transformação, os quais podem ocorrer simultaneamente ou não. Desta forma, por exemplo, durante a mudança de local de uma substância (transporte) pode ocorrer alterações na concentração, isto é diluição, sofrer fotólise (transformação) e ser por fim depositada no sedimento do fundo (transferência de fase) (Mozeto & Zagatto 2006). A Tabela 1 apresenta alguns exemplos desses processos.

Tabela 1. Processos que influenciam no destino dos contaminantes no meio ambiente.

Cartegoria	*Processso*
Transporte	Advecção
	Difusão
	Dispersão
	Transporte através de partícula
Transferência	Dissolução em água
	Sorção (absorção e desorção)
	Deposição (atmosférica e sedimentação)
	Volatização
Transformação	Abiótica: hidrólise, fotólise, reações de oxi-redução
	Biótica: decomposição aeróbica e anaeróbica, metabolismo

Em relação ao ambiente aquático, os principais processos envolvidos no destino das substâncias contaminantes estão ilustradas na Figura 2.

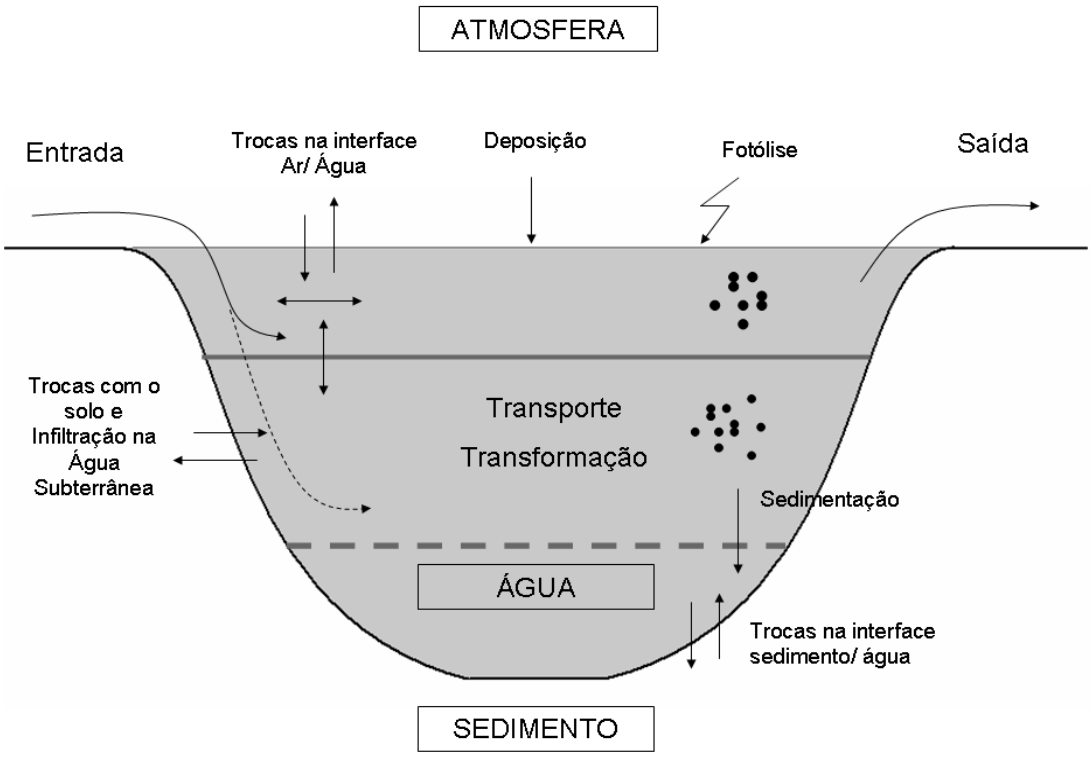


Figura 2. Principais processos envolvidos no destino das substâncias contaminantes no meio ambiente aquático. Adaptado de Fent (2007).

2 O que é Ecotoxicologia

A ecotoxicologia é uma ciência que nasceu da junção da ecologia com a toxicologia, de modo que a ecologia, a química ambiental e a toxicologia foram integradas. Ecotoxicologia se preocupa principalmente com os efeitos e consequências dos compostos químicos no meio ambiente, sendo assim, abrange todos os níveis, de moléculas a ecossistemas (Fent 2007); e inclui o estudo dos tipos de efeitos causados por processos químicos, bioquímicos responsáveis pelos efeitos observados (Chapman 2002).

Assim como para a toxicologia, o axioma da ecotoxicologia é a relação dose-resposta, isto é, que o efeito negativo observado é dependente da dose administrada, ou concentração de exposição. Os efeitos tóxicos podem variar desde reversíveis a letais, dependendo de processos como transformações metabólicas (internas ao organismo) ou biotransformações que ocorrem no meio (externas ao organismo). Desta forma, o efeito tóxico é dependente não só da dose, como do tempo de exposição, determinando exposições agudas ou crônicas.

Fatores que interferem no efeito tóxico de uma substância são tanto dependentes de características intrínsecas a ela como de situações ou estados do meio ambiente e do organismo que entrará em contato com a substância (Fent 2007; Luoma 1989). A Tabela 2 apresenta fatores bióticos e abióticos (internos e externos ao organismo) e intrínsecos à substância que interferem no efeito tóxico observado. Esses fatores determinam três processos básicos da ecotoxicologia: a sensitividade da espécie a uma substância contaminante, as interações do ecossistema e a capacidade de recuperação do organismo ou sistema (Van den Brink 2008).

Tabela 2. Fatores bióticos e abióticos, externos e internos ao organismo e dependentes da substância contaminante que interferem no efeito tóxico.

Fatores dependentes da substância	*Fatores abióticos e abióticos do meio*	*Fatores do organismo exposto*
Características físico-químicas Especie química em que se encontra Biodisponibilidade Concentração (dose) no local de exposição	pH, salinidade, temperatura, salinidade. Condições do ecossistema (estágio sucessional, nível de conservação, etc) Características do ecossistema (resiliência, capacidade suporte, capacidade de tamponamento, etc.); Transformações no ecossistema (bioativação, decomposição, bioturvação, etc).	Espécie Estágio de desenvolvimento /Idade Tamanho Condições nutricionais, de saúde, de estresse, adaptação; Modo de vida Sociobiologia Meio de exposição (através do meio ou do alimento) Tempo de exposição (aguda ou crônica)

3 Relevância ecológica

Uma questão de grande importância na ecotoxicologia diz respeito à relevância ecológica dos dados levantados. Como já mencionado, a ecotoxicologia estuda o efeito tóxico de uma substância no meio ambiente em relação aos diferentes níveis de organização. Isso porque um dos princípios básicos da ecotoxicologia é justamente a interligação do efeito negativo sobre os diferentes níveis hierárquicos de organização (Fent 2007): uma alteração em nível molecular pode levar a uma alteração no *fitness* de um indivíduo, levando a uma alteração no seu sucesso reprodutivo e, possivelmente influindo na população daquela espécie e na estrutura da comunidade, chegando ao nível de ecossistema. Porém, o tempo necessário para que a resposta ao efeito tóxico seja percebida é proporcional ao nível hierárquico avaliado, de modo que quanto maior a complexidade, maior o tempo necessário para que a resposta seja observada. Por esse motivo, os testes ecotoxicológicos em nível celular são mais sensíveis, e menos complexos, enquanto testes em nível de ecossistema são complexos e dependentes de muitas variáveis, e demanda um tempo de estudo maior (anos ou décadas). A Figura 3 ilustra a interdependência entre o nível de complexidade (nível hierárquico) e o tempo de resposta, bem como a relevância ecologia e a sensibilidade da resposta, enquanto a Tabela 3 apresenta os diferentes critérios/ indicadores para a avaliação (*endpoints*) em diferentes níveis de organização.

Tabela 3. Critérios/ indicadores para a avaliação ecotoxicológica em diferentes níveis de organização

Molecular – Celular	*Organismo*	*População – Comunidade*	*Ecossistema*
Gene	Metabolismo	Diversidade	Produtividade
Enzima	Comportamento	Abundância	Cadeia trófica
Proteína	Desenvolvimento	Sucessão	Ciclo dos nutrientes
Alterações celulares	Crescimento	Dispersão	Fluxo de energia
	Estrutura	Relação predador-presa	
	Morfologia		
	Reprodução		
	Mortalidade		

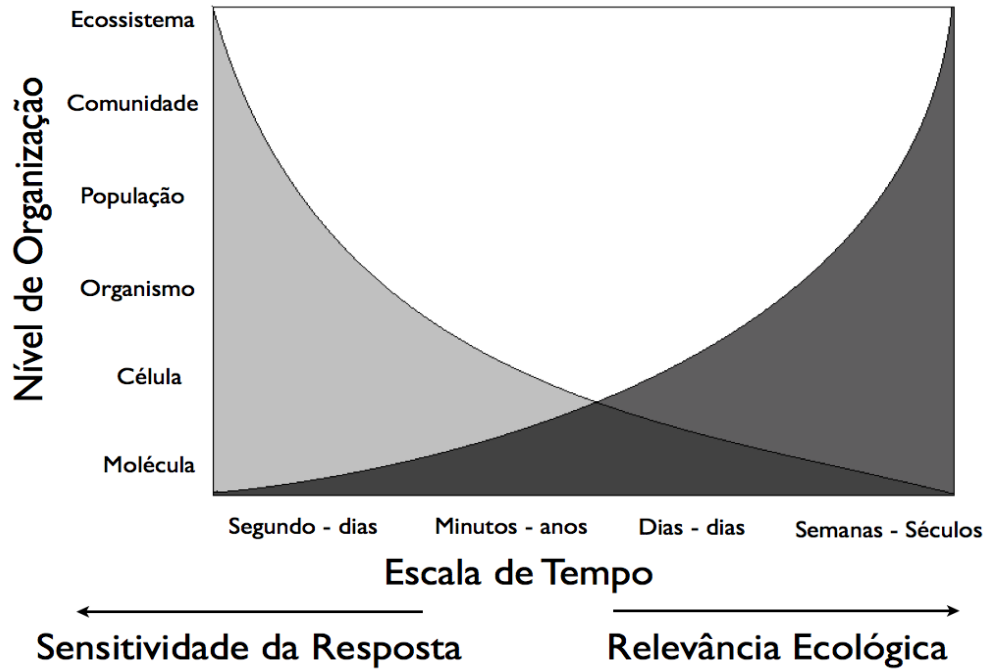


Figura 3. Representação gráfica da relação entre a escala de tempo e o nível de organização, sensitividade da resposta e relevância ecológica. Segundo Fent (2007).

Existem muitos debates a respeito da extrapolação de estudos feitos em níveis organizacionais menores para níveis maiores (Chapman 2002; Chapman et al. 1998; Van den Brink 2008); a condução de testes subcrôniocs e crônicos em microcosmos (em laboratório ou *in situ*) é mais rápida e menos onerosa, além haver disponível maior quantidade de estudos e de metodologias estabelecidas para fins comparativos. Apesar disso, estudos em níveis mais complexos são necessários para melhor avaliação do risco potencial da biota exposta a contaminantes químicos.

4 Análise Integrada (AI) e Avaliação de Risco Ecológico (ERA)

Análise Integrada (AI) pode ser definida como um processo interdisciplinar de combinação, interpretação e comunicação do conhecimento de diversas disciplinas científicas de modo que o a cadeia de causa-efeito como um todo possa ser analisada de uma perspectiva sinóptica com 2 características: (i) deve agregar valor comparado com a avaliação de uma disciplina só; (ii) deve fornecer informação útil para os tomadores de decisão (van der Sluijs 2002).

Avaliação implica na análise e revisão da informação derivada da pesquisa, com o intuito de auxiliar uma pessoa em uma posição de responsabilidade a ponderar possíveis ações ou pensar

sobre um determinado problema. Nesse contexto, avaliação significa resumir, organizar, interpretar e possivelmente conciliar pedaços de conhecimento pré-existente, e comunica-los de forma relevante e útil para um tomador de decisão inteligente, mas não expert (Parson 1995).

Como já mencionado, a gama de contaminantes lançados na natureza é tal que o conhecimento a respeito dos efeitos de cada contaminante individual sobre o ecossistema ainda não é completo. No caso de mistura de substâncias contaminantes, as lacunas são ainda maiores, posto que é preciso considerar efeitos sinergísticos e antagônicos das substâncias no meio ambiente, diferenças inter e intra-específicas além de características como resistência e resiliência do ecossistema.

Mesmo assim, existe a necessidade de se tomar decisões a respeito de gestão e de possíveis interferências em ambientes onde haja suspeita de poluição. Por esse motivo, tem-se desenvolvido mecanismos para se analisar e concatenar as informações disponíveis ou linhas de evidência (Lines of Evidence - LOE), de maneira que sejam úteis e fáceis de serem comunicadas, visando o auxílio a tomadas de decisão.

Desta forma, foi idealizado um processo que envolve uma estrutura ou *framework*, originalmente proposta pela USEPA e revista por diversos especialistas (Dale et al. 2008; Chapman & J. Anderson 2005; USEPA 1992; USEPA 1998), que ficou conhecida como Análise de Risco Ecológica (Ecological Risk Assessment – ERA). Assim como uma AI, ERA é um processo que implica em reunir, organizar e analisar informações ambientais, porém visa estimar o risco de poluição ou um agente estressor para um ecossistema e nortear as decisões de gestão (Jensen & Mesman 2006; Dale et al. 2008; USEPA 1992).

5 *Tríade de Qualidade de Sedimentos e "Weight-of-Evidence" (WOE)*

No que tange avaliação de sedimentos contaminados, uma das primeiras abordagens de avaliação integrada foi proposta por Long e Chapman (1985). Esses autores sugeriram a análise de 3 parâmetros para se avaliar se o sedimento (a) está contaminado, (b) apresenta toxicidade a seres vivos a ele expostos e (c) apresenta toxicidade *in situ*. Para informar quanto à contaminação seriam usados parâmetros químicos, quanto à toxicidade, teste toxicológicos com organismos e quanto à toxicidade *in situ*, dados da comunidade bentônica. Essa abordagem ficou conhecida como Tríade de Qualidade de Sedimento (TQS). Segundo Chapman et al. (1997), TQS consiste em um "sistema conceitual para coletar medidas sinópticas de química, toxicidade e de bentos em sedimentos, e usar essas medidas coletivamente para estimar a qualidade do sedimento" .

Com o avanço no campo da ecotoxicologia, outros testes foram incorporados à TQS como de mutagenicidade, genotoxicidade e testes teratogênicos (Hollert et al. 2002), visando complementar e auxiliar a classificação dos sedimentos, tomando como base uma abordagem de "ponderação das evidências", ou *Weight-of-Evidences* (WOE) (Chapman et al. 1998; Chapman et al. 2002; Chapman & Hollert 2006). A idéia central da TQS permaneceu como paradigma (reunir evidências toxicológicas, químicas e ecológicas ou evidência *in situ*), porém se expandiu no sentido de reunir diferentes linhas de evidências e ponderá-las em uma análise integrada. A Figura 4 apresenta uma ilustração do sistema conceitual da TQS, ou seja, a intersecção entre as três esferas de estudo.

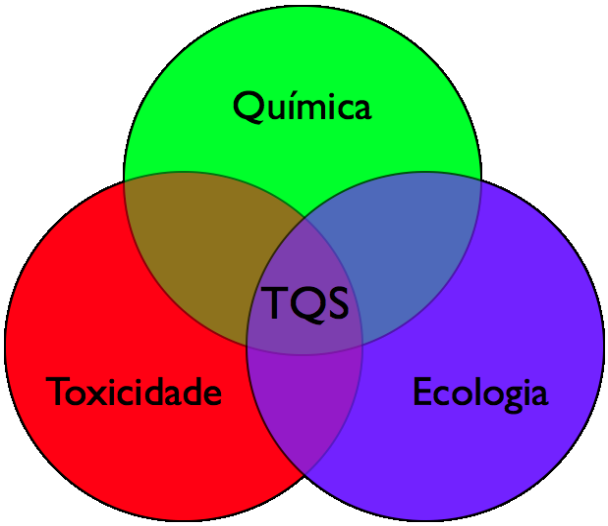


Figura 4. Representação esquemática e conceitual da Tríade de Qualidade de Sedimento (TQS) proposta por Chapman e Long (1985).

WOE é uma das ferramentas que podem ser utilizadas durante o processo de realização de ERA, para se reunir as LOE e ponderá-las, de modo a se avaliar a qualidade dos dados, a existência de lacunas, ou a necessidade de mais informações. Vale ressaltar que o principal objetivo da ERA é a caracterização do risco e o auxílio à tomada de decisão ligada à gestão do risco identificado. Desta forma, ERA é um processo mais abrangente, e tem como objetivo a caracterização do risco ecológico e a gestão desse risco, demandando não somente especialistas cientistas como também políticos e atores sociais envolvidos (Stahl 2001).

6 Escolha do Local de Estudo

O reservatório Rio Grande, localizado no Complexo Billings, na Grande São Paulo, SP, é um exemplo de ambiente submetido a diferentes fatores de estresse e diversas fontes de poluição, em decorrência do uso e ocupação do entorno.

Apesar de ser protegida pela Lei de Proteção dos Mananciais desde a década de 70, a pressão urbana, industrial e minerária na Bacia Hidrográfica do Complexo Billings como um todo cresceu, com perda da cobertura vegetal e aumento de área urbana e de favelas. Com isso, também cresceram os locais de deposição regular e irregular de resíduos sólidos e efluentes líquidos, como lixões, depósitos de entulho, despejo de efluentes domésticos e industriais tratados e *in natura* (Whately et al. 2008; Capobianco 2002). Mesmo assim, o reservatório Rio Grande é de usos múltiplos e serve como fonte de água para a região do Grande ABC.

O item 2 do Capítulo 2 apresenta uma caracterização mais detalhada deste ambiente.

7 Objetivo

O objetivo geral deste trabalho foi contribuir para a avaliação de risco ecológico do reservatório Rio Grande, Complexo Billings, através de análises de diferentes linhas de evidência (LOE) químicas e toxicológicas do sedimento, de integração das informações em uma análise de "ponderação das evidências (Weight-of-Evidences - WOE) e de comunicação das informações em uma forma prática e inteligível para não experts. Não foi objetivo deste trabalho a realização integral de uma ERA, posto que envolve tanto questões científicas quanto políticas e demanda a participação de uma equipe multidisciplinar.

Os objetivos específicos deste trabalho foram:

- Avaliar possível heterogeneidade temporal na concentração de metais do sedimento do reservatório Rio Grande em um curto período de tempo (14 dias);
- Avaliar o potencial tóxico do sedimento em respeito a metais através da comparação com SQGs (Sediment Quality Guidelines) (ver Capítulo 2);
- Avaliar o potencial de toxicidade de amostras de sedimento no desenvolvimento embrionário de peixe;

- Avaliar o potencial de bioacumulação de metais por embriões de peixe após exposição a amostra de sedimento;
- Avaliar o potencial citotóxico de extratos acetônicos de amostras de sedimento;
- Avaliar o potencial genotóxico e mutagênico de extratos acetônicos de amostras de sedimento;
- Avaliar o potencial de indução de atividade de citocromo P450 por extratos acetônicos de amostras de sedimento;
- Avaliar o potencial de toxicidade de PAHs prioritários através de comparação com SQGs e avaliar a contribuição dos PAHs analisados para o potencial de indução de atividade de citocromo P450;
- Analisar, resumir e comunicar as informações referentes aos potenciais tóxicos através de uma análise de WOE.

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Cap.2 General Introduction to Methodology

1 Sediment Quality Guidelines (SQG) and Integrated Assessment

First studies contaminants in sediment attempted to establish background levels, aiming a comparison between "natural" and observed concentrations, hence using enrichment as a parameter for sediment quality. This was particular important for metals, since many organic contaminants are not naturally found in soils and sediments. One example of such attempt was the world mean values of metal concentration, established by Turekian and Wedepohl (1961). However, metal background levels should be established in a local basis, since many factors have influence on its concentration (i.e. soil type, erosion, climate, geology, topography, decomposition, etc). In Brazil, one of the few studies in this direction was done by Nascimento and Mozeto (2008), who established Regional Background Values for metals in sediment of Tiete River Basin.

Further on, more ecological focus was given to sediment quality criteria, and bioavailability was recognized as key-factor not only for metal toxicity but also for many other substances. At the same time, increase of sediment contamination brought about the need for regulation and establishment of maximum acceptable levels for contaminants. For example, US EPA suggested Equilibrium Partition (EqP) for metal assessment (USEPA 2000; USEPA 2005), and CCME established effect levels (Probable Effect Level and Threshold Effect Level) for a list of contaminants (CCME 1999; CCME 2002).

EqP was based on the fact that sulfide forms ans insoluble complex together with metals. Since free metal ions are the most potentially toxic metal specie, meltal sulfide complex would be non-bioavailable to biota (Luoma 1989). It predicts that adverse effect of metals would not happen when sulfide was in excess to total divalent metals, in a stoichiometric relationship. Some studies has proven the ability of such approach to correctly predict non-effect (Di Toro et al. 1990; Di Toro et al. 1992), but some concern was taken regarding the prediction of adverse effect, due to other possible metal complexing phases found in sediment (e.g. organic matter, clay, ion hydroxide, colloids, etc.). After a review of the EqP approach, the USEPA suggested a normalization of the former AVS-SEM equation with total organic carbon (TOC). This improved significantly the prediction of the formula, which is given as follow (USEPA 2005):

$$\frac{\sum [SEM] - [AVS]}{TOC}$$

where,

Σ[SEM] is the sum of molar concentration of divalent metals

[AVS] is the molar concentration of sulfide; and

TOC is Total Organic Carbon.

From this relation, it was postulated that toxicitiy was:

a) likely to occur, when result was > 3,000.0 mmol kg^{-1}

b) uncertain to occur, when result was between 130.0 and 3,000.0 mmol kg^{-1}

c) unlike to occur, when result was < 130,0 mmol kg^{-1}.

The Canadian Council of Ministers of the Environment – CCME used another approach to generate SQG. They created a data bank with information on available ecotoxicological tests from all over Canadda (in situ, in vitro and bioaccumulation) and compared them to contaminant concentration, yielding two reference values: Probable Effect Level (PEL) and Threshold Effect Level or Interim Sediment Quality Guideline (TEL ; ISQG). When a given contaminant is found in the sediment in a concentration bellow ISQG, it is unlikely to have an observed negative effect on biota; when the concentration is above ISQG, negative effect is likely to occur; and when concentration is between ISQG and PEL, negative effect is possible to occur (CCME 1999). With this approach, CCME could establish reference values for most known and common contaminants, not only metals.

Table 5 show SQG suggested by CCME and Nascimento and Mozeto.

Table 4. Sediment Quality Guideline Values suggested by CCME (2003) and Regional Reference Values suggested by Nascimento and Mozeto (2008).

Metal	*ISQG (mg kg^{-1})**	*PEL (mg kg^{-1})**	*RRV (mg kg^{-1})***
Cd	0.6	3.5	0.22
Cu	35.7	197	18
Cr	37.3	90	36
Ni	18	36	23
Pb	35	91.3	61
Zn	123	315	82
PAH***	1610		-

ISQG = Interim Sediment Quality Guideline
PEL = Probable Effect Level
RRV= Reference Regional Value for freshwater sediments of Upper Tiete river basin.
*CCME (2003)
**Nascimento and Mozeto (2008)
***Consensus-based TEC (Treshold Effect Concentration) MacDonald et al. (2002)

Despite such attempts, sediment is a complex system with different processes happening within it. Besides, mixture of substances is subject to synergism and antagonism effect ecosystem interaction with stress is also complex, making it difficult relay ecological assessment of contaminated sediment simply on chemical (Ahlf et al. 2002).

In the 80s, Chapman and some colleagues came with a new idea for sediment assessment (Peter M. Chapman 1990; P. M. Chapman et al. 1996; Peter Chapman & Mann 1999). They suggested the use of three points for sediment toxicity characterization: (a) chemical measure, or the degree of contamination; (b) bioassays, or the degree of toxicity to living organisms; and (c) benthic community integrity, or the degree of *in situ* toxicity. This conceptual framework was known as Sediment Quality Triad (SQT). From them on, SQT has been improved and recently enclosed different techniques for toxicity evaluation in molecular and genomic levels (Hollert et al. 2002; Ahlf et al. 2002), such as genotoxicigy and biomarkers, gaining a weight-of-evidences approach, (Peter M. Chapman et al. 1998; Peter M. Chapman & Hollert 2006). As reviewed by Chapman and Hollert (2006), TQS should become a Tetrad, Pentad or Hexad.

Still, chemical evaluation and SQG are widely used for screening level in a framework of decision for sediment management and are the first step for guiding and directing the need of further investigation (Cornelis et al. 1999; Peter M. Chapman & Anderson 2005; Annicchiarico et al. 2007; Apitz & Power 2002).

2 Sampling Area

The Billings Complex is located inside the São Paulo Metropolitan Area, west side from São Paulo City, between the latitudes 23°43' and 23°45' S and the longitudes 46°27' and 46°42' W, 746.5 m above sea level (Trindade 1988; CETESB 1996a). The drainage basin is 560 km^2 wide, and it is a sub-basin of the Alto Tiete basin (5985 km^2) (CETESB 1996a; CETESB 2005). The dam was built in 1927 and had an additional water source coming from Pinheiros river, which was back pumped. The purpose of the system was regulating the water level of another smaller reservoir, Rio das Pedras, which in turn provided water for a hydropower plant in Cubatao (Henry Borden Plant), making use of the difference in level created by Serra do Mar (720 m).

In 1958, the uses of Billings Complex were no longer restricted to power generation; it was also used for fishing, recreation and public water supply, as an intake water point was installed in Rio Grande location. At the same time, with the development of Sao Paulo city and its metropolitan area, eutrophication became a problem and water quality issue was worsened by the back pumping of Pinheiros river, which received industrial and domestic untreated wastewater from the city.

In the following years, water quality declined and reached such poor level that put water supply at risk. Therefore, Rio Grande branch was isolated from the rest of the Complex in 1981 through the construction of Anchieta Dam. Even nowadays, there is still conflict of interests on multiple uses of Rio Grande Reservoir. In spite of providing water for public consumption, it also receives domestic effluent from 36,352 inhabitants of Rio Grande da Serra, and several clandestine sewages (CETESB 2005) from urban nuclei located in its margins. Local population usually fish for recreation and small scale consumption. Within its catchment area there are mining activities, industries installed (with events of contaminated effluent discharges) and landfill (Whately 2003).

To prevent potential toxic algae bloom, the State Water Company (SABESP) responsible for the water treatment for human consumption in Rio Grande reservoir systematically applies algaecide in the reservoir (Takino & Maier 1986; CETESB 2005; CETESB 2006; CETESB 2007). Algaecide are either hydrogen peroxide or copper sulfide. Figure 1 shows applied algaecide per month in Rio Grande in the year 2006.

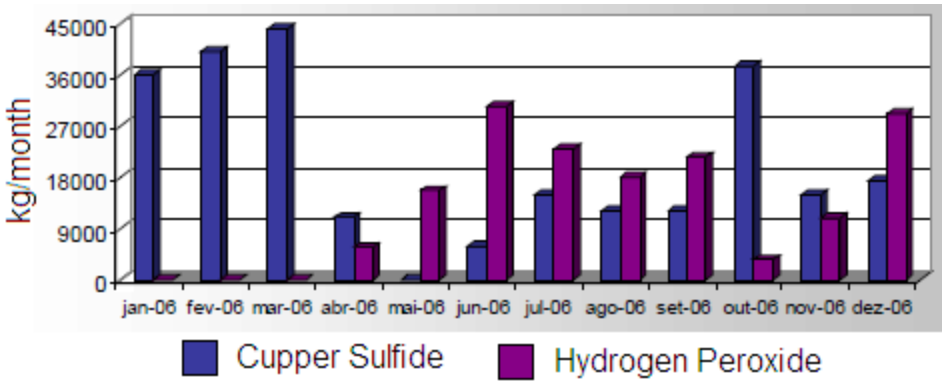


Figure 1. Algaecide applied per month during the year 2006 in Rio Grande Reservoir. Source: SABESP apud CETESB (2007).

Rio Grande reservoir is located in São Bernardo do Campo, Santo André, Ribeirão Pires e Rio Grande da Serra boroughs. It occupies an area of approximately 7.4 km2, and is 9 km long. Table 5 presents some hydromorphological information on Rio Grande Reservoir and Figure 2 shows the location of Rio Grande Reservoir in relation to Billings Complex, Sao Paulo Metropolitan Area and Brazil.

Table 5. Main hydromorphological characteristics of Rio Grande reservoir.

Characteristic	*Data*	*Reference*
Water retention capacity	155 million m^3	(Maier et al. 1985)
Linear extension	12.5 km	(Maier et al. 1985)
Water mirror area	15 km^2	(Maier et al. 1985)
Maximum depth	11.5 m	
Mean depth	10 m	(Beyruth & Pereira, 2002)
Rio Grande sub-Basin Area	188 km^2	(Maier et al. 1985)
Altitude	746.5 m	(Takino & Maier, 1986)
Potential organic load	8,585 kg DBO/day	(CETESB, 2007)
Water supply	1.2 million people	(SABESP, 2005)
Sedimentation rate*	~ 0.857 $cm.y^{-1}$	(Fávaro et al., 2007)

* mean value calculated from 4 dated sediment cores from along the reservoir

A)

B)

C)

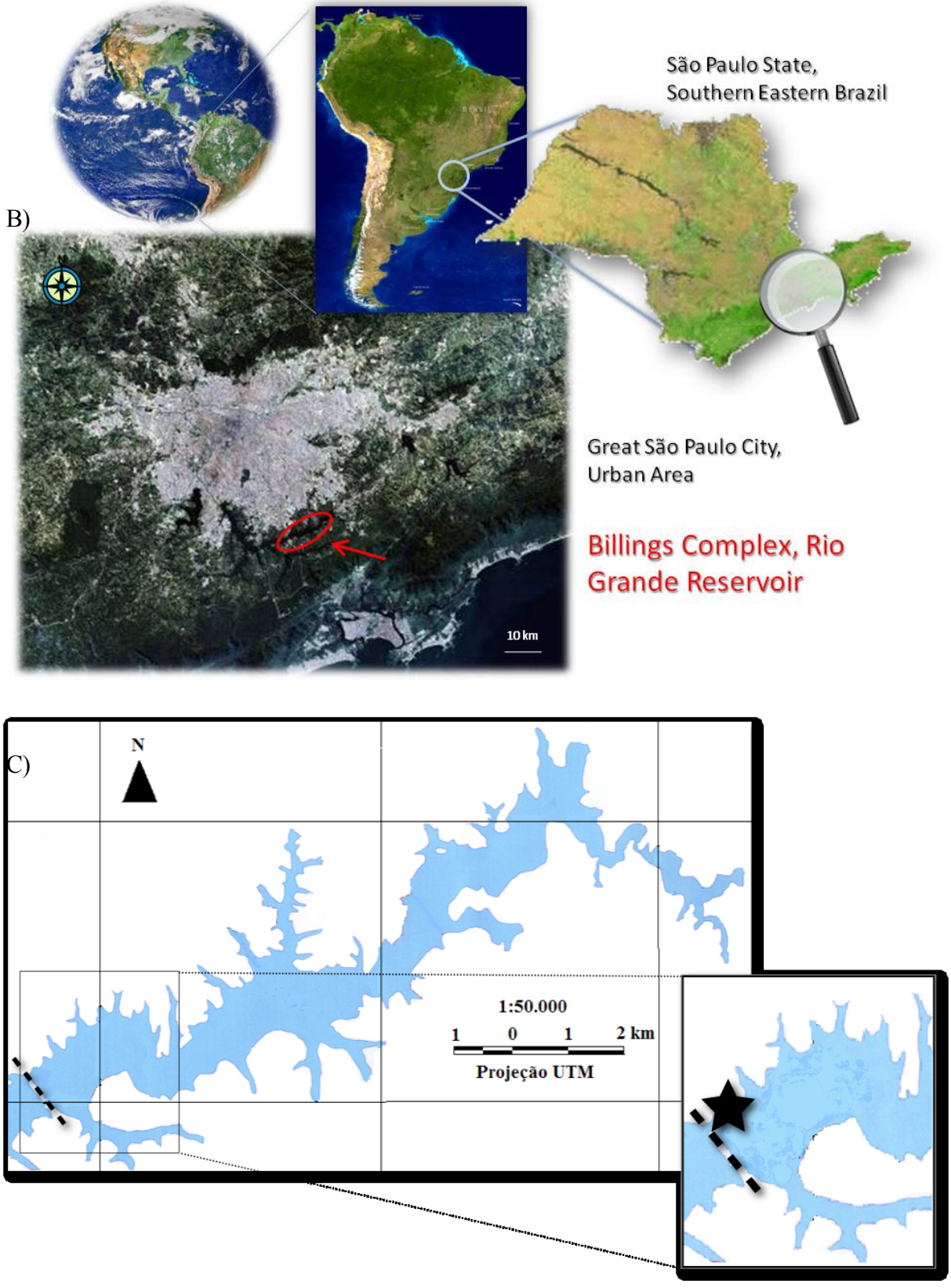


Figure 2. A) Location of Sao Paulo State, B) Satelite picture of Sao Paulo Metropolitan Area; C) Rio Grande Reservoir, and zoom in the studied area close to the dam. Dotted line represent Anchieta Dam; star represents water intake point for public consumption.

Many scientific works have reported sediment metal and organic pollution in Billings Complex and in Rio Grande reservoir (CETESB 1996b; Rocha et al. 1985; Fávaro et al. 2007; Mozeto et al. 2003; Beyruth & H. A. S. L. Pereira 2002; da Silva et al. 2002; Bainy et al. 1996; Bainy et al. 1999; Mozeto et al. 2001; Carvalho et al. 1998; Maier 1985; Maier & Takino 1985; Maier et al. 1985; Maier et al. 1997; Takino & Maier 1986).

Mariani (2006) and Mariani *et al.* (2005) made heavy metal and SVA measurements in the sediment along the Rio Grande reservoir, and found a tendency for increase metal and SVA concentrations from the higher to the lower part of the reservoir. SVA concentrations were much greater than metal concentrations, what indicates that metals are not bioavailable. However, metals were above background values and above Sediment Quality Guidelines.

In addition, Rio Grande reservoir is a shallow water body, with a polymitic dynamic of the water column (Maier et al. 1997; Maier & Takino 1985; Maier et al. 1985; Beyruth & H. A. S. L. Pereira 2002).

3 Methodology

3.1 Digestion and Measurement of AVS and SEM

Extraction was proceeded as indicate by Allen et al. (1993).This methodology comprises a weak acid attack (HCl 1.0 M) at room temperature, allowing sulfide to be quantified, at the same time as weakly-bound metals are solubilized. After sulfide volatilization inside the analytical system (AVS), the quantification of metals is made, therefore, they are called Simultaneously Extracted Metal (SEM). Metal sulfide reacts with HCl and releases H_2S, which is carried by molecular nitrogen (N_2) and trapped in a NaOH solution contained in another compartment. An amount of ~3.0000 g bulk sediment will be weighted in a 5.0 x 5.0 cm peace Parafilm. Both Parafilm and sediment sample will be put inside de balloon flask, added 100.0 mL oxygen free Mili-Q water. Each tube flask will be filled with 80.0 mL 0.5 M NaOH. The system will be switched on and the carrier gas will remove oxygen inside. After 15 minutes, 20.0 mL oxygen free 6.0 M HCl will be inject in the system. During 30 minutes the acid will react with the sediment and emergent H_2S will be trapped in NaOH solution. The sulfide trapped will be quantified by colorimetric process (reaction with N,N-dimetil-p-fenilenedinamina at the presence of $FeCl_3$) (Fonselius 1976; Herbert E. Allen et al. 1993)

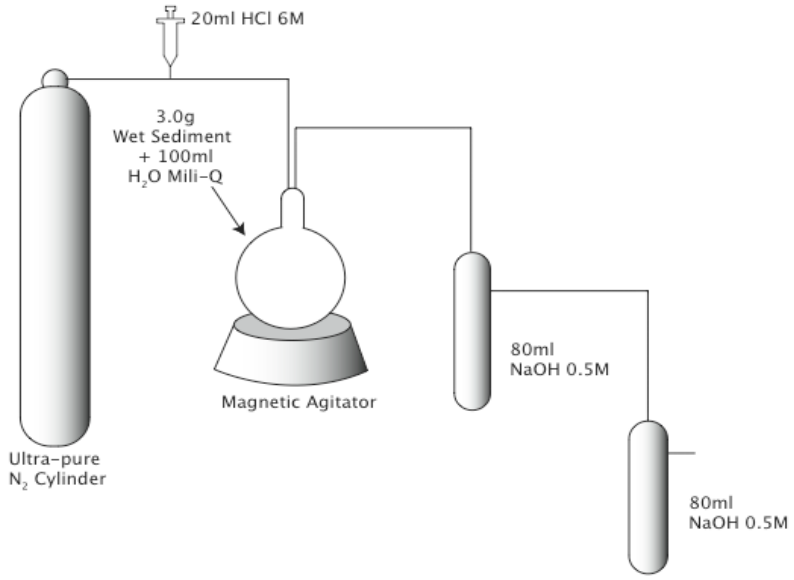


Figure 3. Schematic AVS analytical system.

The solution left in the balloon flask was then filtered and the liquid analyzed using an ICP-AES equipment in order to quantify Cu, Cd, Ni, Pb and Zn. Some of these ions form polysulfide (Bevilacqua 1996), what justified their quantification, even though they are not soluble in HCl.

A sediment aliquot was weighted, dried at 60 °C for 36 hours and weighted again, so moisture content could be measured. Therefore, results were expressed in mg kg^{-1} dry weight.

3.1.1 Organic Carbon Calculation

Organic carbon was indirectly determinated by weight loss through ignition followed by the transformation as the equation:

$$OM = OC\, x\, 1.7$$

where OM is Organic Matter in %, and

OC is Organic Carbon in %

This was based on the assumption that 53% of total organic matter is related to carbon (Meguro 2000). OC expressed in percentage was converted to mg C/kg sediment.

3.2 Water Column

We elected station 1 (coincident to sediment sampling station) to collect water, due to its proximity to water intake for public supply. We sampled water from 3 different depths: bottom water, medium depth water (at ~1.5 m) and surface water. Medium depth water was sampled by means of a Van Dorn bottle. Surface water was sampled by direct diving sampling bottle into the water. Bottom water was sampled by siphoning out from acrylic tube of sediment sample device.

Besides water sampling, we measured temperature in the water column (YSI 63/100 FT), at the beginning sampling procedure.

Water sample storing PVC flaks were previously cleaned (washed with 10% HCl, rinsed with distilled water, 24 h bath in 10% HNO_3, rinsed with distilled then Mili-Q water). Samples were transported on ice, acidified with HNO_3 until pH 2.0 and stored at 4 ^oC.

3.3 Extraction

The extraction was done in Soxhlet apparatus (Figure 4), which was developed in 1879 by the German chemist Franz von Soxhlet. This methodology is broadly used for extraction of organic substances.

Freeze-dried sediment samples were sieved by means of stainless steel sieves. For Rio Grande, a 2.0 mm mesh sieve was used and for Cubatão de Cima 1.25 mm. This difference was necessary because the latter contained more organic matter in large pieces. 10.0 g sediment aliquot was placed inside cellulose thimbles (Whatman, Schleicher & Schuell, Dassel, Germany), stoped with glass wool and extracted with acetone. Soxhlet system was left operating for 14 hours (approximately 8 cycles per hour). Extracts were reduced in volume in a rotatory evaporator (Heidolph, Laborata 4011, Kehlheim, Germany; 400 mbar, 36-38 oC) (Figure 5), then concentrated close to dryness under pure nitrogen stream. The solvent was then changed to dimethylsulfoxide (DMSO) and final concentration was 10.0 g dry sediment/mL DMSO. Table 6 summarizes methodology used for the extraction.

Extracts were then stored in 4 mL ambar bottles with teflon stoppers (Butylred/PTFE grey, VWR International) at -20 ^oC.

We also ran a procedure control for the extract process. It consisted of pure acetone that ran for the same period of time that the actual samples did. The aim of this control was to assure the

quality of the process (glass ware cleanness, no contamination from solvent of thimbles, etc). The procedure control was on tested for cytotoxicity, in order to validate the extraction step.

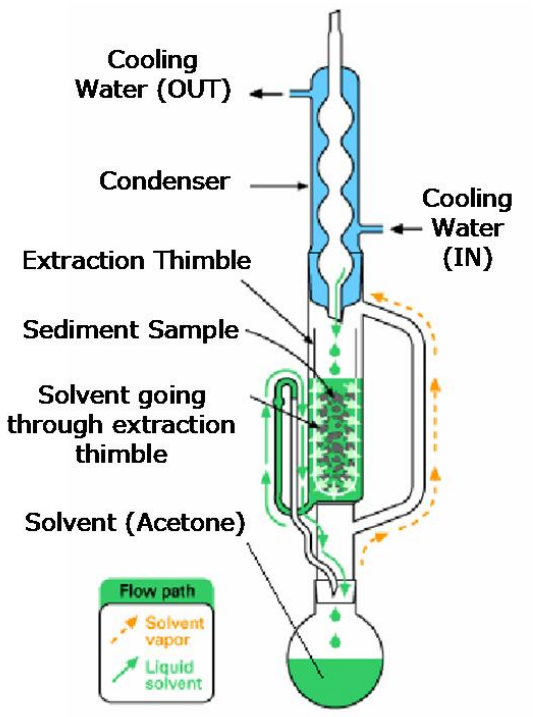


Figure 4.Schematic Soxhlet apparatus (Source: Argonne National Laboratory, adapted from Kosmehl (2003).

A)

B)

Figure 5. Extraction process: A) extracts from Rio Grande (left) and Cubatão de Cima (right) after cycles in Soxhlet Apparatus; B) rotatory evaporatior with extract ready for blowing with nitrogen (volume reduced to size of 1€ coin).

Table 6 Specifications of material and aspects of the methodology used for extractions.

Material / Aspect	*Specifications*
Eluent substance	Acetone
Weight of sediment used	10 g
Volume of eluent substance (acetone)	350 mL
Pressure of rotator evaporator	400 mbar
Temperature of water bad	38-40 °C
Temperature of evaporation	18-22 °C
Final solvent	DMSO
Final concentration	10 g/mL DMSO
Cellulose Thimble	Whatman, Schleicher & Schuell, Dassen, Germany; 47 x 123 mm
Time of extraction	14 hours

3.4 Battery of Bioassays

In vitro cell bioassays have the advantage of providing a rapid, sensitive and are relatively low-cost; they are adequate tool for estimating total biological activity of all compounds that act though the same mode of action present in a environmental sample. They also integrate possible interactions among chemicals and have increased ecotoxicological relevance, for they represent an integrated biological response. However, some disadvantages and limitations of *in vitro* bioassays are: (i) they are specific bioanalytical tools (i.e. do not account for pharmocokinetics, tissue distribution and many biotransformation that occur in vivo), (ii) they account for the metabolic activities and by-substances present inside the studied cell line, so substances toxified by bioactivation may be overlooked by *in vitro* systems; and (iii) non-linear dilution curves, reproducibility, etc have to be addressed (Hollert et al. 2005).

3.4.1 RTL-W1 cell and cell culture

RTL-W1 (Rainbow Trout Line – Waterloo) cell line are permanent cells isolated from liver of rainbow trout (*Oncorhynchus mykiss*) in Waterloo, Canada (L. E. J. Lee et al. 1993). The cells were kept in cell 72 cm^2 culture flasks (Nunclon™ Surface, 50 mL) without additional gassing, in 15 mL sterile medium (Leibovitz's L-15 Medium, Sigma), supplemented with 8% fetal bovine serum (Sigma-Aldrich Chemicals, Deisenhofen, Germany) and 1% penicillin/ streptomycine (Sigma-Aldrich) at 20 °C. The cells suffered passage whenever they became confluent (ca. 6.5-12.0x104 cell/cm2), which happened every 7 days (Klee et al. 2004). Before tests, cells were washed with

PBS (Dulbecco's Phosphate Buffered Saline, Sigma). Figure 6 present a photo of RTL-W1 cells (A) and culture flasks used for cell culture (B).

RTL-W1 cell line is known to keep active their metabolic pathways for biomodification of chemical, i.e. have a high biotransformation capacity when exposed to cytochrome P4501A (CYP1A)-inducing compounds such as PAHs, PCDD/Fs and ß-naphthoflavone. Therefore, biotest with RTL-W1 cell can be undergone without addition of S4 (Behrens et al. 2001; R. F. Lee & Steinert 2003; Kosmehl et al. 2004).

Bioassays carried out with cells RTL-W1, their positive and negative controls and their endpoints are presented in Table 4.

A) B)

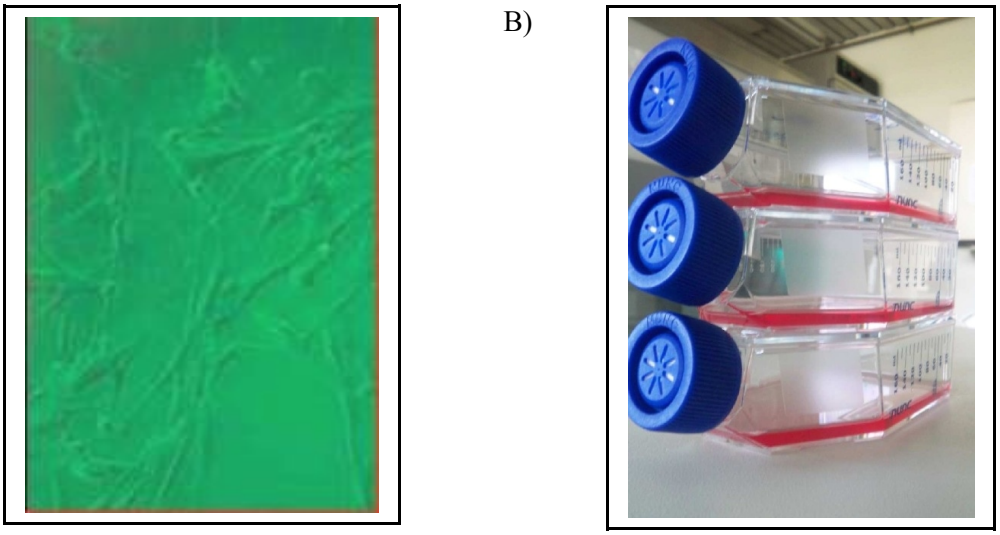


Figure 6. A) Photo of RTL-W1 cells under inversed chamber light microscope; B) Photo of culture flasks containing RTL-W1 cells in L-15 Medium.

Table 7. Biossays carried out with RTL-W1 cell line, the positive and negative control used to validate the test and the Endpoint used to produce the measurements.

Assay	*Positive control*	*Negative control*	*Endpoint*
Cytotoxicity assay	3,5 Dichlorophenol (DCP) 10%	L-15 medium	Lysosome membrane integrity – Neutral Red
Comet assay	4-5 min of UV light	L-15 medium	Oliver's Tail Moment
Micronucleus assay	4-Nitroquinolin-N-oxid (NQO) 190.0 µg L^{-1}	L-15 medium	Frequency of Micronucleus in 2000 cells
EROD	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) 100 pM	L-15 medium	Cytochrome P450 activity

3.4.2 Cytotoxicity Assay – Neutral Red

This test was used for 3 purposes: (a) to estimate the cytotoxicity of the samples; (b) as base to calculate the greatest concentration of extract solution for the other biotests; and (c) to validate the extraction step by testing if the procedure control offered toxicity to the cells.

Acute cytotoxicity proceeded through neutral red assay (2-methyl-3-amino-7-dimethyl-aminophenazine) discriminates viable and dead cells on the basis of membrane integrity or permeability. The dye agent suffers passive diffusion across cell membrane and accumulates within lysosomes. Viable cells accumulate and retain dye in their lysosome, whilst dead cells neither accumulate nor retain the dye. Inside a plate well with a certain cell population, staining intensity is directly proportional to cell viability (Segner & Braunbeck 1992).

Endpoint used was Neutral Red (NR) retention, according to procedure modified by Hollert et al. (2000). In details, Neutral Red solution was added to the cells after 24-hour exposition to sediment extract, and was taken up into the lysosoms of intact cells. NR solution was left for 3 hours in contact with the cells, and then washed away with PBS. The following step was the extraction of NR solution from inside the cells with NR-extraction solution (490 mL ethanol 99%, 10 mL acetic acid, 500 mL bidestiled water) and measurement in spectrophotometer (Genios, Tecan; measurement wavelength: 540 nm; reference wavelength: 690 nm), with help of Magellan 6.0 (Tecan, Austria) software (Figure 7).

Extracts from Rio Grande, Cubatão de Cima and Procedure Control (10.0 mg dry weight sediment/mL DMSO) were put into ultra-sonic bath during 5 min prior to dilution in medium. Another 5-min ultra-sonic bath was done after the first dilution in medium, in order to homogenize the solution. Following concentrations were yield by 1:2 step dilutions.

Maximum concentration of sediment extract was 100.0 mg dry weight sediment/ mL medium (80 µL from extract in 3920 µL of medium), what comprises 1% DMSO (below DMSO non-observable effect concentration for RTL-W1 cells). Viability of exposed cells was expressed as a percentage of the negative control, and data was plotted as concentration-response curves.

A) B)

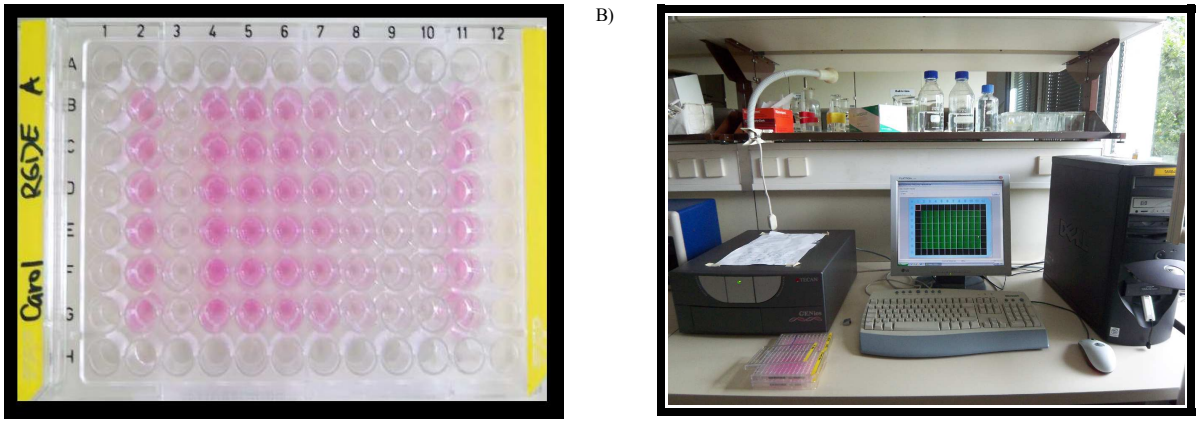


Figure 7. Cytotoxicity Test: A) 96-well microtite plate in final step of Cytotocity Test. It is possible to notice the gradual loss in color due to different treatments (columns 2 and 11 are negative controls, column 3 is positive control, columns from 4 until 10 are dilution steps); B) Spectrophotometer for measurement of absorbance for cytotoxicity tests.

The test was performed three times independently, each time with replicate. For the second and third cytotoxicity test of Rio Grande sample, the highest concentration was 25.0 mg mL^{-1} (40 µL of extract in 7960 µL medium), whilst for Cubatão de Cima remained 100.0 mg mL^{-1}. The need for further dilution of this sample was noticed after a previous cytotoxicity test, and was done in order to establish more accurately NR_{80}.

3.4.3 Alkaline Comet assay

Comet assay is also called single cell gel electrophoresis assay or microgeleletrophoresis assay. It measures DNA strand breakage in single cells, caused by the exposition to genotoxic substances.

RTL-W1 cells were washed with PBS solution and detached from one another using 0.05% trypsin/ 0.02% EDTA solution (Sigma), transferred to 6-well plates, and incubated for 24 hours at 20 ºC. Dissolved extract was added and exposition took place at 20 ºC for 48 hours. Cells were then centrifugated at 150 *g* m^{-1}, at 4 ºC, for 10 min, and then ressuspended in L-15 medium. Maximum concentration of extracts to which the cells were submitted was calculated based 20% survival of the Cytotoxicity assay (NR_{80}). This step was necessary, because the extract should not be in lethal concentration to cells, otherwise cells would die before DNA breakage could be produced. Following concentrations were achieved by 1:2 step dilutions. Table 7 shows the different concentrations to which RTL cells were exposed.

Table 8. Concentration used for the performance of Comet Assay for Rio Grande and for Cubatão de Cima samples.

	Unity	*Highest concentration*	*1st step dilution*	*2nd step dilution*	*3rd step dilution*
Rio Grande	Sediment equivalent	3.5 mg mL^{-1}	1.75 mg mL^{-1}	0.875 mg mL^{-1}	0.4375 mg mL^{-1}
	Extract volume	3.5 µL in 10 mL of medium			
Cubatão de Cima	Sediment equivalent	64.0 mg mL^{-1}	32.0 mg mL^{-1}	16.0 mg mL^{-1}	8.0 mg mL^{-1}
	Extract volume	64.0 µL in 10 mL of medium			

Comet assay was done under alkaline conditions, according to Singh et al. (1988) modified by Schunrstein and Braunbeck (2001). Frosted slides (Langenbrink, Emmendingen, Germany) were washed with 99% (v/v) ethanol, and then coated with 1.0% (w/v) normal melting agarose (NMA – Campex) in PBS. This layer was scraped off and a permanent 0.5% (w/v) NMA in PBS layer was placed, thus assuring better adherence of the agarose to the slide.

Exposed single cell suspension was mixed with 90µL of 0.7% low melting agarose (LMA) and placed over the pre-coated slides; another 0.7% LMA layer was applied. Slides were cooled on ice for 3 min and at 37 °C for 5 min and placed in cell lysis solution (100 mM EDTA, 2.5 M NaCl, 1% Triton X-100, 10% DMSO; pH 13.0) for 2 hours, in the dark, at 4 °C. After incubation, slides where placed in electrophoresis chamber with buffer solution (12.0% (w/v) NaOH, 0.37% (w/v) EDTA in bidestiled water) and left rest for 20 min. Electrophoresis was carried out under 25 V and 310 mV for 20 min. Samples were then neutralized by incubation in 400 mM Tris (pH 7.4) for 2 min and stored in a humid box, at 4 °C, protected from light. Immediately before DNA scoring, 75 µL of 20 mM ethidium bromide solution was added and slide was covered with cover slip.

Image processing was done at 340 x magnification, in fluorescence microscope (Aristoplan, Leica, Germany) equipped with 518 nm filter and image analysis system (Optilas, Munich, Germany) with grey-scale CCD camera (JAI Pulnix TM-765E Kinetic, Glostrup, Denmark). Tail moment (i.e. fluorescence intensity in the tail and the tail length in relation to "comet head") was measured by means of Komet 3.0 software (Kinetic Images, Liverpool, UK). For each concentration, 100 randomly selected cells were scored.

A) B)

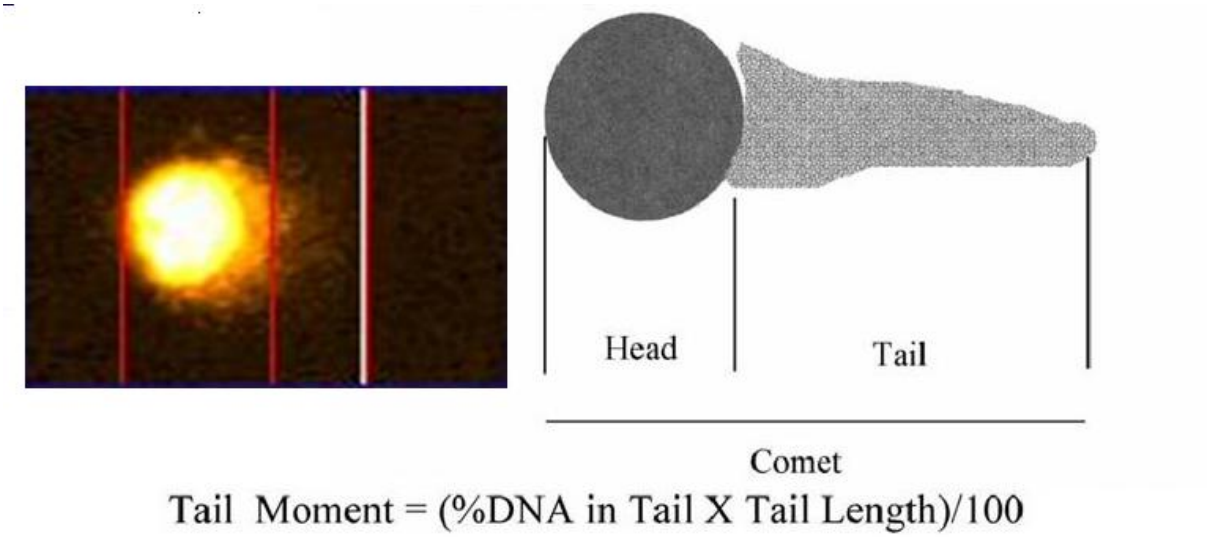


Figure 8. Single cell comet assay. A) Photo taken under fluorescence microscope after addition of Ethidium bromidae; B) Schematic single cell, showing the parts of the comet and the concept under Tail Moment. Adapted from Lee and Steinert (2003).

For Positive Control, cells were left under UV light (320 nm) for either 4 or 5 min, using a TM-36 Transilluminator (4 x 15 watts; Herolab, Wiesloch, Germany). Negative Control was yield with L-15 medium.

3.4.4 Micronucleus assay

Micronucleus assay enables the identification the presence of substances, which causes genotoxicity by inducing Micronucleus formation. The formation of Micronucleus occurs when a chromatin fragment without centromer do not move entirely to the nucleus locus of the daughter cell, but remains in the cytoplasm, where it forms a smaller nucleus (Figure 9). As consequence, errors in cell functions may occur, possibly leading to cell death or to neoplasmatic tissue formation. Therefore, Micronucleus assay can detect non-reparable damages in DNA, such as clastogenic and aneugenic lesions, while Comet assay, for example, can detect recent and reparable lesions, such as breaks and alkali-labile sites. The micronuclei are recognizable under light microcopy using DNA staining solutions, such as Giemsa or Acridine Orange (the latter under fluorescence light).

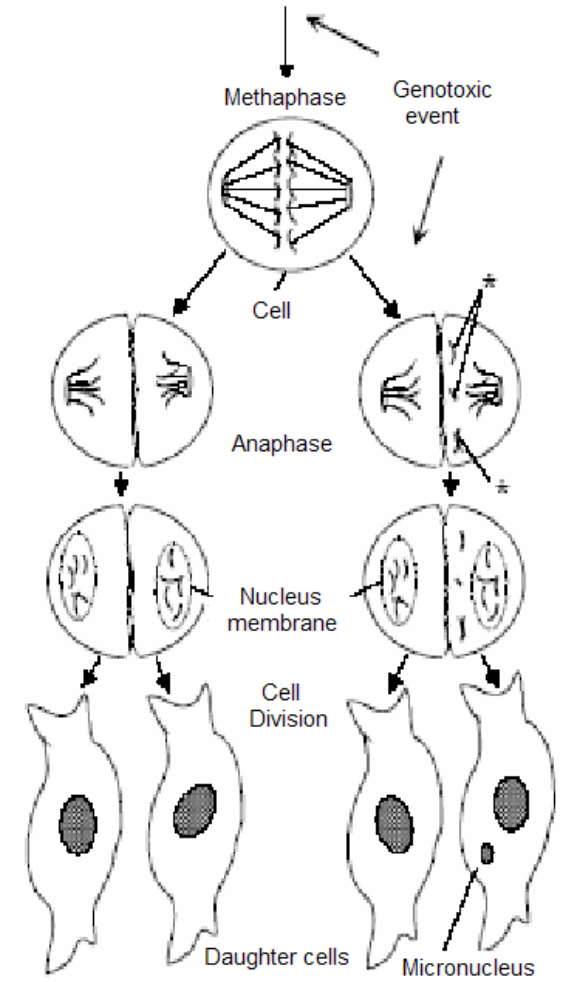


Figure 9. Schematic formation of micronucleus during cell division. * = chromosome fragments without spindle apparatus. Source: Al-Sabti and Matcalfe (1995)

2-mL Aliquots of RTL-W1 cell suspension were transferred to 6-well plates were previously clean glass slips were placed (22 x 22 cm; cleaned with ethanol 99%, left 15 min under UV-light and flamed). Cells were incubated for 12 hours, at 20 ºC, then exposed to acetonic sediment extract diluted in L-15 medium for 48 hours, at 20 oC. The exposition was stopped by replacing the sediment extract with L-15 medium. The cells were again incubated for 72 hours, at 20 oC, in order to undergo cell division, hence producing micronuclei. Cells were fixed with 4:1 (v/v) methanol:acetic acid solution and left to dry. Prior to observation in oil-immersion lens of fluorescence microscopy (Leitz, Germany), 20 µL Acridine Orange (0,004%; 4 mg in 100 mL PBS) was added and micronuclei were identified according to the following criteria: a) color and texture similar to main nucle-

us; b) diameter not more than 1/3 of the main nucleus; c) absence of contact with main nucleus; d) same bidimensional plan as the main nucleus (Figure 10).

Micronuclei were expressed as frequency in 2000 cells and the effect was compared to the correspondent frequency caused by of the positive control (190.0 µmol NQO – 4 Nitroquinoline 1-oxyde – see Figure 11 for molecular structure).

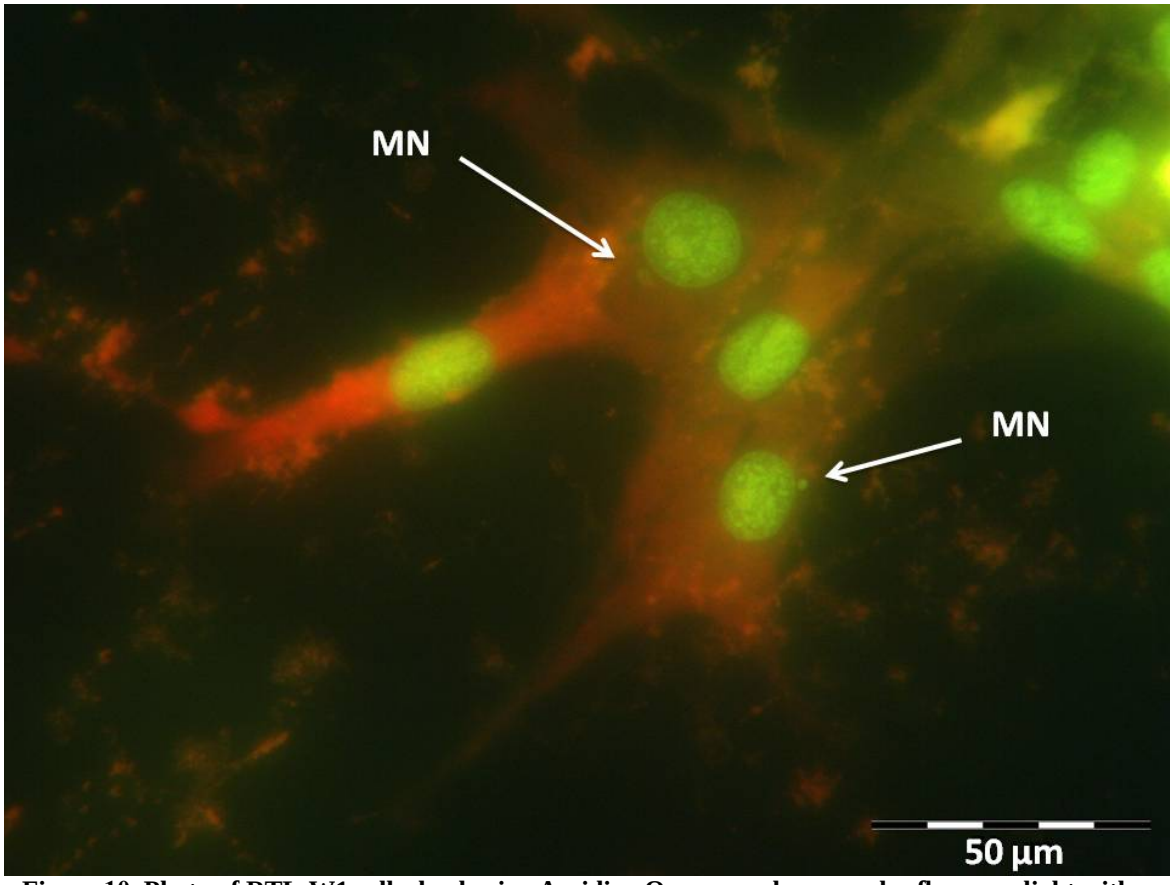


Figure 10. Photo of RTL-W1 cells dyed using Acridine Orange and seen under fluoresce light with a 100 x objective. Cytoplasm is seen in orange and nucleic material in green. Two Micronuclei are to be seen (MN).

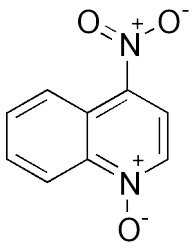


Figure 11. Molecular Structure of NQO (4-Nitroquinoline 1-oxyde).

3.4.5 Ethoxyresorufin-*O*-deethylase (EROD) activity

The aim of this test is to identify dioxin-like activity in acetonic extracts by indirect measure of Cytochrome P450 1A1 (CYP1A1) activity through 7-ethoxyresorufin-O-deethylase (EROD).

CYP1A1 is a member of a multigene family of xenobiotic metabolizing enzymes that oxydize, hydrolyze, or reduce compounds through the insertion of an atom of atmospheric oxygen to the substrate during the reaction cycle. Although the aim is detoxification, CYP1A1 generates active oxygen and mutagenic metabolites (Nebert 1987; Nebert et al. 2000), therefore causing alteration in cell homeostasis. In fish, it is associated with apoptosis and embryo mortality (Cantrell et al. 1996), while in mammals, theses effects include wasting syndrome, tumor promotion and thymic atrophy (Poland & Knutson 1982).

The useful aspect of CYP1A1 for biomonitoring purposes is the positive relation between its concentration and chemical exposure. It means that, the great the enzyme activity, the great the sample toxic potential. Figure 12 shows schematic AhR induction inside the cell.

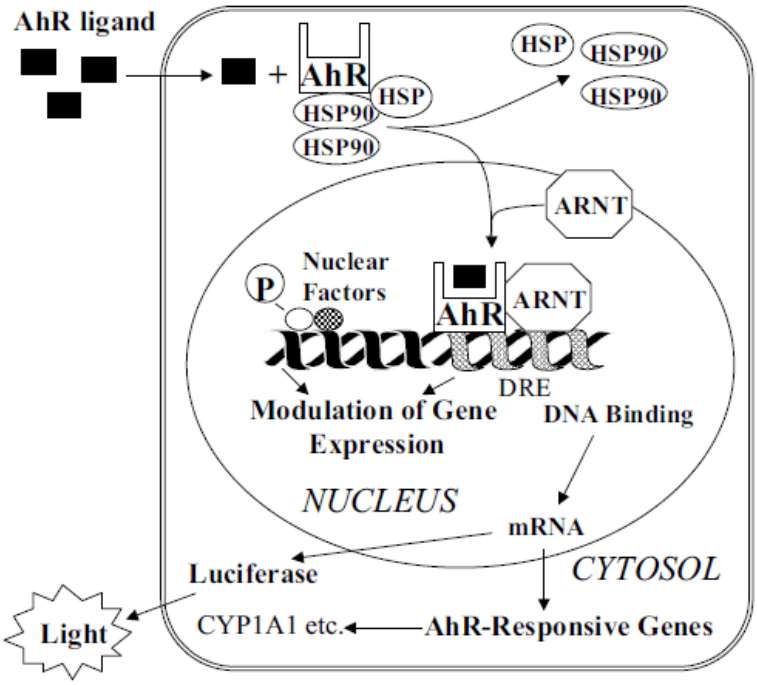
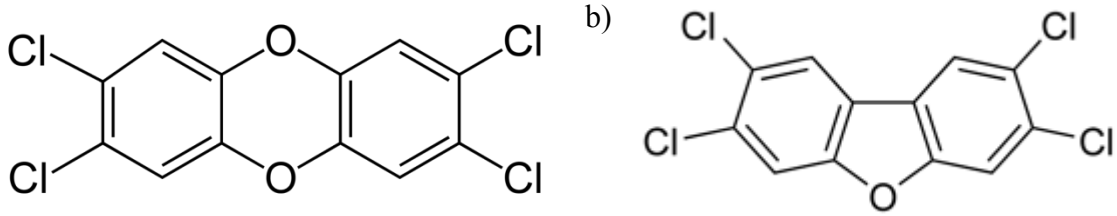


Figure 12. Schematic AhR induction mechanism in cell bioassay. ER = encoplasmic reticulum, CYP1A1 = cytochrome P450 1A1; AhR = aryl hydrocarbon receptor, Hsp = heat shock protein; Arnt = Ah receptor nuclear translocator, mRNA = messenger ribonucleic acid. Source: Hilscherova et al (2000).

Dioxin and furans are among the most toxic substance known so far. They are a group or a class of substances with similar molecular structure, and a range of toxicity, being 2,3,7,8, Tetrachlorodibenzo-p-dioxin (TCDD) the most toxic of them.

Figure 13 shows the molecular structure of one substance from dioxin and one from furans. They are lipophilic, highly stable and formed mainly through industrial process: burn of fossil fuel and waste emission, but biomass combustion also produces dioxin .

a) b)



2,3,7,8, Tetrachlorodibenzo-p-dioxin 2,3,7,8 Tetrachlorodibenzofuran

Figure 13. Molecular structure of a) dioxin; and b) furan.

Usual chemical associated to dioxin-like activity are halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs); both group of compounds are known to act as aryl hydrocarbon receptor (AhR) agonists (Giesy et al. 2002; Seiler et al. 2006; Gustavsson et al. 2004). After the biding of such substances to AhR complex, a series of biochemical reactions are activated, leading to the translocation of AhR into the nucleus, where it dimerises with a second protein and binds to specific DNA recognition sites (dioxin responsive elements – DREs). DREs are present in the upstream region of the cytochrome P4501A and other AhR-regulated genes.

3.4.6 Ames Fluctuation Test

Ames Test is a mutagenicity test that uses genetically modified lines of *Salmonella typhimurium*. Those mutations cause a failure in surviving in a histidine free medium. In presence of a mutagenic compound, the mutation is reversed and the bacteria can then survive. Ames Fluctuation test is an adaptation of the classic Ames test and is less time and material consuming (Reifferscheid et al. 2005). It uses pH sensitive medium as indicator of bacteria survival, meaning reverse mutation occurrence, instead of unities of colonies for survivor bacterial individuals (Maron & Ames 1983; Kosmehl et al. 2004).

The principle of the assay is that the test bacteria and test chemical are mixed in media containing a trace amount of the required histidine and dispensed in around 384- well plates. If a mutation arises during the residual growth in any well, the mutant clone develops and eventually produces enough acid from sugar utilization to turn an acid base indicator yellow (Bridges 1980). When indicator medium changes from purple to yellow, it indicates that pH has changed, hence presence of reverse mutant bacteria.

Two bacteria strains were tested: TA98 (frameshift mutation) and TA100 (base pair substitution), both with and without S9 fraction addition (rat liver homogenate S9-fraction from phenobarbital/ß-naphthoflavon-treated mice, RCC Rossdorf, Germany), aiming the evaluation of metabolic activation. Liver S9 fractions are subcellular fractions that contain drug-metabolizing enzymes including the cytochromes P450, flavin monooxygenases, and UDP glucuronyl transferases. Liver S9 fractions are a major tool for studying xenobiotic metabolism; biotransformation of chemicals may either activate or inhibit toxic response. Bisphenol is an example of substance, whose toxicity is enhanced or initiated with metabolic activation (Yoshihara et al. 2001). Example of partial or total inactivation by metabolism are MX (for 3-chloro-4-dichloromethyl-5-hydroxy-2(5H)- furanone), which is formed by the reaction of chlorine with complex organic matter in drinking-water) (WHO 2004).

Base pair substitution mutagens are agents that cause a base change in DNA. In a reversion test this change may occur at the site of the original mutation, or at a second site in the bacterial genome. Frameshift mutagens are agents that cause the addition or deletion of one or more base pairs in the DNA, thus changing the reading frame in the RNA.

Bacteria were suspended in medium and let grow overnight in a shaking bath at 37 °C. Density was measured by spectrophotometer and adjusted to 1,800 FAU (Formazine Attenuation Units) for TA98 and 450 FAU for TA100.Test was done in 48 wells per replicate (positive and negative controls plus sample dilutions), and 48-hour incubation time, at 37 °C. Positive controls were 4-nitro-*o*-phenylenediamine (20 nM per well) for TA 98 strain without S9, nitrofurantoin (1.67 nM per well) for TA 100 without S9 and 2-aminoanthracene for TA 98 and TA 100 with S9 treatment (0.87 nM per well). DMSO was used as negative control. Tests were valid when mean values of spontaneous revertants in negative controls (counted in well basis) were from 0 to 5 per 48 wells (TA 98) and from 0 to 10 per 48 wells (TA 100) at all testing conditions with both strains, with and without S9. Positive controls were valid when of revertants were equal or greater than 25 per 48 wells as mean values for both bacterial strains with and without S9 addition (Wölz et al. 2009).

Figure 14 shows a schematic representations of the steps in Ames Fluctuation test procedure, and Figure 15 shows a photo of a364-well microtiter plate at the end of the procedure, with the 3 test replicates.

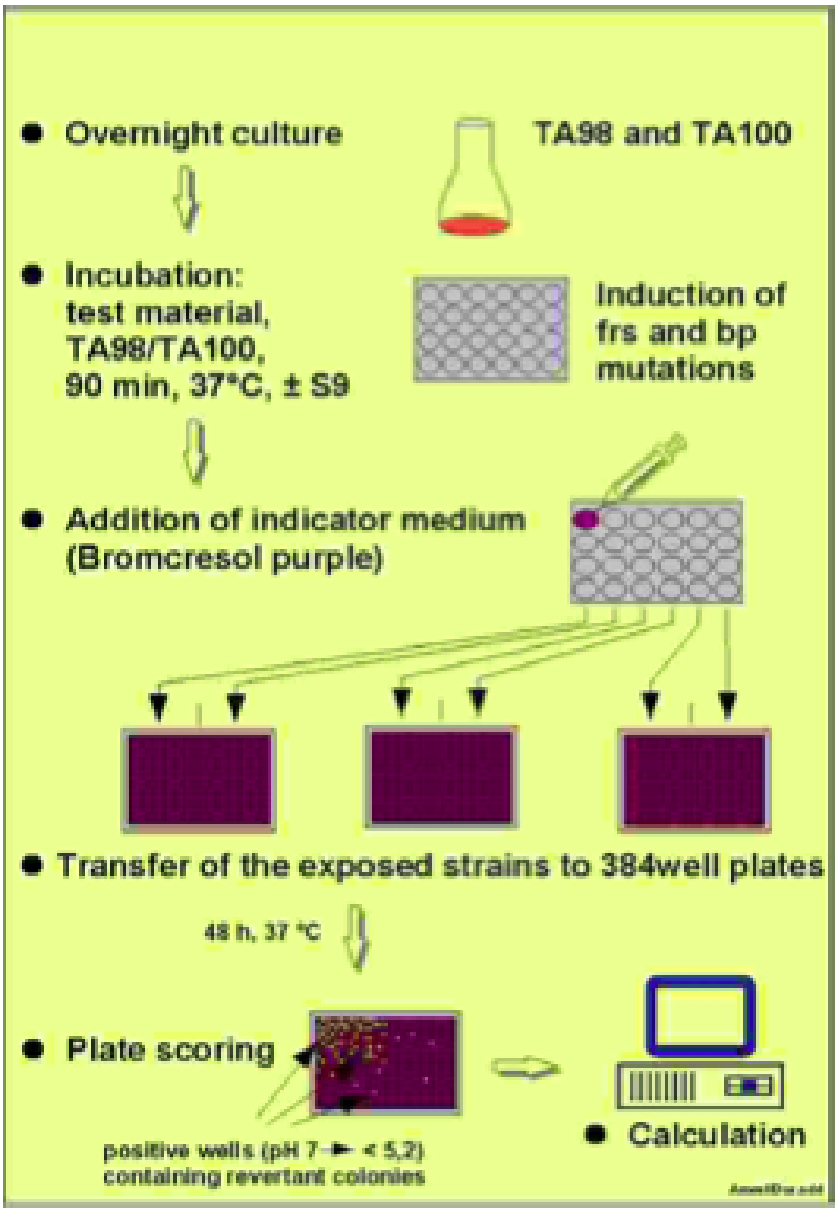


Figure 14. Schematic methodology for Ames Fluctuation Test. Source: www.uni-mainz.de

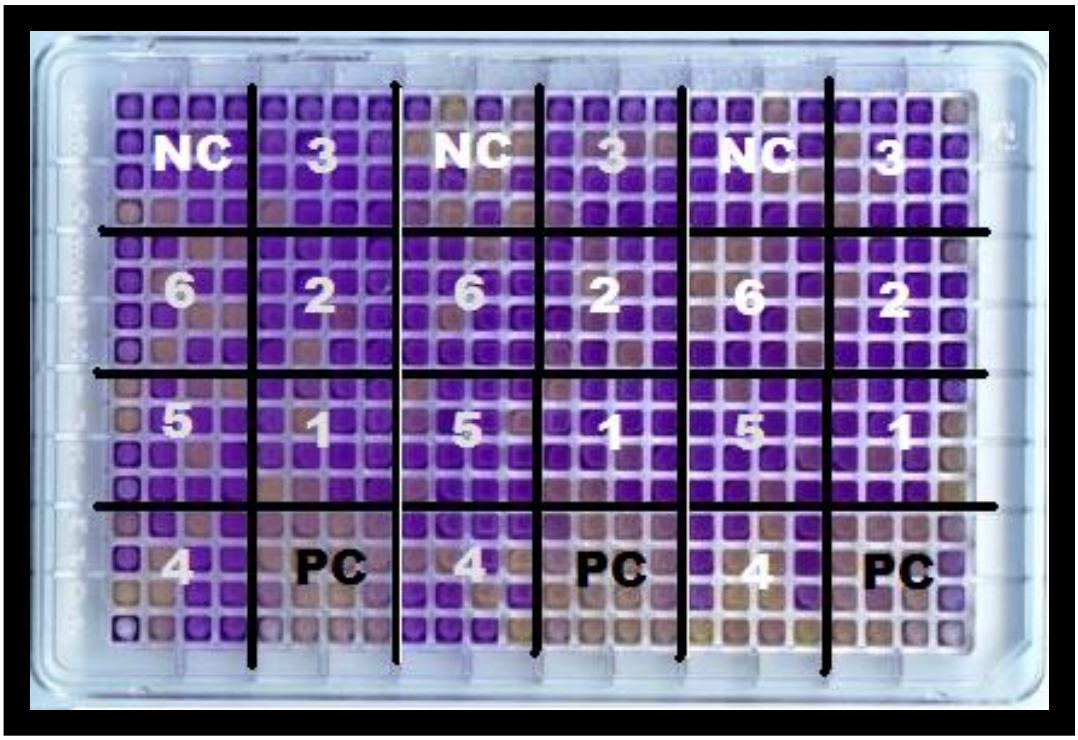


Figure 15. 384-well plate after incubation of exposed bacteria to tested extract for Ames fluctuation test. Triplicates of the test is performed in the same plate. NC = Negative Control; PC = Positive Control; 1 to 6 = tested extract concentration (1 = highest concentration; 6 = lowest concentration). Purple wells contain no reverse mutant bacteria; yellow wells contain reverse mutant bacteria.

3.4.7 Sediment Contact test using *Danio rerio* embryos

Adult zebrafish (*Danio rerio*, Cypriniforms, Cyprinidae) is 3 to 5 cm large and its origin is Asiatic. Among the advantages in using its eggs for toxicity assays are: (a) rapid development (reach adulthood within 3 months); (b) adults do not require much care and reproduce quickly, since they are "r" strategists; (c) eggs can be easily obtained, and in great number, throughout the whole year (one female can lay about 100 eggs per day, which are fertilized by male sperm released into the water); (c) eggs and recently hatched larvae are transparent, allowing easy observation by optic microscope; (e) vast information is available including field studies, development, molecular, genetics, neurobiology and vertebrate biology, what make it a useful model also for molecular level studies (biomarkers, genomics, proteomics, etc) (Nagel 2002; Scholz et al. 2008).

The body plan is laid out after 24 hours pos-fertilization, and embryos hatch after 2-3 days after fertilization. At day 5 pos-fertilization, yolk is completely consumed and external feeding starts.

Furthermore, fish egg tests can replace adult fish tests, what brings advantages regarding animal protection agencies (Nagel 2002; Braunbeck et al. 2005).

3.4.7.1 Fish maintenance and egg production

Sexually mature fish (3-4 years old) were kept in communities of up 150 individuals, in 30-L aquarium under constant water flow. Specifications were with 12/12 dark/light regime, 27.0 ± 0.5 ^{o}C of temperature, 744 µS of conductivity, 379 mg L^{-1} $CaCO_3$ (21.3^{o} d) of hardness, pH 7.5 ± 0.25, 10.5 ± 0.5 mg L^{-1} of dissolved oxygen (95% saturation) (Figure 16A). Ammonia, nitrite and nitrate were kept under detection limits (respectively 0-5, 0.025 and 0-0.140 mg L^{-1}). Diet consisted of commercially available artificial food (TetraMin™ flakes, Tetra, Melle, Germany) twice a day, with occasional supplements of *Artemia nauplii*.

On the previous day of the test, four males and two female fish were placed to lay eggs in breeding chambers, containing water at the same conditions and stimulation subtract, immediately before the dark period (Figure 16 B). Spawning and fertilization happened 30 min after the light onset. The eggs were taken in the morning, and separated, with 1-2 hours old.

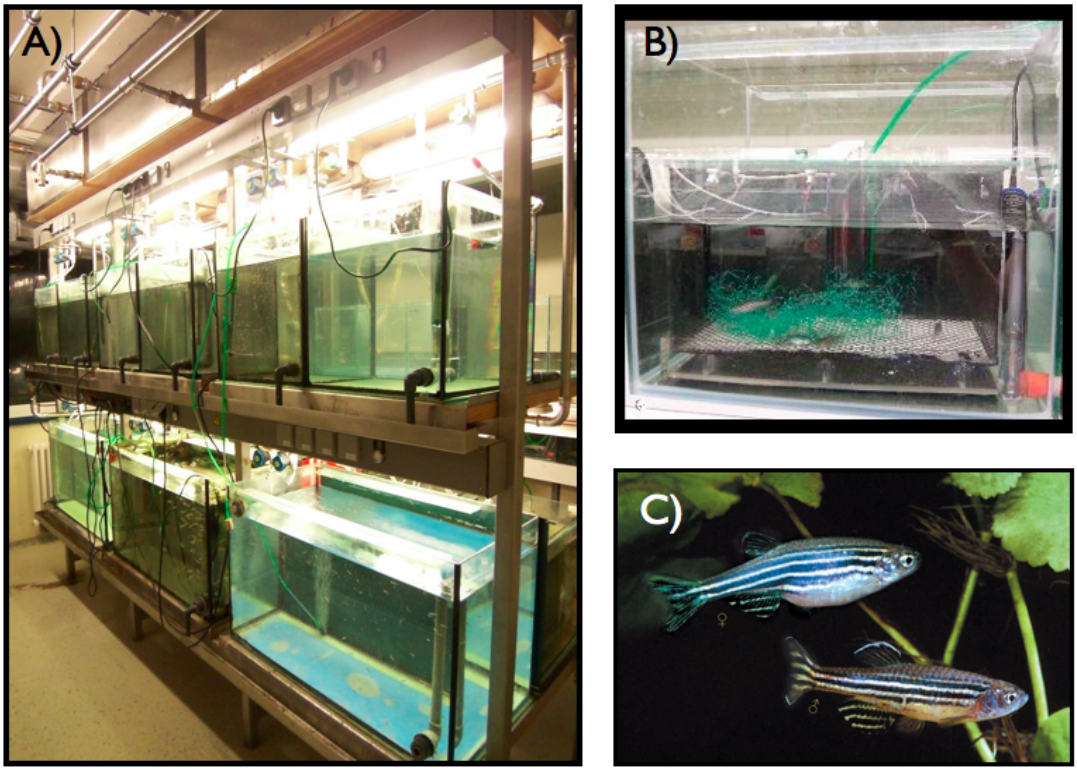


Figure 16. Fish maintainance system. A) Tanks were adult fish are kept under constant water flow and controlled conditions. B) Chamber where adult fish are placed for spawning and fertilization of eggs; C) Zebrafish (Danio rerio): "pregnant" female (above) and male (down).

3.4.7.2 The test

On the previous day of the test, four males and two female fish were placed to lay eggs in breeding chambers, containing water at the same conditions and stimulation subtract, immediately before the dark period (Figure 16B). Spawning and fertilization happened 30 min after the light onset. The eggs were taken in the morning, and separated, with 1-2 hours old.

The test was done according to Hollert et al. (2003). Fertilized eggs were separated from non-fertilized eggs, using a 2 mL plastic pipette with widened opening. Five fertilized eggs were then transferred to each well of 6-well microtiter plattes (TPP Renner, Darmstadt, Germany) containing a mixture of freeze-dried sediment and quartz sand (see Table 8 for proportions) plus 5 mL of artificial water (Hollert et al. 2003; Seitz 2005). Sediment und quartz sand (Quarzwerke, grain size F36, Frenche, Germany) were prior homogenized in ceramic mortar, in order to avoid hotspots inside the well. Artificial water (stock solutions: 58.8 mg L^{-1} $CaCl_2$ x $2H_2O$, 24.6 mg L^{-1} $MgSO_4$ x $7H_2O$, 12.6 mg L^{-1} $NaHCO_3$, 5.5 mg L^{-1} KCl; stock solutions were diluted 1:5 with bidestiled water) was bubbled with air overnight, and pH was adjusted to 7.8 ± 0.2 prior to the test (Kosmehl et al. 2006). The plates were covered with adhesive film to avoid loss of volatile components. To each treatment, 4 replicates were made (i.e. 4 wells, 20 eggs per concentration) and 6 different concentrations. There were 2 positive and 2 negative controls (see Table 9), each with 4 replicates (5 fish eggs per well, 20 eggs in total). Positive control substance was 0.37% (w/v) 3,4 Dichloroaniline. 80% survival of fish eggs in negative control was necessary to validate the test.

Figure 17 shows a photo of a 6-well plate containing different sediment dilutions.

Table 9. Ratio of sediment to quartz sand (per well) used for the dilution of the sediment contact test with fish egg. The total weight of solid material (sediment + sand) was always 3 g, to with 5 mL of artificial water was added (SEITZ, 2005).

	1:1	*1:2*	*1:4*	*1:8*	*1:16*	*1:32*	*1:64*	*1:128*
Conc. in mg/mL H_2O	600	300	150	75	37.5	18.75	9.375	4.688
Sediment in g	3	1.5	0.75	0.375	0.188	0.094	0.047	0.023
Quartz sand	0	1.5	2.25	2.625	2.812	2.906	2.9053	2.977

Table 10. Content *of each well of Positive and Negative Controls for Fish egg test.*

Positive Control	*Negative control*
3,4 Dichloroaniline + Artificial water (5 mL)	Artificial water (5 mL)
3,4 Dichloroaniline + Artificial water (5 mL) + Quartz sand (3.0 g)	Artificial water (5mL) + Quartz sand (3.0 g)

Figure 17. 6-well Plate with mixture of sediment and quartz sand, 5 mL of artificial water and 5 fish egg per well.

The eggs were incubated at 27.0 ± 0.1 ºC in the dark. The eggs were then observed after 24 and 48 of exposition using inverted microscope (CK-40 equipped with an SC-35 camera, Olympus, Hamburg, Germany). Lethality endpoints for 24 hours were: a) lack of somite formation; b) coagulation of embryos and; c) no detachment of tail. For 48 hours, endpoints were: a) no development of eyes; b) no heart beat and; c) no blood circulation (Table 10 and Figure 18).

One pilot (28.Aug.07) and one real test (03.Sep.07) were carried out, with different concentrations for the Rio Grande (Table 11). Pilot test was done with quartz powder (Quartzwerke, grain size W4, Frechen, Germany) and the real test was done with quartz sand (Quartzwerke, grain size F36. Frechen, Germany). For the second test, Dissolved oxygen was measured (Oxy-4 4-channel fiber-optic oxygen meter, PreSens, Regensburg, Germany) in sediment/water interface inside the wells containing the concentration 1:4; 1:8 and 1:16 for Rio Grande and 1:1; 1:2 for Cubatão de Cima sediment samples before the observation of the eggs. This was done in order to assure that the mortality of eggs were not due to lack of oxygen.

Table 11. Endpoints observed in fish embryos after 24 and 48 hours of exposition to whole sediment.

24 hours	*48 hours*
Non-fertilized egg	No pigmentation
Coagulation of egg*	Coagulated egg*
Epibolismus stage	No blood circulation*
No detachment of tail*	No Heart beat*
No spontaneous movement	Edema
Lack of somite formation*	Embryo miss developed
	Embryo underdeveloped
	Embryo without eye locus

* lethality endpoint (i.e. embryos with these observed characteristics were considered dead)

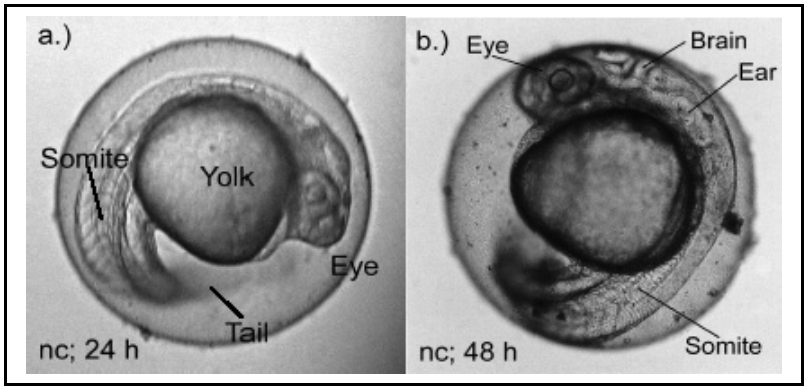


Figure 18. Normal Development of Zebrafish (*Danio rerio*) embryo, after A) 24 and; B) 48 h incubation in artificial water (as negative control), showing main observable structures. Source: Keiter et al. (2006).

Table 12. Proportion of sediment sample and quartz sand or quartz powder used for the pilot test and for following tests using Rio Grande and Cubatão de Cima samples

Sample	*1:1*	*1:2*	*1:4*	*1:8*	*1:16*	*1:32*	*1:64*	*1:128*
Rio Grande (pilot test)	X	X	X	X	X	X		
Rio Grande			X	X	X	X	X	X
Cubatão Cima (pilot test)	X	X	X	X	X	X		
Cubatão Cima	X	X	X	X	X	X		

3.4.7.3 Embryo digestion and metal measurement

After observation, 48-hour non-coagulated eggs were transferred to plates with bi-distilled water and benzocaine (Sigma, Switzerland) in order to be mechanically dechorionated by means of tweezer and needle. This step was done because chorion may act as a barrier for chemicals (Braunbeck et al. 2005). Metals presumably measured in eggs could be only present in the chorion, hence yielding false up take results (Zielke 2007).

Non-coagulated and dechorionated eggs were washed 3 times with bi-distilled water, transferred to 15 mL polypropylene conical tubes (TPP, Switzerland) and stored at -60 °C until digestion.

Digestion was carried out in cooperation with Dr. Zimmer (University hospital of Heidelberg, Department of internal medicine) and performed as suggested by Schramel (Golimowski & Golimowska 1996; Angerer & Schaller 2003). The precision of the method was improved by adding the internal standards Rhodium and Scandium to the samples (blank, calibrators, real samples).

The digestion procedure runs as follows: the organic matrix is destroyed by the OH radicals formed from H_2O_2 added to the sample under the influence of UV radiation. Samples inside the Falcon tubes were brought to a volume of 2.0 mL using bi-distilled water (B. Braun Melsungen AG, Melsungen, Germany), transferred into quartz glas vessels and 0.8 mL of nitric acid (65%, suprapure, from Merck, Darmstadt, Germany) was added. The vessels were placed in the UV-reactor to be irradiated (UV digestion device "UV 1000", Kürner Analysentechnik, Germany) (Figure 19). After 1 hour, 0.4 mL of hydrogen peroxide (30%, from Merck, Darmstadt, Germany) was added. After 3 hours in the UV apparatus, another 0.4 mL of hydrogen peroxide was added and the irradiation ran for another hour. The total duration of digestion was 5 hours.

After digestion, the samples were transferred to 10 mL volumetric flasks and the volume was filled up to 10 mL with bi-distilled water. Samples were then stored in 12 mL sterile PS-Tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) and frozen (-80 °C) until analysis by ICP-MS (Inductively Coupled Plasma – Mass Spectrometer, Perkin Elmer Sciex, ELAN 6100). For calibration of the ICP/MS, a standard solution was used (Mehrelement Sdandardlösung VI für ICP-MS, Merk). The analysis was performed in cooperation with Dr. Lothar Erdinger (University hospital of Heidelberg, Department of Hygiene).

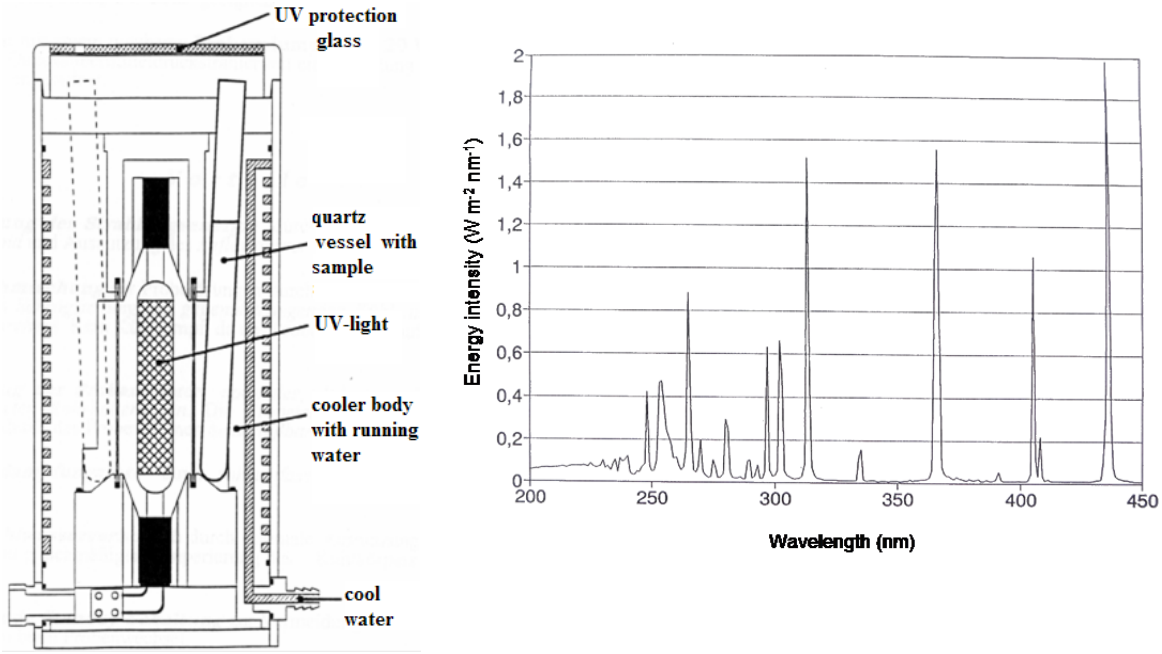


Figure 19. "UV 1000" UV digestion device for the sample preparation of fish eggs (with illustration of the components and assemblies) (left); Energy intensity (W m^{-2} nm^{-1}) against wavelength (nm) of the UV-light in the apparatus (right). Source: Kürner Analysentechnik, Germany.

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Cap.3 Short-time temporal heterogeneity of metals in sediment from a tropical polymitic reservoir and implications for metal dynamics

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Abstract

Sediments are known to act both as sink and source of metals, depending on momentary environmental conditions. In tropical polymitic reservoirs, small changes in temperature may cause water to circulate, and start a series of shot-time changes, which may give rise to heterogeneity in space and time including in bottom sediments. Passage of cold fronts, for example, have been linked to changes in limnological parameters in such environments. The aim of this study was to verify possible temporal heterogeneity in metal content of sediment from a tropical shallow polymitic reservoir (Rio Grande reservoir, Sao Paulo, Brazil) and try to relate it to measured parameters; besides, we aimed to contribute to metal risk assessment of this reservoir, since it serves as human water supply. We proceeded a 14-day sediment survey, with samples taken in alternate days at 3 sampling stations in Rio Grande reservoir; we collected surface sediment and analyzed sulfide (AVS), metals (SEM), organic carbon (OC), dissolved oxygen (DO), pH, redox-potential (E_H) and temperature. At one station we also sampled water in 3 different depths for metal analysis, and measured temperature in water profile. We compared results with 3 Sediment Quality Guidelines (SQG). We used a mixed effect model and backward elimination to test possible heterogeneity and to select dependent variables that helped model adjustment. SQG indicated enrichment in relation to background and probable adverse effect living organisms. Metal concentration in sediment showed temporal heterogeneity, while AVS showed spatial heterogeneity. Peaks of metal concentration in sediment were followed by peaks of metal in the water after 2 – 3 days, suggesting metal release from sediment into the water towards the surface, in a time dependent manner. Changes in overlying water DO coupled with water circulation and cold front occurrence. Despite that, DO was not a significant effect for better adjustment metals and AVS models. OC, followed by temperature, did

contribute to better adjustment of metal models, suggesting OC as key phase in controlling metal bioavailability. Such findings have implication on risk assessment and risk management; choice of SQG and experimental designs for sediment surveys in reservoirs similar to Rio Grande (tropical shallow lake) should be carefully done, and include spatial, temporal heterogeneities and sediment-water interdependency.

Key words: metal dynamics; SQG; Equilibrium Partitioning; tropical reservoirs; cold fronts

1 Introduction

In urban areas, where multi-use is required from the water bodies, reservoirs are subject to a great variety of contaminant sources and contaminant substance. Among those substances, metals are especially hazardous because they do not degrade and may accumulate in the tissues of living organism, leading to toxicity and biomagnifications along the food web. Furthermore, the sources of metals are variable (atmospheric deposition, run off from ore exploration, input from industrial and urban sewage) and its ultimate fate is deposition in sediment.

Sediments are known to act both as source an sink for metals depending on physical, chemical and biological conditions of the surrounding medium, and also under certain climate events such as floods that alters bioavailability (P. M. Chapman et al. 1999; Hollert et al. 2000; Luoma 1989). Some studies point out sulfide as one of key metal complexing fraction, besides organic matter, colloids and clay particles (Lombardi et al. 2005; De Schamphelaere et al. 2004; Jung-Suk Lee & Jong-Hyeon Lee 2005; W. Wang 1987; Doig & Liber 2006; F. Wang & P. M. Chapman 1999).

In the past decades, some approach were developed aiming the establishment of sediment quality guidelines (SQG) for metals (MacDonald et al. 2000; P. M. Chapman et al. 1999). The first approach used as comparison parameter was background values that yield enrichment factor in relation to natural or pre-industrial metal concentration. Such values are best to be established in regional basis, since many geological, pedological and climatological processes have influence on natural occurring metal concentration. Further approaches for SQG intended to aggregate biological relevance, metal bioavailability (instead of total metal content) and adverse effect levels for biota. The US EPA developed an approach based on chemical equilibrium between bivalent metals (Cd, Cu, Cr, Pb and Zn) and sulfide that controlled the partition of metal between sediment and pore water; this approach is known as Equilibrium Partitioning (EqP) (USEPA 2000b). The Canadian Council of Ministers of the Environment (Environmental Canada) used a statistical approach to calculate

threshold and probable effect levels, considering a large data bank of metal concentration versus various toxic endpoints (CCME 1999).

In tropical reservoirs, small changes in wind and temperature may cause water column to circulate and start a series of short-time changes such as pH and dissolved oxygen homogenization in water, phosphorus and metal remobilization from sediment (Jensen & Andersen 1992; Wetzel 2001). All those short time changes may also give rise to heterogeneity in sediment, both in space and time. Heterogeneity in space might be understood as spatial pattern that vary in a systematic way from place to place (Ripley 1981) while temporal heterogeneity refers to one point in space and many point in time (Dutilleul 1993). The scale at which heterogeneity is observed may vary, and a shift of scale may create homogeneity out of heterogeneity, and vice-versa.

For sediments, spatial heterogeneity has been recognized for macro and micro scales, in chemical and biological studies, this last reflected especially on benthic community (M. Chapman & Tolhurst 2007; Mikael Motelica-Heino et al. 2003). Sediment spatial heterogeneity and sediment temporal variation as seasonal basis have been reported (Howard & Evans 1993; van Griethuysen et al. 2005; Padial 2008; Brumbaugb et al. 1994; Mariani & Pompêo 2008).

In this context, Rio Grande reservoir fulfills the characteristics for a good case of study. It is part of the Billings Complex, the greatest body of accumulated water in the São Paulo Metropolitan Region (São Paulo State, Brazil); it is polymictic, inserted inside one of the greatest conurbations of the world, with more than 20 million inhabitants, and water is taken in to supply part of the population at the same that they receive sewage from neighboring cities (Beyruth & Pereira 2002; Maier et al. 1985; Maier et al. 1997).

Different studies carried out in Rio Grande sediment found either sulfide or metal in molar excess (IPT 2005; Leal & Oliveira 2005), or the opposite (Silvério et al. 2005; Mozeto et al. 2003; Mariani & Pompêo 2008), which raises the question of temporal heterogeneity of metal content in this particular sediment. In tropic shallow aquatic environments, short term changes can happen in a much smaller time scale, such as days and weeks (J.G. Tundisi et al. 2004; Maier et al. 1985).

The aim of this study was to verify possible short time temporal heterogeneity in metal concentration of sediment from Rio Grande Reservoir, and try to relate them to possible changes in measured physical and chemical parameters. We also aimed to evaluate the toxic potential of the sediment by comparison with some Sediment Quality Guidelines (SQG). We expect to contribute to the understanding of sediment metal dynamics in such environment and to metal ecological risk assessment.

2 Material and Methods

2.1 Field Procedures

2.1.1 Sediment Sampling

Sediment sampling was performed on the 16th, 18th, 20th, 23th and 25th October 2006. We selected 3 sampling stations close to the dam, as shown in Figure 20. This area was chosen for this temporal evaluation due to the closeness to a water take-in for water supply and because it is the deepest portion, hence the most probable for sedimentation to occur. Stations were georeferenced by means of a Garmin GPS (GPS 72, datum Sad69, central meridian W45°00, UTM).

We used an Ambühl e Bührer (1975) sampler, with and acrylic tube of 7.2 cm diameter, so we could have intact sediment samples. We took off top 3 cm sediment and stored it in zip log bags, previously rinsed with HNO_3 10 % (v/v), withdraw the air and sealed it. We kept the samples at 4 ^{o}C (Allen et al. 1993; Mudroch & Macknight 1994). At each station, sediment sampler was launched 3 times, in order to yield 3 true replicates.

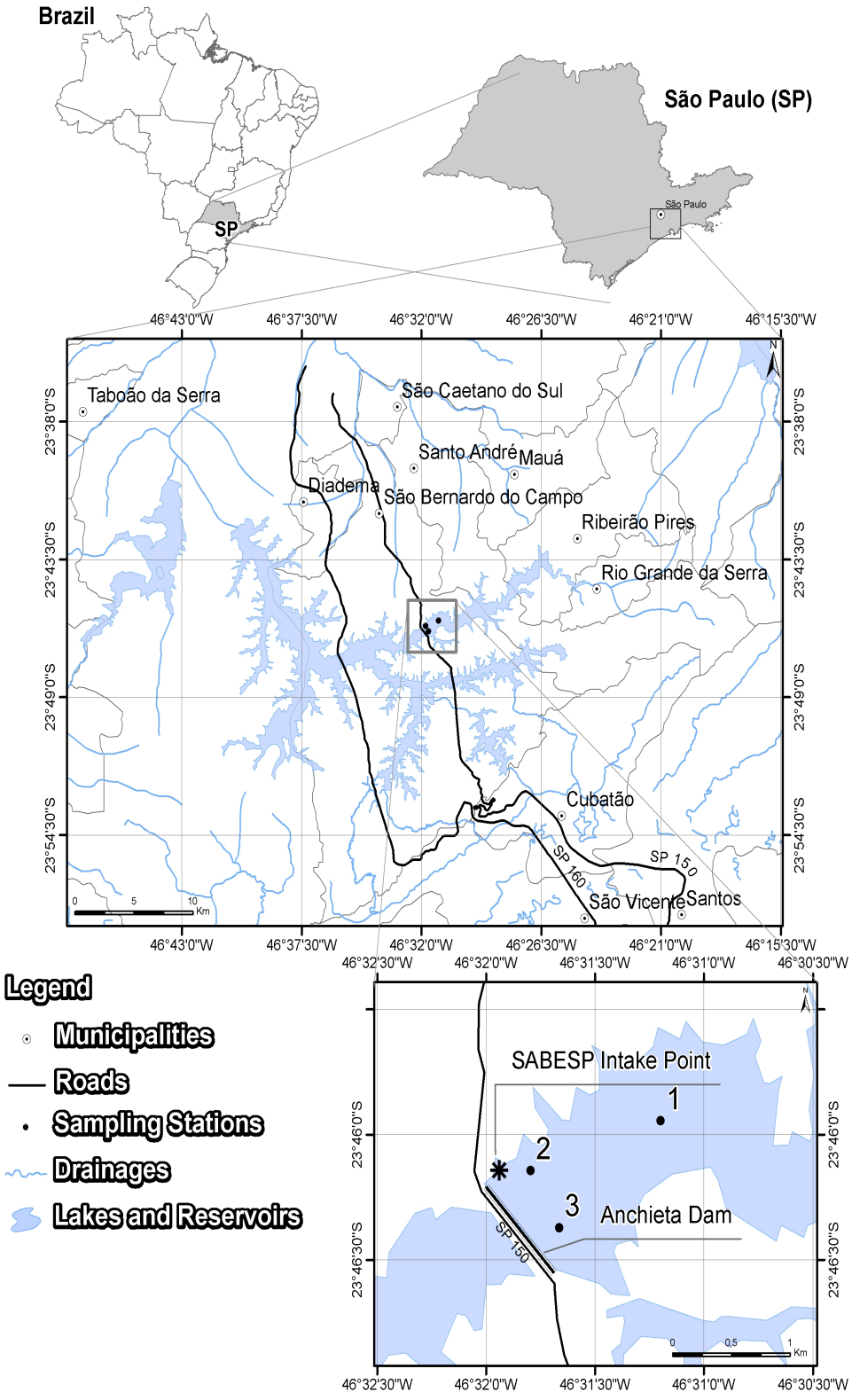

Figure 20. Rio Grande reservoir and the location of sediment sampling stations. In detail are the location of Anchieta Dam, which separates Rio Grande reservoir from the rest of Billings Complex, and the position of the water intake of State Water Company SABESP for human supply.

2.1.2 Sediment i*n situ* measures

After core sampler withdraw out from water, we carried out measurement of temperature, pH, dissolved oxygen (DO) and redox potential (E_H). We inserted probe inside the core, while sediment sample was still inside it. E_H was converted to Standard Hydrogen Electrode (SHE) (Stumm & Morgan 1996).

2.1.3 Water sampling

We elected station 1 (coincident to sediment sampling station) to collect water, due to its proximity to water intake for public supply. We sampled water from 3 different depths: bottom water, medium depth water (at ~1.5 m) and surface water. Medium depth water was sampled by means of a Van Dorn bottle. Surface water was sampled by direct diving sampling bottle into the water. Bottom water was sampled by siphoning out from acrylic tube of sediment sample device.

Besides water sampling, we measured temperature in the water column (YSI 63/100 FT), at the beginning sampling procedure.

Water sample storing PVC flaks were previously cleaned (washed with 10% HCl, rinsed with distilled water, 24 h bath in 10% HNO_3, rinsed with distilled then Mili-Q water). Samples were transported on ice, acidified with HNO_3 until pH 2.0 and stored at 4 ºC.

2.2 Laboratory Analysis

AVS (Acid Volatile Sulfide) and SEM (Simultaneously Extracted Matals) analysis proceeded as indicated by Allen et al (1991; 1993) in a closed system and in triplicates. Sulfide sequestered in NaOH was quantified by colorimetric method using HACH sulfide analysis kit (methylene blue method). Metal ions Cd, Ni, Pb, Cr, Cu e Zn were analyzed by ICP-AES (Spectro Co). A 3.0 g sediment aliquot was dried at 60°C for humidity measurement, so results were expressed in sediment dry weight basis. Organic Matter was quantified through ignition method and expressed as weight loss percentage, then results were transformed to express organic carbon per kg (see item 3.1.1, Cap. 2).

Water samples were acidified with HNO_3 to pH 2 and stored at 4oC. Digestion proceeded by adding 0.5 mL HNO_3 to 10 mL water sample aliquot and heating (105ºC) until volume was reduced

to 2 mL (Clescerl et al. 1999). After filtration (Whatman 41, 125 mm), metals were analyzed by ICP-AES (Spectro Co), the same apparatus used for analyzing metals in sediment samples.

2.3 Data Analysis

2.3.1 Sediment Quality Values (SQV)

We used three different Sediment Quality Guidelines to compare our data: a) Equilibrium Partitioning (EpP) from the USA EPA (2000a; 2005); b) Probable Effect Level (PEL) from the Canadian Environmental Canada (CCME 2002); c) background values established for Alto Tietê Watershed (Regional Background Value – RBV) (Nascimento & Mozeto 2008).

EqP is an approach proposed by EPA, in which sulfide and metals content are compared in order to establish whether or not sulfide is in excess in relation to metals. The extractions of metal and sulfide are made in one step, therefore sulfide is called Acid Volatile Sulfide (AVS) and metals are called Simultaneously Extracted Metals (SEM). Sulfide is able to complex divalent metals as metal sulfide. When AVS is in excess, the probability of metals to be as metal sulfide is great; this means that metals are not in the aqueous form, hence not bioavailable (Allen et al. 1993). AVS is compared to SEM in a molar based, then normalized with Organic Carbon, as following (USEPA 2005):

$$\frac{\sum [SEM] - [AVS]}{OC}$$

where,

Σ[SEM] is the sum of molar concentration of divalent metals

[AVS] is the molar concentration of sulfide; and

OC is Organic Carbon.

PEL was established after statistical analysis that related probability of death (or other endpoints) in several organisms under a range of chemical content, using a data bank from works carried out in Canada (CCME 1999) Chemical concentrations on sediment above PEL are expected to be associated with adverse effect on biota. Application of Canadian SQV also assumes consideration of general characteristic of the studied aquatic environment, effect of local environmental con-

ditions on sediment quality, physical and chemical parameters influencing sediment dynamics and factors that modify toxicity to aquatic organisms.

Regional Background Values (RBV) were established by dating sediment cores withdrawn from within the watershed (Nascimento & Mozeto 2008).

Comparison of metal concentration using EpP approach allows inference on metal bioavailability and probable risk to biota; comparison with PEL also allows inference on probable risk effect to biota, while RBV allows inference on increment in relation to natural conditions.

2.4 Statistical Analyses

We used statistic tools to verify the following questions:

1) If there was any significant difference in metal and AVS average concentration along the sampling dates;

2) If there was any co-variance between the concentration of a given metal or AVS and explanatory variables (pH, DO, reduction potential, OC and temperature);

3) If there was significant co-variance between the concentration of a given metal in the sediment and in the bottom water; and

4) If there was any significant difference regarding DO among the sampling days.

Item 1 and 3 were also tested for the relation SEM-AVS/OC.

We used "The R Project for Statistical Computing", version 2.10.1 (http://www.r-project.org).

We used median for every replicate or triplicate data, in order to yield a data set with equal numbers of entrances, and log transformed data, for better homoscedacity. Sampling stations were not randomly chosen, but repeated every sampling day, what causes a spatial pseudoreplication. Hence, we used a mixed effect model. Mixed effect models are so called because the explanatory variables are a mixture of fixed effects and random effects (Crawley 2002). We used backward elimination in order to reduce the model. For each metal and AVS, we tested the adjustment of the model with explanatory variables, and then we deducted one variable at a time, following the performance of new adjustment test (backward elimination criterion). At this step, we chose among the known paired dependent variables (pH and Eh; OD and Temperature), in order to exclude the effect

of co-variance. At the end, we performed a Shapiro-Wilk test and analyzed the residues of the final model to check on the adjustment of the curve. Significance level was set as 5%.

To test the relationship among metal in water and in sediment, we compared each water data set (i.e. log of median metal concentration from bottom water, from medium depth water and from surface water). We then proceeded Spearman's rank correlation.

Dissolved Oxygen (DO) measured in overlying water was also modeled aiming investigation of possible differences along sampling days, as an indicator of water circulation event. We used mixed effect models with sampling days as explanatory variable, followed by a 2 by 2 Bonferroni's correlation to identify significant differentiation between paired days. Significance was set to 5%.

3 Results

3.1 Water Column

In general, water temperature varied from 18.6 to 23.8 ºC, being lower mean temperature registered on 20th/Oct (20.0 ºC) and higher on 25/oct (20.7 ºC). Stratification was observed on 16th, 18th and 20th/Oct, with thermocline progressively deeper. On the 23th and 25th/Oct, water column showed no thermal stratification, since no changes of at least 1 ºC was observed within 1 m in the water profile (Lampert & Sommer 1997), as observed in Figure 21.

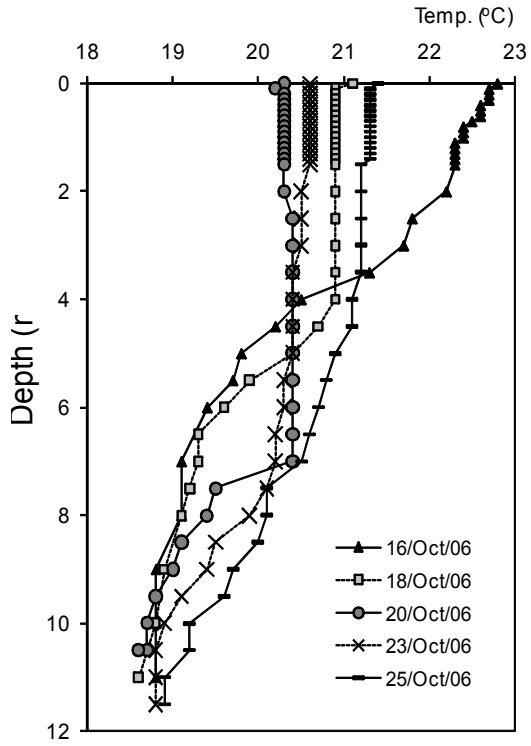

Figure 21. Water column profile on different sampling dates measured at about 9:00 AM.

Due to the rain that occurred previously and during the sampling period, it was possible to observe a rise in water level of the reservoir, reflected both on advance in the margin and on deepening of station 1: difference between the 16th and 25th/October was 0.5 m (data not shown).

3.2 *In situ* measures and Organic Carbon (OC)

Mean values of in situ measures (pH, redox potential - E_H, temperature, dissolved oxygen) and organic matter are presented in Table 13. Mean values obtained for DO per station per sampling day is presented in Figure 24.

Table 13. Mean values of *in situ* measures (pH, redox potential – E_H, temperature – Temp., Dissolved Oxygen – DO) and organic matter - OM per station. Numbers inside parenthesis represent Standard Deviation.

Station	*pH*	*E_H (SHE)*	*Temp. (ºC)*	*DO (mg kg^{-1})*	*OM (%)*
1	6.90 (0.094)	126.08 (22.54)	19.1 (0.59)	5.24 (3.00)	25.5 (1.1)
2	6.93 (0.152)	122.33 (28.03)	19.15 (0.63)	4.33 (2.92)	23.7 (2)
3	7.01 (0.135)	115.54 (25.02)	19.23 (0.52)	3.92 (1.92)	21.3 (1.7)

3.3 Acid Volatile Sulfide (AVS)

AVS concentration varied from 1515.77 mg kg^{-1} (Station 1, 28/Oct) to 143.75 mg kg^{-1} (Station 3, 23/Oct). At station 3 were registered lower mean values of AVS, while at station 3 the higher values (Table 14).

Table 14. Maximum, minimum and mean values of AVS concentration (mg kg^{-1}). Sampling station were maximum or minimum values were observed are within parenthesis.

	Date					
Station	16/10/06	18/10/06	20/10/06	23/10/06	25/10/06	***Mean value per station***
1	665.75	1079.61	720.59	913.58	742.24	831.11
2	893.30	934.65	778.35	586.46	820.17	802.19
3	301.35	260.96	408.40	205.73	707.59	372.89
Mean value per date	644.80	777.54	612.65	569.31	758.11	
Maximum	1287.86 (1)	1515.77 (1)	1291.00 (1)	1393.02 (1)	1368.64 (2)	
Minimum	159.45 (3)	197.80 (3)	160.25 (3)	143.75 (3)	431.16 (2)	

3.4 Simultaneously Extracted Metals (SEM)

Table 15 presents a summary of SEM results through descriptive statistics. Pb values were bellow detection limit (DL) for all studied samples. Cr concentration varied from 492.47 mg kg^{-1} (Station 2, 18th/Oct) to <DL (Station 1, 23th/Oct). Ni maximum value was 308.52 mg kg^{-1} (Station 2, 18th/Oct) and minimum was <DL (Station 1, 16th/Oct and at all three stations on 23 and 25th/Oct. Cu concentration varied from 99.34 mg kg^{-1} (Station 1, 20th/Oct) to 5729.89 mg kg^{-1} (Station 2, 25th/Oct). Zn maximum registered value was 671.71 mg kg^{-1} (Station 2, 16th/Oct) and minimum value was 117.20 mg kg^{-1} (Station 3, 23th/Oct). Cd concentration varied from <DL (Station 1, 23th/Oct) to 31.74 mg kg^{-1} (Station 2, 25th/Oct).

Table 15. Summary table of SEM results – descriptive statistics. Numbers in parenthesis represent station were maxima and minima values were registered.

	Cr	***Ni***	***Cu***	***Zn***	***Cd***	***Pb***
Mean (mg kg^{-1})	105.08	91.36	2415.56	247.57	14.06	<DL
SD	85.81	61.36	1068.27	98.78	6.12	0.00
Maximum (mg kg^{-1})	492.47 (2)	308.52 (2)	5729.89 (2)	671.71 (2)	31.74 (2)	<DL
Minimum (mg kg^{-1})	<DL (1)	<DL (1; 2; 3)	99.34 (1)	117.20 (3)	<DL (1)	<DL
CV (%)	81.7	67.2	44.2	39.9	43.5	0.0

SD = Standard Deviation; CV = Coefficient of Variation; DL = Detection Limit

3.5 SQV

Table 16 presents ratio between mean metal concentration and respective metal reference value – RBV and PEL. Data presented as ratio enables a rapidly evaluation of how many times is metal concentration greater than SQV. Comparison of metal concentration and RBV indicated metal enrichment in sediment, especially regarding Cu and Cd. Concentration of Ni, Cu and Cd were respectively 3.0, 14.2 and 4.5 times above PEL.

Table 16. Ratio between mean metal concentration and reference values PEL, TEL and RBV.

Station	*Cr*		*Ni*		*Cu*		*Zn*		*Cd*	
	PEL	RBV	PEL	RBV	PEL	RBV	PEL	RBV	PEL	RBV
1	1.1	2.5	2.5	3.5	9.3	102.2	0.8	9.6	3.8	61.2
2	1.5	3.6	3.0	4.2	13.3	145.2	0.9	11.0	4.5	71.5
3	0.9	2.2	2.1	2.9	14.2	154.9	0.7	7.9	3.7	59.6

EqP approach showed that sulfide was in excess in relation to metals, and most of calculated Σ SEM-AVS/OC lied within uncertainty range, between 130 and 3,000 mmol kg^{-1}. At station 1, Σ SEM-AVS/OC was bellow 130 mmol kg^{-1} on 20th and 23th/Oct/2006, which indicates no prediction of toxic effect of metal contaminated sediments (Figure 22).

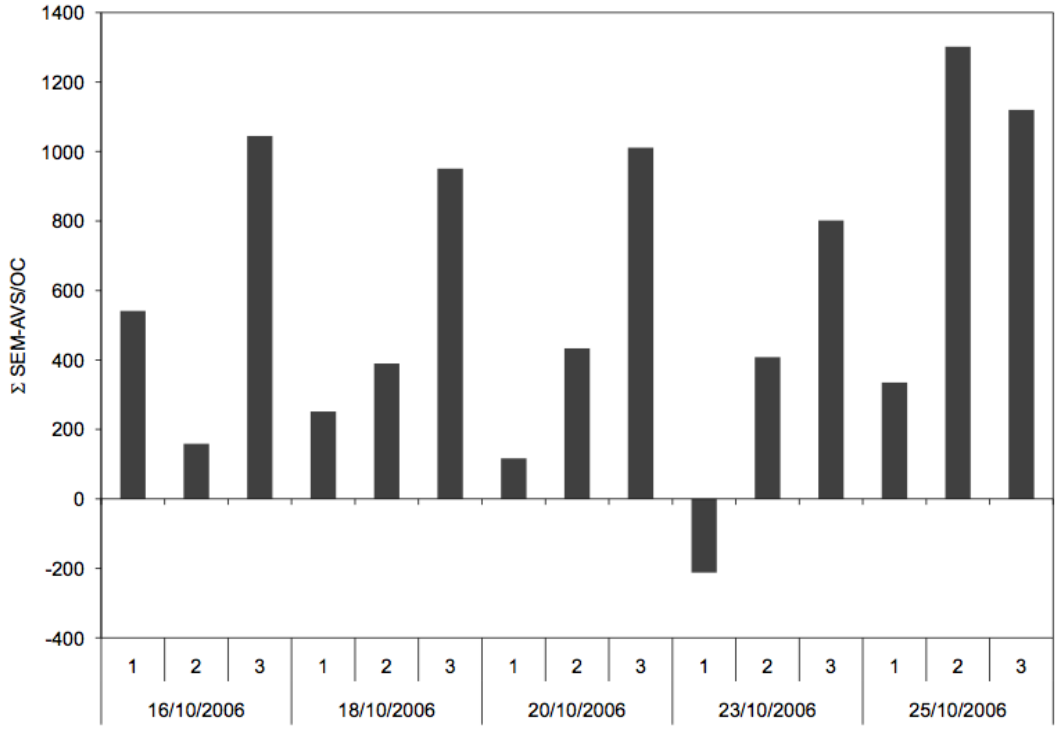

Figure 22. Mean values of EqP approach (Σ SEM-AVS/OC) at different sampling stations (1 to 3) and in the different sampling days.

3.6 Statistical Analyses

Table 17 presents significant effects that composed the final model of each metal, AVS (log-transformed concentration) and the relation SEM-AVS/OC. Respective p-values are also presented.The effect of the days was significant for every tested metal and for MES-AVS/OC; this was not true for AVS. Hence, temporal heterogeneity of metals and MSE-AVS/OC could be observed along the sampling days, but the same was not true for AVS.

Among the tested explanatory variables, OC contributed for the model of all metals with significant p-value, except Cu. Temperature was significant for Cu and Cd, while redox potential was significant for Ni. The relation SEM-AVS/TOC showed co-variance with temperature and pH.

Analysis of correlation (Spearman's correlation coefficient – rho) between concentration of each metal in sediment and in water (at 3 depths) is shown in Table 18.

For Spearman's correlation, the greater the number of observations, the more powerful is the analysis. In the case of data presented, we had 5 observations (n=5), which allows significance only for rho close to ± 1.

Table 17. Final models (statistically significant effects) for log transformed data of metal and AVS concentration and for MES-AVS/OC, with respective p-values.

	Final model	*p-values*
Cr	effect of days	6.7879x 10^{-08} (days)
	OC	0.02700 (OC)
Ni	effect of days	0 (days)
	E_H	0.0254 (E_H)
	OC	0.0045 (OC)
Cu	effect of days	2.8979 x 10^{-05} (days)
	temperature	0.0494 (Temp)
Zn	effect of days	7.7284 x 10^{-05} (days)
	OC	0.0129 (OC)
Cd	effect of days	1.0735 x 10^{-12} (days)
	Temperature	9.3892 x 10^{-05} (Temp)
	OC	1.1695 x 10^{-05} (OC)
AVS	effect of sampling station	4.8951 x 10^{-06}
MES-AVS/OC	Effect of days	5.9615 x 10^{-08} (days)
	temperature	0.0107 (Temp)
	pH	0.0039 (pH)

Table 18. Spearman correlation coefficient (rho) for metal in sediment x metal in water (bottom, medium and surface water). Shaded cells indicate significant rho.

Metal	*Sediment x Bottom water*	*Sediment x Medium depth water*	*Sediment x Surface Water*
Cr	rho -0.4	rho -1	rho -0.8
	p = 0.75	p = 0.08333333	p = 0.3333333
Ni	rho -0.4	rho -0.8	rho -1
	p = 0.75	p = 0.3333333	p = 0.08333333
Zn	rho -0.4	rho -0.4	rho 0.2
	p = 0.75	p = 0.75	p = 0.9166667
Cu	rho 0.6	rho 0.2	rho 0.4
	p = 0.4166667	p = 0.9166667	p = 0.75
Cd	rho 0.8	rho 0.2	rho 0.4
	p = 0.3333333	p = 0.9166667	p = 0.75

Figure 20 shows the variation in median metal concentration along the sampling days. Metal concentration from sediment and water were transformed to assume zero as lower value and one as higher value, hence variation would be more evident, making comparison among them easier. Simple graph analysis points in the direction of further relationships among metal in sediment and in water. One can observe a sequence of metal concentration peaks: first metal concentration in sediment in one day, in bottom water in the next day, and in middle depth water in the following day. This pattern is evident for Cr, Cd, Cu and Ni.

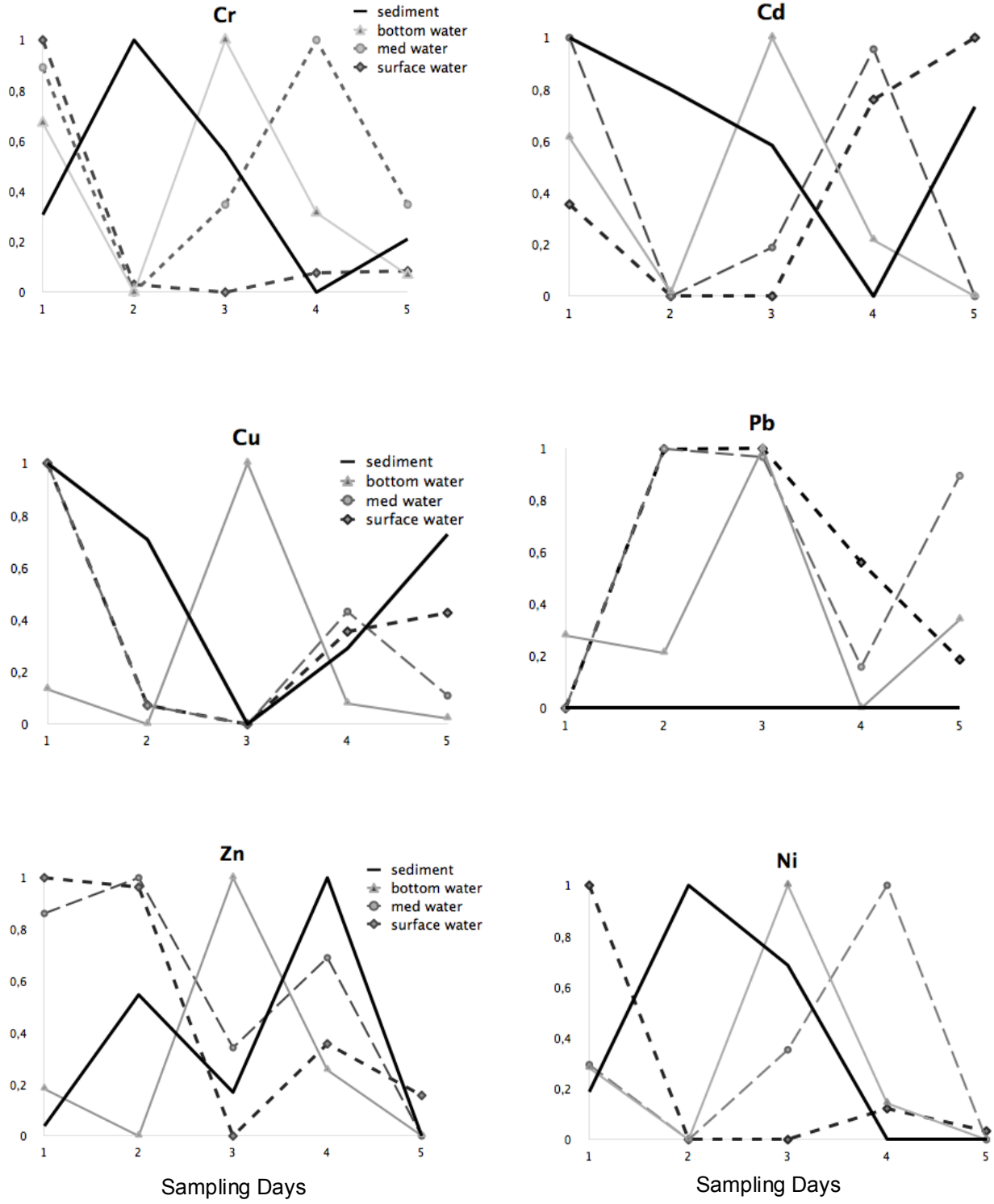

Figure 23. Metal concentration in sediment and water samples (surface, middle depth and bottom water) throughout sampling days. Data was transformed varying from 0 to 1, in order of enhance variance among data sets. Sampling days: 1 = 16th/Oct/06; 2 = 18th/Oct/06; 3 = 20th/Oct/06; 4 = 23th/Oct/06; 5 = 25th/Oct/06.

Global model of DO showed significant differences concentration among sampling days ($p=5.35 \times 10^{-18}$). Result of Bonferroni's correlation represented in Figure 24.

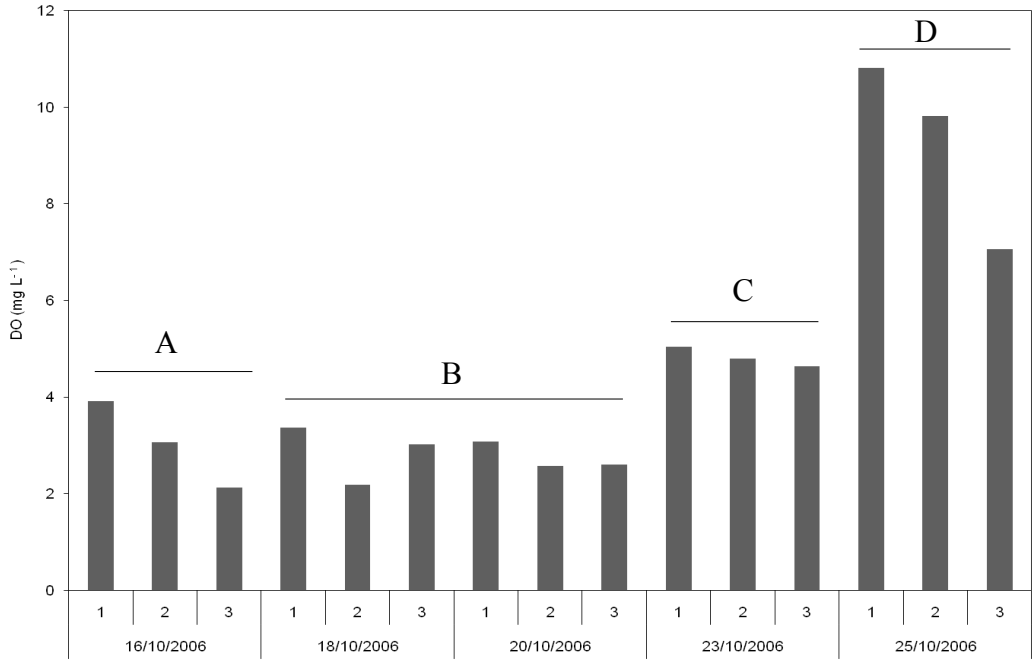

Figure 24. Mean DO concentration in underlying water at the different sampling stations (1 to 3) and in the different sampling days. Capital letters represent similar concentrations (no significant difference in 2 by 2 Bonferroni's comparison).

4 Discussion

Low redox potential is characteristic of reducing environment, which favors reduced chemical species maintenance, such as sulfide. Values of pH in sediment were close to neutral on all sampling stations and days and OC content was high, as expected for eutrophic environments. Similar values of redox potential, pH and OC were previously observed by Mariani and Pompêo (2008) in Rio Grande reservoir, and indicate a favorable environment for sulfide stabilization and metal sequestration by sediment phases.

Likewise this work, Mariani and Pompêo (2008) pointed out Cu, Ni and Cd as priority concern, when compared to PEL, with respective maximum concentration of 3,683.6 ; 167.1 and 14.0 mg kg^{-1}. In comparison to their results, we could observe an increase in metal maximum concentration, except for Pb. Cetesb (2005; 2006) have reported metal concentration above PEL in sediment samples from Rio Grande reservoir, in the same area of the present study, but generally at lower concentration: in 2004 and 2005, Cr was found at 79 and 72.5 mg kg^{-1} respectively, while Cd values

were <0.5 mg kg^{-1} in both years. Cu was an exception, with a concentration of 19,300 mg kg^{-1} in 2004 and 3,670 mg kg^{-1} in 2005.

Pb and Cd are non essential metals with the potential to biomagnify and affect health of biological communities at higher trophic levels or even health of humans consuming contaminated fish, for example. This is relevant and should be taken into account when assessing risk in this environment (P. M. Chapman & Anderson 2005), specially Cd that presented concentrations above RBV and PEL at top 3 cm sediment in this work.

According to Climate Bulletin from Center for Weather Forecasting and National Institute of Spatial Research (INPE/CPTEC 2006) a cold front system entered Brazil on the 13th October 2006 and reached Santos (close to Billings Complex) on the 17th. It moved forward and was strong enough to reach up further until Northeast Brazil. This front contributed to the occurrence of South Atlantic Convergence Zone (SACZ) between the 17th and 20th/Oct, when strong convergence and humidity over Amazon and Center-East and Southeast Regions of Brazil were observed. The SACZ can be described as a region with high variability of convective activity during summertime in eastern South America (L. Carvalho et al. 2004) and is usually associated to more extreme meteorological phenomena.

The formation of SACZ caused temperature to drop, as observed in Figure 25. Air temperature registered at the time of sampling accompanied that drop. It was also registered precipitation at the time of the SACZ formation and cold front passage, which is also shown in Figure 23.

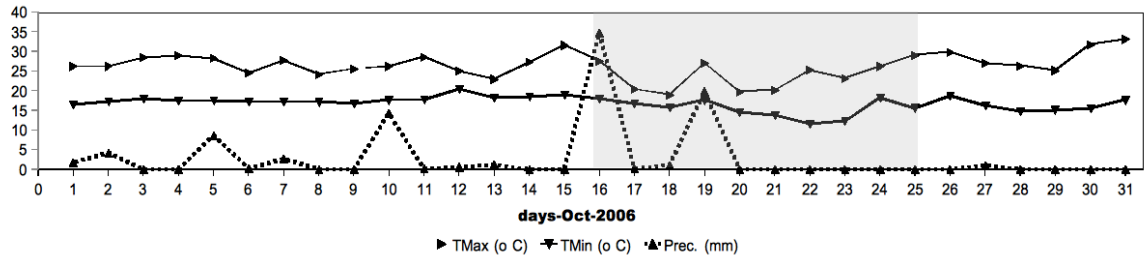

Figure 25. Temperature (maxima and minima; °C) and precipitation (mm) registered in Billings region. Shaded area highlights study period. INMET automatic station. Source: Agritempo (Embrapa 2009).

In tropical aquatic environment, stratification is characterized when changes in at least 1 °C in water temperature is observed within 1 m of the water profile (Lampert & Sommer 1997). Such difference in water temperature was observed on the 16th, 18th and 20th/Oct. Therefore, water column was stratified those days, but not on the 23th and 25th/Oct/06. Water circulation event registered was coincident with cold front occurrence in Rio Grande reservoir and with SACZ formation. Besides,

during the last 2 sampling days, DO concentration in bottom water was significant higher than the previous days, indicating that circulation event reached sediment overlying water.

Tundisi et al. (2004) studied the influence of cold front occurrences on limnological parameters measured by an automatic station in Lobo reservoir (Broa, SP, Brazil), a shallow tropical reservoir, likewise Rio Grande reservoir. Physical, chemical and biological data in vertical profile were obtained with a continuous system located in the deepest part of the reservoir (12 m) during a 32-day period in July and August 2003. This information was compared to climatological satellite images. During the periods of cold fronts passage, air temperature and solar radiation were lower, while wind speed was higher. In response to that, reservoir presented vertical mixing, following a period of stability and thermal, chemical and biological (chlorophyll-a) stratification, after the dissipation of the cold fronts.

Such water circulation events may bring oxygen to the bottom water, causing sulfide to oxydize and release metals. Besser et al. (1995) found significant difference in bioaccumulation of Cu of larvae of the midge, *Chironomus tentans,* between two sediment incubation treatments (oxic and anoxic conditions), corresponding to differences in concentrations of AVS and SEM:AVS ratios. Concentrations of AVS were significantly less in sediments incubated with oxic overlying water than in sediments incubated under anoxic conditions. SEM and overlying water characteristics were also different comparing the two treatments. From their experiment in laboratory, those authors indicated the need for temporal evaluation of AVS. In field experiment, Howard and Evans (1993) compared three lakes in Canada, and were able to identify spatial and temporal heterogeneity of AVS and SEM in the sediment, which they related to changes DO in bottom water and with turnover events. Carvalho et al. (1998) related oxic/ anoxic conditions of incubated sediment to metal remobilization and consequent toxicity to *Daphnia similis;* tested sediment were sampled from Billings Complex.

In the particular case of Rio Grande, previous studies that used EqP approach reached different conclusions regarding excess of AVS in relation to metal (IPT 2005; Silvério et al. 2005; Mariani & Pompêo 2008), including this work, what confirms sulfide and metal does vary over space and time in this environment. We found most of SEM-AVS/CO results lying within the uncertainty range, and one observation pointed out probable negative effect on biota. So, spatial and temporal heterogeneity of metal and sulfide does create conditions favorable for metal to become bioavailable in sediment of Rio Grande reservoir.

Moreover, such heterogeneity happen over a short-time period, as observed in the present work. Modeling statistical approach reveled significant effect of sampling days in log-transformed concentration of metals measured in sediment of Rio Grande reservoir, meaning those metal vary not only along the year or along the seasons of the year, but also in a 14-day time basis.

For AVS, on the contrary, our results show no effect of time, but do show differences in space, since sampling station was a significant effect for the adjustment of AVS model. As mentioned above, oxic environments do not favor sulfide stability (Besser et al. 1995; Howard & Evans 1993). Water circulation observed in the last 2 sampling days in Rio Grande reservoir was accompanied by rising of DO concentrations in bottom water, which were significant different from the previous days. Despite that, DO did not contribute significantly to better adjustment of AVS nor metal models, hence, no co-variance was observed between OD and AVS or between metals and OD.

What we did observe was an effect of OC on log-transformed metal concentration. Co-variance of metal and organic matter was also reported by Mariani and Pompêo (2008) when analyzing spatial heterogeneity in Rio Grande sediment. Many studies have demonstrated the importance of organic matter for metal bioavailability and toxicity to organism (Santos et al. 2008; Lombardi et al. 2005; Gouvea et al. 2005; Bouezmarni & Wollast 2005; Di Toro et al. 2001; Kunz & Jardim 2000). Therefore, our results emphasize organic matter as a key phase in controlling metal bioavailability, even more important than sulfide. In the context of polymitic tropical shallow reservoir such as Rio Grande (Maier & Takino 1985), this might be a relevant finding and should be object of further studies and taken into consideration when using EpP model as reference for metal bioavailability prognosis. In such environment, changes in water column plus stratification break down happen many times during the year and rapidly; high temperatures imposes high metabolic rates, hence affecting physical-chemical and biological processes of the whole ecosystem: organic matter dynamics may be accelerated, causing higher sulfide production (reduction of sulfur into sulfide is bacteria mediated), accelerated metabolism of organisms (accelerating metal efflux and afflux, possibly uptake and toxicity) and accelerate sorption and other physical-chemical processes. The consequences of this dynamics is not yet fully understood in a ecosystem level. Temperature had also significant direct effect on metal concentration, but was restrict to Cu and Cd.

Metal variation in sediment along time influenced metal concentration in water. Spearman's correlation coefficient was significant for Cr (sediment x middle depth water) and for Ni (sediment x surface water). For this statistical analyzes gains power directly in relation to sample size, signi-

ficance could only be established when rho was close to maximum (+ 1 or -1). Another possible reason why no further co-variance could be observed between sediment and water metal concentration is the delay in water response to changes in sediment. Graph analysis demonstrated that a peak in metal concentration in sediment was followed by a peak in bottom water metal concentration after 2 – 3 days; subsequent peak in metal concentration was observed in middle depth water. This was particularly clear for Cr, Cd, Cu and Ni (Figure 20). Those results suggest remobilization of metal from sediment into the water and a time dependent transport towards the surface. Other authors have suggested metal remobilization from sediment, either through correlation or direct measured using specific techniques (Avila-Pérez et al. 1999; Naylor et al. 2006).

Therefore, we demonstrated an interdependency between sediment and water; water column circulation had an effect on sediment overlying water physical and chemical parameters, while changes in metals concentration in sediment had an effect on metal concentration in the water at different depths. Sediment and water, though consist different compartments in the system, can not be studied independently, especially regarding an environment with tropical and polymitic characteristics.

Rio Grande reservoir in particular is of public interest, since its resources are used for human consumption (water and fish). As management tools, monitoring program should be designed to account for spatial and temporal scales, and, at the same time, couple water and sediment evaluations.

5 Conclusions

Metal in sediment was above RBV and PEL, what indicates metal enrichment in relation to background and probable adverse effect living organisms. EqP approach also pointed out possible risk to biota due to lack of sulfide complexation phase, hence metals are probably in a bioavailable form.

Metal concentration in sediment varied along 14-day survey (temporal heterogeneity), while AVS did not vary in time, but in space (spatial heterogeneity). Peaks of metal concentration in sediment were followed by peaks of metal in the water after 2 – 3 days, suggesting metal release from sediment into the water towards the surface, in a time dependent manner.

We observed changes in overlying water DO coupled with water circulation and cold front occurrence. Despite that, DO was not a significant effect for better adjustment metals and AVS models. On the other hand, OC did contribute to better adjustment of log-transformed metal con-

centration models, suggesting it as key phase in controlling metal bioavailability. Temperature also appeared as significant effect for Cu and Cd models. This is an important finding, since temperature and organic matter interferes in ecosystem metabolism; besides, Rio Grande is an eutrophic polymitic reservoir, thus changes in temperature happen many times throughout the year, and the whole system in enriched with organic matter.

EqP should be considered with regard for metal risk assessment in Rio Grande reservoir, both because it did not vary along with metals and because different studies in this environment reached a broad spectrum of conclusions. Organic Matter appeared as a key phase in metal complexation, thus its dynamics should be object of further research; this is particularly important in a tropical shallow polymitic aquatic environment, where temperature has a direct and rapid influence on type, quantity and physical properties of organic matter.

An interdependency could be observed between sediment and water: water circulation event was coupled with DO rising in bottom water; metal concentration in sediment affecting metal concentration in the water.

Such findings have implication on risk assessment and risk management, since experimental designs for sediment surveys in reservoirs similar to Rio Grande (tropical shallow lake) should be carefully done, and include spatial, temporal heterogeneities and sediment-water interdependency.

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Cap.4 Ecotoxicity of sediment through cytotoxicity and fish embryo assays with emphasis on metals

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Abstract

Many processes influence on bioavailability of substances to organisms, hence bioassays should be choosed based on different exposure scenario. Whole sediment exposure protocols represent a more realistic condition, while sediment acetonic extract exposure provides worst-case study and a broader range of comparison, since it is more widely used for testing sediment toxicity. In order to assess sediment quality from a Rio Grande reservoir (Billings Complex, Brazil) we collected sediment from Rio Grande reservoir and from Cubatao de Cima (also located in Billings Complex, in a more protected area). We carried out cytotoxicity on RTL-W1 permanent cell line derived from rainbow trout (*Oncorhynchus mykiss*) liver. We also proceeded sediment contact test with zebrafish eggs (*Danio rerio*), followed by measurement of metals in non-coagulated embryo tissue (digestion: H_2O_2 and HNO_3 under UV light radiation following measurement in ICP/MS). The sediments showed high cytotoxicty (NR_{50} = 10.37 mg sediment equivalent /mL). For fish embryo tested on whole-sediment samples from Rio Grande reservoir, mean EC_{50} 24h was 17 mg/mL (n=2) and mean EC_{50} 48h was 12 mg/mL; no mortality was observed after 48h in embryos tested on Cubatao de Cima sediment sample. No metal could be detected in the non-coagulated fish eggs, what suggests that another class of contaminants was responsible for fish egg mortality.

Key words: metal; bioaccumulation; sediment contact test; *Danio rerio*; cytotoxicity test; bioassay

1 Introduction

Bioavailability of substances to organisms in sediment is a complex matter, since it is a result of many physical, chemical and biological processes and factors. Some examples are particle-compound interaction, solubilization, partition coefficient, degradation, organic matter and clay content, bioturvation, uptake and exposure roots (Luoma 1989; Ravichandran et al. 1995; Heise & Ahlf 2005).

In current bioassay protocols for testing sediment toxicity, intact organisms or *in vitro* systems are exposed to sediments using different exposure scenarios, which has a direct effect on the conclusions one can have, since chosen tested phase has implications on bioavailability. Test phases can be categorized as (a) organically extractable phases (in solvents other than water), (b) elutriate phase (water extractable), (c) interstitial water phase (pore water), (d) whole sediment, and (e) *in situ* assays (Burton 1991).

Native-whole sediment exposure protocols represent a more realistic scenario to simulate *in situ* exposure conditions in the laboratory, thus mimic normal bioavailability (Hollert et al. 2003; Heise & Ahlf 2005; Kosmehl et al. 2006). On the other hand, acetonic extracts are well established method for organic compound extraction, and provides advantages for storing, testing and comparing results. Bioassays proceeded with organic extract represent worst-case scenario, because compounds are virtually dissolved, bioavailable and concentrated.

In vitro cytotoxicity tests have the advantage of reduced complexity; hence can be designed for detecting specific and/or general damages at cellular or subcellular levels. *In vitro* cytotoxicity cell lines can offer similar alternative for testing ecotoxicologically relevant substances on fish, by using *in vivo* acute lethality endpoint such as neutral red (Babin & Tarazona 2005). Acute cytotoxicity can be quantified via uptake and retaining of neutral red stain in lysosomes; damaged cells loses stain retention capacity, since mechanism of retention depends on membrane transportation (Borenfreund & Puerner 1985; Babich & Borenfreund 1991; Wölz et al. 2008).

In contrast to cellular based experiment, the fish embryo assay offers a complex multicellular system of a vertebrate integrating the interactions of various tissues and differentiation process, which means it is suitable also for inferring about implications on humans (Scholz et al. 2008).

The chosen study area is Rio Grande Reservoir, Billings Complex, a strategic water body for Sao Paulo Metropolitan Area, located in Southern eastern Brazil. It has been used for multiple purposes, including water intake for public supply, although it surfers a variety of anthropogenic impacts. Previous studies in this reservoir have documented sediment contamination by metals: con-

centration of cadmium, cooper, zinc, lead, nickel and chromium in sediments are above background levels (Nascimento & Mozeto 2008) and also many times higher than Canadian reference value PEL (Probable Effect Level), meaning sediment has potential for causing toxicity to biota (Mozeto et al. 2003; Mariani & Pompêo 2008). Despite high mental content in sediment from Rio Grande reservoir, physical and chemical characteristic of sediment measured in those studies (low E_H, neutral pH, high sulfide and organic matter content) pointed towards no bioavailability of metals. Since it also receives domestic and industrial sewage, and many other point and diffused sources of contamination, organic substances may also play an important role in ecotoxicity.

In the present work, we applied fish embryo (*Danio rerio*) sediment contact assay, followed by metal measurement in viable egg tissue, to test metal bioavailability and bioaccumulation. We also applied cytotoxicity assay using permanent cell line RTL-W1 (L. E. J. Lee et al. 1993) derived from rainbow trout (*Oncorhynchus mykiss*) liver and sediment acetonic extract aiming toxicity evaluation of organic contaminants.

2 Material and Methods

2.1 Sediment Sampling

Sampling was done in Rio Grande reservoir (25th/Oct/07), and in Cubatão de Cima region (17th/Jan/07), both sites located in Billings Complex, a major water reservoir near Sao Paulo City, Southern Eastern Brazil. The latter was designed to serve as reference to Rio Grande, since it is of restrict access and has better protected margins. Sediment sampling in Rio Grande was done by means of Eckman-Birge gripper, whilst in Cubatão de Cima, with a shovel. Sediment was transferred to plastic bucket and left to settle down in the shadow for about 1 hour. The surface (and therefore fine-grained) layer was transferred to aluminum trays (previously washed with Extran 10%) and transported on ice to the laboratory, where they were frozen and freeze-dried (Thermo Savant, ModulyoD-115). Samples were transferred to polyethylene pots (previously washed with HNO_3 20% and Extran 10%), and stored at 4 ^{o}C in the dark.

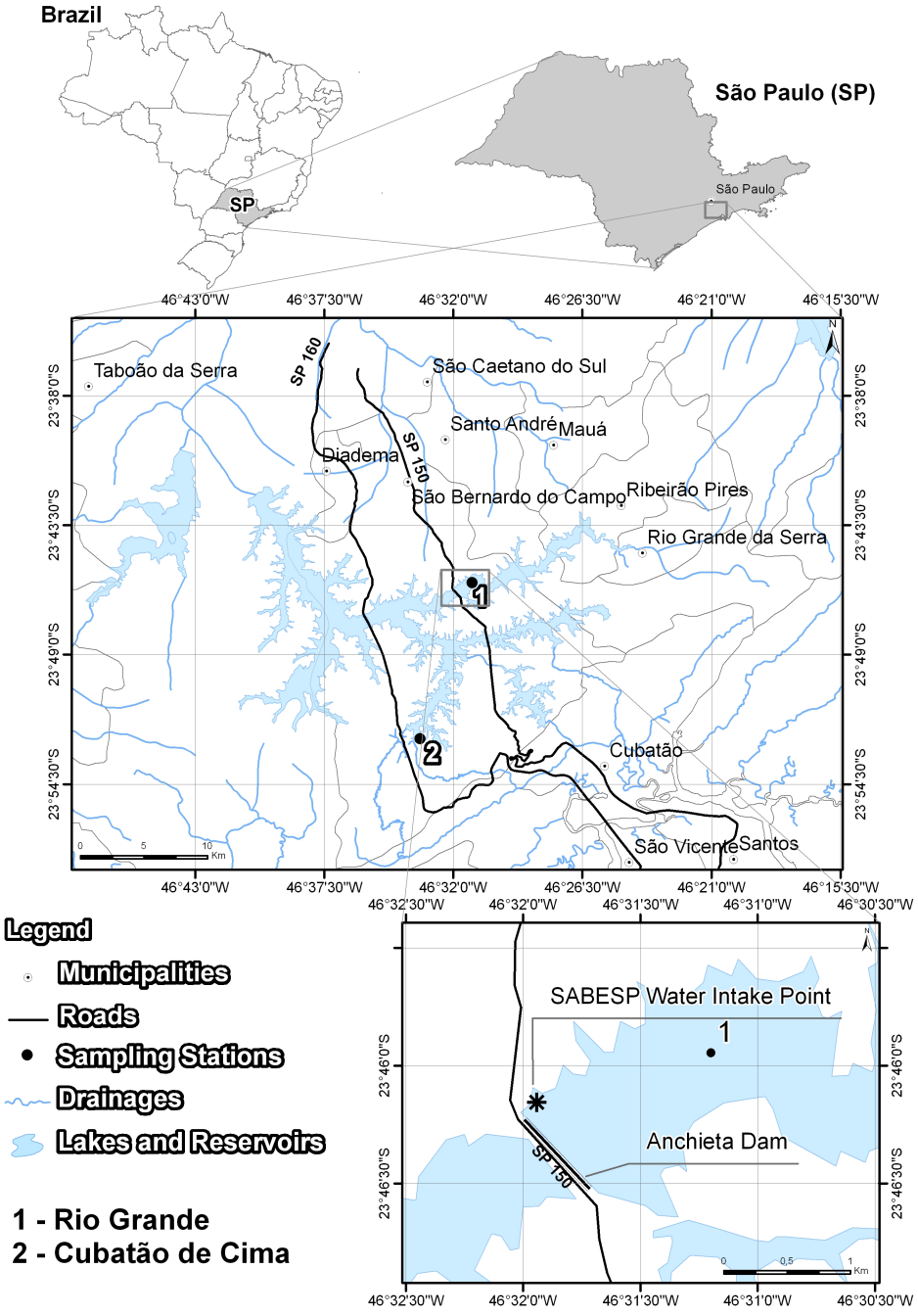

Figure 26. Location of Rio Grande reservoir in Billings Complex, Sao Paulo Brazil, with indication of sampling stations.

2.2 Sediment contact test with embryo and metal bioaccumulation in embryo

2.2.1 The fish, maintenance and egg production

Zebrafish (*Danio rerio*, Cypriniforms, Cyprinidae) is 3 to 5 cm large when adult and its origin is Asia. Zebrafish embryos is considered a good model for ecotoxicity assays, since its biology is well documented in the literature (feeding, behavior, genetic sequence, metabolism, etc) and it possesses a series of practical advantages (easy to grow in laboratory, eggs are transparent, it has a short life cycle and its female lays up to 100 eggs per day) (Braunbeck et al. 2005; Scholz et al. 2008; E. Lammer et al. 2009).

Fish were grown in the laboratory at the Department of Zoology, University of Heidelberg, Germany. ^{o}C of temperature, 744 µS of conductivity, 379 mg/L $CaCO_3$ (21.3^{o} d) of hardness, pH 7.5 ± 0.25, 10.5 ± 0.5 mg/L of dissolved oxygen (95% saturation). Ammonia, nitrite and nitrate were kept under detection limits (respectively 0-5, 0.025 and 0-0.140 mg/L). Diet consisted of commercially available artificial food (TetraMin™ flakes, Tetra, Melle, Germany) twice a day, with occasional supplements of *Artemia nauplii*.

On the previous day of the test, four males and two female fish were placed to lay eggs in breeding chambers, containing water at the same conditions and stimulation subtract, immediately before the dark period. Spawning and fertilization happened 30 min after the light onset. The eggs were taken in the morning, and separated, with 1-2 hours old.

2.2.2 Sediment Contact Assay

The assay was performed according to Hollert et al.(2003). Fertilized eggs were separated from non-fertilized eggs, using a 2 mL plastic pipette with widened opening. Five fertilized eggs were then transferred to each well of 6-well microtiter plates (TPP Renner, Darmstadt, Germany) containing a mixture of freeze-dried sediment and quartz sand plus 5 mL of artificial water (Hollert et al. 2003; Seitz 2005). Sediment test-concentrations were 600, 300, 150, 75, 37.5, 18.75, 9.375 and 4.688 mg/mL. Sediment und quartz sand (Quarzwerke, grain size F36, Frenche, Germany) were prior homogenized in ceramic mortar, in order to avoid hotspots inside the well. Artificial water (stock solutions: 58.8 mg/ L CaCl2 x 2H2O, 24.6 mg/ L MgSO4 x 7H2O, 12.6 mg/L NaHCO3, 5.5 mg/L KCl; stock solutions were diluted 1:5 with bidestiled water) was bubbled with air overnight, and pH was adjusted to 7.8 ± 0.2 prior to the test (Kosmehl et al. 2006). The plates were covered

with adhesive film (Renner, Darmstadt, Germany) to avoid loss of volatile components. To each treatment, 4 replicates were made (*i.e.*, 4 wells, 20 eggs per concentration) and 6 different concentrations. Four test quality control were performed as following: (a) for negative control: artificial water only and artificial water plus quartz sand; (b) for positive control: 0.37% (w/v) 3,4 Dichloroaniline (3,4 DCA) only and 3,4 DCA solution plus quartz sand. Each test control was done in 4 replicates (5 fish eggs per well, 20 eggs in total). 80% survival of fish eggs in negative control was necessary to validate the test.

Embryos were incubated at 27.0 ± 0.1 ºC in the dark, and then observed after 24 and 48 of exposition using inverted microscope (CK-40 equipped with an SC-35 camera, Olympus, Hamburg, Germany). Observed endpoints for 24 and 48 hours are shown in Table 19.

One pilot (28.Aug.07) was carried out, in order to establish higher concentration for subsequent tests. Dissolved oxygen was measured (Oxy-4 4-channel fiber-optic oxygen meter, PreSens, Regensburg, Germany) in sediment/water interface inside the wells containing the concentration 1:4; 1:8 and 1:16 for Rio Grande and 1:1; 1:2 for Cubatão de Cima sediment samples before the observation of the eggs. This was done in order to assure that the mortality of eggs were not due to lack of oxygen.

Table 19 Endpoints observed in fish embryos after 24 and 48 hours of exposition to whole sediment.

24 hours	*48 hours*
Non-fertilized egg	No pigmentation
Coagulation of egg*	Coagulated egg*
Epibolismus stage	No blood circulation*
No detachment of tail*	No Heart beat*
No spontaneous movement	Edema
Lack of somite formation*	Embryo miss developed
	Embryo underdeveloped
	Embryo without eye locus

* lethality endpoint according to DIN 38415-T6 (2001) (i.e. embryos with these observed characteristics were considered dead)

2.2.3 Embryo digestion and Metal Measurement

After observation, 48-hour non-coagulated eggs were transferred to plates with bi-distilled water and benzocaine (Sigma, Switzerland) in order to be mechanically dechorionated by using tweezer and needle. This step was necessary because chorion may act as a barrier for chemicals and absorb them (Braunbeck et al. 2005), hence masking results. Metals that were presumably measured

in embryos could be only present in the chorion, hence yielding false result of bioaccumulation and metal uptake (Zielke 2007).

Non-coagulated and dechorionated eggs were washed 3 times with bi-distilled water, transferred to 15 mL polypropylene conical tubes (TPP, Switzerland) and stored at -60 ºC until digestion.

Samples inside the Falcon tubes were brought to a volume of 2.0 mL using bi-distilled water (B. Braun Melsungen AG, Melsungen, Germany), transferred into quartz glass vessels and 0.8 mL of nitric acid (65%, suprapure, from Merck, Darmstadt, Germany) was added. The vessels were placed in the UV-reactor to be irradiated (UV digestion device "UV 1000", Kürner Analysentechnik, Germany). After 1 hour, 0.4 mL of hydrogen peroxide (30%, from Merck, Darmstadt, Germany) was added. After 3 hours in the UV apparatus, another 0.4 mL of hydrogen peroxide was added and the irradiation ran for another hour. The total duration of digestion was 5 hours.

The digestion procedure ran as follows: the organic matrix was destroyed by the OH radicals formed from H_2O_2 added to the sample under the influence of UV radiation (Golimowski & Golimowska 1996; Angerer & Schaller 2003). The precision of the method was improved by adding Rhodium and Scandium as internal standards to the samples (blank, calibrators, real samples).

After digestion, the samples were transferred to 10 mL volumetric flasks and the volume was filled up to 10 mL with bi-distilled water. Samples were then stored in 12 mL sterile PS-Tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) and frozen (-80 ºC) until analysis by ICP-MS (Inductively Coupled Plasma – Mass Spectrometer, Perkin Elmer Sciex, ELAN 6100). For calibration of the ICP/MS, a standard solution was used (multiple elements standard solutions VI for ICP-MS, Merk).

2.3 Cytotoxiciy test with RTL-W1 cell line

Cytotoxicity assay was performed on permanent cell line RTL-W1 derived from rainbow trout (*Oncorhynchus mykiss*) liver (L. E. J. Lee et al. 1993). RTL-W1 cells have been reported as suitable for replacing fish test, since it has shown correspondence with fish test and have metabolic transformation capacity; hence they are able to turn chemicals into a metabolite (L. E. J. Lee et al. 1993; R. F. Lee & Steinert 2003; L. E. J. Lee et al. 2008). Cell cultures was carried out as described by Klee *et at.* (2004).

2.4 Sediment Extract

Freeze-dried sediment samples were sieved by means of stainless steel sieves. For Rio Grande sediment sample, a 1,25 mm mesh sieve was used and for Cubatão de Cima sample a 2.00 mm one. This difference was necessary because the latter contained more organic matter in large pieces. 10.0 g sediment aliquot was placed inside cellulose thimbles (Whatman, Schleicher & Schuell, Dassel, Germany), stopped with glass wool and extracted with 350 mL acetone (p.a. grade, Reidel-de-Haën, Seelze, Germany). Soxhlet system was left operating for 14 hours (approximately 8 cycles per hour). Extracts were reduced in volume in a rotator evaporator (Heidolph, Laborata 4011, Kehlheim, Germany; 400 mbar, 36-38 ºC), then concentrated close to dryness under pure nitrogen stream. The solvent was then changed to dimethylsulfoxide (DMSO, Sigma, Deisenhofen, Germany). Extracts were then stored in 4 mL ambar bottles with Teflon stoppers (Butylred/PTFE grey, VWR International) at -20 ºC. Final concentration of extract was 10.0 g dry sediment/mL DMSO.

We also ran a procedure control for the extract process. It consisted on pure acetone that went through the same process that the actual samples did. The aim of this control was to assure the quality of the process (glass ware cleanness, no contamination from solvent of thimbles, etc). The procedure control was on tested for cytotoxicity, in order to validate the extraction step.

2.5 Cytoxocitity assay

We use Neutral red (2-methyl-3-amino-7-dimethylaminophenazine) as end point for testing acute cytotoxicity according to procedure modified by Hollert *et al.* (2000). Neutral red retention discriminates viable and dead cells on the basis of membrane integrity or permeability. The dye agent suffers passive diffusion across cell membrane and accumulates within lysosomes. Viable cells accumulate and retain dye in their lysosome, whilst dead cells neither accumulate nor retain the dye. Inside a plate well with a certain cell population, staining intensity is directly proportional to cell viability (Segner & Braunbeck 1992).

Neutral Red solution was added to the cells after 24-hour exposition to sediment extract, and was taken up into the lysosomes of intact cells. NR solution was left for 3 hours in contact with the cells, and then washed away with PBS. The following step was the extraction of NR solution from inside the cells with NR-extraction solution (490 mL ethanol 99%, 10 mL acetic acid, 500 mL

bidestiled water) and measurement in spectrophotometer (Genios, Tecan; measurement wavelength: 540 nm; reference wavelength: 690 nm), with help of Magellan 6.0 software (Tecan, Austria).

Extracts from Rio Grande, Cubatão de Cima and Procedure Control (10.0 g dry weight sediment/mL DMSO) were put into ultra-sonic bath during 5 min prior to dilution in medium. Another 5-min ultra-sonic bath was done after the first dilution in medium, in order to homogenize the solution. Sediment extracts were serially diluted in L15 medium along seven wells in six replicates of a 96-well microtiter plate (TPP, Trasadingen, Switzerland).

Maximum concentration of sediment extract for Cubatão de Cima and Procedure Control samples was 200 mg dry weight sediment/ mL medium, what comprises 1% DMSO (below DMSO non-observable effect concentration for RTL-W1 cells). For Rio Grande sample maximum concentration was 100.0 mg mL^{-1}, since pilot test reveled greater toxic potential of this sample.

3,5-Dichlorophenol (Riedel-de-Haën) was used as a positive control at a maximum concentration of 80 mg/L medium. Confluent cultures of RTL-W1 cells were trypsinized and the resulting cell suspension was added to each well of the microtiter plate. After incubation at 20°C for 48 h, cells were incubated with Neutral Red for 3 h, and Neutral Red retention was measured at 540 nm with a reference wavelength of 690 nm using a Spectra™ III multiwell plate reader (Tecan, Crailsheim, Germany)

Viability of exposed cells was expressed as a percentage of the negative control, and data was plotted as concentration-response curves (Sigmoidal Dose-response Curve). Non linear regression analysis was performed using Prim 5 (GraphPad, San Diego, USA), which allowed the calculation of the concentration inducing 50% of mortality after 48 hours (NR_{50}).

3 Results

3.1 Sediment Contact Assay

Pilot test provided an approximated mortality rate, hence yielding a better calculation of EC_{50} for the following tests. For the pilot test, 100% mortality was observed for the 5 highest concentrations of sediment from Rio Grande Reservoir after 48 hours of exposition (data not shown), therefore, maximum chosen concentration was set to 150.0 mg/mL.

Mean EC_{50} 24h calculated from two experiments was 17 mg/mL and mean EC_{50} 48h was 12 mg/mL. Figure 27 shows data from each experiment, after 24 and 48 hours of exposition, and calculated EC_{50}.

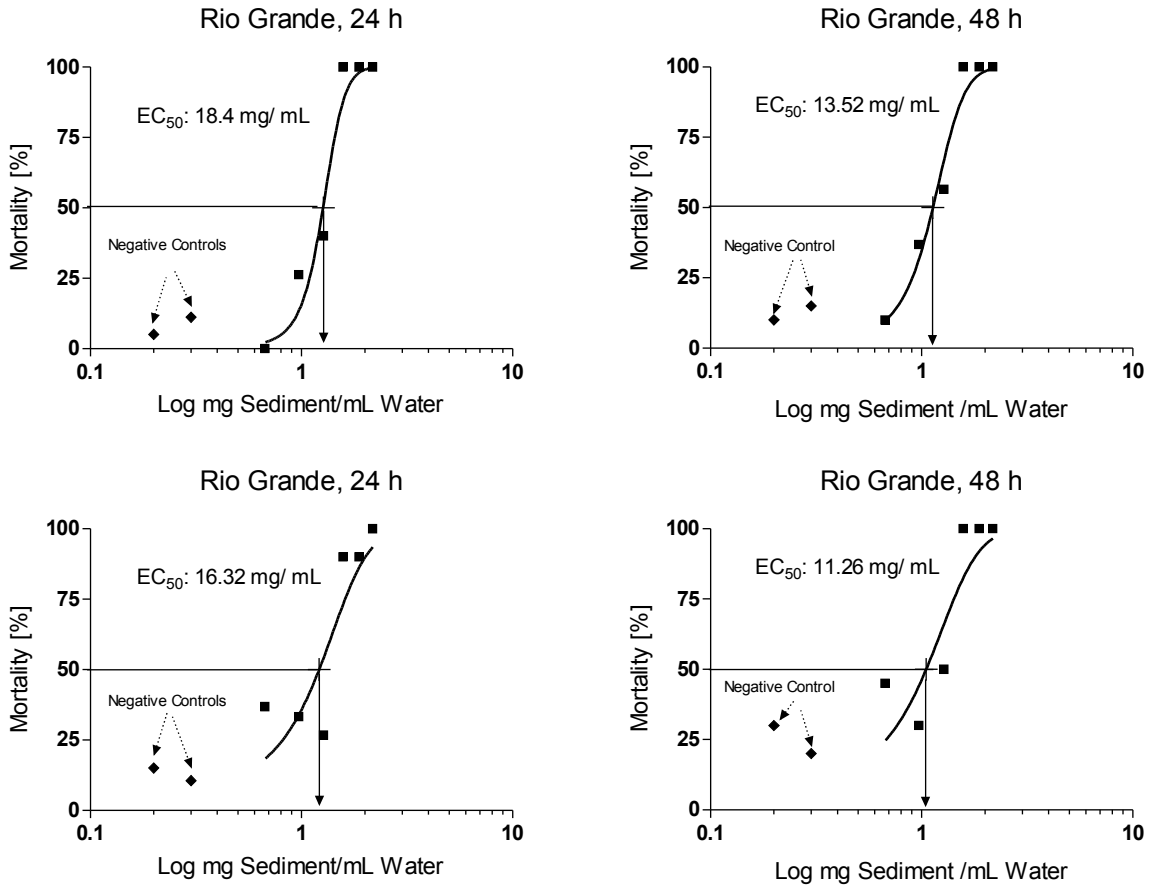

Figure 27. Zebrafish (*Danio rerio*) embryo toxicity for sediment contact test (whole, solid phase) after 24 and 48 hours incubation time. Sediment samples from Rio Grande. Data is presented as percentage of mortality (n=20) per log mg sediment/mL water. Graph intersection represents 50% mortality. EC_{50} values are not expressed as logarithms.

Pilot test for Cubatão de Cima revealed no significant mortality, even for the higher concentration, although some lack of pigmentation was observed after 48 hours for concentration as high as 300.0 mg/mL. For the subsequent sediment contact test with Cubatão de Cima sediment samples, no effect was observed on the eggs: mortality was fewer than 20%, what is comparable to the Negative Control (Figure 28).

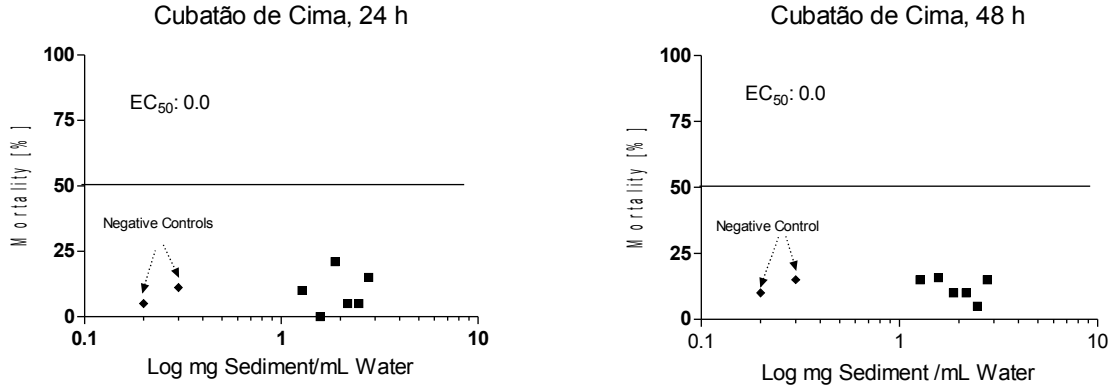

Figure 28. Zebrafish (*Danio rerio*) embryo toxicity for sediment contact test (whole, solid phase) after 24 and 48 hours incubation time. Sediment samples are from Cubatão de Cima. Data is presented as percentage of mortality (n=20) per log mg sediment/mL water. 3 g SiO powder (grain size W4) were used as negative controls.

After 24 hours, those embryos exposed to the three highest concentrations (150.0, 75 and 37.5 mg/mL) were almost all coagulated. The most common observed effect in non-coagulated embryos were development retardation (embryo still in epiboly stage) and no detachment of the tail. After 48 hours, edema was frequently observed for concentrations 18.75 9.375 and 4.688 mg/mL, along with no blood circulation and no heart beating. Misdevelopment was also observed.

Oxygen was not limiting factor for eggs; oxygen as low as 2.0 mg/L has shown no consequences to zebrafish embryos development (Braunbeck et al. 2005). Minimum measured oxygen concentration was 4.06 mg/L; and maximum 6.11 mg/L.

3.2 Metals in the embryos

Metal measured in digested fish embryos were below detectable level both for embryos exposed to Rio Grande and Cubatão de Cima sediment samples. For each treatment a total of 6 to 18 dechorionated embryos were measured. This variation was due to difference in survival for each treatment, since only non-coagulated embryos were considered for digestion and metal measurement.

3.3 Cytotoxicity assay with RTL-W1 cell line

Mean NR_{50} for Rio Grande was 16 ± 5.8 mg/mL and mean NR_{80} was 6.7 ± 1.6 mg/mL. For Cubatão de Cima, mean NR_{50} was 202.7 ± 51.7 mg/mL and mean NR_{80} for Cubatão de Cima was

136.8 ± 35.0 mg/mL. Four valid tests were used for mean calculation for each sediment sample. Figures 6 and 7 present results from valid tests on Rio Grande and on Cubatão de Cima sediment samples.

Cytotoxicity assay performed with procedure control validated the extraction step, since this sample recorded no cytotoxicity (Figure 26).

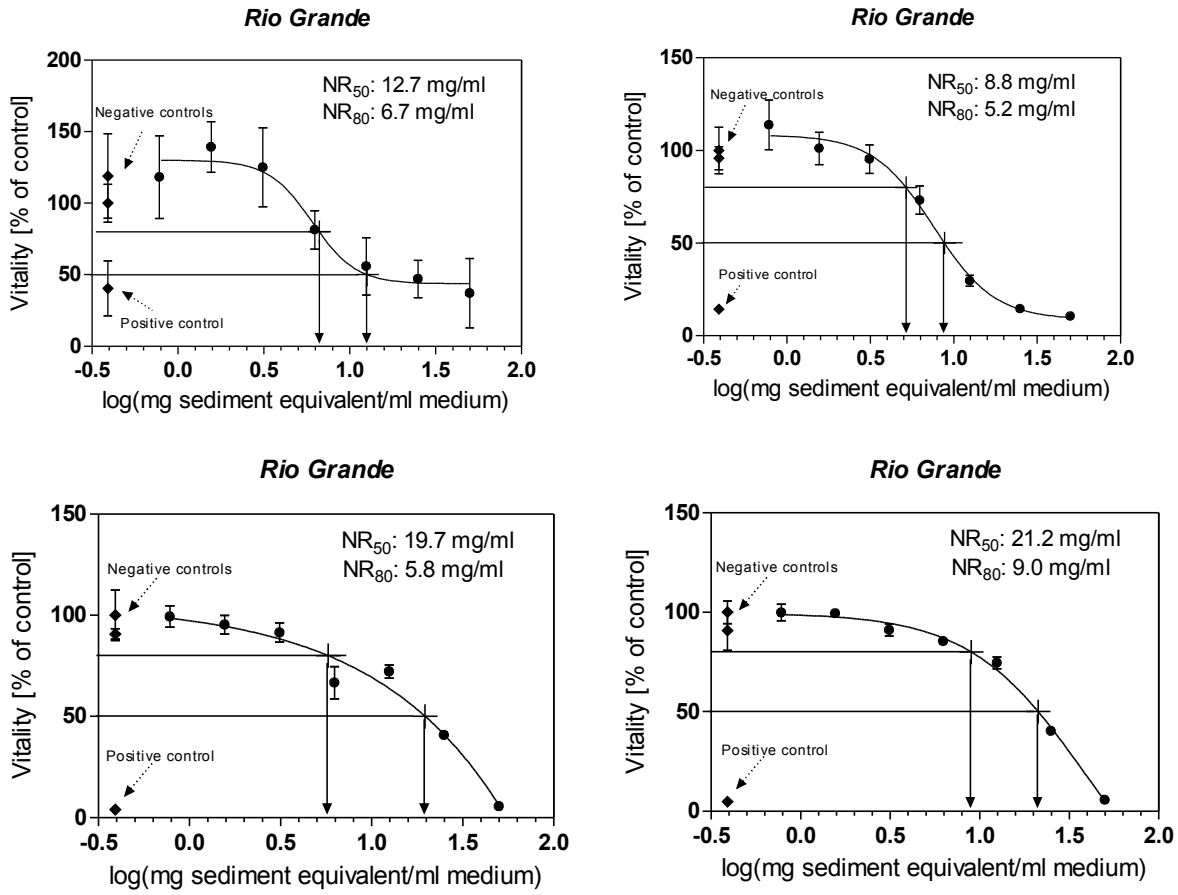

Figure 29. Cytotoxicity assays using acetonic extract of Rio Grande sediment tested on RTL-W1 cells and Neutral Red retention as endpoint. Data is presented as percentage of mortality in comparison to negative control per log mg sediment/mL. Sigmoidal Dose-Response (variable slope) Model was used to yield NR_{50} and NR_{80}. Tests were considered valid when the 2 negative controls did not differ more than 20% from one another

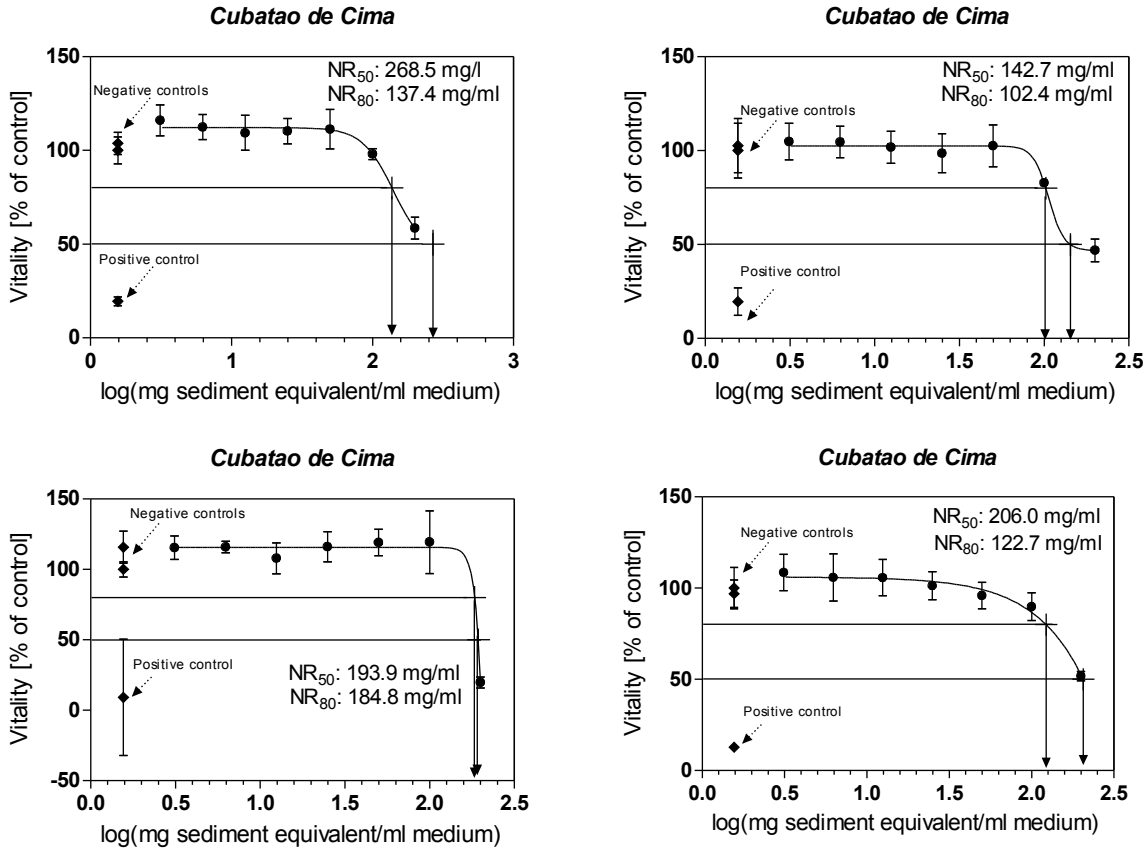

Figure 30. Cytotoxicity assays using acetonic extract of Cubatão de Cima sediment tested on RTL-W1 cells and Neutral Red retention as endpoint. Data is presented as percentage of mortality in comparison to negative control per log mg sediment/mL. Sigmoidal Dose-Response (variable slope) Model was used to yield NR_{50} and NR_{80}. Tests were considered valid when the 2 negative controls in the plate did not differ more than 20% from one another.

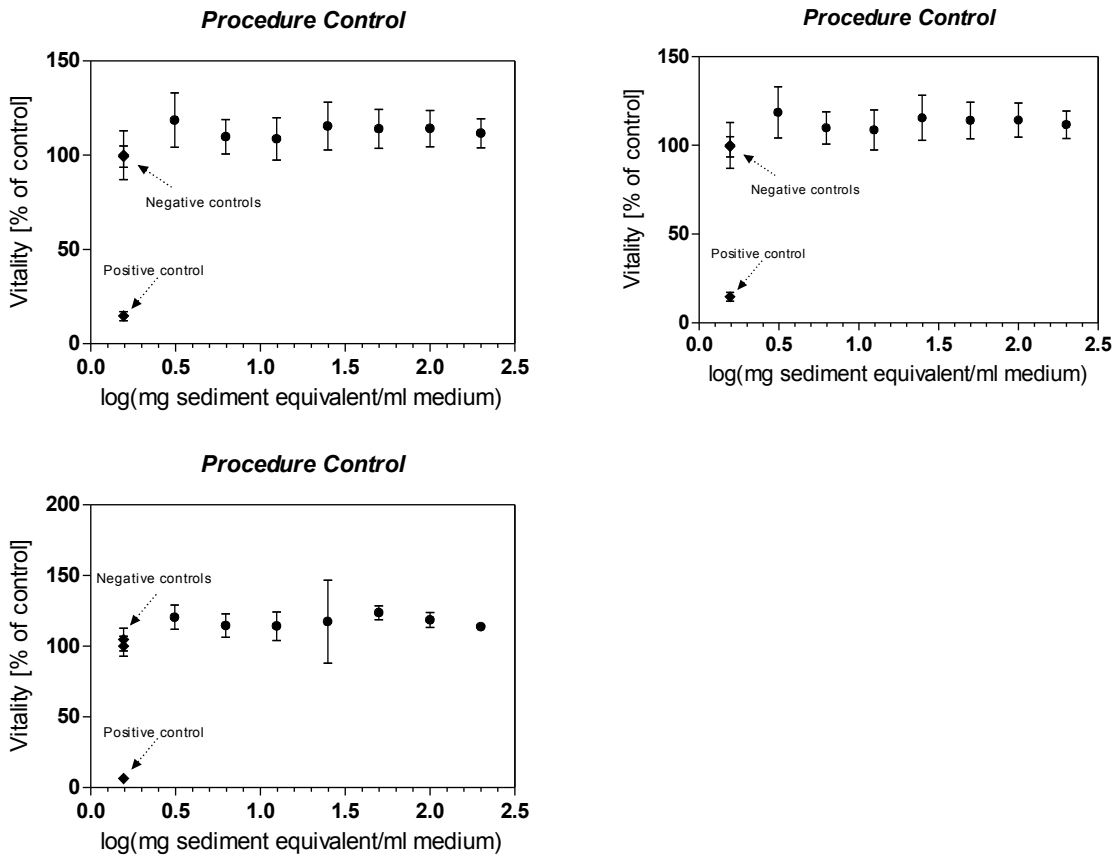

Figure 31. Cytotoxicity assays performed on RTL-W1 cells using acetonic procedure control, in order to validate the extraction step.

4 Discussion

Cubatão de Cima sediment samples showed low cytotoxic potential to RTL-W1 cells, and no toxic potential to zebrafish embryo, since tests revealed mortality under 20% for highest concentration. On the other hand, Rio Grande sediment sample showed high toxicity to zebrafish embryos and high cytotoxicity for RTL-W1 cells. Yet no metal was detected in embryo tissue.

For sediment contact assay with Rio Grande samples mortality reached 100% at test-concentration of 35.5 mg/mL and above after 48 hour exposure. Non-detachment of the tail, one of most observed 24-hour effect on non-coagulated embryos, was detected at concentration as low as 4.688 mg/mL. Hollert et al. (2003) studied sediment from Neckar river area (Germany), a place where sediments are recognized as polluted. Authors performed sediment contact test with zebrafish embryos and observed non-detachment of the tail at test-concentration of 231 mg/mL. The same study showed 100% mortality after 48 hours only at test-concentration of 600 mg/mL. Hence, sediment

from Rio Grande expresses similar effect on fish embryo as sediment from Neckar River, but in a much stronger way, since lower concentration of the former caused complete mortality of tested embryos.

Oxygen depletion was not the cause of embryo mortality, since minimum measured dissolved oxygen was 4.06 mg/L, above critical level (2.0 mg/L). Experiments with oxygen levels under identical test conditions identified 0.5 mg/L dissolved oxygen as sufficient for normal zebrafish embryo development (Hollert et al. 2003). Hence, observed effect on fish embryos had to be due to particle-bound substances.

Despite Rio Grande sediment toxicity and its known high metal content, no metal could be detected in dechorionated non-coagulated embryos, which suggests that metal was not the primary cause of toxicity to fish embryos.

Regarding toxicokinetics (the rate of absorption, distribution and excretion a toxicant) in fish eggs, one major physical barrier has to be considered, which is the egg shell, or chorion. Chorion can potentially modulate toxicity, and serve as a barrier, since the toxic substance must pass through it in order to exert the toxic effect on the embryo (Scholz et al. 2008).

Burnison *et al.* (2006) studied bioaccumulation of Cd in zebrafish eggs and the effect of dissolved organic matter (DOM). They concluded that Cd content of zebrafish eggs increased with time and Cd-concentration in the surrounding medium, and that most of Cd was associated with the chorion (61% of the Cd was bound to the chorion of the zebrafish eggs). In the present work, dechorionation was performed prior to digestion; thus, one could argue that chorion might have prevented metal to enter embryo tissue while embryos were exposed to sediment, whilst metals retained in chorion were not accounted for measurement. Nevertheless, given the high concentration of metal in sediment, we believe that metal would have crossed chorion barrier. Hence, bioavailability should be the key for explaining why no bioaccumulation of metals was detected in fish embryos.

Still citing to Burnison et al. *(2006)* a 5-h exposure to metals was enough for zebrafish eggs to show a steady increase in Cd-accumulation. But, in the presence of DOM at 16.9 ppmC, accumulation of Cd decreased significantly (an order of 52 to 60% in the embryo), compared with the untreated controls.

Doig and Liber (1996) also studied the influence of organic matter on nickel bioavailability and toxicity to *Hyalella azteca*, and concluded that DMO concentration played great role in determining nickel speciation; in the presence of DOM, bioavailability of nickel was significantly reduce,

hence its toxicity to *H. azteca*. Santos *et al.* (2008) studied copper acute toxicity and bioaccumulation in *Ceriodaphnia silvestrii* in the presence and absence of humic substances (HS). They concluded that the addition of HS lowered measured free Cr^{2+} ion in solution, which caused LC_{50} of *C. silverstrii* to rise significantly.

Therefore, the reason why no metal was detected in embryo tissue in the present work could not be due to exposure time, since fish embryos were kept more than 5 hours in contact with the sediments, period long enough for bioaccumulation of metals to take place (Burnison et al. 2006). On the other hand, analysis on Rio Grande sediment sample showed that 25.5% of total dry weight is organic matter (OM; loss on ignition - data not shown). This high OM content may be the reason why no metal uptake by embryos could be observed, since OM complex ion metals in such a way that, despite metal high concentration, it was not in a bioavailable form. The importance of OM in metal dynamics of sediment from Rio Grande reservoir was raised by Mariani and Pompêo (2008) when they studied metal spatial heterogeneity; those author found great correspondence of metals to OM, even more than with sulfide, for example, in a PCA statistical analysis.

Cytotoxicity effect observed on RTL-W1 cell for Rio Grande sediment extract had a mean NR_{50} of 15.6 ± 5.8 mg/mL , which is comparable to cypermethrin, a highly toxic pesticide (NR_{50}<18 mg/mL) (Babin & Tarazona 2005). Industrial sludge sample extracted with dichloromethane tested on RTL-W1 cell line was NR_{50} = 9 mg/mL (Klee et al. 2004). Over the past 2 decades fish population been declining in Upper Danube river possibly due to ecotoxicological reasons. Sediment extract from that area were tested on RTL-W1 cells and induced cytotoxicity with NR_{50} varying between 24 and 40 mg/mL (Keiter et al. 2006). Therefore, cytotoxicity of acetonic extract from Rio Grande reservoir may be considered high.

Rocha et al. (2009) studied sediment genotoxicity along Tietê river, including Billings Complex. Authors carried out *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*). Acetonic extracts from sediment of Billings caused the highest observed genotoxic effect through comet assay (given as tail moment), and documented a strong increase in genotoxicity from Tietê River's spring to Billings (São Paulo city region), followed by decrease further downstream. High correlation between *in situ* and *in vitro* results indicated the relevance of the results. The study also pointed out the genotoxic potential of sediment-bound contaminants present in the discharges from São Paulo city.

The idea behind applying extraction of sediment matrix is to yield as much toxic compound as possible aiming the assessment of the entire hazardous potential and the reproducibility of data. Furthermore, some specific endpoints, such as those related to permanent cell lines, can only be reached through liquid phase exposure. Acetone is an organic solvent, but it may also dissolve more polar substances, yielding a more toxic extract. Comparing solvent extraction done with three different organic solvents (acetone, acetonitrile and methanol), acetonic extract the most toxic in Microtox assay (Ho & Quinn 1993).

The use of acetone as organic solvent and Soxhlet apparatus for extraction of organic substances from sediment matrix may be considered as "worst-case scenario"; the assessment of ecotoxicity by means of such procedure simulates chronic intoxication, and refers to toxic potential under massive disturbance of the system (Seiler et al. 2008). It also provides a non-selective separation process, since it represents an entire toxic potential of the sediment sample.

In contrast with that, sediment contact test provides a more realistic approach, because it simulates natural exposure and accounts for sediment complex mixture and dynamics, e.g. sorption and desorption, complexation, synergism, antagonism and bioavailability.

In this work, sediment from Rio Grande reservoir induced toxicity both through acetonic extract and sediment contact test. The fact that no metal was detected in embryo tissue after direct whole sediment exposure suggests that metal was not the primary cause of toxicity, despite its high concentration in sample. Cytotoxicity was observed after exposure to acetonic extract, which is an organic solvent, what corroborates the idea of organic xenobiotics as mandatory regarding toxic effect.

Bainy et al. (1996; 1999) studied cytochrome P450 and b5 activities, as bioindicator of aquatic contamination of fish (Nile tilapia - *Oreochromis niloticus*) by polynuclear aromatic hydrocarbons (PAHs), planar polychlorinated biphenyls congeners (PCBs), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 2,3,7,8-tetrachlorodibenzofuran (TCDF). Fish were collect in Billings complex. Those authors found high levels of multiple cytochrome P450 forms, indicating metabolism of xenobiotics by sampled fish individuals, and where able to related to high PCBs detected in fish tissue to oxidative stress.

Total PAH was also found in high concentration in acetonic extract from Rio Grande reservoir. Such extracts were able to cause specific endpoint effects (genotoxicity and dioxin-like activity) on RTL-W1 cells (see Cap.5).

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Cap.5 Battery of Bioassays for genome level toxicity assessment of sediment contaminated with chemical -mixture

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Abstract

Rio Grande reservoir is located in Sao Paulo Metropolitan Area, thus it is an aquatic environment subject to multiple stresses and its sediment is contaminated is a mixture of substances. Assessment of toxic potential of sediment extracts from Rio Grande was performed by means of bioassays and chemical analysis (USEPA target PAHs), using RTL-W1 cell line (EROD, comet assay and micronucleus test) and *Salmonella* tester strains TA100 and TA98, with and without S9 mix (Ames Fluctuation test). EROD assay showed dioxin-like activity, with $EC_{25\ TCDD}$ = 0.82 mg of SEQ/mL, and Bio-TEQ as high as 1884 pg/g (n=6). Sum of target PAH in sediment extract was 763.6 µg/kg, though PAH-TEQ accounted for only 4.6% of Bio-TEQ. Ames Fluctuation test and comet assay reveled high potentials for mutageno- and genotoxicity, with IF_{max}=11.5 (TA98-S9) and 2.5, respectively. Result comparison with reference site Cubatao de Cima corroborates toxicity of Rio Grande sediment sample in relation to observed ecotoxicological effects. Further studies should prioritize chronic exposure of organisms to sediment samples from Rio Grande; non-target PAHs, PCBs and dioxins should be considered as possible EROD activity inducer; sediment fractionation technique should also be applied in order to better understand which class of organic compound is responsible for observed toxic effect.

Key words: EROD; Ames Fluctuation test; comet assay; RTL-W1 cell; Rio Grande reservoir; genotoxicity.

1 Introduction

Most of contaminants entering aquatic ecosystems, when not processed in the water column, precipitate and have their ultimate fate in sediments. In reservoirs, deposition usually supplants suspension, due to slow down of water current, which causes sediment from such environment to become an even more concentrated pool (Baudo & Muntau 1990; Ahlf et al. 2002) and a useful phase for assessing potential hazards to ecosystem and human health (Hollert et al. 2002; M. Maier et al. 2006). Thinking of the watershed, sediment from reservoirs may reflect the uses of the whole catchment area, and accumulate substances from point and non-point sources, such as industrial and domestic wastewater, agriculture run-off, mine or solid waste areas and airborne pollutants (Apitz et al. 2005; Netzband 2007; Perkins et al. 2000).

When the uses of the watershed comprise different kinds of human activities, contamination in sediments possibly is due to a mixture of substances, varying from inorganic (e.g. metals, phosphate, etc) to organic (PCBs, PAHs, organic matter, etc.). Hence, chemical analysis as only tool is not sufficient for assessing hazard, because it does not provide information on biological effects of such pollutants and can not consider all biogeochemical processes (Power et al. 1998). Besides, it does not account for synergism, antagonism or adding factors acting in the ecosystem; isolate acute toxicity test also offer poor insights in that direction. A more useful tool is the analysis of different lines of evidences, throughout a battery of bioassays coupled with chemical analysis, in a weight-of-evidence approach (Chapman 2002; Chapman & Hollert 2006; Chapman & Anderson 2005)

Within the range of available bioassays, molecular based ones, also known as biomarkers, allow identification of toxicity in low levels of organization, thus leading to an early identification of potential hazard to the ecosystem. Mutagenicity, genotoxicity and detection of cytochrome P450 1A (CYP1A) induction are examples of ecotoxicological tests at molecular level.

The Ames Fluctuation test is a modification of the classic Ames Plate incorporation assay, a well known mutagenicity test. Likewise the classic one, test organisms are strains of *Salmonella typhimurium* with known specific mutations that cause failure in surviving in a histidine free medium. In the presence certain mutagenic compounds, mutation is reversed and bacteria became able to grow in histidine free medium (Maron & Ames 1983). The Ames assay has been widely used for assessing mutagenicity both in water and sediment (Klee et al. 2004; Kosmehl et al. 2004; Wölz et al. 2009; Pérez et al. 2003; Chen & White 2004).

The comet assay is also called single cell gel electrophoresis assay or microgel eletrophoresis assay. It measures DNA strand breakage in single cells, caused by the exposition to genotoxic substances (R. F. Lee & Steinert 2003; Mitchelmore & Chipman 1998). The Micronucleus assay enables the identification of substances that cause mutagenicity by inducing micronucleus formation (Tates et al. 1980). Micronucleus assay can detect non-reparable damages in DNA, such as clastogenic and aneugenic lesions, while comet assay, for example, can detect recent and reparable lesions, such as breaks and alkali-labile sites (P. S. Rocha et al. 2009).

The EROD assay identifies dioxin-like activity e.g. in acetonic extracts by indirect measure of Cytochrome P450 1A1 (CYP1A1) activity through the activities of the 7-ethoxyresorufin-*O*-deethylase (EROD) (Sanderson et al. 1996). The useful aspect of CYP1A1 for biomonitoring purposes is the positive relation between its concentration and chemical exposure. It means that, the great the enzyme activity, the great the sample toxic potential. Usual chemical associated to dioxin-like activity are dioxins, furans, halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs); those group of compounds are known to act as aryl hydrocarbon receptor (AhR) agonists (Giesy et al. 2002; Gustavsson et al. 2004; Seiler et al. 2006).

Nevertheless, combining bioassays and target chemical analysis, in an effort to quantify levels of known genotoxic substances, is a promising approach towards determination of the proportion of the total bioassay-measured mutagenic hazard that can be accounted for such substances (Chen & White 2004), thus revealing characterization of possible hazard.

As a case of study, Rio Grande reservoir is a good example of aquatic environment under multiple stress. It is located in Sao Paulo Metropolitan Area, one of the biggest conurbations of the world, having a population of almost 20 million people. It is of multiple uses, including recreation, water supply for human consumption, fishing and receptor of industrial and domestic effluent, much of it *in natura*, among other non-point source of pollutants (Capobianco 2002).

Previous studies have reported sediment and water contamination with metals and organic chemicals (Mariani & Pompêo 2008; Fávaro et al. 2007; Beyruth & Pereira 2002; CETESB 2006; CETESB 2007), and also expression of toxicity on bioassays (see Cap. 3 and 4).

The aim of this work was to evaluate ecotoxicological potentials from sediments of Rio Grande reservoir through genotoxic and CYP P4501A induction potentials, and to evaluate priority PAHs contribution to EROD induction of such sample, as part of an integrated assessment of Rio Grande reservoir sediments.

2 Material and Methods

2.1 Sampling Procedure

Sampling was done in Rio Grande reservoir (25th/Oct/07), and in Cubatao de Cima region (17th/Jan/07), both sites located in the Billings Complex, a major water reservoir near Sao Paulo City, Southern Eastern Brazil. The latter was chosen to serve as reference site to Rio Grande, since it is of restrict access and has better protected margins. Sediment sampling in Rio Grande was done by means of Eckman-Birge gripper, whilst in Cubatao de Cima, with a stainless steel shovel. Sediment was transferred to polyethylene plastic bucket and left to settle down in the shadow for about 1 hour. The surface (and therefore fine-grained) layer was transferred to aluminum trays (previously washed with Extran 10%) and transported on ice to the laboratory, where they were frozen and freeze-dried (Thermo Savant, ModulyoD-115). Samples were transferred to polyethylene pots (previously washed with HNO_3 20% and Extran 10%), and stored at 4 ^{o}C in the dark.

Figure 32 shows the location of Rio Grande and Cubatao de Cima sediment sampling stations. It also highlights the location of water intake point from Sabesp (Sao Paulo Water Company) for water supply.

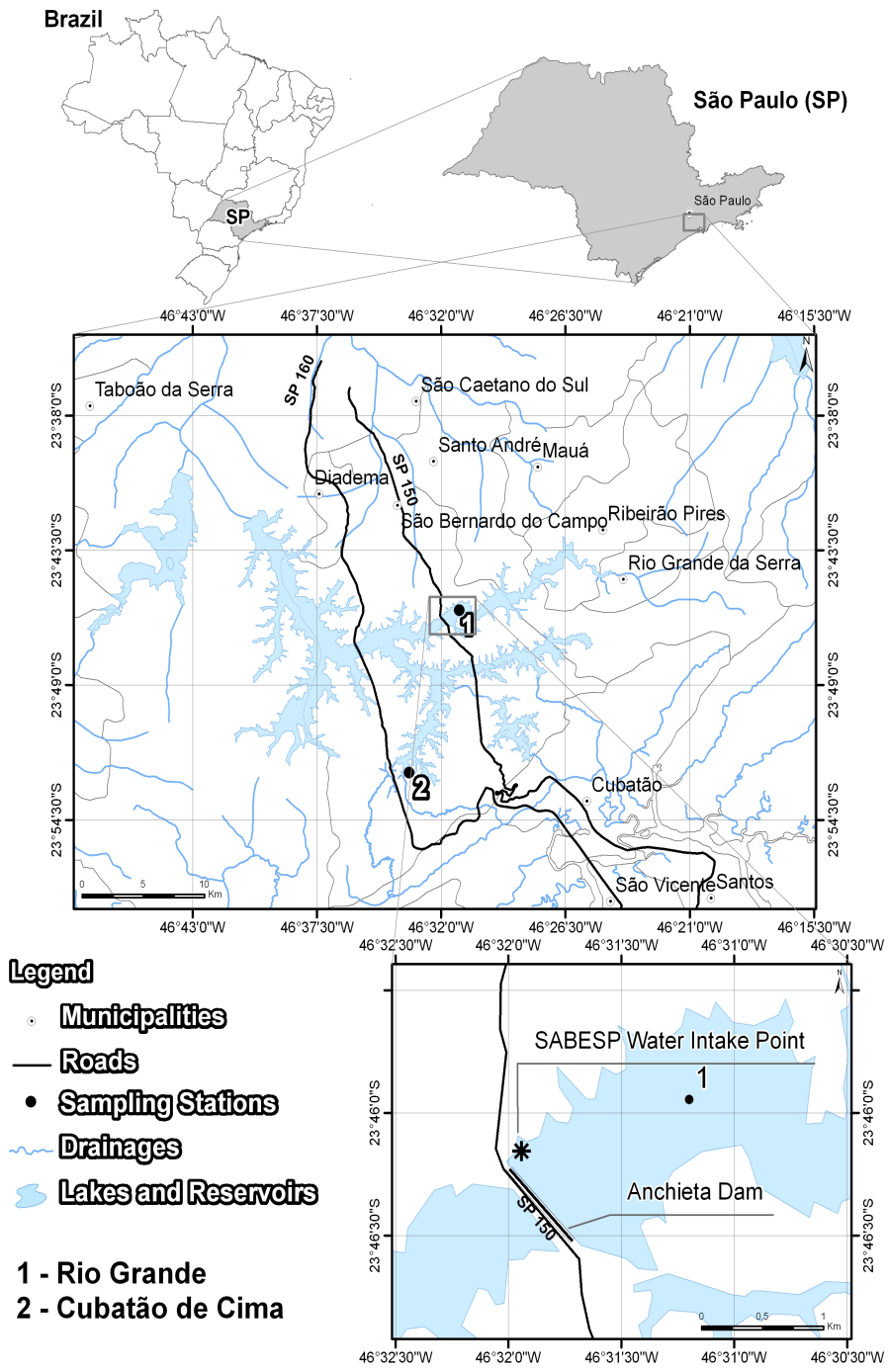

Figure 32. Location of Billings Complex in Sao Paulo State and sampling stations in Rio Grande reservoir and Cubatao de Cima region. A zoom is given near Anchieta Dam, in Rio Grande reservoir, and location of water intake point for water supply.

2.2 Extractions

Freeze-dried sediment samples were sieved by means of stainless steel sieves. A 10.0 g sediment aliquot was placed inside cellulose thimbles (Whatman, Schleicher & Schuell, Dassel, Germany), stoped with glass wool and extracted with either acetone. Soxhlet system was left operating for 14 hours (approximately 8 cycles per hour). Extracts were reduced in volume in a rotatory evaporator (Heidolph, Laborata 4011, Kehlheim, Germany; 400 mbar, 36-38 ^{o}C), then concentrated close to dryness under pure nitrogen stream. The solvent was then changed to dimethylsulfoxide (DMSO).

2.3 RTL-W1 cell line culture maintenance

RTL-W1 (Rainbow Trout Line – Waterloo) cell line are permanent cells isolated from liver of rainbow trout (*Oncorhynchus mykiss*) (L. E. J. Lee et al. 1993). RTL-W1 cell line is known to keep active their metabolic pathways for biomodification of chemical (Behrens et al. 2001; R. F. Lee & Steinert 2003). Cells were purchased by Drs. L. Lee and N. Bols, University of Waterloo, Canada.

Cells were kept in 75cm^2 culture flasks at 20 ^{o}C, with L15 medium (Leibowitz medium, Sigma-Aldrich) supplemented with 10% bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin solution. Cells suffered passage whenever they became confluent (ca. 6.5-12.0x10^4 cell/cm^2), which happened approximately every 7 days (Klee et al. 2004). For use in assays cells were previously trypsinized (0.05% trypsin/0.02% EDTA) and rinsed with PBS (Kosmehl et al. 2004).

2.4 Micronucleus assay

The micronucleus assay was performed according to the methods given by Rocha et al. (2009) and Böttcher et al (Boettcher et al. In prep). RTL-W1 cells were transferred to 6-well plates (TPP, Trasadingen, Swizerland) with cover slips placed on the bottom and incubated for 12 hours, when they became attached to the cover slips. Cells were exposed to 6 step dilutions of the acetonic extract for 72 hours. Highest concentration was defined through cytotoxicity test, as the concentration inducing 20% mortality after 48-h exposure. Exposure was stopped with addition of methanol and acetonic acid at a 4:1 mixture and cover slips were left to air dry.

The micronuclei were scored under light fluorescence microcopy using DNA staining solution Acridine Orange and oil-immersion lens.

Micronuclei were identified according to the following criteria: a) color and texture similar to main nucleus; b) diameter not more than 1/3 of the main nucleus; c) absence of contact with main nucleus; d) same bidimensional plan as the main nucleus (Titenko-Holland et al. 1998). Micronuclei were expressed as frequency in 2000 cells and the effect was compared to the correspondent frequency caused by of negative control (only medium) and a positive control (190.0 μmol NQO – 4 Nitroquinoline 1-oxyde). In order to validate the test, negative control should have a micronucleus frequency of less than 3%, while positive control, more than 3% (Reifferscheid et al. 2008).

2.5 Alkaline Comet assay

Comet assay was done under alkaline conditions, according to Singh et al. (1988) modified by Schnurstein and Braunbeck (2001). For Positive Control, cells were left under UV light (320 nm) for either 4 or 5 min, using a TM-36 Transilluminator (4 x 15 watts; Herolab, Wiesloch, Germany). Negative Control was yield with L-15 medium.

Image processing was done at 340 x magnification, in fluorescence microscope (Aristoplan, Leica, Germany) equipped with 518 nm filter and image analysis system (Optilas, Munich, Germany) with grey-scale CCD camera (JAI Pulnix TM-765E Kinetic, Glostrup, Denmark). Tail moment (i.e. fluorescence intensity in the tail and the tail length in relation to "comet head") was measured by means of Komet 3.0 software (Kinetic Images, Liverpool, UK). For each concentration, 100 randomly selected cells were scored. Kruskal-Wallis One Way Analysis of Variance on Ranks (ANOVA on Ranks) was performed and a *post-hoc* test (Dunn's) was used to identify groups with significant difference from negative control (SigmaStat 3.1; Systat). The Induction Factor (IF) was calculated by dividing the median of each concentration by the median of the corresponding negative control (Kosmehl et al. 2006).

2.6 Ames Fluctuation test

Ames Fluctuation test is an adaptation of the classic Ames test (Maron & Ames 1983), modified by Reifferscheid et al. (2005). Test bacteria are exposed to testing substance and incubated in 384-well microtitre plate in reversion indicator medium containing bromocresol purple as pH indic-

ator of sugar conversion (Bridges 1980). Two bacteria strains were used: TA98 (frameshift mutation) and TA100 (base pair substitution), both with and without S9 fraction addition (rat liver homogenate S9-fraction from phenobarbital/ß-naphthoflavon-treated mice, RCC Rossdorf, Germany), aiming the evaluation of metabolic activation. A more detailed description of the method can be found elsewhere (Bridges 1980; Maron & Ames 1983; Kosmehl et al. 2004; Wölz et al. 2009).

In brief, bacteria were suspended in medium (Oxid Nutrient Broth No. 2 + ampicilin) and let grow overnight in a shaking bath at 37 ºC. Density was measured by spectrophotometer and adjusted to 1,800 FAU (Formazine Attenuation Units) for TA98 and 450 FAU for TA100. Test was done in 48 wells per replicate (positive and negative controls plus sample dilutions), and 48-hour incubation, at 37 ºC. Positive controls were 4-nitro-*o*-phenylenediamine (20 nM per well) for TA 98 strain without S9, nitrofurantoin (1.67 nM per well) for TA 100 without S9 and 2-aminoanthracene for TA 98 and TA 100 with S9 treatment (0.87 nM per well). DMSO was used as negative control at 2% concentration. Tests were valid when mean values of spontaneous revertants in negative controls (counted in well basis) were from 0 to 5 per 48 wells (TA 98) and from 0 to 10 per 48 wells (TA 100) at all testing conditions with both strains, with and without S9. Positive controls were valid when of revertants were equal or greater than 25 per 48 wells as mean values for both bacterial strains with and without S9 addition (Wölz et al. 2009). Mutagenic activity was considered statistically significant when $p < 0.05$ after Fisher's Exact Binomial test.

We calculated maximum Induction Factor (IF_{max}), by dividing mean maximum effect by the corresponding concentration of sediment that caused observed effect (Wölz et al. 2009). This allows direct comparison of genotoxic potentials of samples from different sites (P. S. Rocha et al. 2009).

2.7 Concentration-dependent Induction Factor (CDI)

The CDI was developed as an index aiming the integration of important information regarding genotoxicity potential in the comet assay, providing a basis for general comparison (Seitz et al. 2008). It is calculated by integrating all concentrations and the respective induction factors, as following.

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

where IF*i* = Induction Factor at concentration *i*; c*i* = concentration *i*; n = concentrations

Although CDI was developed for comet assay, we calculated it also for Ames Fluctuation test, in order to yield one unique comparison index.

2.8 Ethoxyresorufin-*O*-deethylase (EROD) activity

Induction of 7-ethoxyresorufin-*O*-deethylase (EROD) was tested using RTL-W1 cells, a CYP1A-expressing cell line (L. E. J. Lee et al. 1993) according to the method of Gustavsson et al. (2004) with the modifications given by Keiter et al. (2008). RTL-W1 cells were suspended in medium, placed in 96-well plates (TPP, Trasadingen, Swizerland) and incubated for 72 h for cell confluence. After that, cells were exposed to 8 extract step dilutions and incubated again for 72 h. Negative and positive controls were set with DMSO and TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), respectively. The latter was serially diluted until a final range concentration of 3.13 to 100 pM TCDD (TCDD, Promochem, Wesel, Germany). Exposure ended with cell lyse through freeze shock at -80 ^{o}C. Production of resorufin was measured in fluorescence GENios plate reader (Tecan, Crailsheim, Germany) at excitation at 544 nm, emission at 590 nm, after 10 min; protein fluorescence was measured at 355 nm excitation and 590 nm emission.

Highest tested concentration was set to previously established NR_{80}, meaning the concentration that caused 20% effect on RTL-W1 cells through cytotoxicity test, having neutral red retention as endpoint.

Concentration-response curves for EROD induction were drawn using GraphPad Prim 4 (GraphPad, San Diego, USA) aided dose-response curve to yield $EC_{25\ TCDD}$. This value is related to the maximum response concentration in the TCDD standard curve (Brack et al. 2000). Sample concentration was given on mg equivalent/ml basis, and can be understood as the amount of dry sediment extracted in the final test medium that caused a certain EROD induction.

The enzyme-inducing potentials were converted into Bio-TEQ (toxicity equivalent values relative to 2,3,7,8 TCDD), according to the following formula (Engwall et al. 1999):

$$BioTeq(pg/g) = \frac{TCDD\,EC_{25}(pg/mL)}{sample\,extract\,EC_{25\,TCDD}(g/mL)}$$

We used mean values of each sample out of 3 independent replicates (n=3) and mean $EC_{25\ TCDD}$ value derived from 24 independent replicates (plates). Such normalization to an equivalent factor of positive control is a useful tool for comparison, since it accounts the variability of individual experiments with primary cell cultures and resumes them into a single number with the same comparison pattern (Engwall et al. 1999; Brack et al. 2000; Hollert et al. 2005)

2.9 Chemical Analysis

PAHs in the sediment extracts were quantified by GC–MS analysis according to the method given by Rocha et al. (2010). We included EPA priority PHAs: 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.

Sample extracts were first subjected to clean-up using Florisils (Merck, Darmstadt, Germany) and, as an eluent, iso-octane:toluene 95:5 (v/v); occasionally, the clean-up was performed twice. Resulting samples were then 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 (30 m x 0.25 mm, film thickness: 0.25 mm; Machery-Nagel, Düren,Germany). Reference compounds (16 EPA priority pollutants mixture) were provided by Promochem (Wesel, Germany).

PAH-TEQs, which account for the EROD- inducing potencies of analyzed PAHs relative to TCDD, were calculated. We used the relative equivalency potency values (REPs) given by Bols et al. (1999). Each resulting PAH concentration was multiplied by the corresponding TCDD-related toxic equivalency factor (TEF), synonym of equivalency potency values (REPs), in order to estimate PAH-TEQ. A direct comparison between PAH-TEQ and the corresponding Bio-TEQ values allows an elucidation of the percentage of measured PAH responsible for the induction in EROD assay in tested extracts.

We also compared PAH concentration with Interim Sediment Quality Guidelines (ISQG) suggested by CCME (2002) and consensus-based Threshold Effect Concentration (TEC) suggested by MacDonald et al. (2000).

3 Results

3.1 Comet Assay

The results from comet assay are presented in Figure 34. The test shows genotoxic effects of sediment sample from Rio Grande and Cubatao de Cima. For Cubatao de Cima, an effect can be noticed with a minimum of 16.0 mg sediment equivalent/ mL medium; for Rio Grande, the lower concentration with effect was 0.873 mg sediment equivalent/ mL medium. Mean IF_{max} was 2.12 for Rio Grande sample and 1.925 for Cubatao de Cima.

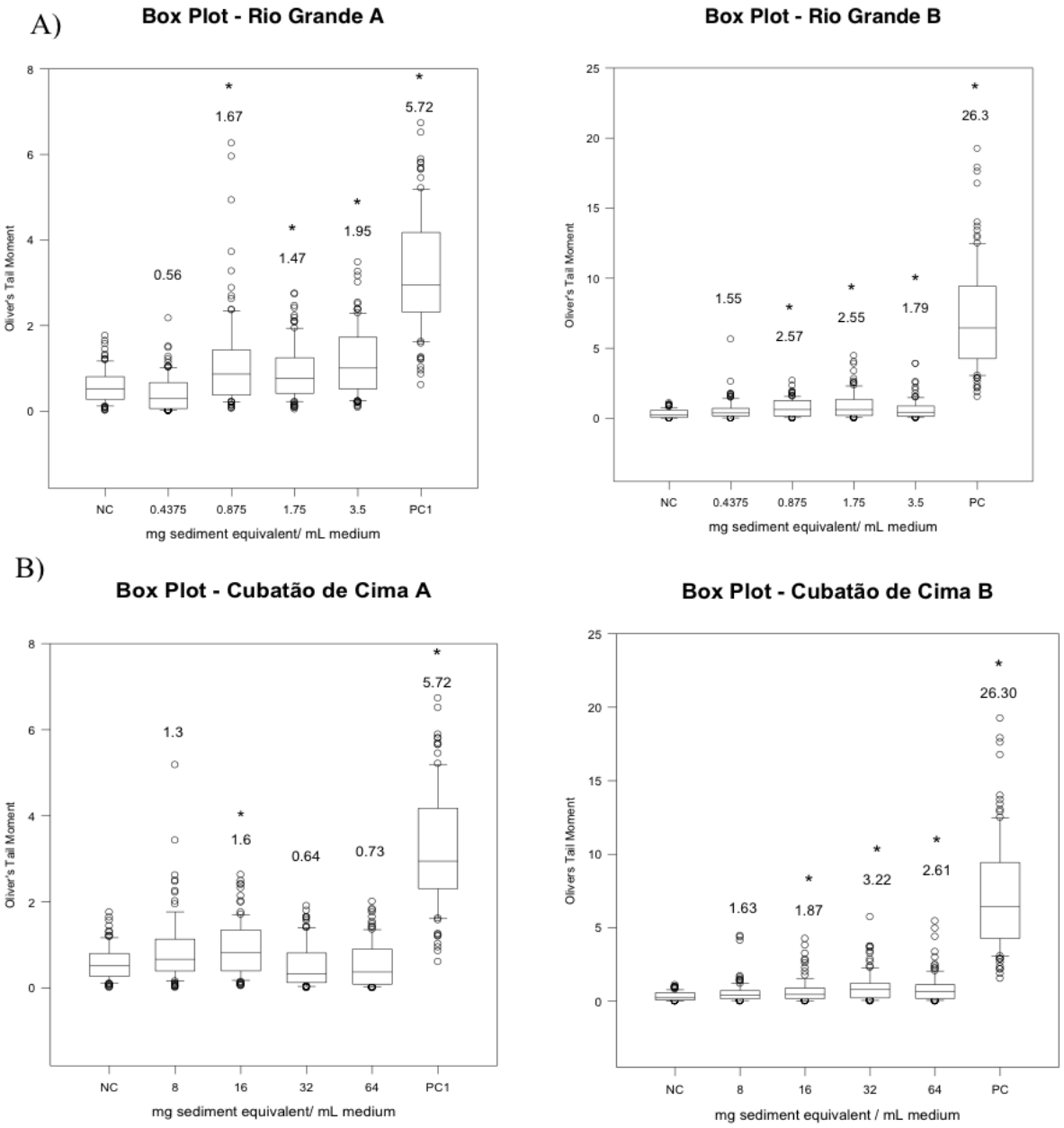

Figure 33. Genotoxic effects induced by acetonic extracts from Rio Grande and Cubatao de Cima sediments in the comet assay with RTL-W1 cells. Data are given as box plots of tail moments (n=100 on two replicates) and induction factors (data given above the boxes). Significant difference against negative control (ANOVA on ranks followed by post-hoc test according to Dunn, $p<0.05$)is indicated with (*) above the IF. NC = Negative Control and PC = Positive Control. Tests were carried out in 2 different days and given the letter A and B for identification. A) Rio Grande sediment sample; B) Cubatao de Cima sample.

3.2 Micronucleus Assay

Results from Micronucleus assay are shown in Figure 34. T-test analysis revealed no significant difference between individual tested concentration and negative control. Validation of test was compromised, because micronucleated cells in negative control exceeded 3% frequency.

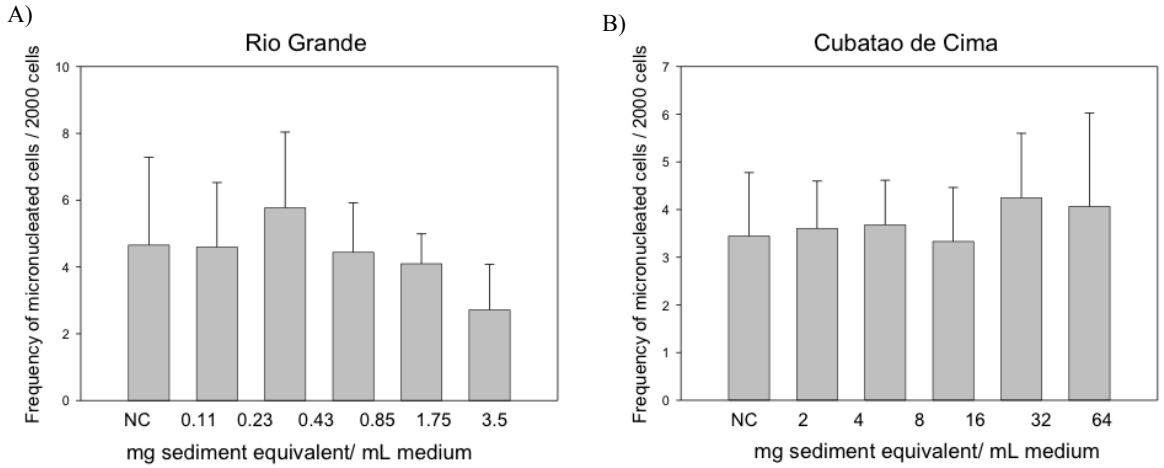

Figure 34. Frequency of micronucleated cells scored for 2000 RTL-W1 cells exposed to 6 step dilutions of sediment extract. Bars represent mean value and standard deviation from 4 test replicates. Highest concentrations were determined by Cytotoxicity tests for Rio Grande and Cubatao de Cima extracts. T-test reveled no significant difference between MN frequency of each individual test concentration and Negative Control (NC). A) MN frequency for Rio Grande sediment extract; B) MN frequency for Cubatao de Cima sediment extract.

3.3 EROD

Results from EROD are presented in Figure 35 to Figure 37. Mean calculated EC_{25} for Cubatao de Cima sediment extract was 10.7± 6.8 mg/mL. For Rio Grade reservoir, mean calculated EC_{25} was 0.87 mean calculated EC_{25} was 0.87 ± 0.11 mg/mL, showing approximately 10 times higher dioxin-like activity when compared to Cubatao de Cima. No EROD effect was observed in Procedure Control, hence no EC_{25} was calculated for this sample.

Mean TCDD EC_{25} was 5.14 pM, derived from 24 single measurements (plates). BioTEQ from Rio Grande was 1,884.18 pg Bio-TEQ/g sediment and Bio-TEQ from Cubatao de Cima was 155.22 Bio-TEQ/g sediment.

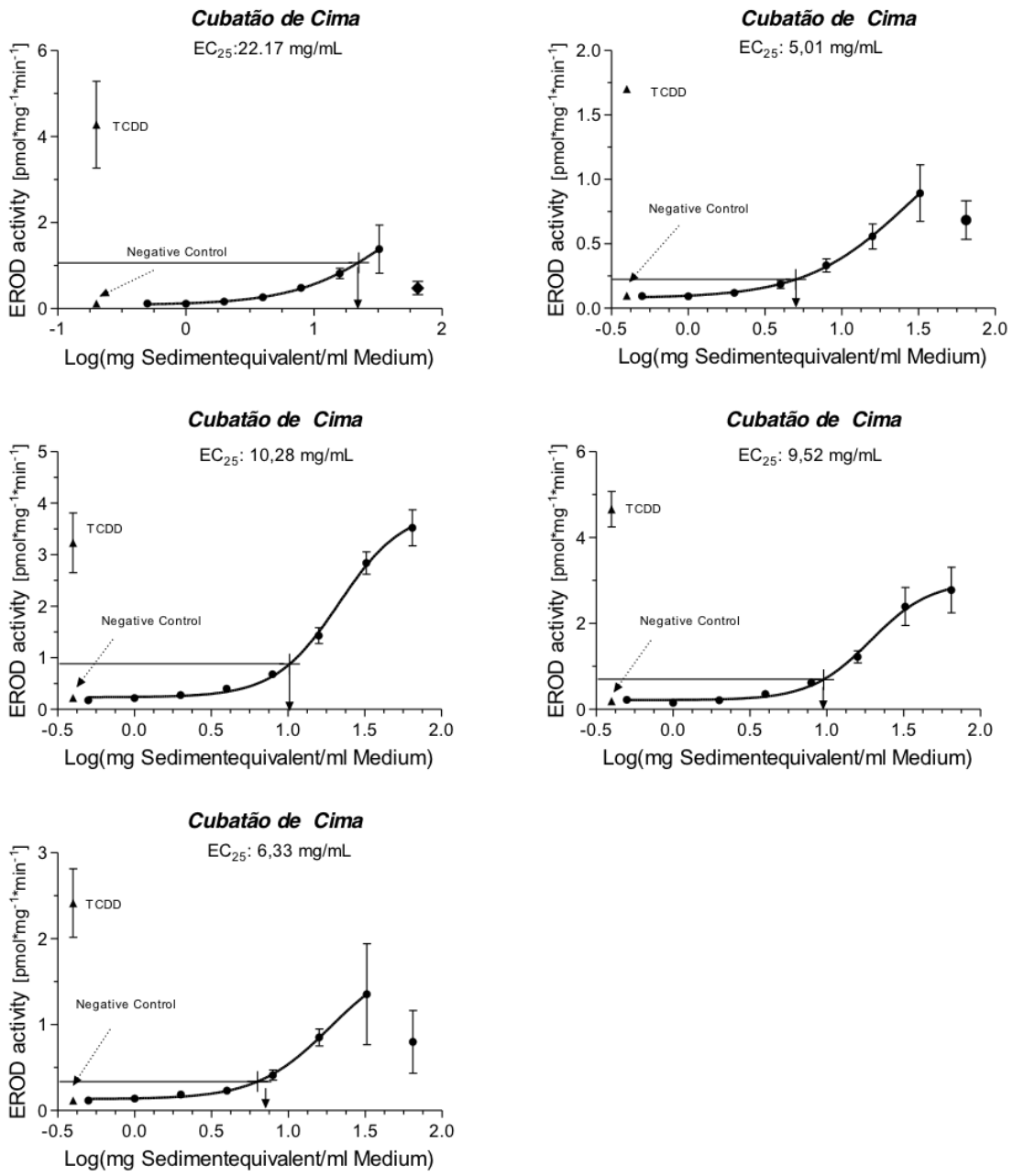

Figure 35. EROD induction potential of sediment samples from Cubatao de Cima in RTL-W1 cells. Data are given as mean EC_{25TCDD} (concentration of each sample which caused 25% of TCDD-induced maximum EROD activity) values from 5 independent measurements. Dashed lines on the diagrams indicate the intersections 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.

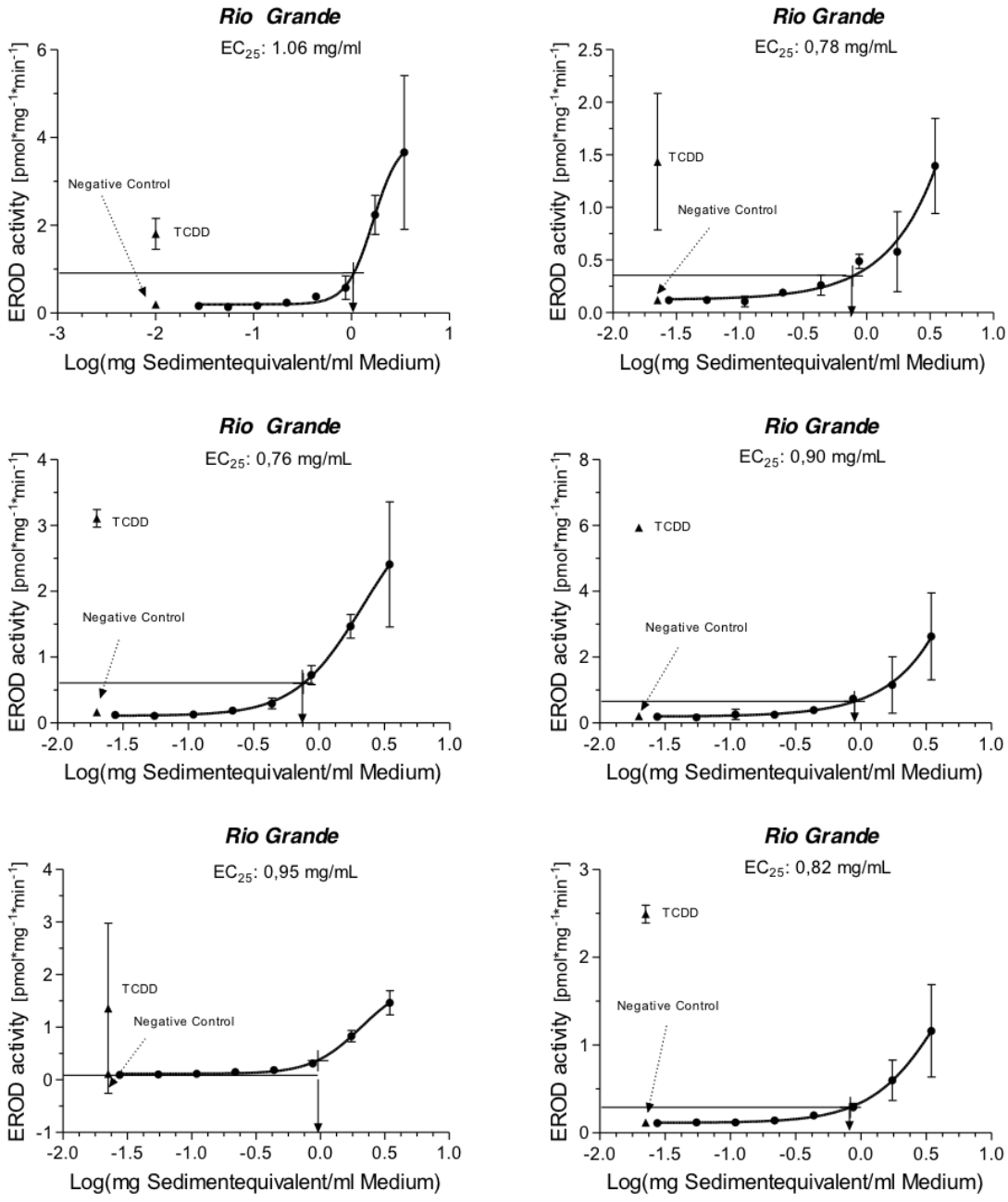

Figure 36. EROD induction potential of sediment samples from Rio Grande reservoir in RTL-W1 cells. Data are given as mean EC_{25TCDD} (concentration of each sample which caused 25% of TCDD-induced maximum EROD activity) values from 6 independent measurements. Dashed lines on the diagrams indicate the 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.

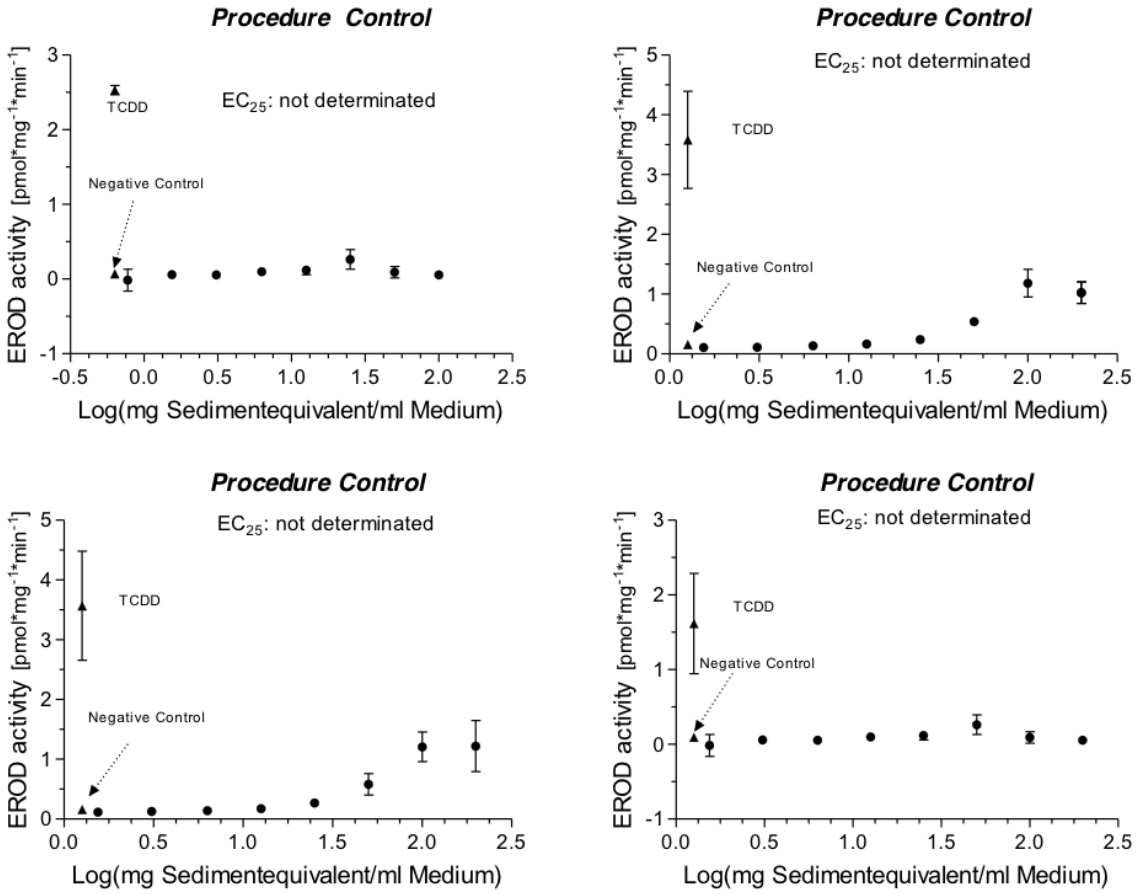

Figure 37. EROD induction potential of the procedure control in RTL-W1 cells. EC_{25} was not determined. Data are given as mean EC_{25TCDD} (concentration of each sample which caused 25% of TCDD-induced maximum EROD activity) values from 4 independent measurements. Dashed lines on the diagrams indicate 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.

3.4 Ames Fluctuation test

Results from Ames Fluctuation test using the tester strains TA98 and TA100 (with and without S9 addition) are presented in Figure 38 (Rio Grande reservoir sediment extract) and in Figure 39 (Cubatao de Cima sediment extract). Table 20 presents IF_{max} calculated for the two bacteria tester strains, with and without S9 addition, for both sediment sample extracts.

For Rio Grande sediment sample, TA98 bacteria strain showed a clear cut increase in the revertants, especially with addition of S9 fraction, indicating the presence of transformation-dependent toxicity. Even the lowest concentration (0.3 mg SEQ/ml) presented was significantly different

from negative control, when S9 was added; IF_{max} was up to 10 (11.5 - Table 20) with revertant number comparable to positive control, indicating a very strong mutagenic effect. Lowest concentration with induced mutation in TA98 without S9 addition was 1.3 mg SEQ/mL. On the other hand, IF_{max} was higher to TA98 without S9 addition.

For TA100, lowest concentration that induced increase of mutations was 0.6 mg SEQ/mL. No tested concentration of Rio Grande sediment sample induced significant alterations of mutation on TA100 without S9 addition.

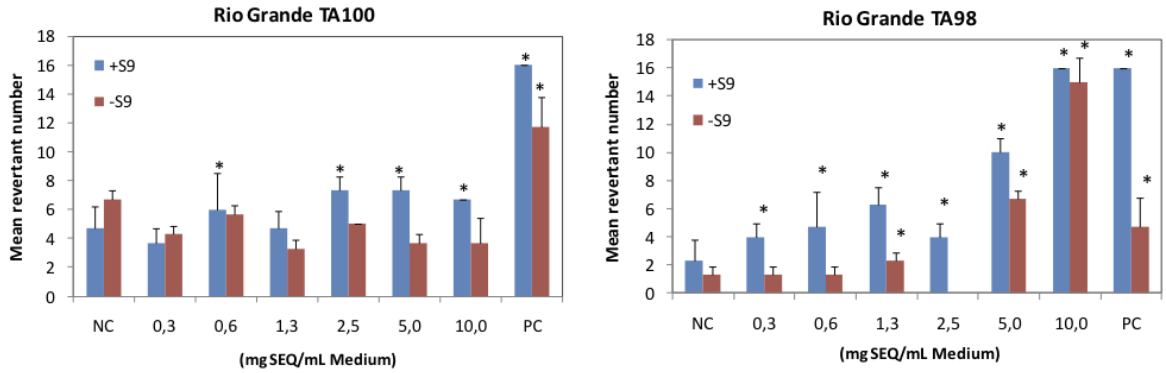

Figure 38. Results from the Ames Fluctuation test after exposure to Rio Grande sediment samples. TA100 and TA98 bacteria tester strains, with and without S9 fraction. Concentrations marked with (*) are significantly different from negative control.

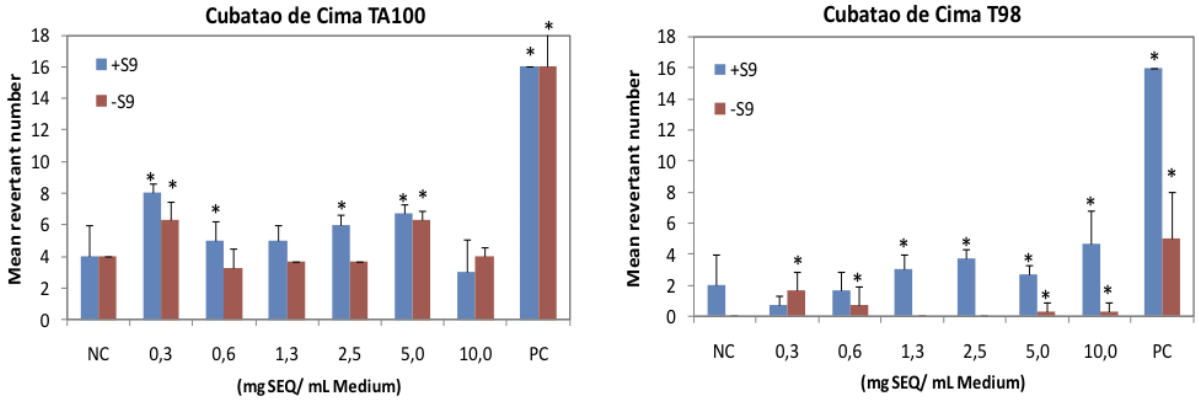

Figure 39. Results from the Ames Fluctuation test after exposure to Cubatao de Cima sediment samples. TA100 and TA98 bacteria tester strains, with and without S9 fraction. Concentrations marked with (*) are significantly different from negative control.

Mutation induction in response exposure to Cubatao de Cima sediment extract was somewhat inverse to Rio Grande. Lowest significant induction was observed without S9 addition for the TA98 tester strain (0.3 mg SEQ/mL); however, IF_{max} was higher with S9 addition for both bacteria strains.

In contrast to Rio Grande sediment sample, Cubatao de Cima sediment extract induced mutation on TA100 tester strain at a concentration as low as 0.3 mg SEQ/mL. (with and without S9) addition.

Table 20. Maximum Induction Factors (IF_{max}) results from the Ames Fluctuation test after exposure to sediment samples from Rio Grande and Cubatao de Cima. TA98 and TA100 bacteria tester strain, with and without S9 fraction.

	TA100 +S9	*TA100 -S9*	*TA98 +S9*	*TA98 -S9*
Rio Grande	1.6	0.9	7.0	11.5
Cubatao de Cima	2.0	1.6	2.4	1.7

Both for Rio Grande and Cubatao de Cima sediment samples, TA98 showed greater IF_{max}, meaning that this bacterium strain was more sensitive to mutagenic substances in the extract. In general, addition of S9 fraction enhanced toxic response of sediment extract, indicating the presence of transformation-dependent mutagenic substances in the sediment extract from both samples.

3.5 Concentration-Dependent Induction Factor (CDI)

Aiming an integrated comparison of valid genotoxicity assays (comet and Ames Fluctuation tests), we calculated CDI for those tests, as shown in Figure 36.

Highest CDI was observed for Rio Grande sediment extract in the Ames Fluctuation test using TA98+S9 (13.28). Lowest CDI was observed for Cubatao de Cima extract, comet assay A (0.30). In general, Rio Grande sediment extract showed higher CDI in comparison to Cubatao de Cima sample, apart from Ames Fluctuation test using TA100 bacteria tester strain.

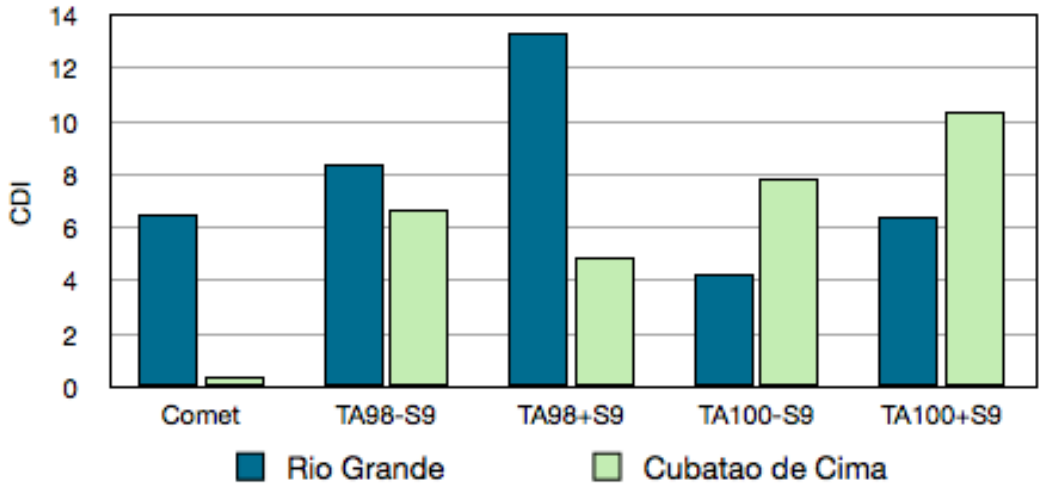

Figure 40. CDI calculated for sediments from the Rio Grande and Cubatao de Cima in the comet assay (mean value; n=2) and Ames Fluctuation test (mean value; n=3; TA98 and TA100 bacteria tester strains, with and without S9).

3.6 Priority PAH concentration, Bio-TEQ and PAH-TEQ

Table 21 presents the results from PAHs measurements in sediment extracts of Rio Grande and Cubatao de Cima compared to reference values suggested for sediment quality guidelines. It also presents calculated REFs (Relative Equivalency Potentials), PAH-TEQ, Bio-TEQ and the percentage of Bio-TEQ related to PAH-TEQ.

Some of PAHs measured in Rio Grande sediment extract were above ISQG values (Interim Sediment Quality Guidelines) suggested by CCME (2002) – Canadian Council of Ministers of the Environment, meaning those PAHs are in such concentration that that cause probable adverse effect on biota. Those were naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene and anthracene. On the other hand, these specific PAHs were not identified as EROD inducers by Bols et al. (1999); EROD-inducer PAHs were found in low concentration in sediment extract from Rio Grande. Therefore, 4.64% of observed EROD effect, calculated as Bio-TEQ, could be explained by PAHs, calculated by PAH-TEQ. Total analyzed PAH was 763.65 µg/kg SEQ, bellow consensus-based threshold effect concentration (TEC), suggested by MacDonald et al. (2000).

For Cubatao de Cima, no PAH were detected in concentrations above respective Interim Sediment Quality Guideline (ISQG) from CCME (2002); total target PAH concentration was 123.74 µg/kg SEQ and percentage of PAH-TEQ in relation to Bio-TEQ was 9.9 %.

Table 21. Concentration of chemically analyzed priority EPA-PAHs in sediment extracts from Rio Grande and Cubatao de Cima (GC-MS analysis), as well as respective calculated TEQs (Bio-TEQ and PAH-TEQ) and contribution of PAHs (percentage) to the EROD induction in each sediment extract. PAH data are given in µg/kg SEQ.

		Consensus based				*Rio Grande*		*Cubatao de Cima*	
	unity	*TEF**	*TEC***	*ISQG****	*PEL****	*PAH*	*REF*****	*PAH*	*REF*****
Naphthalene	µg/kg	NI	176	34.6	391	74.126	-	12.13	-
Acenaphthylene	µg/kg	NI		5.87	128	12.91	-	ND	-
Acenaphthene	µg/kg	NI		6.71	88.9	17.00	-	5.61	-
Fluorene	µg/kg	NI	77.4	21.2	144	25.34	-	7.05	-
Phenanthrene	µg/kg	NI	204	41.9	515	104.26	-	20.18	-
Anthracene	µg/kg	NI	57.4	46.9	245	16.48	-	7.96	-
Fluoranthene	µg/kg	NI	423	113	1494	88.70	-	11.72	-
Pyrene	µg/kg	NI	195	153	1398	84.55	-	12.44	-
Benz(a)anthracene	µg/kg	0.000043	108	74.8	693	36.72	0.0015	8.57	0.0003
Chrysen	µg/kg	0.000047	166	108	846	68.86	0.0034	7.90	0.0004
Benzo(b)fluoranthene	µg/kg	0.000193				92.79	0.0965	9.42	0.0098
Benzo(k)fluoranthene	µg/kg	0.001039				31.68	0.0060	8.93	0.0017
Benzo(a)pyrene	µg/kg	0.000302	150	88.8	763	47.87	0.0144	11.78	0.0035
Dibenz[a. h]anthracene	µg/kg	0.000350	33	6.22	135	ND		ND	
Benzo(ghi)perylene	µg/kg	NCI				ND		ND	
Indo('1.2.3-cd)pyrene	µg/kg	0.000278				62.3102	0.0173	ND	
Total PAH	µg/kg		1610			763.64		123.74	
PAH-TEQ	pg/g						87.42		15.4
BioTEQ	pg/g						1888.18		155.2
Percentage	%						4.64		9.92

* TEF = Toxic Equivalent Factor relative to TCDD – Bols et al. (1999).
** MacDonald et al. (2000). TEC = Threshold Effect Concentration.
*** ISQG = Interim Sediment Quality Guidelines ; PEL = Probable Effect Level (CCME 2002)
**** REF = Relative Equivalency Potencies (PAH x TEF) - Bols et al. (1999).
Shaded cells indicate concentration above ISQG values; ND = not detectable;
NI = no induction (PAHs without EROD induction potency); NCI = no constant induction (PAHs with inconstant EROD induction).

Figure 33 presents comparison of results from Bio-TEQ (biological response in EROD) and PAH-TEQ (calculated by multiplying compound concentration and related TEF).

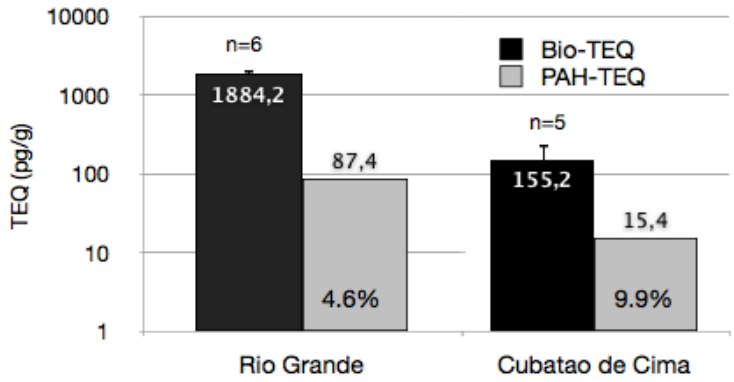

Figure 41. Comparison of the total biological response in the EROD assay (Bio-TEQs) and PAH-TEQs (calculated by multiplying compounds concentrations and relative equivalency potencies) and the calculated contribution of PAH-TEQ to the EROD induction (in percent) in each sediment extract. Error bars = standard deviation ; n = number of independent replicates.

4 Discussion

Sediment Quality Guidelines have been reviewed many times, since new information and additional endpoints allow a more refined evaluation of such values. Furthermore, different governmental institutions around the globe have provided their own regulatory values, in order to better adjustment of national particularities, such as climate conditions and occurring aquatic species. Hence, a great range of values are now available, most of them developed for temperate regions. According to Burton (2002), total PAHs threshold effect values may vary from 870 to 4090 µg/kg. MacDonald et al. (2000) made an effort towards a consensus-based sediment quality guideline, and suggested 1610 µg/kg as maximum value for total PAHs in sediment. In the present work, total target PAH in Rio Grande sediment extract was 763.64 µg/kg, which is bellow suggested threshold effect concentration, though not low, especially considering that only EPA target PAH were analyzed. Total target PAH in Rio Grande is of the same order of magnitude as previous sediment studies: the Environmental Company of Sao Paulo, Brazil - Cetesb registered a concentration of 445 and 281µg/kg in 2005 and 2006, respectively, in sediment samples this area (CETESB 2007; CETESB 2006).

Rocha et al. (2009; 2010) studied sediment collected along Tiete river, including a site close to its spring and Billings reservoir. Some of their results are presented in Table 22 for comparison purposes. The sampling site close to Tietê spring (used as reference site) pointed out better sediment quality and lower toxic potential compared Billings and Rio Grande sediment samples. Cubatao de Cima (reference site for the present work) showed very similar BioTEQ value compared to sediment from near Tiete spring, and lower total PAHs.

Total analyzed PAH concentration in Billings sediment extract were lower than Rio Grande's; one would expect the contrary to happen, since Billings receives direct influence from Pinheiros river, hence a much greater load of potential hazard effluent coming from Sao Paulo city. To illustrate Pinheiros river sediment hazard potential, calculated Bio-TEQ through EROD assays was as high as 24,169.73 pg/g (P. S. Rocha et al. 2010). Despite its higher total analyzed PAH concentration and PAH-TEQ, EROD induction of sediment from Rio Grande was lower than sediment from Billings: calculated Bio-TEQ for Billings was about 2.5 times greater. This difference is due to the fact that sediment from Rio Grande contains mostly non-EROD inducing PAHs (see Table 22), and to a possible greater concentration of other EROD-inducer substances rather than target PAH in sediment from Billings reservoir (P. S. Rocha et al. 2010).

Bio-TEQ from sediment extract of Rio Grande was also lower than those observed for suspended particulate matter (SPM) tested in RTL-W1 cells during flood events of Neckar river (8,341 pg/g) and for long-term SPM of Rhine river 3,693 pg/g (Wölz et al. 2008). Sediment from those two rivers in Southwest Germany contain chlorinated hydrocarbons such as PCBs, PCDDs and PCDFs. Other studies with sediments from Neckar river found that less than 6% of Bio-TEQ was explainable by PAH-TEQ (Hollert et al. 2002); while for sediment of upper river Danube, a maximum of 50% of BioTEQ could be explained by PCBs, PAHs and PCDD/F together (Keiter et al. 2008).

Table 22. Results of PAH measurement, EROD and comet assays from this study (Rio Grande / Cubatao de Cima sediment samples) and other studies (Tiete river Spring / Billings sediment samples).

	unity	*Rio Grande*	*Cubatao de Cima*	*Tiete Spring*	*Billings*
PAH and EROD					
total PAH	µg/kg	763.65	123.74	204.27 (₮)	627.56 (₮)
PAH-TEQ	pg/g	87.42	15.4	91 (₮)	72.03 (₮)
Bio-TEQ	pg/g	1884.18	155.2	158.79 (₮)	5077.73 (₮)
%	-	4.64	9.92	5.73 (₮)	1.42 (₮)
Comet Assay					
Lowest concentration with significant effect	mg SEQ/mL	0.875	16	> 100 (※)	1.5 (※)
IFmax	-	2.12	1.925	0.01 (※)	0.9 (※)
CDI*	-	6.5	0.37	0.14 (※)	4.32 (※)

***** CDI and IF_{max} Comet for Rio Grande and Cubatao de Cima samples are expressed as mean value from 2 replicates
References: ₮Rocha et al. (2010) ; ※ Rocha et al. (2009)

Oxidative stress and CYP450 activity in tilapia (*Oerochromis niloticus*) were studied in Billings reservoir by Bainy et al. (1999; 1996), who found levels of hepatic microsomal EROD and MROD as high as 20 times reference site. Unusual levels of total microsomal cytochrome P450 in tilapia collected in Billings Complex in November/1995 were associated with high PCB concentration found in fish tissue, leading to oxidative stress (Bainy et al. 1996). Araújo et al. (2006) carried out a TIE (Toxicity Identification Evaluation) in Rasgao reservoir, one of Tiete cascade reservoirs located down stream Billings Complex, and related PCBs to mutagenotoxicity on *Salmonella/*microsome assay.

CYP450 activity can be induced not only by priority PAHs, but also by non-priority PAHs, PCBs, TCDD and TCDF. PAH-TEQ (of the measured priority PAHs) represent 4.64% of Bio-TEQ from sediment extract of Rio Grande reservoir, what indicate that other aryl hydrocarbon receptor agonists rather than PAHs are responsible for most of observed EROD inductions. PCBs were already linked to toxic effect on biota along Tiete river, so further studies are required in order to elucidate the role of PCBs to potential hazard in sediment from Rio Grande reservoir. A possible ap-

proach towards better characterization of toxicity from organic compounds is sediment extract fractionation followed by further EROD-activity testing (Brack et al. 2005; Brack et al. 2008; Wölz et al. 2008).

Table 22 also presents a summary of results from comet assays carried out with sediment extracts of Rio Grande, Cubatao de Cima (this work), Tiete river spring and Billings (P. S. Rocha et al. 2009). Calculated indexes show greater expression of comet in Rio Grande sediment extracts, thus greater genotoxic potential among all samples, followed by Billings. Cubatao de Cima showed higher genoxotic potential than the site near Tiete spring, but still much lower than Rio Grande or Billings sediment samples. As already mentioned, Billings reservoir receives direct influence from Pinheiros river, which in turn receives wastewater from Sao Paulo city. Treated sludge samples from two Sludge Treatment Plants in Sao Paulo city have been proven to induce genotoxicity to Clone #4430 and *Tradescantia pallida* (Trad-MN assay) in experiments with 3 months exposure in the field; those Plants receive urban and industrial effluent from São Paulo metropolitan region, including from chemical industries and dye factories (Mielli et al. 2009). Hence, one would expect sediment extract from Billings to be more genotoxic than from Rio Grande, what was not true.

Klee et al. (2004) studied untreated sludges from a wastewater treatment plant in Sweden that received sludge from industries such as pharmaceuticals, chemical intermediates and explosives. They performed Comet assays on RTL-W1 cells and found an IF_{max} as high as 2.7, which is comparable to IF_{max} found in sediment extract from Rio Grande reservoir (this work). These authors also tested mutagenicity through classic Ames test. Results from untreated sludge reveled IF_{max} ranging from 37 to 45 (TA98+S9) and from 28 to 36 (TA98-S9). Pérez et al. (2003) carried out Ames Fluctuation test on sludge samples and found no reverse mutation without S9 metabolic activation; on the other hand, TA98 strain tested with S9 addition showed good relationship with PAH concentration measured in sludge.

At the present work, TA98 was more sensitive than TA100; for sediment extract from Rio Grande, higher CDI was observed in test using TA98 with S9 mix; on the other hand, higher IF_{max} was observed in test using TA98 without S9 fraction. Cardozo et al. (2006), when studying effect of river water samples from South Brazil on *Salmonella*/microsome test, also reported higher sensitivity of TA98 bacteria tester strain and higher Mutagenesis Index without S9 mix.

Cubatao de Cima samples showed higher mutagenic potentials in TA100 tests compared to Rio Grande, both with and without S9 mix. This indicate a difference in mutagenic mode of action,

hence differences in mutagenic components within sediment extracts, since TA100 bacteria tester strain have base-pair mutation, while TA98 have frameshift mutation.

Classic Ames test using TA98-S9 reveled acute toxicity in the water from Rio Grande reservoir on July/2006 and 2007 (CETESB 2008; CETESB 2007). From 2004 to 2007, water samples from Rio Grande showed chronic toxicity in 10.3% of tested samples, and acute toxicity in 13.8% of them, many of which correlated well with high levels of cooper, according to monitoring program of Sao Paulo State Government (CETESB 2008). Mariani et al. (see Cap. 3) have found high metal concentration in sediment from Rio Grande; they have observed temporal heterogenicity regarding metals and indicated possible metal remobilization into the water. On the other hand, Mariani et al. (see Cap. 4) have measured metals in dechorionated embryos exposed to whole bulk sediment from Rio Grande and could not detect metal uptake by fish, what suggest that metals were not priority cause of toxicity observed in fish embryo test. Vargas et al. (2001) studied fractionated sediment from Sinos river basin (South Brazil), and concluded that non-polar to medium-polar fractions were responsible to observed genotoxicity effect, while polar fraction to cytotoxicty. The possibility of polar fractions to participate on observed negative effect of Rio Grande sediment extract should not be completely discharged, since uptake of metals have been registered by fish embryos exposed to sediment acetonic extracts (Zielke 2007).

As reference site, Cubatao de Cima was acceptable: genotoxic (comet assay) and EROD induction potentials were similar to site near Tiete spring; higher mutagenic potential was registered for Rio Grande sediment sample than for Cubatao de Cima using TA98 bacteria tester strain. On the other hand, Ames Fluctuation test with TA100 showed higher mutagenic potential for Cubatao de Cima than for Rio Grande sediment sample, both with and without S9. However, IF_{max} of 1.6 and 2.0 does not account for high mutagenic potentials, what indicate low mutagenic induction on TA100 bacteria tester strain for both sediment samples.

Micronucleated cell frequency with tested concentrations of sediment extract from Rio Grande and Cubatao de Cima were not significant different from negative control; besides, micronucleus in negative controls were above 3%. A probable systematic error is the most feasible cause of such failure, since many studies have proven micronucleus test to be a suitable endpoint for identification of mutagenic substances in environmental samples (Al-Sabti & Metcalfe 1995; Cardozo et al. 2006; Chen & White 2004; Giller et al. 1995; Grisolia & Starling 2001; P. S. Rocha et al. 2009; Tates et al. 1980; Villela et al. 2007; Ali et al. 2008).

Sediment from Rio Grande reservoir is a complex chemical mixture, what makes ecotoxicological results more difficult to interpret; confirmation of the suspected toxicants as contributing to the effect of the occurring mixture is not straightforward task, due to chemical interactions (such as concentration addition, independent action, and simple interaction) or presence of unidentified chemicals (either emerging or not considered for analysis) (Donnelly et al. 2004; Chapman 2006; Rolf Altenburger et al. 2004; Belden, Gilliom & Lydy 2007). In fact, many researchers have demonstrated that presence of multiple toxicants generally results in greater toxicity than individual components (Rolf Altenburger et al. 2003; Rolf Altenburger et al. 2004; Belden, Gilliom, Martin et al. 2007).

Nevertheless, direct observations from laboratory experiments with complex mixtures are necessary to completely understand the potential joint action and interactions for specific mixtures that have not been previously studied. Although numerous toxicity studies have been performed with complex mixtures, the amount of data available is small compared to the large number of mixtures that occur in the environment (Belden, Gilliom & Lydy 2007).

In the case of Rio Grande sediment, we could report high genotoxic, mutatoxic and EROD induction potentials, though neither target PAHs or heavy metals could be directly linked to observed effects. A great range of chemicals have so far been identified as mutagenotoxic: heavy metals, PAHs, heterocyclic amines, pesticides (Ohe et al. 2004; Chen & White 2004). For EROD induction, PAHs, PCBs and TCDDs are recognized as aAhR agonists (Gustavsson et al. 2004; Brack et al. 2005). Further studies should aim evaluation of community endpoints, long-term exposure, and no-observed-effect levels. Also, techniques with decreased complexity of the mixtures, such as fractionation, should be applied to consider reducing number of components within the identified mixtures as well as identifying a few specific combinations that constitute the majority of mixtures within the sample (Brack et al. 2008; Brack et al. 2005; Brack et al. 1999; Kammann et al. 2005; Olsman et al. 2007; Wölz et al. 2008).

5 Conclusions

Sediment from Rio Grande reservoir has shown great genotoxic and EROD induction potentials. Contribution of PAHs to Bio-TEQ was minimal and total PAH concentration was bellow threshold levels, what suggest that other class of chemical is responsible for observed effect. Mutagenic potentials calculated as IF_{max} from Ames Fluctuation test results were comparable to industrial

effluents; genotoxicity expressed as DNA breakage (comet assay) was higher than sediment from Billings Complex.

For a better understanding of possible EROD induction substances present in Rio Grande sediment extract, we suggest a fractionation approach. PCB should be considered for further studies aiming possible association of observed EROD effect. We also suggest chronicle exposure approach in order to better understand toxicity dynamics over a lager exposure time.

6 Acknowledgment

Thanks to FAPESP and CAPES/DAAD for PhD scholarship both in Brazil and Germany granted to the first author; special thanks to Alexandre Ungar, Markus Brinkmann, Thomas-Benjamin Seiler, and Breylla Carvalho for contributing with this paper; the authors acknowledge Niels C. Bols and Lucy Lee for providing the permanent cell line RTL-W1.

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Cap.6 Integrated Assessment of Sediment from Rio Grade Reservoir

Abstract

Assessment of complex mixture sediments is a great challenge, especially for reservoirs located near urban areas where a multitude of pollution sources is present, such as Rio Grande reservoir. Aiming an integrated assessment, we performed a battery of bioassays on sediment from Rio Grande reservoir, using permanent cell line RTL-W1 and bacteria exposed sediment acetonic extracts (cytotoxicity test, EROD assay, comet assay, micronucleus assay and Ames Fluctuation test) and *Danio rerio* embryos exposed to whole sediment samples. After exposure, we measured metal (ICP-MS) in dechorionated non-coagulated fish embryos, as a bioaccumulation indicator. Target PAHs were analyzed in extracts and metal dynamics was evaluated as temporal heterogeneity and remobilization to water column. Ames Fluctuation test and comet assay reveled high potentials for mutageno- and genotoxicity; EROD assay also indicated high CYP1A1 induction potential. Studied metals appear not to be the cause of primary acute effect observed in toxicity tests, nor seam target PAHs. Yet, we recorded high metal content in sediment, short-time temporal heterogeneity of metals and possible remobilization into the water column, what indicates high metal bioavailability potential *in situ*. WOE indicates the need of management actions in sediment from Rio Grande, though further studies should be conducted for better risk characterization, and establishment of causality, so action could address the problem. This study could be used as part of an ERA framework.

Key words: ERA; bioassays; Integrated Assessment; Decision-making; sediment; complex mixture

1 Introduction

Assessment of complex mixture sediments is a great challenge, especially for reservoirs located near urban areas where a multitude of pollution sources is present. Rio Grande reservoir is an example of such environment. It is inserted in Sao Paulo Metropolitan Region, and receives domestic and industrial sewage, runoff from landfills areas, at the same time that water is taken in for

human consumption. Previous studies have addressed hazard potential of sediments regarding metal and organic contamination (Mariani & Pompêo 2008; Fávaro et al. 2007; Beyruth & H. A. S. L. Pereira 2002; A. A. Rocha et al. 1985; Maier et al. 1985; Nishimura et al. 2008).

Weight-of-evidence (WOE) "is the process of combining information from multiple lines of evidence to reach a conclusion about an environmental system or stressor" (Burton Jr. et al. 2002). It has been widely used under different names for the study of ecosystem impairment. It comprises gathering information from different lines of evidence (LOE), incorporate judgments of quality, extent and congruence, then integrate them in a weighted manner.

WOE can also be used as a tool in Ecological risk assessment (ERA); ERA is a process that evaluates the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors (USEPA 1992). It was first suggested by USEPA and its framework is shown in Figure 42.

The aim of this chapter was to summarize the results from this work and integrate the information of different LOEs, in a WOE manner, in order to evaluate (a) if data gathered so far is enough for assessing sediment quality from Rio Grande, (b) which conclusion can be withdrawn with available data, and (c) what kind of information is still needed. The results can be used in an ERA process, though it was not our object to perform one.

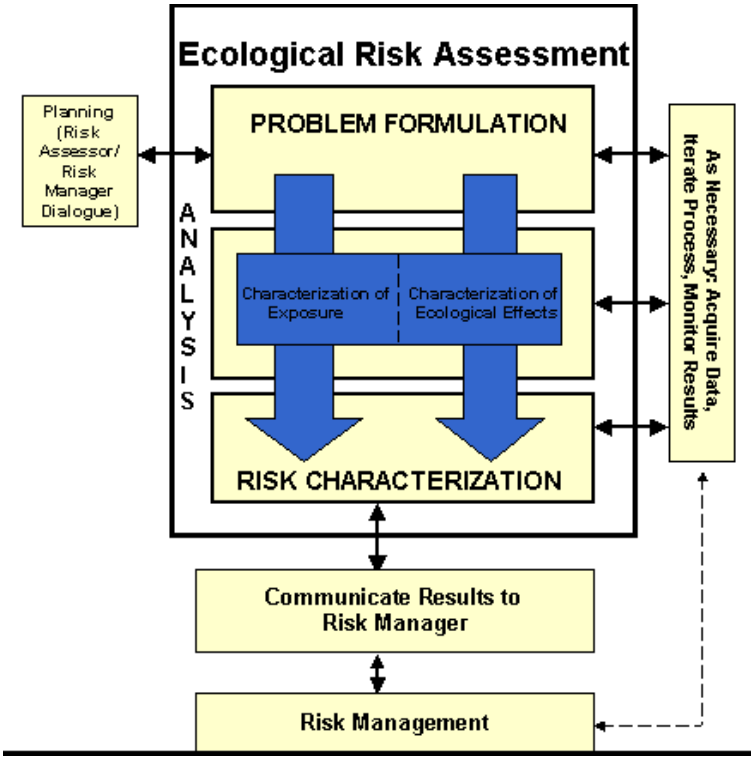


Figure 42. USEPA framework for Ecological Risk Assessment (ERA).

2 Material and Methods

We proceeded chemical and ecotoxicological analysis, as summarized in Figure 43, Figure 44 and Figure 45. More details on methodology can be found in Chapters 2, 3, 4 an 5. Each LOE result was compared to a parameter, as shown in Table 23, and then transformed into qualitative categories: low, medium or high toxic potential, according to the criteria presented in Table 24. We attributed semaphoric colors to the different toxic potential levels, to better visualize those potentials.

Table 23. Analyzed LOEs and parameters used as reference.

	LOE	*Parameters*
Chemical Analysis	Metal	SQG (TEL and PEL)
	PAHs	SQG (TEC ; PEL and ISQG)
	Metal dynamics	SQG (EqP) ; temporal heterogeneity
Toxiciy Assays	Cytotoxicity test	NR_{50} from reference sites*
	comet	CDI from reference sites*
	Ames	IF_{max} from reference sites*
	EROD	Bio-TEQ from reference sites*
	Fish embryo test	EC_{50} from reference sites*
Bioaccumulation	Metals measurement in fish embryo tissue	metal concentration

* Reference sites are Cubatao de Cima (this work) and near Tiete spring site (P. S. Rocha et al. 2010).

Table 24. Criteria used for categorization of Toxic Potential of each LOE.

LOE	*Criteria*	*Categorization of Toxic Potential*
Metal	< ISQG	low
	> ISQG ; < PEL	medium
	> PEL	high
PAHs	all individual PAH < ISQG	low
	individual PAH > ISQG; total PAH < TEC	medium
	total PAH > TEC ; individual PAH > TEL	high
Metal dynamics	EqP < 130,0 ; no temporal heterogeneity ; no remobilization	low
	130 < EqP < 3,000 ; or temporal heterogeneity ; or remobilization from sediment	medium
	EqP > 3,000 ; and/or temporal heterogeneity ; and/or remobilization from sediment	high
Toxicity Assays	< respective Parameters	low
	< 3 x respective Parameters	medium
	> 3 x respective Parameters	high
Bioaccumulation	< LD	low
	> LD	high

LD = Limit of Detection
PEL = Probable Effect Level
ISQG = Interim Sediment Quality Guideline
TEC = Threshold Effect Concentration
EqP = AVS-SEM/OC (mmol/kg)

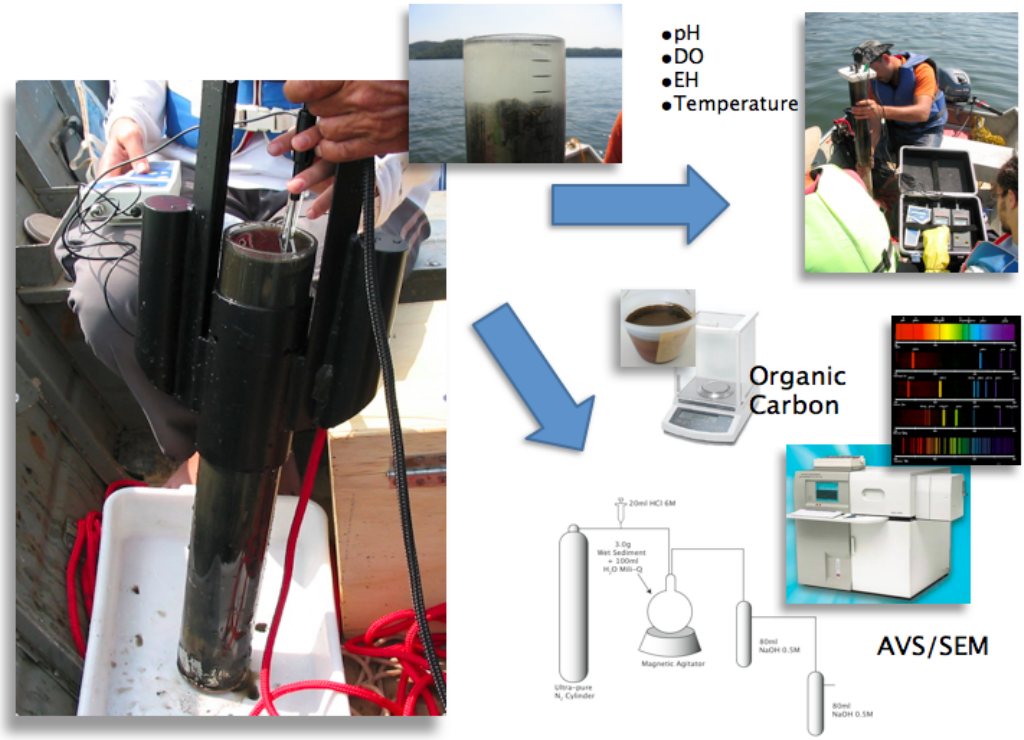


Figure 43. Sediment sampling proceedure, in situ measurements and chemical analysis carried out in the laboratory.

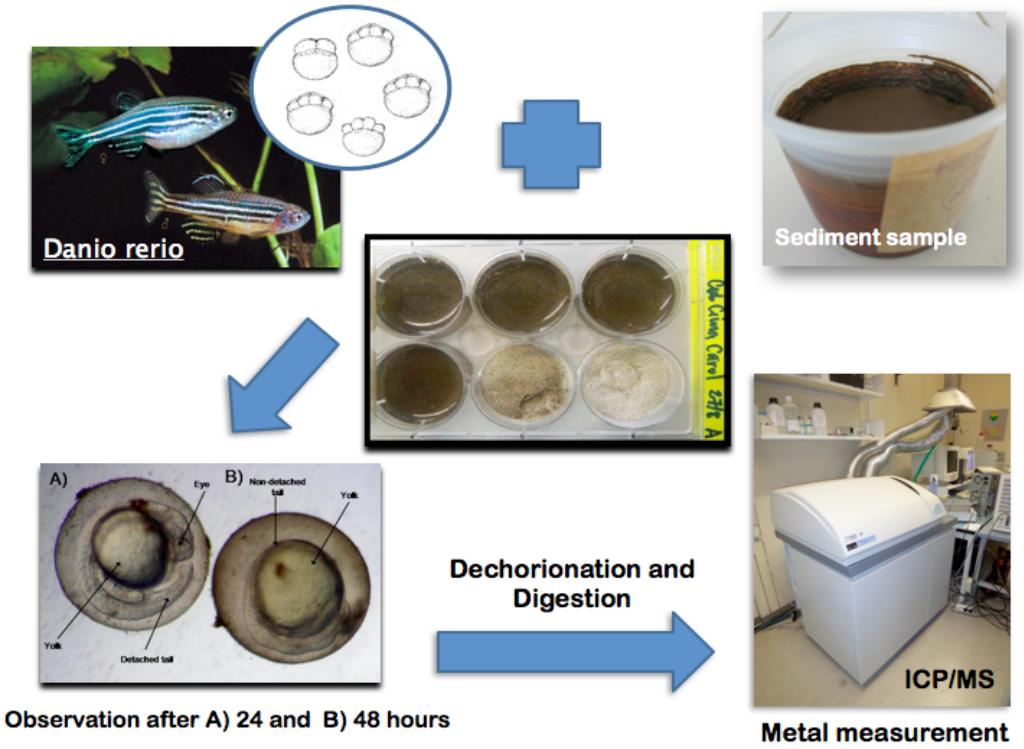


Figure 44. Fish embryo assay exposed to whole sediment, followed by bioaccumulation test (analysis of metals in non-coagulated and dechorianated embryos).

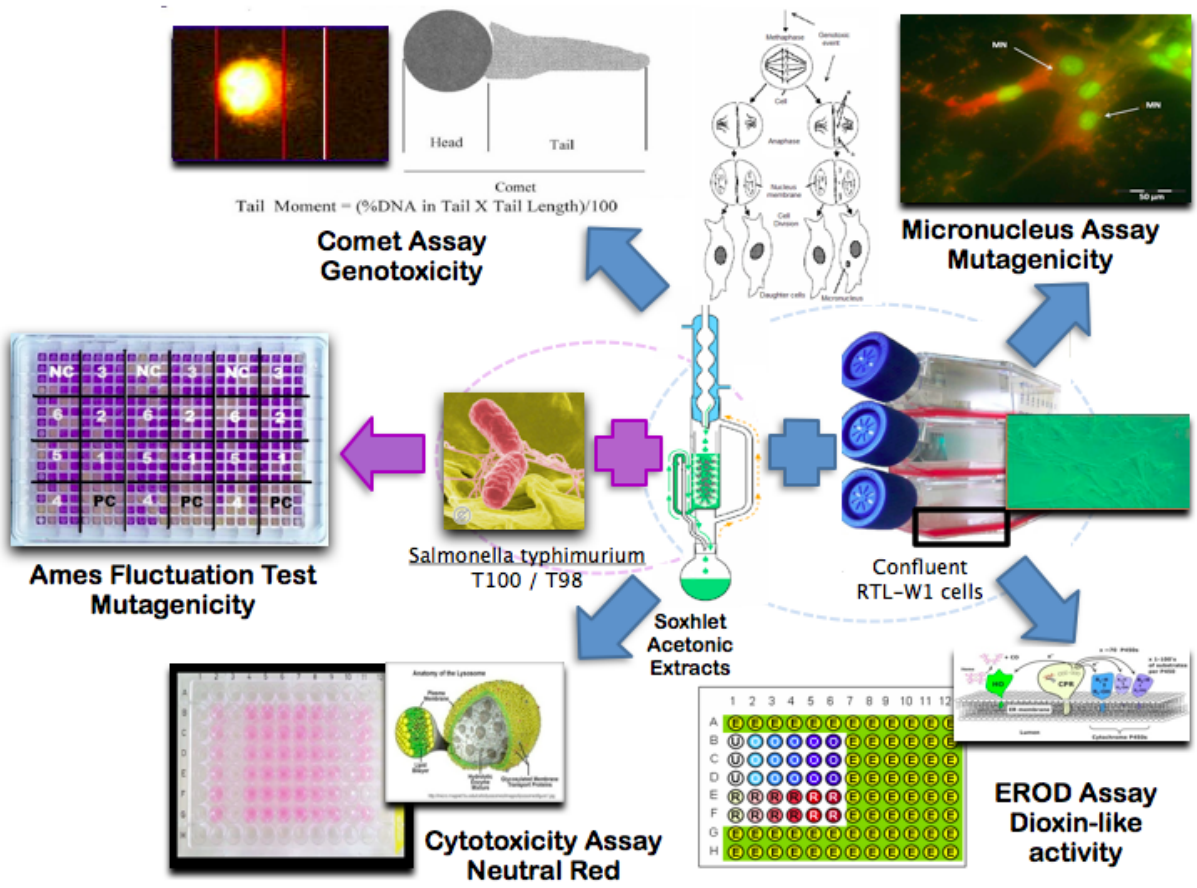


Figure 45. Representation of toxicity tests carried out using sediment extract and RTL-W1 cells (blue arrows) and Salmonella (purple arrow).

3 Results

Results from LOEs and evaluation of toxic potentials are presented in Table 25 (chemical analysis) and in Table 26 (ecotoxicological tests).

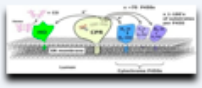
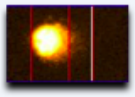
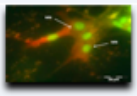
Table 25. Results from analyzed chemical LOEs and respective categorization of toxic potential.

CHEMICAL PARAMETER	RESULT	REFERENCE*	TOXIC CATEGORY
Metals	Cu = 2118.8 mg/kg Ni = 115.2 mg/kg Cr = 143.5 mg/kg Cd = 12.4 mg/kg Zn = 267.0 mg/kg	Cu = 197 mg/kg Ni = 36 mg/kg Cr = 90 mg/kg Cd = 3.5 mg/kg Zn = 315 mg/kg	
Metal dynamics	mean EqP = 576 mmol/kg variation along time remobilization	130 < EqP < 3,000 mmol/kg	
Total target PAHs	763.6 µg/kg	1610.0 µg/kg	

* Reference for metals: ISQG – Interim Sediment Quality Guideline (CCME 2002) ; Reference for metal dynamics EqP: ∑SEM-AVS/OC (USEPA 2000); Reference for metal variation and remobilization: Statistics (see Cap. 3); Reference for PAHs: consensus-based TEC – Threshold Effect Concentration (MacDonald et al. 2000).

EqP = SEM-AVS/OC

Table 26. Results from analyzed ecotoxicological test LOEs and respective categorization of toxic potential.

		BIOASSAY	RESULT*		TOXIC CATEGORY
Acetonic Extract and permanent fish cell line (RTL-W1)		Cytotoxicity assay Neutral Red	NR_{50} = 16 mg/mL		
		EROD CYP1A1 dioxin-like activity	1884 pg BioTEQ/g sediment		
		Comet assay Genotoxicity	CDI = 6.5 LOC = 0.875 mg/mL		
		Micronucleus assay Genotoxicity	Not conclusive		
		Ames Fluctuation Test Mutagenicity	IFmax	11.5 (TA98+S9) 7.0 (TA98-S9) 0.85 (TA100+S9) 1.55 (TA100-S9)	
Whole sediment contact Test		Fish embryo contact Test	LC_{50} 24h = 17 mg/mL LC_{50} 48h = 12 mg/mL (100% lethality at 35.5 mg/mL ; 48h)		
		Analysis of metals in dechorionated embryos	No metal detected		

LEGEND:

High Toxic Potential **Medium Toxic Potential** **Low Toxic Potential**

NR_{50} = Concentration at which 50% neutral red retention is observed; meaning 50% cell mortality
Bio-TEQ = toxicity equivalent values relative to 2,3,7,8 TCDD
CDI = Concentration-dependent Induction Factor
LOC = Lowest Observed effect Concentration
IF_{max} = Maximum Induction Factor
LC_{50} = Effect Concentration at which 50% survival is observed

4 Discussion

Chapman and Anderson (2005) proposed a matrix for WOE categorization, as part of a decision-making framework. They suggested four main types of LOE, which are: benthos alteration, biomagnification potential, bulk sediment chemistry and toxicity. They built 16 scenario by combin-

ing qualitative results from LOE, and then used WOE to provided the related decision for each scenario.

Regarding benthic community, Rio Grande reservoir is part of the Environmental Company of Sao Paulo, Brazil – CETESB monitoring program. Data on Rio Grande reservoir is available from 2003 to 2004 in sampling station RGDE 02900 (site coincident with sampling site from this work) and from 2006 to 2008 in GADE 2900 (site up stream the reservoir, in a riverine area). Results are summarized in a Benthic Community Index (BCI), which accounts for specie richness, number of sensitive species and dominance of tolerant species, and vary from "excellent" to "poor" quality (5 classes: excellent, good, regular, bad, poor). In 2003, BCI from Rio Grande indicated "regular" quality and in 2004 "bad" quality of benthic community. Those results were influenced by dominance of tolerant organisms (76%; mainly *Limnodrilus hoffmeisteri*, *Pristina*, *Pristinella* and *Chironomus*) and no record of sensitive species, though richness (S = 16) and diversity (S = 13,24) were equivalent to a better quality BCI class. In 2006 and 2007, benthic community from GADE 2900 was classified as "bad" and "regular", respectively, again mainly due to dominance of tolerant species (CETESB 2004; CETESB 2005; CETESB 2006; CETESB 2007).

As guidance for the decision-making framework, Chapman and Anderson (2005) considered biomagnification potential in a broad sense, by conservatively modeling the concentration in the sediments and predicting uptake by sediment-dwelling organisms and predators of those organisms. In this concept, metal dynamics as measured in the present work could be considered as biomagnification potential indicator, together with bioaccumulation test, since EqP, variation of metal concentration along short time and remobilization into the water are indicator of free soluble ion formation, which is bioavailable and passive of bioaccumulation.

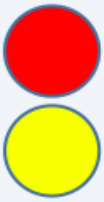
Assuming BCI from CETESB data basis as benthic community quality, bioaccumulation potential as described in the previous paragraph, toxicity as bioassays from this work and bulk sediment chemistry as PAH and metals compared to respective SQG, it is possible gather information on the four LOE suggested by Chapman and Anderson (2005) to build a WOE matrix such as the one shown by those authors

To fit the 3 established categories of toxic potential, benthos alteration was categorized as "medium toxic potential", since BCI reported by CETEB was "regular" to "bad". To yield biomagnification toxic potential, we combined attributed categories of metal dynamics (high toxic potential - Table 25) and bioaccumulation test with fish embryo (low toxic potential - Table 26), thus we attributed the category "medium toxic potential". Toxicity LOE was considered as high, since all

bioassays from this work presented "high toxic potential". Finally, we attributed the category "high toxic potential" to sediment chemistry; we considered the category "high toxic potential" from metals and "medium toxic potential" from total target PAHs; since no category in between was established, we considered "high toxic potential", for some individual PAH concentration were above ISQG (see Cap. 5).

Table 27 presents the scenario built on WOE basis using results from this work and criteria presented in Table 24, and the respective best fitted scenario suggested by Chapman and Anderson (2005). The assessment related to this scenario is the requirement for management actions, hence contaminated sediments may pose an environmental risk. However further assessment must be done before definite decision is made, in order to link biological observed effect to measured chemicals, and ensure that management action address the problem.

Table 27. Scenario built based on WOE by combining categorized LOE suggested by Chapman and Anderson and the scenario built with the present results.

SCENARIO	SEDIMENT CHEMISTRY	TOXICITY	BENTHOS ALTERATION	BIOMAGNIFICATION POTENTIAL	ASSESSMENT
16*					Management actions required
this work					

LEGEND:

*Source: Chapman and Anderson (2005) with adaptation regarding semaphoric representation

According to Ecological Risk Assessment (ERA) framework, risk characterization precedes risk management. ERA has three phases of risk assessment: problem formulation, analysis, and risk

characterization, and is based on two major elements: characterization of effects and characterization of exposure (USEPA 1998).

In an ERA context, the present work can be used for characterization of effect (genotoxicity, mutagenicity, EROD activity, cytotoxicity and fish embryo mortality), also to characterization of exposure, for we have demonstrated short-time variation in metal content in sediment. Still, in order to achieve risk characterization, further research should be carried out, covering other endpoints, such as endocrine disruptors, extending the period of exposure, such as life-cycle tests, and connecting observed in vitro toxicity observation to field parameter. Also, chemical analysis should be expanded to other class of organic chemicals, e.g., non-target PAHs, PCBs, dioxin and furans.

Regarding causality, extract fractionation followed by bioassays may contribute to identification of organic contaminant class responsible for most observed effect, and give some insights on causality and chemicals of priority concern in this environment (Brack et al. 2005; Brack et al. 2007).

5 Conclusions

Sediment extract revealed high mechanism-specific toxicity, such as dioxin-like activity, cytotoxicity, mutagenicity, and genotoxicity. Whole-sediment exposure also reveled high toxic potential in acute fish embryo test. Chemical parameters indicated high toxic potential regarding metals, and medium toxic potential regarding target PHAs. Comparing weighted lines-of-evidence (WOE) from our results with decision-making framework suggested by Chapman and Anderson (2005), sediment management is requested, though, in an ERA context, further studies must be done in order to better characterize risk, including testing other endpoints (e.g. endocrine disruptors), field toxicity evaluation and chronic exposure tests (e.g. life-cycle). Chemical analysis should embrace a broader range of organic compounds. Besides, sediment extract fractionation may contribute to better understanding of toxic organic pollutants present in this sediment and may give some insights on causality. This work could be used as part of an ERA framework.

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Cap.7 Conclusões e Considerações Finais

CONCLUSÃO 1: Heterogeneidade temporal e dinâmica de metais no sedimento do Reservatório Rio Grande

- ✔ Os sedimentos do reservatório Rio Grande apresentam heterogeneidade temporal com relação aos metais, em um curto espaço de tempo (14 dias);
- ✔ A Matéria Orgânica foi mais importante que os sulfetos para explicar a variação dos metais;
- ✔ A comparação da concentração de metais nos sedimentos e na coluna d'água (em diferentes alturas da mesma) aponta para possível remobilização de metais e interdependência entre os dois compartimentos;
- ✔ As alterações na coluna d'água e nos sedimentos foram coincidentes com a passagem de uma frente fria.

A constatação da existência de heterogeneidade temporal relativa aos metais no sedimento do reservatório Rio Grande exige uma mudança do modo como é concebido o monitoramento: os sedimentos não são estáveis, portanto uma amostragem por ano não é representativa para o período de um ano, nem mesmo para uma determinada estação do ano; desta forma, um desenho experimental ideal para este ambiente deve considerar diversas campanhas de amostragem, em diversos pontos ao longo do reservatório.

A relação observada entre sedimento e massa d'água indica a existência de interdependência entre esses dois compartimentos. Desta forma, o estudo dos sedimentos precisa estar vinculado ao estudo da coluna d'água, visando ao entendimento do sistema como um todo.

A passagem de uma frente fria provocou alterações na coluna d'água, que foram coincidentes com alterações nos sedimento. Portanto, outros estudos são necessários para o estabelecimento mais preciso da dinâmica de transformações desse sistema. Essa é uma informação que pode ser utilizada para a gestão do recurso hídrico, em especial devido ao seu uso para abastecimento público. Como exemplo, esses dados poderiam nortear ajustes no tratamento da água e na altura da tomada d'água.

Os resultados obtidos com o presente estudo podem ser expandidos para um questão mais abrangente, que é referente a ambientes aquáticos rasos localizados na região dos trópicos: esses ambientes, em geral, possuem padrão polimítico de ciclos de desestratificação/ estratificação da

massa d'água, muitas vezes desencadeados pelas mudanças climáticas decorrentes da passagem de frentes frias. Em contraponto com ambientes temperados, essas mudanças ocorrem em uma escala temporal muito menor, e demandam diferenças de temperatura também menores. A temperatura é um fator determinante do metabolismo do sistema, consequentemente, as transformações refletem nos processos abióticos e bióticos. Isso tem implicações não somente na estratégia de amostragem como também na eficácia da importação de padrões e modelos desenvolvidos em ambientes temperados que, muitas vezes, são transplantados para os ambientes tropicais sem que contudo haja uma avaliação prévia da aplicabilidade dos mesmos.

CONCLUSÃO 2: Potencial de efeitos negativos referentes avaliados nos biotestes

- ✔ Os extratos acetônicos de sedimentos do reservatório Rio Grande apresentaram alto potencial para indução de efeitos citotóxicos, genotóxicos, mutagênicos e de atividade de EROD. Os sedimentos também apresentaram alta toxicidade a embriões de peixe em teste agudo.

Os estudo realizados *in vitro* apontaram para um risco potencial para a biota residente em testes realizados em nível molecular e também com embriões de peixe (nível de organismo); contudo, existe necessidade de estabelecimento mais estreito entre o potencial e a real probabilidade de que os efeitos negativos sejam observados nos organismos aquáticos, com a realização de testes que revelem o risco potencial *in situ*.

CONCLUSÃO 3: Correspondência entre os efeitos observados e as análises químicas realizadas no sedimento e nos extratos orgânicos

- ✔ Não foi possível estabelecer inter-relação direta entre os compostos químicos analisados (metais e HPAs prioritários) e os efeitos negativos observados por meio dos biotestes.

Isso traz a necessidade de ampliação da gama de compostos químicos analisados, e a ampliação dos critérios de avaliação de toxicidade, com por exemplo exposições de organismos por um período prolongado (exposição crônica) e desruptores endócrinos. Outra abordagem possível é o fracionamento do extrato orgânico, seguido de biotestes com cada uma das frações resultantes, visando à

elucidação da classe de composto orgânico mais provável de desencadeia os efeitos negativos observado, em uma tentativa do estabelecimento de causalidade.

Vale ressaltar que os sedimentos do reservatório Rio Grande são compostos de uma mistura de substâncias contaminantes, o que dificulta a avaliação e o estabelecimento de causalidade, em virtude de sinergismo e antagonismo entre as substâncias, de características inerentes aos testes de toxicidade (sensibilidade do organismo-teste, tempo de exposição e modo de ação, etc) e do ecossistema em si (resistência e resiliência).

CONCLUSÃO 4: Análise Integrada dos LOEs

- ✔ A avaliação integrada, através da ponderação das linhas de evidência estudadas, indicou a necessidade de manejo do sedimento;
- ✔ Antes de uma ação efetiva, é necessária a realização de estudos complementares, visando à caracterização do risco, de modo que a ação escolhida seja realmente eficaz na solução do problema.

A caracterização do risco seria a próxima etapa, em um estudo contextualizado dentro de uma estrutura de Análise de Risco Ecológico (ERA). Os dados levantados pela CETESB relativos à comunidade bentônica mostraram haver comprometimento da qualidade ambiental *in situ*. O presente trabalho pode ser utilizado para compor uma ERA. No entanto, mais dados são necessários, como a análise de outros critérios toxicológicos (exposição crônica, desruptores endócrinos, estudo de ciclo de vida, etc) e a ampliação da gama de análises químicas realizadas no sedimento, no sentido de caracterização do risco. Novamente sugere-se a realização de fracionamento do extrato orgânico, visando ao estabelecimento de causalidade.

Considerações sobre o Reservatório Billings

Vale também ressaltar que nos últimos anos, diversas iniciativas surgiram visando à conservação e ao uso sustentável desse reservatório, a saber os estudos realizados por ONGs com o Instituto Socioambiental, os esforços dos meios de comunicação local (ex. Diário do Grande ABC), coo-

perações entre os governos e a JICA (Agência de Cooperação Internacional do Japão), dentre outros.

Um marco relativamente recente foi a aprovação da Lei Específica a Billings (Lei Estadual nº 13.579, de 13 de julho de 2009) que "define a Área de Proteção e Recuperação dos Mananciais da Bacia Hidrográfica do Reservatório Billings - APRM-B, e dá outras providências correlatas" e do Decreto Estadual nº 55.342, de 13 de janeiro de 2010, que "regulamenta dispositivos da Lei nº 13.579, de 13 de julho de 2009, que define a Área de Proteção e Recuperação dos Mananciais da Bacia Hidrográfica do Reservatório Billings - APRM-B, e dá providências correlatas". Esses documentos legais delimitam faixas de uso e aproveitamento do entorno do reservatório, e prevêem mecanismos de gestão, como um SGI (Sistema de Gestão Integrada) específico para a sub-bacia do Reservatório Billings, metas de redução de carga poluidora e de preservação da vegetação, e programas de compensação ambiental para a manutenção das áreas de mata. Porém, a legislação também é polêmica no sentido de que possibilita a regularização de propriedades e ocupações irregulares no entorno da represa, com o argumento de diminuir o lançamento de esgotos clandestinos.