UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Programa de Pós-Graduação em Toxicologia e Análises Toxicológicas

Ocorrência, genotoxicidade e risco ecotoxicológico de corantes no ambiente aquático

Francine Inforçato Vacchi

Tese para obtenção do Título de DOUTOR

Orientadora: Profa. Dra. Gisela de Aragão Umbuzeiro

São Paulo
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pelo amor incondicional que sempre me deram,
pois sem amor não somos nada.

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“L’essentiel est invisible pour les yeux…”
(“O essencial é invisível aos olhos…”)

Antoine de Saint-Exupéry

RESUMO

VACCHI, F. I. **Ocorrência, genotoxicidade e risco ecotoxicológico de corantes no ambiente aquático.** 2016. 135f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2016.

Corantes são utilizados na coloração de diferentes substratos, incluindo papel, couro e plásticos, mas o uso mais importante é o têxtil e 1 a 5% destes corantes podem ser descartados no ambiente. Em geral, os corantes do tipo azo são tóxicos para os organismos aquáticos e alguns tipos de corantes podem ser mais tóxicos que outros. Mas, embora estes compostos e seus produtos de transformação reduzidos e/ou clorados podem ser encontrados no ecossistema aquáticos, não existem dados sobre genotoxicidade em organismos aquáticos até o momento. Muitos estudos têm demonstrado que avaliar danos ao DNA representa um biomarcador de exposição muito sensível em espécies aquáticas, que pode ser estudado utilizando ensaios *in vivo* e *in vitro*, como no caso das linhagens de células de peixe. Os objetivos deste trabalho foram: avaliar a ocorrência de corantes dispersos em amostras ambientais; avaliar a mutagenicidade dessas amostras utilizando o ensaio de *Salmonella*/microssoma com as linhagens TA98 e YG1041, e a genotoxicidade com o ensaio do cometa em culturas celulares de peixe RTL-W1. HPLC-MS/MS foi utilizada para verificar a ocorrência de corantes em amostras do Rio Piracicaba à montante e à jusante do Ribeirão Quilombo e do descarte de uma Estação de Tratamento de Efluentes (ETE), localizados no Estado de São Paulo, Brasil. Foram detectados seis corantes dispersos nas amostras de águas superficiais e efluentes. O corante Disperse Red 1 foi o composto mais frequente, detectado em 8 das 16 amostras, porém sua contribuição para a mutagenicidade total foi baixa; os corantes Disperse Blue 373 e Disperse Violet 93 foram os que mais contribuíram. A genotoxicidade do Rio Piracicaba, avaliada pelo ensaio de *Salmonella*/microssoma e ensaio do cometa, aumentou após o lançamento do Ribeirão Quilombo e do efluente ETE, mostrando uma possível contribuição destes na genotoxicidade do Rio Piracicaba.

Palavras-chaves: Corantes; Mutagenicidade; Genotoxicidade.

ABSTRACT

VACCHI, F. I. **Occurrence, genotoxicity and ecotoxicological risk of dyes in the aquatic environment.** 2016. 135f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2016.

Dyes are used in the coloration of different substrates, including paper, leather and plastics, but the most important use is on textiles and 1 to 5% of these dyes might be lost into the environment. Azo dyes are the most important class, accounting for over 50% of all commercial dyes, and this class has been the most studied. In general, azo dyes are toxic to aquatic organisms and some types of dyes are more toxic than others. But although these compounds as well as their reduced/chlorinated transformation products can be found in aquatic ecosystems, no mutagenicity data are available until now in aquatic organisms. This remark remains of value, as well, regarding genotoxicity potential of such dyes towards aquatic organisms. Many studies have demonstrated that DNA damage measurement represents a very sensitive biomarker of exposure in aquatic species that can be studied both in vivo and in vitro using for example fish cell lines. The objectives of this work were evaluate the occurrence of disperse dyes in environmental samples; evaluate the mutagenicity of this samples using the Salmonella/microsome assay with strains TA98 and YG1041; evaluate the genotoxicity using the comet assay with fish cell lines RTL-W1. HPLC-MS/MS was used to verify the occurrence of dyes in samples of Piracicaba River upstream and downstream the discharge of Quilombo River and Wastewater Treatment Plant (WWTP) effluent, located in São Paulo State, Brazil. Six dyes were detected in samples of water and effluents. Disperse Red 1 dye was detected in 8 of 16 samples, but its contribution for the mutagenicity was low. Disperse Blue 373 and Disperse Violet 93 were the major contributors for the mutagenicity found in the samples. The genotoxicity of Piracicaba River, evaluated with Salmonella/microsome assay and comet assay, increased after the discharges of Quilombo River and the effluent of WWTP, showing a contribution of this discharges on the river genotoxicity.

Keywords: Dyes; Mutagenicity; Genotoxicity.

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1. INTRODUÇÃO

Os corantes estão presentes em praticamente todos os produtos que utilizamos diariamente, como, vestuários, alimentos, couros, papéis, plásticos, e o seu uso mais importante é na indústria têxtil (BAFANA; DEVI; CHAKRABARTI, 2011). Os corantes têxteis são caracterizados pela sua habilidade em absorver a luz visível (400 a 700 nm) e podem ser classificados de acordo com sua estrutura química ou de acordo com o método pelo qual ele é fixado à fibra têxtil. Considerando o método de aplicação à fibra, eles podem ser classificados como corantes ácidos, corantes diretos, corantes básicos, corantes reativos, corantes dispersos, corantes vat, e corantes sulfurosos (GUARATINI; ZANONI, 2000). Considerando a estrutura química, os corantes são classificados de acordo com o grupo cromóforo presente em sua molécula, os quais podem ser azo, antraquinona, indigóide, ftalocianina, nitro, nitroso, xanteno, trifenilmetano. No entanto, o grupo mais representativo e largamente empregado pertence à classe azo, que se caracterizam por apresentarem pelo menos uma ligação azo (-N=N-) ligados a sistemas aromáticos (HUNGER, 2003).

As indústrias têxteis estão entre as indústrias que mais consomem água e o efluente gerado tem composição química variada, dependendo da etapa de processamento, sendo que sua composição é um dos maiores responsáveis pela grande dificuldade em tratá-los. Corantes dispersos, por exemplo, são utilizados para colorir fibras de poliéster, são geralmente pouco solúveis em água e são aplicados como uma fina dispersão aquosa. A fim de fazer esta dispersão, estes corantes poderão conter surfactantes em sua formulação, como por exemplo, lignina sulfonada (CHRISTIE, 2001). Depois que um corante disperso é utilizado no processo industrial, 1 a 5% do corante não é aproveitado e permanece no banho aquoso (RIBEIRO; UMBUZEIRO, 2014). Este tipo de efluente normalmente é tratado com lodo ativado e o efluente líquido é descartado no ambiente aquático. O tratamento por lodos ativados do corante azo Disperse Blue 79 foi estudado pela Agência de Proteção Ambiental dos EUA e foi verificado que o corante não foi degradado num processo de lodo ativado convencional e que 85% do corante permaneceram no sistema (USEPA, 1990).

Corantes azo podem ser mutagênicos sob condições oxidativas e/ou redutivas (PRIVAL; MITCHELL, 1982; PRIVAL et al., 1984). Quando o nitrogênio da ligação azo é reduzido, aminas aromáticas ou outros compostos aromáticos são formados, tais como nitrocompostos, dependendo da estrutura do corante. Muitas aminas aromáticas, amidas e nitrocompostos são de uma classe de produtos químicos que produzem tumor em uma ampla variedade de tecidos de animais experimentais. No homem, a exposição às algumas aminas aromáticas está associada com tumores de bexiga urinária e pélvis renal. A sua atividade biológica é dependente de ativação metabólica *in vivo.* A carcinogenicidade das aminas aromáticas é dependente da oxidação de derivados N-hidroxi e a carcinogenicidade de nitrocompostos aromáticos está ligada com sua redução a hidroxilaminas (BARTSCH, 1981).

Os corantes azo, se entrarem no corpo humano por meio da ingestão, serão metabolizados em aminas aromáticas principalmente pelas enzimas azoredutases dos microrganismos intestinais. Enzimas redutoras no fígado podem também catalisar a clivagem redutora das ligações azo, no entanto evidências indicam que a azoredutase presente na microbiana intestinal é mais importante (CHUNG; STEVENS; CERNIGLIA, 1992). A geração de espécies reativas de oxigênio também parece estar envolvida na genotoxicidade de aminas aromáticas (SWEENEY; CHIPMAN; FORSYTHE, 1994).

Alguns trabalhos relatam que efluentes de fábricas têxteis podem apresentar atividade mutagênica. Na região metropolitana de São Paulo, Brasil, descobriu-se que a indústria têxtil foi a categoria industrial com a maioria das respostas positivas, entre vários efluentes analisados (SANCHEZ et al., 1988), e que o efluente de uma planta de processamento de corantes foi um dos contribuintes para a atividade mutagênica encontrada no rio Paraíba, São Paulo (VALENT et al., 1993). Um estudo mostrou que a água subterrânea em Tirupur, Tamilnadu, Índia estava contaminada com compostos genotóxicos, provavelmente aminas aromáticas, como consequência da descarga de efluentes têxteis no solo e na água, e também apresentavam danos ao DNA em peixes sob a influência dessas descargas (RAJAGURU et al., 2002).

Amostras do Ribeirão dos Cristais, Brasil, foram analisadas utilizando o ensaio de focos de criptas aberrantes em ratos Wistar. Os resultados mostraram que os grupos tratados com as duas maiores concentrações do efluente *in natura* (1% e 10%) apresentaram um aumento estatisticamente significativo de lesões pré-neoplásicas (ALVES DE LIMA et al., 2007). Outro estudo mostrou que os corantes encontrados no

Ribeirão dos Cristais, Disperse Blue 373, Disperse Orange 37 e Disperse Violet 93, também foram capazes de induzir focos de criptas aberrantes, quando os ratos foram expostos por via intraperitoneal (UMBUZEIRO et al., 2005).

Tsuboy et al. (2007) encontraram efeitos genotóxicos e mutagênicos do corante comercial Disperse Blue 291 em células de mamíferos. Os autores testaram o corante utilizando uma linhagem celular derivada de fígado humano (HepG2) com o teste do cometa, teste micronúcleo (MN) e teste de viabilidade celular. O corante induziu a formação de micronúcleos (MN) e a fragmentação do DNA e aumentou o índice apoptótico em células HepG2.

Muitos estudos têm demonstrado que a avaliação de danos ao DNA representa um biomarcador de exposição muito sensível em espécies aquáticas, que pode ser estudada *in vivo* e *in vitro*, como por exemplo, as linhagens de células de peixe (DEVAUX; PESONEN; MONOD, 1997; BONY et al., 2008; JHA, 2008; BONY; GAILLARD; DEVAUX, 2010). Além disso, estudos recentes realizados tanto em peixes e invertebrados de água doce têm demonstrado claramente que os eventos genotóxicos em espécies aquáticas podem prejudicar a reprodução, posteriormente afetando a dinâmica populacional (DEVAUX et al., 2011; LACAZE et al., 2011).

Modelos *in vitro* alternativos, como as linhagens de células de peixe, representam um sistema padronizado de fácil uso, que podem ser conduzidos em ambiente totalmente controlado, dando resultados rápidos, baratos e eticamente aceitáveis (SCHIRMER, 2006). O uso desses modelos na toxicologia ambiental tem sido positivamente aceito em relação à avaliação da citotoxicidade e da genotoxicidade de substâncias puras e de amostras ambientais (CASTAÑO et al., 2003). Testes de genotoxicidade *in vitro* frequentemente utilizam organismos procarióticos como o padronizado teste de Ames (OHE; WATANABE; WAKABAYASHI, 2004). As linhagens de células de peixe representam um interessante modelo eucariótico, mantendo as características fisiológicas de peixes (ectotermia, tolerância à variação da pressão osmótica, capacidade metabólica e de reparo do DNA).

Em geral, corantes do tipo azo são tóxicos para organismos aquáticos (WALTHALL; STARK, 1999; GOTTLIEB et al., 2003; NOVOTNÝ et al., 2006; BAE; FREEMAN, 2007a, 2007b; LIU et al., 2007; IMMICH; ULSON DE SOUZA; ULSON DE SOUZA, 2009; WANG et al., 2009; FERRAZ et al., 2011) e alguns tipos de corantes são mais tóxicos do que os outros. O corante Disperse Red 1 tem sido estudado e apresentou alta toxicidade para organismos aquáticos e mutagenicidade no teste de

Ames e em outros sistemas celulares (CHEQUER et al., 2009; FERRAZ et al., 2011; RIBEIRO; UMBUZEIRO, 2014). *Daphnia similis* foi o organismo aquático mais sensível a este corante e sua CE50 foi de 130 µg L^{-1}. Quando o corante foi clorado, ficou menos tóxico aos organismos aquáticos, mas ficou mais mutagênico no teste de Ames quando comparado ao corante; e o principal composto gerado foi 1-cloro-4-nitrobenzeno (VACCHI et al., 2013).

Quanto à ocorrência de corantes em ambientes aquáticos, em um estudo realizado no rio Yamaska em Quebec, Canadá, foram detectados 15 corantes em amostras de águas, sólidos em suspensão e sedimento do rio, à jusante de descargas de indústria têxtil. Os autores identificaram apenas 3 dos 15 corantes detectados: Disperse Blue 79, Disperse Blue 26 e Disperse Red 60. As concentrações dos corantes nas amostras de água variaram de 2,4 a 17,1 µg L^{-1}. Ao total, foram investigados 23 corantes de diferentes classes: ácidos, básicos, diretos, dispersos, mordentes e reativos, utilizados nas indústrias têxteis do Canadá (MAGUIRE; TKACZ, 1991).

Estudos realizados no Ribeirão dos Cristais, São Paulo, Brasil, revelaram a presença dos corantes Disperse Blue 373, Disperse Orange 37 e Disperse Violet 93 neste corpo d'água devido ao descarte de efluente de indústria têxtil, os quais estavam contribuindo para a mutagenicidade observada nas amostras de água superficial (UMBUZEIRO et al., 2005; OLIVEIRA et al., 2007). Posteriormente, estes mesmos corantes foram quantificados no efluente industrial bruto e tratado, no corpo d'água e na água tratada para abastecimento público, em concentrações que variaram de 0,002 a 360 µg L^{-1} (CARNEIRO et al., 2010). Recentemente, os corantes Disperse Red 1, Disperse Blue 373 e Disperse Violet 93 foram detectados em amostras do Rio Piracicaba e do Ribeirão dos Cristais, ambos localizados no Estado de São Paulo, Brasil, em concentrações de 0,08 a 3,5 µg L^{-1} (ZOCOLO et al., 2015), mostrando que o descarte de corantes através de despejos de efluentes de indústrias têxteis ainda está acontecendo nos rios do Brasil.

2. OBJETIVOS

Os objetivos principais deste trabalho foram avaliar a ocorrência de corantes e aminas aromáticas em amostras ambientais e avaliar o risco eco/genotoxicológico causados por estes compostos para a vida aquática.

Os objetivos específicos foram:

- Avaliar a ocorrência dos corantes Disperse Blue 291, Disperse Blue 373, Disperse Orange 1, Disperse Orange 30, Disperse Orange 37, Disperse Red 1, Disperse Violet 93, Disperse Yellow 3 e Disperse Yellow 7 em amostras ambientais;

- Avaliar a ocorrência das aminas aromáticas 2,4-Diaminotoluene, 2,5-Methoxymethylaniline, 3,3-Dichlorobenzidine, 4,4-Diaminodimethane, 4-Chloroaniline, 4-Nitroaniline e Aniline em amostras ambientais;

- Avaliar a genotoxicidade das amostras ambientais utilizando o teste *Salmonella*/microssoma e o ensaio do cometa com culturas celulares de peixe;

- Avaliar a mutagenicidade dos corantes individualmente e verificar a contribuição destes na mutagenicidade das amostras analisadas;

- Avaliar o risco ecotoxicológico do corante Disperse Red 1 para a vida aquática.

3. METODOLOGIA

3.1 Coleta e preparo de amostras

As amostras foram coletadas em quatro pontos incluindo amostras de águas superficiais do Rio Piracicaba e Ribeirão Quilombo e do efluente final da Estação de Tratamento de Efluentes (ETE) Carioba, que trata principalmente efluentes do parque têxtil de Americana e é lançado também no rio Piracicaba. O acondicionamento das amostras foi feito em frascos de vidro âmbar de 1 litro que foram lavados com uma solução de ácido nítrico (v/v) 10%. Após a coleta e transporte, as amostras foram processadas imediatamente.

Tanto para as análises químicas como para os testes de mutagenicidade e genotoxicidade foram feitas extrações orgânicas. Os corantes do tipo dispersos não tem mostrado boa adsorção em colunas, então, optou-se por utilizar extração líquido-líquido. Foram feitos alguns testes comparativos e verificou-se que a eficiência de extração de corantes utilizando o método líquido-líquido é maior (dados não publicados).

A extração líquido-líquido das amostras de águas superficiais e efluentes foi realizada empregando-se os solventes Metanol (CAS 67-56-1, Sigma-Aldrich, grau HPLC, pureza ≥ 99,9%) e Diclorometano (CAS 75-09-2, Macron, grau HPLC, pureza 99,5%) na proporção 1:2,5 (v/v) como fase extratora. Para cada litro das amostras foram adicionados 160 mL de fase extratora, agitado por 10 minutos e recolhido a fase orgânica. Tal procedimento foi repetido por mais duas vezes. Ao total, foi realizada a extração de 3 litros de cada amostra. Os extratos foram concentrados em evaporador rotativo até atingir cerca de 2-3 mL. Esse volume foi transferido para frascos de vidro de 4 mL e seco em fluxo de gás nitrogênio (White Martins, pureza 4.6 FID).

3.2 Cromatografia líquida acoplada à espectrometria de massas

Para identificar os compostos presentes nos extratos orgânicos foram realizadas análises por Cromatografia Líquida Acoplada à Espectrometria de Massas (HPLC-MS/MS), em cooperação com a Profª Drª Maria Valnice Boldrin Zanoni, do Instituto de Química, Unesp, Araraquara/SP.

Os extratos secos obtidos da extração líquido-líquido foram diluídos em 1mL de uma solução Metanol/Água (1:1, v/v) e submetidos as análises de HPLC-MS/MS, onde 20 µL de cada amostra foram injetados. A coluna utilizada durante as análises foi Phenomenex Kinetex PFP 5µm (4,6 x 150 mm). O método desenvolvido utilizou os solventes A (água) e B (Acetonitrila), ambos contendo 0,1% de ácido fórmico. O modo gradiente de eluição foi aplicado sendo 0,01 – 1,0 min (5% B), 1,0 – 5,5 min (5 – 9% B), 5,5 – 6,5 min (9 – 25% B), 6,5 – 9,5 min (25 – 40% B), 9,5 – 11,5 min (40 – 45,5% B), 11,5 – 16,5 min (45,5 – 60,5% B), 16,5 – 18,5 min (60,5 – 100% B), manteve-se em 100% de B por 4 min e condicionou-se a coluna por mais 6 min, totalizando o método em 30 min. O fluxo utilizado foi de 1500 µL min^{-1} e a temperatura da coluna foi de 40ºC. O modo gradiente de eluição foi desenvolvido baseado na alta retenção dos compostos, já observado no trabalho de ZOCOLO et al. (2015).

A detecção via espectrometria de massas (3200 QTRAP, ABSCiex) ocorreu através do experimento de reações múltiplas selecionadas (SRM), onde duas transições entre o íon precursor e seus íons fragmentos mais abundantes foram monitorados para cada composto. Para a otimização dos parâmetros, utilizou-se a infusão direta de cada padrão individualmente no fluxo de 10 µL min^{-1} através da seringa automática. A concentração dos padrões utilizadas para a otimização foi de 1 mg L^{-1} em metanol:água (1:1, v/v) contendo 0,1% de ácido fórmico. A ionização foi obtida com uma fonte de TurboIon Spray (*electrospray*) operando no modo positivo. Os parâmetros da fonte de ionização comum a todos os compostos de interesse foram: IonSpray: 5000 V, Temperatura: 600ºC, Gas 1: 45 psi, Gas 2: 45 psi e Curtain Gas: 15 psi.

As curvas analíticas para a quantificação dos compostos foram realizadas em amostras reais fortificadas das águas do rio (uma mistura de todos os pontos estudados). Depois de extraídas, as águas foram fortificadas com os analitos de interesse em diferentes níveis de concentração (0,05, 0,1, 0,5, 1, 5, 10, 25, 50, 100 e 200 ng L^{-1}). Devido a ausência de padrões, a quantificação foi realizada por curvas de padrão externo.

Para os estudos de recuperação do método, utilizou-se água ultrapura e uma mistura de amostra real de cada ponto do rio coletado. Um total de 250 mL foram fortificados com os corantes padrões para a concentração de 100 µg L^{-1} e submetidos à extração líquido-líquido. A recuperação foi, então, via comparação dessas amostras com os respectivos controles analisados, ou seja, amostra fortificada após extração.

A construção da curva de calibração foi realizada com no mínimo 6 pontos, onde a linearidade foi mantida, como estabelece a Diretiva Européia (2002/657/CE), seguida neste trabalho. Para a quantificação dos analitos nas amostras, utilizou-se os parâmetros descritos na Diretiva Européia (2002/657/CE), onde duas transições de SRM foram monitoradas para cada composto, sendo a SRM1 a transição utilizada para a quantificação e a SRM2 para a confirmação do analito. A relação das áreas entre as duas transições também foram monitoradas, uma vez que se trata de um requisito para a quantificação dos compostos, sendo que tal relação deve se manter sempre constante. Outro requisito exigido é o tempo de retenção dos compostos, que permaneceram constantes entre ambas as transições. Dessa forma, o método atinge os critérios para a quantificação e identificação dos compostos por HPLC-MS/MS. O cálculo dos limites de detecção e quantificação foram de 3 e 10 com relação ao sinal/ruído, como prevê a mesma diretiva. O software utilizado para o processamento dos dados foi Analyst 1.5.1 da AB SCiex.

3.3 Ensaio *Salmonella*/microssoma

A avaliação da mutagenicidade dos extratos orgânicos foi realizada utilizando o ensaio *Salmonella*/microssoma, empregando o protocolo de microssuspensão (KADO; LANGLEY; EISENSTADT, 1983), com as linhagens TA98 e YG1041 da bactéria *Salmonella typhimurium* em ausência e presença de ativação metabólica exógena (S9). Este protocolo é recomendado quando se dispõe de pouca quantidade de amostra ou quando se pretende usar maior número de linhagens.

Utilizou-se nos testes culturas pernoite concentradas 5 vezes, por centrifugação a 10000 g, por 10 minutos, a 4 °C. Os extratos orgânicos secos foram ressuspendidos em dimetilsulfóxido (DMSO, CAS 67-68-5, Sigma, pureza 99,5%) em diferentes doses e adicionou-se 5 µL de cada dose aos tubos de ensaio contendo 50 µL da linhagem e 50 µL da mistura S9. Para o teste em ausência de sistema de metabolização utilizou-se igual volume de tampão fosfato diluído 1/13 (50 µL). A mistura foi incubada a 37 °C por 90 minutos, e depois desse tempo, foi adicionado 2 mL de ágar de superfície (acrescido de biotina/histidina), fez-se o plaqueamento em 20 mL de ágar mínimo. As placas foram incubadas invertidas, durante 66 horas em temperatura de 37 ºC. Após o tempo de incubação procedeu-se então a contagem do número de colônias revertentes em todas as placas.

O controle negativo utilizado nos ensaios foi dimetilsulfóxido (DMSO, CAS 67-68-5, Sigma, pureza 99,5%). O controle positivo utilizado para a linhagem TA98 foi 2-aminoantraceno (2AA) a 0,625 µg/placa com ativação metabólica, e 4-nitroquinolina-1-óxido (4NQO) a 0,125 µg/placa em ausência de ativação metabólica. O controle positivo utilizado para a linhagem YG1041 foi 2-aminoantraceno (2AA) a 0,03125 µg/placa com ativação metabólica e 4-nitro-o-diamino-fenilina (4NOP) a 2,5 µg/placa sem ativação metabólica.

O número de revertentes no controle negativo é a taxa de reversão espontânea obtida para a linhagem nas condições do ensaio. Ao expor essa linhagem em doses crescentes de um composto com atividade mutagênica, o número de revertentes por placa aumenta, proporcionalmente ao aumento das doses, até atingir um valor máximo, no qual as doses posteriores causam letalidade. Espera-se, portanto, uma relação dose resposta entre o número de revertentes por placa e as doses de uma amostra com atividade mutagênica.

A razão de mutagenicidade (RM) foi calculada para cada dose analisada através da divisão da média de revertentes na placa teste (espontâneos e induzidos) pela média de revertentes na placa do controle negativo (espontâneos). A análise de variância (ANOVA) é aplicada para verificação de diferenças estatísticas entre o controle negativo e as doses aplicadas, seguida de regressão linear. A potência da amostra é expressa pela inclinação da parte linear da curva dose resposta. Quando a ANOVA e a dose resposta foram significativas e o valor da RM for maior ou igual a 2, a amostra foi considerada positiva. Quando a ANOVA e/ou a dose resposta não foram significativos, os resultados foram considerados negativos. As análises de variância e regressão linear foram realizadas no programa SALANAL, utilizando o modelo de Bernstein (BERNSTEIN et al., 1982), que consiste na retirada de uma ou mais doses da análise usando somente os resultados que representam a porção linear da curva dose resposta.

3.4 Ensaio do cometa e citotoxicidade

Esta parte do estudo foi realizada durante estágio no exterior, em colaboração com Prof Dr Alain Devaux e Profª Drª Sylvie Bony do "Laboratoire d'Ecologie des Hydrosystèmes Naturels et Anthropisés", Université de Lyon I, Lyon, França.

Os experimentos foram realizados utilizando a linhagem RTL-W1 (LEE et al., 1993), de células de fígado de truta arco-íris, com alta capacidade de biotransformação, representando um interessante modelo eucariótico, e mantendo as características fisiológicas específicas de peixes. As células foram cultivadas em meio Leibovitz (L15, Sigma) suplementado com 10% de soro fetal bovino (FBS), penicilina (100 IU/mL) e estreptomicina (100 μg/mL), em frascos de cultura celular de 75 cm^2 e mantidas a 20 ºC em incubadora livre de CO_2. As suspensões celulares foram obtidas a partir de tripsinização das células dos frascos de cultura utilizando 0,25% tripsina com 0,03% EDTA.

Para determinar as maiores concentrações a serem utilizadas no ensaio do cometa, foi avaliada a citotoxicidade das amostras através do ensaio de viabilidade celular com o reagente Cell-Titer-Blue® (Promega, France). Os extratos orgânicos secos foram ressuspendidos em DMSO e as concentrações foram preparadas com meio de cultura L15 sem soro bovino para evitar complexação dos corantes com as proteínas do soro, não ultrapassando 0,1% de DMSO do extrato. Vinte e quatro horas antes da exposição, 200.000 cel mL^{-1} foram transferidas para placas pretas de 96 poços. A citotoxicidade foi avaliada 24 horas após a exposição às amostras.

A viabilidade celular foi avaliada através da intensidade da fluorescência do reagente Cell-Titer-Blue reduzido (590 nm), sendo a intensidade proporcional à quantidade de células vivas. A viabilidade das células resultantes de cada tratamento foi avaliada comparando com as células do controle negativo. Em seguida, o modelo de Hill (software RegTOX) foi aplicada às curvas dose/resposta obtidas, a fim de calcular a CI10 para cada amostra. Foram realizados três testes independentes para cada amostra, cada teste com 7 doses e 6 poços por dose.

Para os ensaios do cometa, as placas de 24 poços foram preparadas 24 horas antes da exposição e contendo 200.000 cel mL^{-1} em cada poço. O dano primário ao DNA foi avaliado após exposição de 24 horas e para evitar falsos positivos (DNA danificado de células mortas) foram utilizadas apenas concentrações abaixo do CI10.

O teste do cometa modificado com a enzima Fpg foi realizado sob condições alcalinas baseado no método proposto por COLLINS et al. (1996) e modificado por KIENZLER et al. (2012). Rapidamente, após o período de exposição, as células foram tripsinizadas e misturadas com igual volume de agarose de baixo ponto de fusão a 1%. Quarenta µL desta mistura foram dispostos em lâmina de microscópio pré-revestidas com agarose normal (0,8%) e cobertos com lamínulas de 22x22 mm (2 géis por lâminas, 2 lâminas por amostra). Depois da solidificação da agarose (4°C, 5 min), as lamínulas foram retiradas e as lâminas foram colocadas em uma solução de lise (2.5 M NaCl, 0.1 M Na_2EDTA, 10mM Tris, 10% DMSO, 1% Triton X100; pH 10) a 4°C durante 1h30 no escuro. As lâminas foram lavadas, três vezes de 5 minutos cada, com tampão (0.1 M KCl, 0.5 mM Na_2EDTA, 40 mM HEPES KOH, 0.2 mg mL^{-1} albumina de soro bovino, pH 8.0), e depois foram incubadas por 30 minutos com a enzima Fpg a 37ºC (New England Biolabs, 0.4 UI/gel). As lâminas foram lavadas, apenas uma vez, com tampão PBS e colocadas em cuba de eletroforese contendo a solução de 0.3M NaOH e 1 mM Na_2EDTA durante 40 minutos para permitir o desenrolamento do DNA. A eletroforese foi realizada com 0.6 V cm^{-1} durante 24 min. As lâminas foram neutralizadas (0.4 M Tris-HCl, pH 7.5) com três lavagens de 5 minutos cada, desidratadas em etanol absoluto por 20 minutos e secas em temperatura ambiente.

Antes de realizar a leitura por microscopia de fluorescência (Axioskop 40, Zeiss), as lâminas foram coradas com 25 µL de uma solução de brometo de etídio a 0,02%, e as imagens foram analisadas pelo software Comet 4.0 (Perceptive Instruments). Para cada concentração, a porcentagem da intensidade da cauda do cometa de 50 células selecionadas aleatoriamente foi registrada por gel (2 géis por amostra). Os resultados foram expressos como a média e o desvio padrão de três experimentos independentes. Os dados foram analisados estatisticamente com o teste de Kruskal-Wallis (Statistica©-StatSoft, Inc.) e com o teste não-paramétrico Mann-Whitney para identificar os tratamentos diferentes significantemente do controle.

3.5 Avaliação do risco ecotoxicológico

Dados toxicológicos

Para a derivação de critérios para proteção da vida aquática para o corante Disperse Red 1 foram utilizados dados toxicológicos da literatura (VACCHI, 2012; VACCHI et al., 2013; RIBEIRO; UMBUZEIRO, 2014). Apenas o teste crônico com *Daphnia similis* foi realizado para complementar os dados e refinar a derivação de critérios, uma vez que *D. similis* foi o organismo mais sensível ao corante Disperse Red 1 em testes de toxicidade aguda (VACCHI, 2012).

O teste crônico foi realizado de acordo com LAMEIRA (2008), utilizando neonatas de *D. similis* com menos de 24 horas de vida, expostas a diferentes concentrações da substância-teste durante 14 dias. Para cada concentração, inclusive para o controle negativo (água de cultivo), foram preparadas 10 réplicas com 50 mL de amostra e um organismo por recipiente. O ensaio foi realizado em ambiente com temperatura controlada entre 23 e 25 ºC, e com fotoperíodo de 16 horas de luz e 8 horas de escuro. Diariamente, os organismos foram alimentados com algas (*Pseudokirchneriella subcapitata*) e, a cada dois dias, foram realizados a contagem de neonatas nascidas e a troca total da solução-teste. No término do ensaio, foram somados os números de neonatas nascidas em cada concentração e no controle. Atentando-se que, para a validação dos resultados ao término do ensaio, a mortalidade das matrizes do controle não deve ultrapassar 20%, até 60% das matrizes do controle devem apresentar 3 posturas e a média de neonatas das matrizes do controle deve ser ≥23. Os dados foram analisados no programa ICp (versão 2.0) (NORBERT-KING, 1993) que compara a fecundidade das matrizes do controle e das amostras.

Derivação de critérios para proteção da vida aquática

A derivação de critérios que visam proteger as diferentes formas de vida aquática dos ecossistemas de água doce e marinha dos efeitos adversos causados por agentes químicos, é um importante passo na avaliação do risco ecotoxicológico. A base de dados toxicológicos para cada substância para a qual um critério será derivado deve ser proveniente de ensaios com protocolos padronizados. Para derivar um critério, conhecido internacionalmente como *Predicted No-Effect Concentrations* (PNEC),

devem ser utilizados, preferencialmente, dados crônicos de espécies de diferentes níveis tróficos, representando uma grande variedade de grupos taxonômicos e espécies sensíveis, embora dados agudos possam também ser utilizados (EUROPEAN COMMISSION, 2011; SBMCTA, 2011).

Os valores de PNECs para o corante Disperse Red 1 foram derivados de acordo com normativa da União Européia (EUROPEAN COMMISSION, 2011), utilizando a abordagem determinística que visa utilizar o menor *endpoint* toxicológico e aplicar um fator de avaliação (*Assessment Factor* – AF) para extrapolar a uma concentração ambientalmente protetiva. A fim de abranger efeitos gerados de exposições a curto e longo prazos, dois PNECs são normalmente recomendados de acordo com esta norma: um critério de proteção a longo prazo baseado em dados toxicológicos crônicos e um critério de proteção a curto prazo baseado em dados toxicológicos agudos.

Avaliação do risco ecotoxicológico

O quociente de risco (*Risk Quotient* – RQ) é a primeira etapa para a avaliação do risco de substâncias químicas para o ambiente aquático e pode ser realizado através da comparação da concentração medida no ambiente (*Measured Environmental Concentration* – MEC) com o PNEC (EMA, 2006). Os valores de MEC podem ser baseados na concentração máxima ou na média dependendo da abordagem utilizada. Se a relação MEC/PNEC for maior que 1, o risco ecotoxicológico deve ser considerado. Assim, para o cálculo do RQ para o corante Disperse Red 1 foram utilizadas as concentrações determinadas nas análises químicas deste trabalho, considerando a concentração máxima e a concentração média obtidas.

4. RESULTADOS

Os resultados obtidos neste trabalho foram organizados em três capítulos, apresentados a seguir, e correspondem aos três artigos que resultaram deste trabalho. O Capítulo I - "*Quantifying the contribution of dyes in the mutagenicity of environmental samples under the influence of textiles activities*" apresenta a avaliação da ocorrência de corantes dispersos em amostras ambientais com influência de atividades têxteis e a contribuição dos corantes na mutagenicidade encontrada nas amostras.

O Capítulo II - "*Combining different assays and chemical analysis to characterize the genotoxicity of waters impacted by textile discharges*" compreende a avaliação da genotoxicidade das amostras e a comparação das respostas obtidas nos ensaios do cometa e *Salmonella*/microssoma com as análises químicas.

E, finalmente, o Capítulo III - "*Occurrence and risk assessment of an azo dye - the case of Disperse Red 1*" apresenta a avaliação do risco ecotoxicológico do corante Disperse Red 1, bem como a derivação de critérios para proteção da vida aquática baseados na avaliação da toxicidade do produto comercial contendo este corante.

4.1 Capítulo I

Quantifying the contribution of dyes in the mutagenicity of environmental samples under the influence of textiles activities

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Abstract

Dyes are used in the coloration of different substrates, but the largest use is on textiles industries. Dyes are emergent contaminants and have being found in the environment, especially in Brazil, India and China. The aim of this study was to analyze the occurrence of dyes in surface water under the influence of textile industries discharges; and evaluate the contribution of dyes in the mutagenicity of those samples. Samples were collected in Piracicaba river before WWTP discharge, Quilombo river, WWTP treated effluent, and Piracicaba river after WWTP, in Americana city, SP State, Brazil. Samples were organically extracted by liquid-liquid using dichloromethane/methanol (2.5:1 v/v) and analyzed by a Linear Ion Trap Quadrupole LC-MS/MS. Extracts were tested in the Salmonella/microsome assay using microsuspension method with strains TA98 and YG1041, in the absence and presence of exogenous metabolic activation (S9). Six disperse dyes were detected in in the samples and the most frequent dyes were Disperse Red 1, Disperse Blue 291, Disperse Blue 373, Disperse Violet 93 and Disperse Yellow 3. Samples were mutagenic in Salmonella/microsome assay and the highest potencies were found in Piracicaba River after the discharges with YG1041 with S9. The dyes Disperse Blue 373 and Disperse Violet 93 are the major contributors to the mutagenicity of the samples, corresponding more than 40% in Quilombo river and more than 30% in Piracicaba river after the discharges, both cases with YG1041 with S9. We can conclude that dyes are present and contributing for the mutagenicity of Piracicaba River; and the discharges of Quilombo River and WWTP effluent are increasing in 10 times the mutagenicity of Piracicaba River.

Key-words: Dyes; mutagenicity; surface water.

1. Introduction

The combination of chemical analyses and bioassays allows the identification of potentially mutagenic in several types of samples (Brack 2003). There is increasing interest in the application this approach for water quality monitoring, since the biological tools have to be carefully selected with respect to their ability to detect specific effects and their significance in hazard assessment. This approach offers opportunities to reveal and prioritize pollutants with potential health impact that are currently not included in monitoring programs (Brack et al 2016).

Bioassays, such as the Salmonella/microsome assay, produce an interesting response to a complex mixture evaluation without prior knowledge of the chemical sample composition (Claxton et al 2004). This assay can be considered an important bioanalytical tool and the responses can be linked to specific adverse outcome pathways when the ultimate goal is to protect the quality of the environment at the population level (Altenburger et al 2015). Several studies have been identified water contaminants by integrating chemical screening and the Salmonella/microsome assay (Gallampois et al 2013; Liu et al 2015).

Azo dyes are the most widely used synthetic organic dyes in textiles (Bafana et al 2011) and showed genotoxic and mutagenic activity in mammalian and bacterial systems (Rajaguru et al 1999; Umbuzeiro et al 2005a; Tsuboy et al 2007; Chequer et al 2009; Oliveira et al 2010). Waters under the influence of textile discharges can exhibit genotoxic and mutagenic activity, which has been related to the presence of certain dyes and aromatic amines. However in the Cristais River case study, mutagenic compounds were not quantified in the samples and it was not possible to know the contribution of each one in the mutagenicity of the river (Umbuzeiro et al 2005b; Oliveira et al 2007). Considering this, the aim of this study was to identify the occurrence of azo dyes in environmental samples under the influence of textile discharges and to verify their contribution to the mutagenicity of those samples.

2. Materials and methods

2.1 Study area and sampling

The largest textile industrial conglomerate of Brazil is located in the city Americana, in São Paulo State. A Wastewater Treatment Plant is responsible for the collection and treatment of several of those industrial effluents. It uses a biological

treatment and the final effluent is released in one of the most important rivers in São Paulo, the Piracicaba River. Unfortunately this type of biological treatment alone is poorly efficient for the removal of disperse dyes (USEPA 1990) and Piracicaba River is supposed to support aquatic life and human consumption, according to Brazilian law. Furthermore, the WWTP does not treat all industrial effluents and several facilities discharge their effluents directly to a tributary of Piracicaba River, called Quilombo. Four sampling campaigns were performed during 2013 and samples were collected from the WWTP outflow, Quilombo River, Piracicaba River upstream and downstream the discharges (Figure 1). Four liters of each sample were collected in amber glass vials and transported in ice to the laboratory where they were immediately processed (APHA 1999).

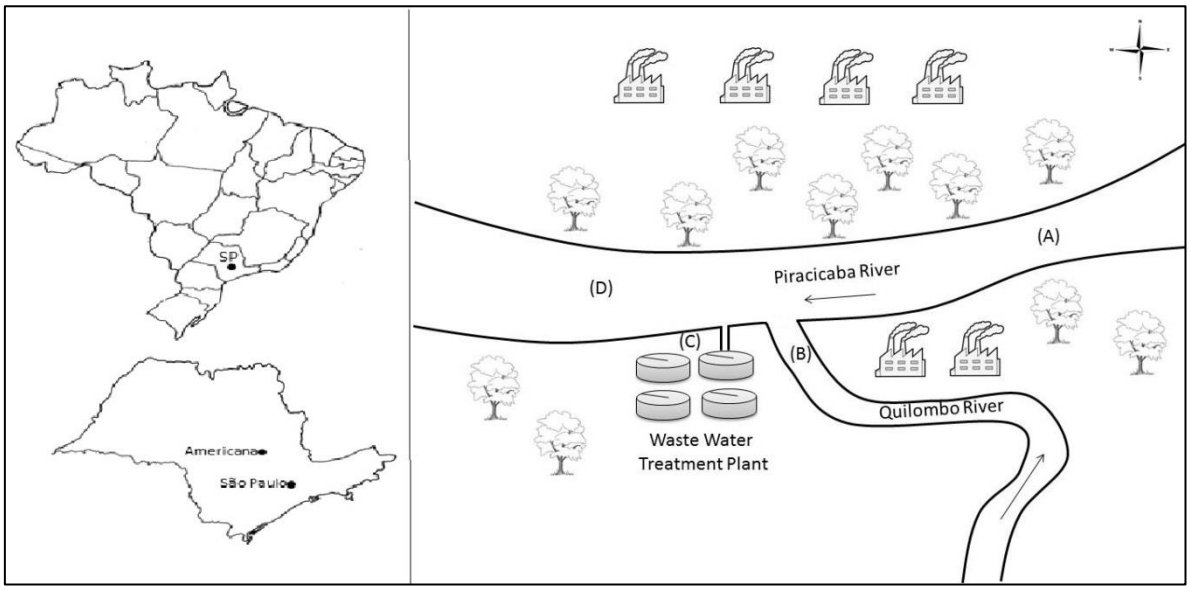

Figure 1. Sites of sampling at Piracicaba River upstream (A), Quilombo River (B), Wastewater Treatment Plant (C) and Piracicaba River downstream (D) in Americana city, São Paulo State, Brazil.

2.1 Liquid–liquid extraction/concentration procedure

Environmental samples usually need to be prepared before testing in *in vitro* biological systems that uses bacteria or cell culture. When the compounds of interest belong to the organic class, solid phase or liquid-liquid extractions are employed. The resultant extract is then dried and then re-dissolved in compatible solvents to allow bio-testing. In this work, extraction was done by liquid-liquid processing using

dichloromethane (DCM) and methanol (2.5:1) as organic phase because this procedure had been proved to be effective in other studies related to textile industries (Umbuzeiro et al 2004). The extract volume was reduced in a rotary evaporator and then completely dried using pure nitrogen gas. Extracts were carefully kept frozen at -20°C in amber glass vials. Before testing, a minimal volume of DCM was added just to facilitate dissolution along with the needed amount of DMSO. DCM was then completely evaporated using pure nitrogen gas before the beginning of the tests. A blank of this last drying procedure was prepared to make sure it does not produce toxicity in the selected tests.

2.2 HPLC-MS/MS analyses

Chemical analyses were performed in the same extracts using a high performance liquid chromatography (HPLC) Agilent 1200 system (Waldbronn, Germany) coupled to an AB Sciex 3200 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (MS). The extracts were diluted with methanol:water (50:50, v/v) containing 0.1% formic acid. Chromatographic separation was accomplished using a Kinetex PFP analytical column (150 mm x 4.6 mm; 5 µm, Phenomenex). The mobile phase constituted of water (A) and acetonitrile (B), both spiked with 0.1% formic acid, at flow rate of 1.5 mL min^{-1} with the following gradient program for water/acetonitrile: 0 - 1 min, 5% B; 1 - 5.5 min, 5 - 9% B; 5.5 - 6.5 min, 9 - 25% B; 6.5 – 9.5 min, 25 – 40% B; 9.5 – 11.5 min, 40 – 45.5% B; 11.5 – 16.5 min, 45.5 – 60.5% B; 16.5 – 18.5 min, 60.5 – 100% B, 18.5 - 23 min, 100% B and re-established by 5% B over 7 min, total run length was 30 min. Column temperature was set to 40 °C and injection volume was 20 µL. The chromatography system was coupled to the 3200 QTRAP via an electrospray ionization (ESI) source, operating in positive ion mode with the following ionization parameters: spray voltage, 5500 V; capillary temperature, 650 °C; the nebulizing gas (Nitrogen, 45 psi); the heating gas (Nitrogen, 45 psi) and the curtain gas, 15 psi. Identification of analytes was performed in the selected reaction monitoring (SRM) mode, where two SRM transitions were selected to eliminate any false result. Fragmentation parameters were optimized by direct infusion of solutions of the individual compounds prepared at concentration of 0.1 mg L^{-1} in methanol/water (50:50,v/v) containing with 0.1% formic acid. The flow used in this analysis was 10 µL

min^{-1}. In this step the following parameters were analyzed: collision energy (CE), declustering potential (DP), entrance potential (EP), cell entrance potential (CEP) and collision cell exit (CXP). All parameters of each compound analyzed are resumed in Table 1.

Table 1. Optimized parameters to identify disperse dyes by HLPC-MS/MS, using SRM mode.

	SRM			**MS/MS**				
Compounds	**Precursor Ion (*m/z*)**	**Fragment Ion (*m/z*)**	**Dwell Time (ms)**	**DP (V)**	**EP (V)**	**CEP (V)**	**CE (V)**	**CXP (V)**
Disperse Blue 291	511	207	5	56	8.5	22	39	4
	511	192	5	56	8.5	22	47	4
Disperse Blue 373	533	260.4	5	66	5	24	25	4
	533	245.4	5	66	5	24	33	4
Disperse Orange 1	319	169.2	5	56	4	24	35	4
	319	122.2	5	56	4	24	29	4
Disperse Orange 30	450	87	5	46	7.5	24	49	4
	450	132	5	46	7.5	24	33	4
Disperse Orange 37	392	351	5	51	5	32	21	6
	392	133	5	51	5	32	51	4
Disperse Red 1	315	134	5	51	4	24	33	4
	315	255	5	51	4	24	29	4
Disperse Violet 93	481	191	5	56	7.5	20	37	4
	481	206	5	56	7.5	20	25	4
Disperse Yellow 3	270.2	107.2	5	41	5.5	18	33	4
	270.2	108.1	5	41	5.5	18	39	4
Disperse Yellow 7	317.1	77	5	51	4	22	47	4
	317.1	105.1	5	51	4	22	29	4

(SRM) selected reaction monitoring; (MS) mass spectrometer; (DP) declustering potential; (EP) entrance potential; (CEP) cell entrance potential; (CE) collision energy and (CXP) collision cell exit.

2.2 *Salmonella*/microsome microsuspension assay

We employed the microsuspension method of the *Salmonella*/microsome assay because of its high sensitivity when limited amount of sample is available (Kado et al 1983). The strain TA98 (*his*D3052, *rfa*, Δ*bio*, Δ*uvr*B, and pKM101) was selected because it has been the most used in monitoring studies and provides the majority of the positive responses for surface water testing (Umbuzeiro et al 2001; Ohe et al 2004). It was also decided to include the strain YG1041 (a derivative of the TA98 that overproduces nitroreductase and O-acetyltransferase) because of its higher sensitivity to compounds containing nitro and amine groups (Hagiwara et al 1993), usually present in dyeing textile plant effluents (Umbuzeiro et al 2005b; Oliveira et al 2006; Freeman 2013). If a marked increase of the YG1041 response is observed in relation to its parental TA98, this indicates that nitroaromatics (response without S9) and aromatic amines (response with S9) are present in the samples (Umbuzeiro et al 2011). Here the application of two strains aims to confirm of what type of compounds are causing the observed effect.

Overnight cultures of each strain were concentrated by centrifugation at 10,000g at 4 ºC for 10 min and resuspended in 0.015 M sodium phosphate buffer. Six different doses ranging from 0.07 to 40 mL equivalent/plate of samples were tested, along with negative and positive controls. Each concentration was tested in duplicates. Tests were performed in the presence and absence of a 4% (v/v) lyophilized Aroclor-1254-induced rat liver S9 fraction (Moltox Inc., Boone, NC) with cofactors, using dimethylsulfoxide (DMSO) as a solvent and negative control. Volumes of 50 µl of cell suspension, 50 µl of 0.015M sodium phosphate buffer (or S9 mix) and 5 µl of each extracts dilution were added to each tube and incubated at 37 ºC for 90 min without shaking. After incubation, 2 mL of molten agar was added, the mixture was poured onto a minimal agar plate, and the plates were incubated at 37 ºC for 72 h. Colonies were manually counted under a stereoscope. Toxicity was also evaluated by visual observation under a stereoscope. Preliminary testing was performed (data not shown) and when toxicity was observed, only non-toxic doses were included in the final experiment. Positive controls for TA98 were 0.125 µg/plate of 4-nitroquinoline-oxide (4NQO) (Sigma-Aldrich) without S9 and 0.625 µg/plate of 2-aminoanthracene (2AA) (Sigma-Aldrich) with S9, both dissolved in DMSO. For YG1041, 2.5 µg/plate of 4-nitro-*ortho*-phenylenediamine (4NOP) (Sigma-Aldrich) without S9 and 0.03125 µg/plate of 2-aminoanthracene (2AA) (Sigma-

Aldrich) with S9, dissolved in DMSO. Results were statistically analyzed using an ANOVA followed linear regression using the Bernstein model (Bernstein et al 1982). Samples were considered positive when significant ANOVA ($p < 0.05$) and positive dose response were obtained ($p < 0.05$). Model was considered acceptable when $p > 0.05$.

3. Results & Discussion

3.1 Chemical analysis

The methodology developed in this study was efficient in separation and identification of dyes in environmental matrices, since 6 different dyes were detected and quantified in the samples in a range of 0.01 to 6.81 µg L^{-1} (Table 2). The dyes Disperse Red 1 and Disperse Blue 373 were the most frequents dyes, present in 50% and 43.75%, respectively, of the samples analyzed; and they were also detected in Piracicaba River and Cristais River in previously studies (Zocolo et al 2015; Vacchi et al 2016). Disperse Orange 1, Disperse Orange 37 and Disperse Yellow 7 were not quantified in any sample, because their concentrations were lower than the limit of quantification (LOQ).

Table 2. Recovery (%), limits (µg L^{-1}), concentrations (µg L^{-1}) and frequency (%) of azo dyes analyzed in environmental samples

Dyes	CAS number	Recovery (%)	Limits (µg L^{-1})		Sampling 1 (µg L^{-1})				Sampling 2 (µg L^{-1})				Sampling 3 (µg L^{-1})				Sampling 4 (µg L^{-1})				Frequency (%)
			LOD	LOQ	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	
Disperse Blue 291	56548-64-2	107.2	0.0022	0.0075	-	-	-	-	-	-	-	-	0.04	-	0.05	-	-	-	-	-	12.50
Disperse Blue 373	51868-46-3	129.8	0.0016	0.0054	-	-	0.35	-	-	-	0.08	-	-	1.38	0.15	3.13	-	0.54	-	0.28	43.75
Disperse Orange 1	2581-69-3	88.7	0.0022	0.0072	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Disperse Orange 30	5261-31-4	61.4	0.0128	0.0427	-	-	-	-	-	-	-	-	-	0.14	-	-	-	-	-	-	6.25
Disperse Orange 37	13301-61-6	166.1	0.0136	0.0228	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Disperse Red 1	2872-52-8	120.0	0.0003	0.0010	0.52	-	0.03	-	-	-	0.19	-	-	0.09	0.08	0.11	-	0.15	-	0.13	50
Disperse Violet 93	52697-38-8	90.7	0.0064	0.0214	-	0.08	-	-	-	0.08	-	-	-	2.81	-	6.81	-	-	-	-	25
Disperse Yellow 3	2832-40-8	88.5	0.0020	0.0066	-	-	-	-	-	-	-	-	-	0.01	-	0.03	-	0.41	-	0.02	25
Disperse Yellow 7	6300-37-4	80.7	0.0010	0.0032	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0

- < LOQ
LOD: Limit of Detection
LOQ: Limit of Quantification
A: Piracicaba River upstream
B: Quilombo River
C: WWTP effluent
D: Piracicaba River downstream

3.1 *Salmonella*/microsome microsuspension assay

Organic extracts were tested with strains TA98 and YG1041. TA98 is the most used in mutagenicity studies and it was chosen due to the fact that it is considered a sensitive and reliable tool in the detection of mutagenic activity in aquatic environment (Ohe et al 2004). YG1041 presents high levels of acetyltransferase and nitroreductase enzymes and is more sensitive to of nitroaromatics, in the absence of S9 and aromatic amines, which require S9 to reveal its mutagenic effects (Hagiwara et al 1993). Mutagenicity was detected in 94% of the samples with strain YG1041, with higher responses with S9. Only one sample was positive with TA98, without S9 (Table 3).

In Figure 2 is shown the mutagenic potencies of each site in all samplings and is possible to see that, besides the higher variation of responses between the four samplings, Quilombo River and WWTP discharges are contributing to the mutagenicity of Piracicaba River, which was increased 10 times.

The increase in the response with YG1041 in relation to TA98, especially in the presence of S9 is characteristic of aromatic amines compounds, which are the class of azo dyes we analyzed in this study. Therefore we suspected that those azo dyes were contributing to the mutagenicity observed in the samples. To evaluate their contribution we selected the responses of YG1041 with S9, which provided the most relevant responses.

Table 3. Mutagenic potencies (rev L^{-1}) of environmental samples tested with strains TA98 and YG1041, without (-S9) and with (+S9) metabolic activation

		TA98 (rev L^{-1})		YG1041 (rev L^{-1})	
		-S9	+S9	-S9	+S9
Sampling 1	A	ND	ND	6,000	4,000
	B	ND	ND	ND	68,000
	C	ND	ND	ND	145,000
	D	ND	ND	9,000	10,000
Sampling 2	A	ND	ND	30,000	13,000
	B	ND	ND	2,000	34,000
	C	ND	ND	82,000	65,000
	D	ND	ND	41,000	28,000
Sampling 3	A	ND	ND	3,000	ND
	B	5,500	ND	5,800	50,550
	C	ND	ND	ND	85,000
	D	ND	ND	3,400	149,000
Sampling 4	A	ND	ND	8,000	ND
	B	ND	ND	ND	ND
	C	ND	ND	ND	37,000
	D	ND	ND	21,000	11,000

A: Piracicaba River upstream
B: Quilombo River
C: WWTP effluent
D: Piracicaba River downstream
ND: not detected

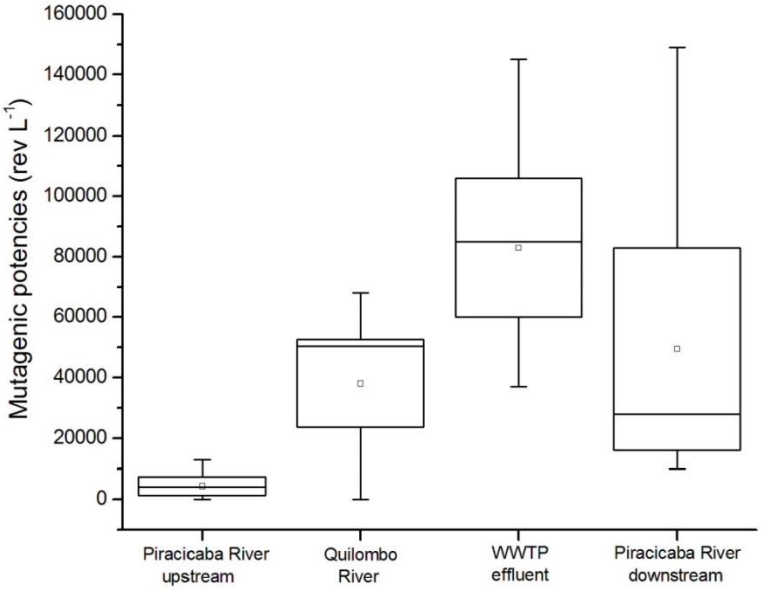

Figure 2. Mutagenic potencies (rev L^{-1}) of Piracicaba River upstream, Quilombo River, WWTP effluent and Piracicaba River downstream to strain YG1041 with S9.

3.2 Contribution of dyes in the mutagenicity

Considering the concentration of dyes in each environmental sample and the mutagenic potency of each dye, it was possible to calculate the individual contribution of the dyes on the most mutagenic scenario (YG1041, with S9). Mutagenicity of dyes Disperse Blues 291, Disperse Blue 373, Disperse Violet 93 and Disperse Red 1 was previously studied for YG1041 and their potencies for this strain with S9 were 180, 6300, 4600 and 207 rev μg^{-1}, respectively (Umbuzeiro et al 2005a; Umbuzeiro et al 2005b; Vacchi et al 2013). No data was found in the literature for the dyes Disperse Orange 30 and Disperse Yellow 3, so we tested each one for strain YG1041 in order to complete the evaluation of the contribution and their potencies for this strain with S9 were 5.9 and 5.5 rev μg^{-1}, respectively.

Disperse Red 1, besides the most frequent dye detected in the samples, their contribution on the mutagenicity of samples was low (0.004 – 2.7%). However, even Disperse Red 1 presents low mutagenic profile and low mutagenic contribution, this dye have been studied and showed high ecotoxicity (Ferraz et al 2011; Vacchi et al 2013; Ribeiro and Umbuzeiro 2014). The concentrations found for Disperse Red 1 in the samples are higher than the Predicted No-Effect Concentration (PNEC) of 60 ng L^{-1} (Vacchi et al 2016), so this dye can pose a potential risk to freshwater biota.

Disperse Blue 373 and Disperse Violet 93 were the major contribution for the mutagenicity, counting with more than 40% of the mutagenicity of Quilombo River considering all samplings (Figure 3), also they were the compounds found in the most higher concentration in the samples, especially in Piracicaba river downstream, and they presented the most higher mutagenic potencies.

Several studies are using the combination of chemical analyses and bioassay in order to identify mutagenic compounds in water and sediment samples, and most of them applying a fractionation step of the samples according to different physicochemical properties in order to reduce the chemical complexity of fractions and facilitates chemical analysis (Brack 2003). Highly potent nitro-PAHs such as dinitropyrene (DNP) isomers, 3-nitrobenzanthrone (3-NBA) and nitrobenzo[a]pyrenes have been detected in samples of sediment of industrial area in Germany; and, in some fraction of the samples, the compounds 1,8-DNP and 3-NBA explain together more than 70% of the mutagenicity (50% 1,8-DNP and 21% 3-NBA) (Lübcke-von Varel et al 2012).

Considering this, in our work we didn't apply a fractionation of the samples and we could explain more than 40% of the mutagenicity. The use of strains with different metabolic capacities gives indications to the class of mutagens present and in this study we knew what classes of compounds were used in industrial area of sampling site, since Americana city has the biggest textile production of Brazil. So we used this previously knowledge in combination of diagnostic strain to guide the mutagenicity tests without fractionation and we could explain the mutagenicity by the presence of dyes with success.

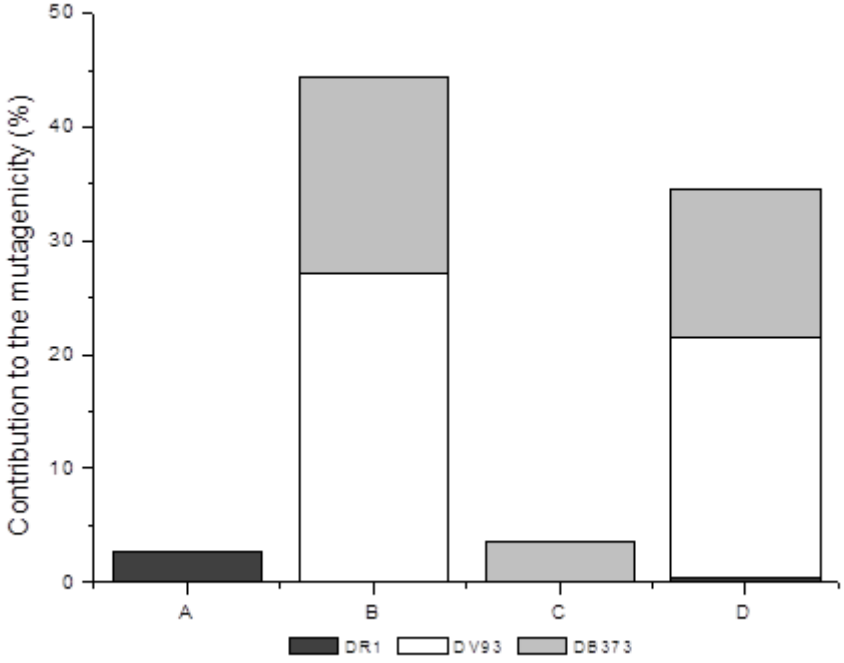

Figure 3. Contribution of the dyes Disperse Red 1 (DR1), Disperse Violet 93 (DV93) and Disperse Blue 373 (DB373) to the mutagenicity of samples Piracicaba River upstream (A), Quilombo River (B); WWTP effluent (C) and Piracicaba River downstream (D) using data with strain YG1041 (+S9).

4. Conclusion

The dyes Disperse Blue 291, Disperse Blue 373, Disperse Orange 30, Disperse Red 1, Disperse Violet 93, and Disperse Yellow 3 were detected in samples of Piracicaba River, Quilombo River and WWTP effluent. Disperse Red 1, besides the most frequent dye detected in the samples, their contribution on the mutagenicity of samples was low. Disperse Blue 373 and Disperse Violet 93 are the major contributors to the mutagenicity of the samples, corresponding more than 40% in Quilombo river and more than 30% in Piracicaba river after the discharges, both cases with YG1041 with S9.

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4.2 Capítulo II

Combining different assays and chemical analysis to characterize the genotoxicity of waters impacted textile discharges

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Abstract

Waters receiving textile discharges can exhibit genotoxic and mutagenic activity, which has been related to the presence of dyes and aromatic amines as synthesis precursors or byproducts. The aim of this study was to identify dyes and aromatic amines in water samples impacted by textile discharges, and to evaluate the genotoxic responses of these samples using the Salmonella/microsome assay in strains TA98 and YG1041, and the Fpg-modified comet assay in the RTL-W1 fish cell line. The genotoxicity of river samples downstream of the discharge was greater than the upstream samples in both of the Ames tests. The Fpg-modified comet assay detected similar levels of DNA damage in the upstream and downstream samples. Mutagenicity was not detected with TA98, except for the Quilombo River samples, but when YG1041 was used as the tester strain mutagenicity was detected for all sites with a very different profile in upstream sites relative to the other sites. The mutagenic response strongly indicated that aromatic amines or dyes were contributing to the mutagenic activity downstream. The impact of textile discharges was also confirmed by chemical analysis, because the highest concentrations of azo dyes and aromatic amines were detected in the river downstream. This study shows the value of combining assays measuring complementary endpoints to better characterize the mutagenicity of environmental samples, with the advantage that this approach provides an indication of what classes of compounds are responsible for the effect.

Key-words: genotoxicity, mutagenicity, azo dyes, aromatic amines.

1. Introduction

Waters receiving textile discharges can exhibit genotoxic and mutagenic activity, which has been related to the presence of certain dyes and aromatic amines (Oliveira et al., 2007; Umbuzeiro et al., 2005b). Several dyes, especially disperse azo dyes that are among the most widely used synthetic organic dyes in textiles (Bafana et al., 2011), have clear-cut genotoxic and mutagenic activity in mammalian and bacterial systems (Chequer et al., 2009; Oliveira et al., 2010; Rajaguru et al., 1999; Tsuboy et al., 2007; Umbuzeiro et al., 2005a). Aromatic amines are used as precursors in the synthesis of dyes, remaining as impurities in the final product, and they can be formed from the cleavage of the dyes after they are discharged into the environment (Freeman, 2013). A select group of aromatic amines has been prohibited in dye production because of their carcinogenic properties (ETAD, 2014). Nevertheless, non-prohibited aromatic amines exhibiting genotoxic and mutagenic properties (Josephy et al., 2016) remain of public health concern because they have been found in environmental samples (Oliveira et al., 2007) and clothing textiles (Brüschweiler et al., 2014).

The genotoxicity of environmental contaminants, such as dyes and aromatic amines, is not only a concern for humans but also for aquatic life. Genotoxicity endpoints are considered of high relevance in predicting chronic ecotoxicity, because they can lead to delayed adverse consequences at population levels if deleterious mutations occur. Recent studies carried out in fish and invertebrates have demonstrated that primary DNA damage in gametes of aquatic species lead to reproduction impairment including high mortality and abnormality rates in the early life stages of progeny, and impairment in population dynamics in the long term (Devaux et al., 2015; Lacaze et al., 2011; Santos et al., 2013).

Azo dyes (Carneiro et al., 2010; Maguire, 1992; Umbuzeiro et al., 2005b; Zocolo et al., 2015) and aromatic amines (Ohe et al., 1999; Oliveira et al., 2007) have been found in aquatic ecosystems, and data on acute and chronic toxicity in aquatic organisms show possible effects on fitness endpoints such as survival, reproduction, and growth (Bae and Freeman, 2007; Ferraz et al., 2011; Immich et al., 2009; Liu et al., 2007; Luna et al., 2014; Novotný et al., 2006; Vacchi et al., 2013). However, studies in aquatic organisms dealing with the genotoxic potency of environmental samples containing azo dyes and aromatic amines are still scarce (Al-Sabti, 2000; Cavaş and Ergene-Gözükara, 2003; Zhang et al., 2014), despite the fact that such compounds in their pure or commercial form have proved to be genotoxic and mutagenic in bacteria

and mammalian cells (Chequer et al., 2011, 2009; de Oliveira et al., 2016; Ferraz et al., 2011; Oliveira et al., 2007, 2006).

The Salmonella/microsome assay (Ames test) is one of the most used in vitro tests to assess genotoxicity in waters impacted by textile discharges (Claxton et al., 2010). In Brazil, Sanchez et al. (1988) and Valent et al. (1993) found that effluents from textile factories produced the greatest number of positive responses in the Salmonella/microsome assay among several industrial effluents analyzed in São Paulo State. Umbuzeiro et al. (2004) found that Cristais River, São Paulo, Brazil, was contaminated with mutagenic compounds released by a dye processing plant. River water, sediment, and sludge from the Waste Water Treatment Plant and drinking water derived from this river all had mutagenic activity. In this case study (Umbuzeiro et al., 2005b) azo dyes, their reduced/chlorinated derivatives (Oliveira et al., 2006), and aromatic amines (Oliveira et al., 2007) were at least partially responsible for the mutagenic activity. Mathur et al. (2012) also observed mutagenic activity in surface water of the Bandi River, and groundwater from Pali, Rajasthan, India, due to substances present in textile wastewater.

The Ames test has been used with strains that are very sensitive to the mutagenicity of azo dyes (Umbuzeiro et al., 2005a) and aromatic amines (Hagiwara et al., 1993). Differential mutagenicity in these strains indicates the possible presence of these substances in environmental samples (Umbuzeiro et al., 2011). However, this in vitro assay uses bacteria that have limited ecological relevance. Fish in vivo assays are a logical choice if the aim is to protect aquatic life, but there is an increasing emphasis on the replacement of in vivo tests with in vitro alternatives (e.g., fish cell lines) for both economic and ethical reasons (i.e., the 3Rs principle: Replace, Reduce, Refine) (Russell and Burch, 1959). In eco-genotoxicity, alternative in vitro models can be very useful for monitoring studies and chemical regulatory testing because large numbers of samples can be evaluated in a short period of time to provide the information necessary for enforcement and prevention actions.

There are several standardized easy-to-use fish cell line systems that avoid the use of animals and can be conducted in a fully controlled environment, giving fast and affordable results (Schirmer, 2006). These biological models have been used for acute toxicity measurements (using cell proliferation and cell viability as endpoints). They have also been employed to evaluate the genotoxic potential of pure substances and environmental matrices using endpoints such as primary DNA damage with the comet

assay or chromosome damage using the micronucleus test (Brinkmann et al., 2014; Castaño et al., 2003). Modified versions of the comet assay have already been performed with a fish cell line and have proven to be a good way of enhancing assay sensitivity, especially for oxidative and alkylating DNA damage. The modification involves the addition of an enzymatic digestion step, using, for example, the enzyme formamidopyrimidine DNA glycosylase (Fpg) (Kienzler et al., 2012).

The aim of the present study was to combine two assays in order to characterize the genotoxic potential of waters impacted by textile discharge: the Salmonella/microsome assay using several diagnostic strains and the comet assay in its standard and Fpg-modified versions applied to a liver fish cell line. Additionally, chemical analyses were performed to identify carcinogenic and mutagenic azo dyes and aromatic amines in the samples.

2. Materials and methods

Study area and sampling

The largest textile industrial conglomerate of Brazil is located in Americana City in São Paulo State. A Wastewater Treatment Plant (WWTP) is responsible for the collection and treatment of several of those industrial effluents. It uses a standard biological treatment (activated sludge) without any additional tertiary process. The treated effluent is released into the Piracicaba River, a river whose water quality is protected by law to ensure the safety of aquatic life, livestock, irrigation and human consumption. Unfortunately, biological treatment alone is known to be ineffective for the removal of disperse dyes (USEPA, 1990). Furthermore, the WWTP does not treat all industrial effluents and in particular, several factories discharge their untreated effluents directly into a tributary of the Piracicaba River, called the Quilombo River. Samples were collected at the WWTP outflow, the Quilombo River, and the Piracicaba River upstream and downstream of the discharges (Figure 1). Four liters of each sample were collected in amber glass vials and transported on ice to the laboratory where they were immediately processed (APHA, 1999).

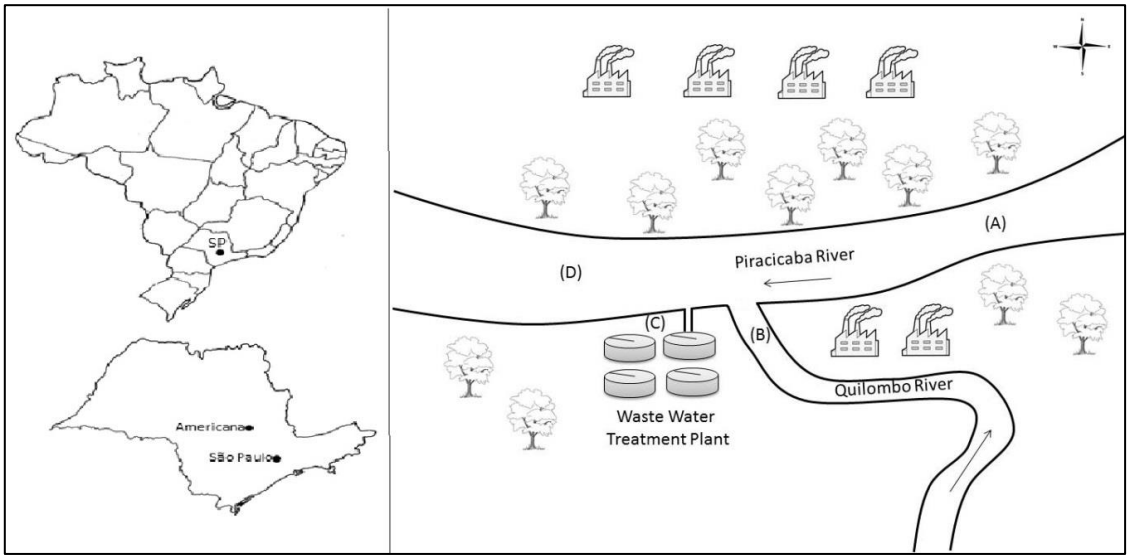


Figure 1. Sites of sampling at Piracicaba River upstream (A), Quilombo River (B), Waste Water Treatment Plant outflow (C) and Piracicaba River downstream (D) in Americana city, São Paulo State, Brazil.

Organic extract preparation

Environmental water samples usually need to be prepared before testing in in vitro biological systems that use bacteria or cell culture. When the compounds of interest are organic, solid phase or liquid-liquid extractions are employed. The resultant extract is usually dried and then re-dissolved in compatible solvents to allow bio-testing. In this work, extraction was done by liquid-liquid processing using dichloromethane (DCM) and methanol (2.5:1) as the organic phase because this procedure has been effective in other studies related to textile industries (Umbuzeiro et al., 2004). The extract volume was reduced in a rotary evaporator and then completely dried using pure nitrogen gas. Extracts were carefully kept frozen at -20°C in amber glass vials. Before testing, a minimal volume of DCM was added to facilitate dissolution along with the needed amount of DMSO. DCM was then completely evaporated using pure nitrogen gas before the beginning of the tests. A blank control sample employing this last drying procedure was prepared to evaluate any adverse effects that it might produce in the tests.

Chemical Analysis

Chemical analysis was performed on the extracts using a high performance liquid chromatography (HPLC) Agilent 1200 system (Waldbronn, Germany) coupled to

an AB Sciex 3200 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (MS). The extracts were diluted with methanol:water (50:50, v/v) containing 0.1% formic acid. Chromatographic separation was accomplished using a Kinetex PFP analytical column (150 mm x 4.6 mm; 5 µm, Phenomenex). The mobile phase constituted of water (A) and acetonitrile (B), both spiked with 0.1% formic acid, at flow rate of 1.5 mL min-1 with the following gradient program for water/acetonitrile: 0 - 1 min, 5% B; 1 - 5.5 min, 5 - 9% B; 5.5 - 6.5 min, 9 - 25% B; 6.5 – 9.5 min, 25 – 40% B; 9.5 – 11.5 min, 40 – 45.5% B; 11.5 – 16.5 min, 45.5 – 60.5% B; 16.5 – 18.5 min, 60.5 – 100% B, 18.5 - 23 min, 100% B and re-established by 5% B over 7 min; total run length was 30 min. The column temperature was set to 40 °C and the injection volume was 20 µL. The chromatography system was coupled to the 3200 QTRAP via an electrospray ionization (ESI) source, operating in positive ion mode with the following ionization parameters: spray voltage, 5500 V; capillary temperature, 650 °C; nebulizing gas (Nitrogen) 45 psi; heating gas (Nitrogen) 45 psi; and curtain gas, 15 psi. Identification of analytes was performed in the selected reaction monitoring (SRM) mode, where two SRM transitions were selected to eliminate any false result. Fragmentation parameters were optimized by direct infusion of solutions of the individual compounds prepared at a concentration of 0.1 mg L-1 in methanol/water (50:50, v/v) containing 0.1% formic acid. The flow used in this analysis was 10 µL min-1. In this step the following parameters were evaluated: collision energy (CE), declustering potential (DP), entrance potential (EP), cell entrance potential (CEP), and collision cell exit (CXP). All analytical parameters for each compound are shown in Table S1, Supplementary Material I.

Salmonella/microsome microsuspension assay

The microsuspension method of the Salmonella/microsome assay was chosen because of its high sensitivity when limited amounts of sample are available (Kado et al., 1983). Strain TA98 (hisD3052, rfa, Δbio, ΔuvrB, and pKM101) was selected because it has been the most used in monitoring studies and provides the majority of the positive responses for surface water testing (Ohe et al., 2004; Umbuzeiro et al., 2001). The diagnostic strain YG1041 (a derivative of TA98 that overproduces nitroreductase and O-acetyltransferase) was also included because of its high sensitivity to compounds containing nitro and amine groups (Hagiwara et al., 1993) often present in the effluent of textile plant that use or produce dyes (Freeman, 2013; Oliveira et al., 2006;

Umbuzeiro et al., 2005b). If a marked increase in the YG1041 response is observed in relation to its parental TA98, this indicates that nitro-aromatics (response without S9) or aromatic amines (response with S9) are present in the sample (Umbuzeiro et al., 2011). Here, the application of various strains provides insight into what types of compounds are causing the observed effects.

Overnight cultures of each strain were concentrated by centrifugation at 10,000g at 4ºC for 10 min and resuspended in 0.015M sodium phosphate buffer. Six different doses ranging from 0.07 to 40 ml-equivalent/plate of water samples were tested, along with negative and positive controls. These doses correspond to 0.7% to 400% equivalent of raw samples. These units were used for comparisons with the fish cell line tests. The conversion was performed considering the concentration tested in each tube, which was pre incubated before being poured onto agar plates. Each concentration was tested in duplicate. Tests were performed in the presence and absence of a 4% (v/v) lyophilized Aroclor-1254-induced rat liver S9 fraction (Moltox Inc., Boone, NC) with cofactors, using dimethylsulfoxide (DMSO) as a solvent and negative control. Volumes of 50 µl of cell suspension, 50 µl of 0.015 M sodium phosphate buffer (with or without S9 mix) and 5 µl of each extract dilution were added to each tube and incubated at 37ºC for 90 min without shaking. After incubation, 2 ml of molten agar was added, the mixture was poured onto a minimal agar plate, and the plates were incubated at 37ºC for 72 h. Colonies were manually counted and toxicity evaluated by visual observation of the background bacterial lawn under a stereo microscope. Preliminary testing was performed (data not shown) and when toxicity was observed, only non-toxic doses were included in the final experiment. Positive controls for TA98 were 0.125 µg/plate of 4-nitroquinoline-1-oxide (4NQO) (Sigma-Aldrich) without S9 and 0.625 µg/plate of 2-aminoanthracene (2AA) (Sigma-Aldrich) with S9, both dissolved in DMSO. For YG1041, 2.5 µg/plate of 4-nitro-ortho-phenylenediamine (4NOP) (Sigma-Aldrich) without S9 and 0.03125 µg/plate of 2-aminoanthracene (2AA) (Sigma-Aldrich) with S9, dissolved in DMSO. Results were analyzed statistically using an ANOVA followed linear regression using the Bernstein model (Bernstein et al., 1982).

Comet assay in fish cell line

RTL-W1 (Lee et al., 1993), a non-transformed and non-cancerous rainbow trout cell line derived from liver, was chosen because of its wide range of biotransformation capacities (Thibaut et al., 2009), including reductase activity toward the model

genotoxicant 4-nitroquinoline-1-oxide (4NQO) (Nehls and Segner, 2001, 2005). RTL-W1 (gift of Drs. N. Bols and L. Lee, Waterloo University, Canada) was cultured routinely in 75 cm2 culture flasks at 20 °C in Leibovitz's (L-15) medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL) and streptomycin (100 µg/mL) in an ambient air atmosphere incubator. Cells were trypsinized once or twice a week using 0.25% trypsin in sterile PBS containing 0.03% EDTA and replaced at lower densities to maintain cell growth.

Cytotoxicity was measured in a cell survival assay using the Cell-Titer-Blue® viability assay (Promega). Cell viability was recorded after 24h exposure by measurement of the fluorescence intensity of the reduced CTB reagent (Ex: 560nm, Em: 590nm), the latter being proportional to the number of living cells. Cells (2x104 cells/well, 6 wells /dose) were seeded in 96 black multiwell plates. After 24h, the culture medium was removed and replaced by L15 medium (without FBS) containing dilutions (in the range of 5 to 200 % equivalent of the raw samples) of the four studied extracts. Cell viability resulting from each treatment was evaluated as a percentage relative to control untreated cells. Then, the Hill model (RegTOX, Excel macro TM) was applied to the dose-response curves in order to calculate the IC15 and the IC50 for each sample. Three independent experiments were performed. Sub-cytotoxic concentrations (≥85 % viability) were evaluated in addition to the IC50 in order to define the concentrations to be tested in the comet assay to avoid false positive results.

Primary DNA damage was evaluated after 24h-exposure to increasing concentrations of the environmental sample extracts added to L15-culture medium without FBS in order to maximize the bioavailability of organic compounds. Twenty four hours prior to exposure, 200,000 cells/mL were seeded into 24-well transparent plates. At the end of the exposure, cell suspensions were prepared from each well by trypsinization and both the standard and Fpg-comet assays were performed immediately on each sample to evaluate primary DNA damage.

The modified comet assay was performed with and without Fpg enzyme under alkaline conditions based on the method proposed by Collins et al. (1996) and modified by Kienzler et al. (2012). Briefly, after exposure, trypsinized cells were mixed with an equal volume of 1% low melting point agarose. Forty µL of this mixture were spread on a frosted microscope slide pre-coated with normal agarose (0.8%), and covered with a 22x22 mm coverslip (two gels per slide, two slides per sample). After agarose polymerization (4°C, 5 min), coverslips were slid off and the slides placed in a lysis

solution (2.5 M NaCl, 0.1 M Na2EDTA, 10 mM Tris, 10% DMSO, 1% Triton X100; pH 10) at 4°C for 1h in the dark. The slides were washed three times for 5 min with an enzyme buffer (0.1 M KCl, 0.5 mM Na2EDTA, 40 mM HEPES KOH, 0.2 mg/mL bovine serum albumin, pH 8.0), and then incubated with Fpg enzyme (New England Biolabs, 0.4 UI/gel), 15 min at 37°C and then 10 min at 4°C in the dark in order to stop the enzymatic cleavage and to reinforce the gel polymerization. Slides without Fpg treatment (standard procedure) were incubated in the same way, but using the enzyme buffer without Fpg. At the end of the enzymatic digestion step, slides were washed once with cold PBS and placed in an electrophoresis tank in a 0.3 M NaOH and 1 mM Na2EDTA solution (pH>13) for 40 min to allow for DNA unwinding. Electrophoresis was carried out at 0.65 V/cm (300 mA) for 24 min. Slides were then neutralized three times for 5 min (0.4 M Tris-HCl, pH 7.5), drained, dried in absolute ethanol for 20 min and stained with 25 µL of a 0.02% ethidium bromide solution.

Comets were viewed by fluorescence microscopy (Axioskop 40, ZeissLtd). Image analyses were performed with the Comet 4.0 software (Perceptive Instruments Ltd). For each concentration, the parameter used was the average of the 2 median values of comet tail intensity (%) obtained by counting two sets of fifty cells per duplicate gel (100 cells/experiment, 3 independent replicated experiments) from each treatment and experimental condition (with/without Fpg). Results were expressed as the mean and standard error of the mean (SEM) of three independent experiments. Data were analyzed statistically with the Kruskal-Wallis test (Statistica©-StatSoft, Inc.). The non-parametric Mann-Whitney test was performed to identify groups that differed significantly from the control.

Tail intensity (%) is considered a relevant parameter to assess the level of DNA damage using the comet assay (Collins et al., 2008). Additionally, comet data were used according to Seitz et al (2008) to calculate a "concentration-dependent induction factor" (CDI), this parameter being proposed by these authors as a straightforward, precise and realistic assessment of the genotoxic potential of field samples.

3. Results & Discussion

Salmonella/microsome microsuspension assay

River and WWTP samples were not mutagenic in the Salmonella/microsome microsuspension assay with TA98, with or without S9, except for the Quilombo River sample, which induced 5,500 revertants per liter without S9 (Tables 1-4). According to current water mutagenicity classification (Ohe et al., 2004; Umbuzeiro et al., 2001) this sample falls into the extreme activity category (> 5,000). When YG1041 was used, mutagenicity was produced by all of the samples analyzed (Tables 1-4). This indicates that the extracts contained mutagenic compounds such as nitro/aromatic amines because this strain overproduces nitroreductase (NR) and O-acetyl transferase (OAT). Without the addition of S9, NR reduces the NO2 group of nitroaromatic molecules to a hydroxylamine, which is further acetylated by OAT, generating very potent metabolites that can cause DNA adducts (Arlt et al., 2002; Bartsch, 1981). If these adducts are not properly repaired they will lead to mutations in the next cell generation. When S9 is added to the mixture, NH2 groups are oxidized by CYP enzymes generating hydroxylamines that become substrates for OAT as explained above. The Piracicaba River upstream sample was only mutagenic for YG1041 without S9 (3,000 revertants per liter). Conversely, the mutagenic potency of the discharges (WWTP and Quilombo River) and Piracicaba River downstream were much higher when S9 was added. Surprisingly, the most mutagenic sample was Piracicaba River downstream, producing 150,000 revertants per liter. These data strongly suggest that aromatic amines are one of the causes of the mutagenicity of these samples, and that the discharges are increasing the contamination of the Piracicaba River downstream, especially the Quilombo River. The discharges seem to be responsible for the increase in mutagenicity of the Piracicaba River, and YG1041 was the most sensitive strain for detecting this mutagenicity.

Fpg-modified comet assay

When assaying primary DNA damage by the comet assay, it is important to establish a concentration range ensuring more than 85% of cells remain viable at the end of exposure since DNA fragmentation is also a major event in necrotic or apoptotic cells. For determining the limit concentration for our assays, IC15 values were calculated from cytotoxicity experiments and it was found that only the Piracicaba upstream sample could be tested up to the equivalent of 100% effluent. For the 3 other

samples, IC15 values limited testing to between 99 and 70%. Subsequently, a 75% concentration was used as the highest test concentration for those 3 samples in order to avoid false positive results.

The level of primary DNA damage induced by a 24 hr exposure of the RTL-W1 fish cell line to the test samples is shown in Figure 2 (standard comet assay) and Figure 3 (Fpg-modified comet assay). Overall, the absolute values of % tail DNA are rather low in all samples, but significant increases above control values were obtained with both assays starting with the 50% (x 0.5) equivalent effluent concentration in the two discharge samples (Quilombo River and WWTP effluent) and in Piracicaba River downstream. In the Piracicaba River upstream sample, the sole significant genotoxic effect was obtained with the 100% (x1) equivalent concentration when evaluated with the Fpg-modified assay. Despite the high degree of dilution of the two discharges when reaching the Piracicaba River, it is probable that they both contribute to the genotoxicity downstream. Considering the concentration-dependent induction factors (CDI) calculated for the various sampling sites, the Quilombo River and WWTP effluent produced the strongest genotoxic potential, and quite surprisingly, this result was obtained with the standard version of the comet assay (Figure 4). Despite some previous evidence that use of the Fpg-modified version of the assay can improve the detection of genotoxic potential for pure substances (Kienzler et al. 2012, Azqueta et al. 2013), it seems that the gain depends on the type of DNA lesion (oxidation, N-alkylation) and those may not have been the prevalent types of DNA lesions observed here.

It is noticeable that in this study, the Fpg-modified comet assay actually resulted in greater net increases in % tail DNA than the standard comet assay, but the net responses were all similar (Figure 2 and Figure 3), i.e. the same amount of Fpg-sensitive sites that are dose dependent but not site dependent (Figure 4). This indicates that ROS-inducing compounds are probably produced during exposure; however, these effects are probably not specific to the azo dyes contained in the samples and result from the highly complex chemical composition of the tested samples. The production of ROS from hydroxy aromatic amine products has been suggested after reduction of azo dyes (Sweeney et al., 1994). Since rather stable DNA adducts are formed following N-reduction and O-acetylation of azo dyes, the comet assay may not be the best assay to reveal such types of DNA damage as previously shown for high molecular weight PAH compounds (Brinkmann et al., 2014). The rather low level of primary DNA damage observed in the RTL-W1fish cell line may also be the result of a low level of metabolic

activity for activating aromatic amines and azo-compounds, although DT-diaphorase, azo and nitroreduction enzymes occur in some fish (Schlenk et al., 2008). Our results could also be related to partial repair of early primary damage, since the 24 h exposure could have been long enough to allow such events. These results demonstrate the need for further investigations to better characterize the capacity of the fish cell lines to be used as a safe alternative to in vitro/in vivo testing in the field of genotoxicity. In this specific study, the standard assay seemed to be the better option for differentiating between samples with different amounts of azo/amino compound contamination in relation to the Fpg-modified version of the comet assay.

Table 1. Salmonella/microsome results of Piracicaba River upstream for strains TA98 and YG1041, with and without S9.

Piracicaba River upstream																
Doses (L-eq/plate)	Number of revertants/plate and mean															
	TA98								YG1041							
	-S9				+S9				-S9				+S9			
	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean
Negative control	24	31	30	28	22	21	27	23	192	178	189	186	152	129	139	140
0.00125	31	28		30	25	28		27	202	204		203	152	172		162
0.0025	34	25		30	25	29		27	182	193		188	199	134		167
0.005	31	24		28	23	24		24	189	191		190	145	128		137
0.01	34	40		37	21	31		26	214	205		210	158	194		176
0.02	45	33		39	21	21		21	262	251		257	174	183		179
0.04	30	22		26	19	22		21	229	263		246	198	196		197
Positive control	1012	840		926	342	556		449	1197	1083		1140	1767	1425		1596
ANOVA p-value				0.268				0.330				< 0.001				0.123
Bernstein p-value				0.204				0.606				0.173				0.437
Dose response p-value				0.604				0.966				< 0.001				0.008
Potency (revertants/L-eq)				0				0				3000				0
Minimal Effective Concentration (MEC) (L-eq)				>0.04				>0.04				0.029				>0.04

Table 2. Salmonella/microsome results of Quilombo River for strains TA98 and YG1041, with and without S9.

Quilombo River																
Doses (L-eq/plate)	Number of revertants/plate and mean															
	TA98								YG1041							
	-S9				+S9				-S9				+S9			
	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean
Negative control	24	31	30	28	22	21	27	23	192	178	189	186	152	129	139	140
0.00125	32	35		34	30	31		31	226	207		217	256	291		274
0.0025	40	46		43	24	36		30	218	228		223	356	272		314
0.005	55	56		56	24	28		26	202	226		214	370	634		502
0.01	55	56		56	34	20		27	257	251		254	604	543		574
0.02	56	65		61	27	26		27	393	402		398	352	279		316
0.04	54	58		56	31	35		33	334	358		346	208	302		255
Positive control	1012	840		926	342	556		449	1197	1083		1140	1767	1425		1596
ANOVA p-value				< 0.001				0.517				< 0.001				< 0.001
Bernstein p-value				0.998				0.602				0.131				0.341
Dose response p-value				< 0.001				0.107				< 0.001				< 0.001
Potency (revertants/L-eq)				5500				0				5800				51000
Minimal Effective Concentration (MEC) (L-eq)				0.0026				>0.04				0.0168				0.0016

Table 3. Salmonella/microsome results of WWTP Effluent for strains TA98 and YG1041, with and without S9.

WWTP Effluent																
Doses (L-eq/plate)	Number of revertants/plate and mean															
	TA98								YG1041							
	-S9				+S9				-S9				+S9			
	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean
Negative control	24	31	30	28	22	21	27	23	192	178	189	166	152	129	139	131
0.00007									176	131		154	139	122		131
0.00015									143	176		160	129	131		130
0.0003									140	151		146	132	158		145
0.0006									179	170		175	170	172		171
0.00125	29	25		27	24	25		25	179	173		176	228	268		248
0.0025	31	25		28	25	25		25	174	183		179	350	308		329
0.005	40			40	28	27		28								
0.01	24	38		31	28	27		28								
0.02	33	24		29	27	30		29								
0.04	22	32		27	38	30		34								
Positive control	1012	840		926	342	556		449	1297	1397		1347	3805	3121		3463
ANOVA p-value				0.797				0.052				0.394				< 0.001
Bernstein p-value				0.722				0.844				0.562				0.871
Dose response p-value				0.618				0.001				0.059				< 0.001
Potency (revertants/L-eq)				0				0				0				85000
Minimal Effective Concentration (MEC) (L-eq)				>0.04				>0.04				>0.04				0.0074

Table 4. Salmonella/microsome results of Piracicaba River downstream for strains TA98 and YG1041, with and without S9.

Piracicaba River downstream																
Doses (L-eq/plate)	Number of revertants/plate and mean															
	TA98								YG1041							
	-S9				+S9				-S9				+S9			
	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean
Negative control	24	31	30	28	22	21	27	23	192	178	189	186	152	129	139	140
0.00125	33	34		34	34	27		31	230	207		219	341	386		364
0.0025	34	30		32	30	35		33	207	231		219	466	455		461
0.005	37	21		29	39	36		38	246	204		225	883	983		933
0.01	26	26		26	30	25		28	228	230		229	1069	1254		1162
0.02	45	32		39	22	30		26	255	259		257	527	601		564
0.04	35			35	39			39	341			341	340	320		330
Positive control	1012	840		926	342	556		449	1197	1083		1140	1767	1425		1596
ANOVA p-value				0.654				0.065				0.024				< 0.001
Bernstein p-value				0.701				0.057				0.485				0.284
Dose response p-value				0.153				0.103				< 0.001				< 0.001
Potency (revertants/L-eq)				0				0				0				150000
Minimal Effective Concentration (MEC) (L-eq)				>0.04				>0.04				>0.04				0.0005

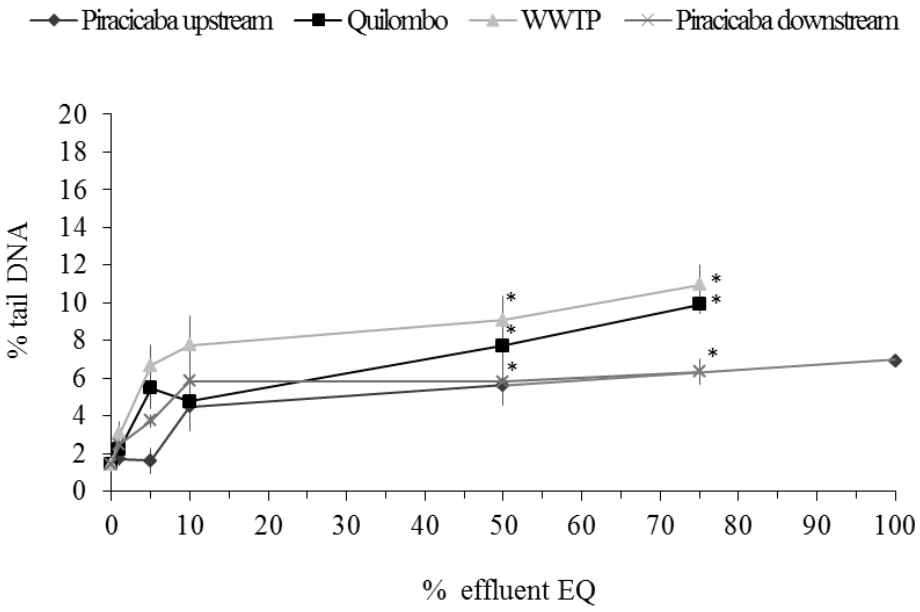


Figure 2. DNA damage level obtained with the standard comet assay in RTL-W1 cells exposed for 24h to various dilutions of the organic extract from the 4 contaminated sites. n=3 ± SEM, *: significantly different from the corresponding control value.

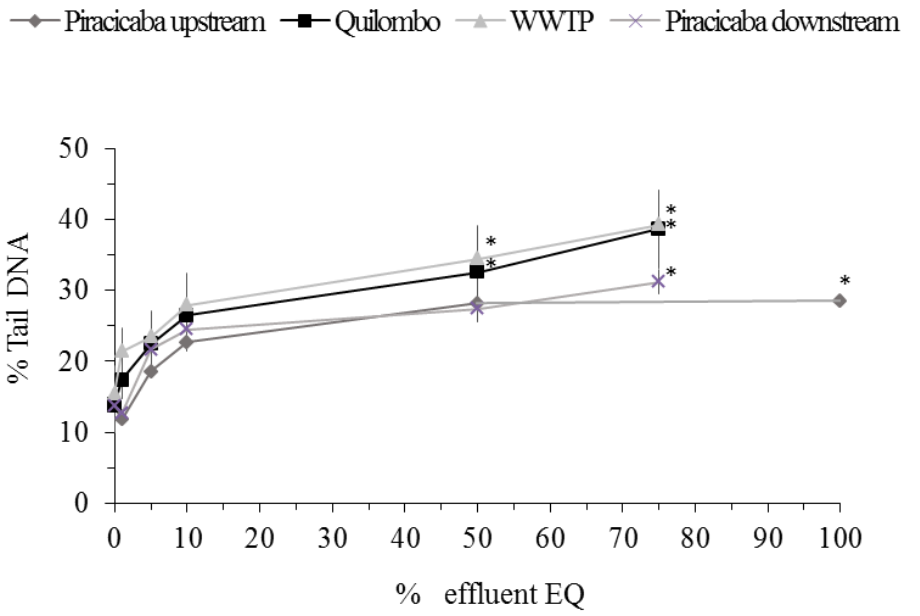


Figure 3. DNA damage level obtained with the Fpg-modified comet assay in RTL-W1 cells exposed for 24h to various dilutions of the organic extract from the 4 contaminated sites. n=3 ± SEM, *: significantly different from the corresponding control value.

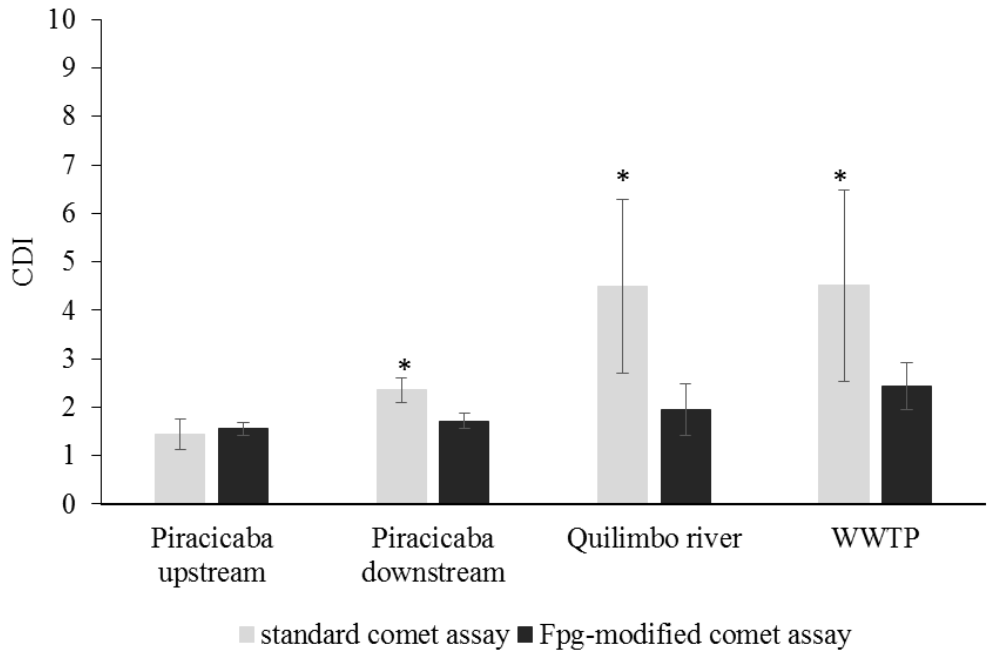


Figure 4. Genotoxicity concentration dependent induction factor (CDI) according to the comet assay protocol applied to each sample (standard or Fpg-modified) (n = 3 ± sd); * indicate a significant difference ($p<0,05$) compared to Piracicaba upstream (Mann-Whitney test).

Chemical analysis

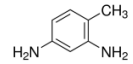
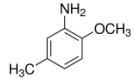
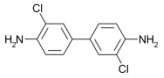
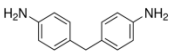
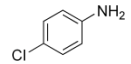
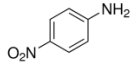
We identified carcinogenic and mutagenic aromatic amines and azo dyes in the samples. Among the aromatic amines detected (Table 5), the majority are known to be mutagenic in the Salmonella/microssome assay (some of them are carcinogens), and some of them (4-chloroaniline and 4-nitroaniline) also were detected in raw and treated textile industry effluent released into the Cristais River (Oliveira et al., 2007). 2,4-diaminotoluene, 3,3-dichlorobenzidine, and 4,4-diaminodimethane are reasonably anticipated to be carcinogenic according to National Toxicology Program of U.S. Department of Health and Human Services (NTP, 2014). 2,4-diaminotoluene, 2,5-methoxymethylaniline, 3,3-dichlorobenzidine, 4,4-diaminodimethane, and 4-chloroaniline are prohibited from being used in the European Community because they are identified as risks to human health according to Directive 2002/61/EC.

Six azo dyes were detected in the samples (Table 6) and the majority of them were detected in the Quilombo River (Site B). Disperse Blue 373 and Disperse Violet 93 were detected at high concentration in the Quilombo River (Site B) and Piracicaba River downstream (Site D) samples; these azo dyes were also detected in other studies in the Cristais River and Piracicaba River (Umbuzeiro et al., 2005b; Zocolo et al., 2015).

Disperse Red 1 was detected in all sites, except in Piracicaba River upstream samples, confirming the results of a previous study that also detected this dye only in WWTP effluent and Piracicaba River downstream (Zocolo et al., 2015) samples. Disperse Yellow 3, classified a group 3 carcinogen by IARC (IARC, 1990), was detected in Quilombo River (Site B) and Piracicaba River downstream (Site D) samples.

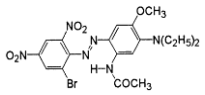
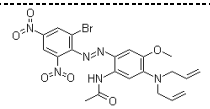
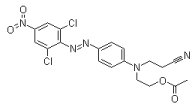
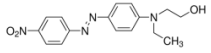
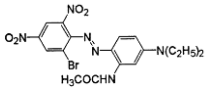
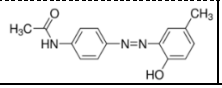
Considering the total amount of compounds detected at each site, azo dyes were detected at higher concentrations in the Quilombo River when compared to the aromatic amines, probably because this river receives untreated effluents from textile industries. Conversely, more aromatic amines were detected in WWTP effluent indicating that the biological treatment probably leads to cleavage of the azo bond of dyes, although azo dyes were also found in this sample. Both azo dyes and aromatic amines were found at higher concentrations in the Piracicaba River downstream than upstream, showing that the two discharges are increasing the contamination of the Piracicaba River with carcinogenic and mutagenic compounds. It is likely that such a load of contaminants can negatively affect the aquatic life of the Piracicaba River at both the individual and population levels as described for aquatic species living in highly polluted areas (Devaux et al., 2015; Maier et al., 2014).

Table 5. Carcinogenic and mutagenic aromatic amines identified in environmental samples using HPLC-MS/MS.

Aromatic amines	**CAS number**	**Chemical structure**	**LOD (µg L^{-1})**	**LOQ (µg L^{-1})**	**Site A (µg L^{-1})**	**Site B (µg L^{-1})**	**Site C (µg L^{-1})**	**Site D (µg L^{-1})**	**Mutagenicity**	**Carcinogenicity**
2,4-Diaminotoluene*	95-80-7		0.0033	0.0110	0.12	0.12			+TA98, +TA100 (Sayama et al., 1991) +TA98 (Watanabe et al., 1989)	2B (IARC, 1987) Reasonably Anticipated (NTP, 2014)
2,5-Methoxymethylaniline*	120-71-8		0.0014	0.0045			0.79		+TA98, +TA100 (Zeiger et al., 1988)	2B (IARC, 1987) Reasonably Anticipated (NTP, 2014)
3,3-Dichlorobenzidine*	91-94-1		0.0062	0.0208			0.13		+TA98, +TA98DPN6, +TA100 (You et al., 1993) +TA98 (Prival et al., 1984)	2B (IARC, 1987) Reasonably Anticipated (NTP, 2014)
4,4-Diaminodimethane*	101-77-9		0.0056	0.0188	0.06	0.43	0.08	0.78	+TA98, +TA100 (Takagi et al., 1995)	2B (IARC, 1987) Reasonably Anticipated (NTP, 2014)
4-Chloroaniline*	106-47-8		0.0016	0.0053				0.06	+TA98 (CCRIS, 2010)	2B (IARC, 1993)
4-Nitroaniline	100-01-6		0.0009	0.0030		0.15	0.09	0.23	+TA98, +TA100 (Aßmann et al., 1997) +TA98 (Dellarco and Prival, 1989)	-
Aniline	62-53-3		0.0030	0.0100			0.09	0.15	-	3 (IARC, 1987)

(*) prohibited by Directive 2002/61/EC; (LOD) Limit of detection; (LOQ) Limit of quantification; (-) unknown.

Table 6. Carcinogenic and mutagenic azo dyes identified in environmental samples using HPLC-MS/MS.

Azo dyes	CAS number	Chemical structure	LOD (µg L^{-1})	LOQ (µg L^{-1})	Site A (µg L^{-1})	Site B (µg L^{-1})	Site C (µg L^{-1})	Site D (µg L^{-1})	Mutagenicity	Carcinogenicity
Disperse Blue 291	56548-64-2		0.0022	0.0075	0.04		0.05		+MN HepG2 (Tsuboy et al., 2007) +TA1537, +TA1538, +TA98, +TA100, +TA98DNP6, +YG1021, +YG1024, +YG1041 (Umbuzeiro et al., 2005a)	-
Disperse Blue 373	51868-46-3		0.0016	0.0054		1.38	0.15	3.13	+TA98, +YG1041 (Umbuzeiro et al., 2005b)	-
Disperse Orange 30	12223-23-3		0.0128	0.0427		0.14			-	-
Disperse Red 1	2872-52-8		0.0003	0.0010		0.09	0.08	0.11	+TA98, +YG1041, +TA100, +YG1042 (Ferraz et al., 2011) +TA98, +YG1041 (Vacchi et al., 2013)	mouse germ cells (Fernandes et al., 2015)
Disperse Violet 93	52697-38-8		0.0064	0.0214		2.81		6.81	+TA98, +YG1041 (Umbuzeiro et al., 2005b)	-
Disperse Yellow 3	2832-40-8		0.0020	0.0066		0.01		0.03	+TA100, +TA97, +TA98 (Zeiger et al., 1988)	3 (IARC, 1990)

(LOD) Limit of detection; (LOQ) Limit of quantification; (-) unknown.

4. Conclusions

The higher sensitivity of the diagnostic strain YG1041 compared to TA98 in the Salmonella/microsome assay strongly indicates that aromatic amines or other compounds that require S9 to become active are contributing to the observed mutagenic activity downstream. This conclusion was corroborated by the chemical analysis since seven aromatic amines and six azo dyes were identified in the samples. It is important to highlight that in this case study, the use of the diagnostic strain YG1041 was central to revealing the mutagenic potential at the study sites. Regarding primary DNA damage, the standard comet assay performed on a eukaryotic model (fish cell line) produced evidence that textile contaminated discharges (WWTP and Quilombo River) contribute to the genotoxicity of the Piracicaba River downstream. Surprisingly, the Fpg-modified comet assay did not improve the sensitivity of detection of the genotoxic response because a non-specific response was obtained at all sites, with a rather constant amount of Fpg-sensitive sites found in the DNA of cells treated with samples from all the different sites. The influence of the textile discharges into the river waters was also confirmed by the chemical analysis, because several azo dyes and aromatic amines were found in the river downstream. These observations increase the possibility that genotoxic effects occur in fish at the population level, which can negatively affect the aquatic life of the Piracicaba River.

In conclusion, it is important to use assays based on complementary endpoints to characterize the genotoxicity of environmental samples, as this approach has the advantage of identifying what classes of compounds are responsible for the effects. But in this specific study, the Salmonella/microsome assay with YG1041 seems to be particularly effective at identifying possible hot spots of contamination with mutagenic aromatic amines and azo dyes.

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6. Supplementary Material

Table S1: Optimized parameters to identify disperse dyes and aromatic amines by HLPC-MS/MS, using SRM mode.

Compounds	SRM		MS/MS					
	Precursor Ion (*m/z*)	Fragment Ion (*m/z*)	Dwell Time (ms)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Aniline	94	77,1	8	46	11,5	10	21	4
	94	51	8	46	11,5	10	39	4
4-chloroaniline	128	93,2	8	41	7,5	10	23	4
	128	75,1	8	41	7,5	10	41	4
2,4-diaminotoluene	123,1	106,1	8	41	7	12	19	4
	123,1	108,2	8	41	7	12	21	4
2,5-methoxymethylaniline	138,1	123,1	8	36	5,5	10	21	4
	138,1	122,6	8	36	5,5	10	33	4
4-nitroaniline	139,1	122,2	5	31	6,5	10	19	4
	139,1	92,2	5	31	6,5	10	27	4
4,4-diaminodimethane	199,2	106,3	50	41	3	14	29	4
	199,2	77,2	50	41	3	14	57	4
3,3-dichlorobenzidine	253,2	217,1	8	61	4	20	29	4
	253,2	182	8	61	4	20	29	4
Disperse Yellow 3	270,2	107,2	5	41	5,5	18	33	4
	270,2	108,1	5	41	5,5	18	39	4
Disperse Red 1	315	134	5	51	4	24	33	4
	315	255	5	51	4	24	29	4
Disperse Orange 30	450	87	5	46	7.5	24	49	4
	450	132	5	46	7.5	24	33	4
Disperse Blue 373	533	260,4	5	66	5	24	25	4
	533	245,4	5	66	5	24	33	4
Disperse Blue 291	511	207	5	56	8,5	22	39	4
	511	192	5	56	8,5	22	47	4
Disperse Violet 93	481	191	5	56	7,5	20	37	4
	481	206	5	56	7,5	20	25	4

(SRM) selected reaction monitoring; (MS) mass spectrometer; (DP) declustering potential; (EP) entrance potential; (CEP) cell entrance potential; (CE) collision energy and (CXP) collision cell exit.

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4.3 Capítulo III

Occurrence and risk assessment of an azo dye - the case of Disperse Red 1

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Abstract

Water quality criteria to protect aquatic life are not available for most disperse dyes which are often used as commercial mixtures in textile coloration. In this study, the acute and chronic toxicity of the commercial dye Disperse Red 1 (DR1) to eight aquatic organisms from four trophic levels was evaluated. A safety threshold, i.e. Predicted No-Effect Concentration (PNEC), was derived based on the toxicity information of the commercial product and the purified dye. This approach was possible because the toxicity of DR1 was accounting for most of the toxicity of the commercial mixture. A long-term PNEC of 60 ng L^{-1} was proposed, based on the most sensitive chronic endpoint for *Daphnia similis*. A short-term PNEC of 1,800 ng L^{-1} was proposed based on the most sensitive acute endpoint also for *Daphnia similis*. Both key studies have been evaluated with the new "Criteria for Reporting and Evaluating ecotoxicity Data" (CRED) methodology, applying more objective criteria to assess the quality of toxicity tests, resulting in two reliable and relevant endpoints with only minor restrictions. HPLC-MS/MS was used to quantify the occurrence of DR1 in river waters of three sites, influenced by textile industry discharges, resulting in a concentration range of 50 - 500 ng L^{-1}. The risk quotients for DR1 obtained in this work suggest that this dye can pose a potential risk to freshwater biota. To reduce uncertainty of the derived PNEC, a fish partial or full lifecycle study should be performed.

Keywords: Daphnia; Algae; Fish; Dyes; Mixture; PNEC.

1. Introduction

Disperse dyes are synthetic colorants for hydrophobic substrates and are commonly applied as commercial mixtures in textile coloration. They are often used in great quantities and due to the huge amount of water involved in the associated dyeing processes and the high proportion of the dye that remains in the water bath, large volumes of wastewater can be generated (Hunger, 2003). It is also known that the conventional treatment of these wastewaters, involving aerobic lagoons or activated sludge, are not efficient in the removal or biological degradation of these dyes and alternative treatment process are necessary to achieve this removal (da Silva Leite et al., 2016; USEPA, 1990). Therefore, the unreacted dyes are often still present in the wastewaters and sludges from textile plants (Umbuzeiro et al., 2005). Although disperse azo dyes are poorly water-soluble compounds, they become dispersed in water because their commercial formulations contain surfactants needed for the dyeing process. One of the main surfactants used for disperse dyes is sulfonated lignin, because of its low cost and easy availability (Tehrani-Bagha and Holmberg, 2013).

Disperse dyes have been found in rivers and sediments worldwide (Maguire, 1992; Zocolo et al., 2015) and mutagenic activity of surface waters and sediments were attributed to the presence of these compounds (Oliveira et al., 2006; Umbuzeiro et al., 2005). Moreover, synthetic dyes are assumed to be toxic to aquatic organisms (Ribeiro and Umbuzeiro, 2014). For example, Disperse Red 1 (DR1) is representative for phenylazoaniline dyes and there are more than 50 commercial products on the market (Colour Index, 2011). This dye also has previously been shown to have a high acute toxicity to the water flea *Daphnia similis*, both when it was tested as pure compound as well as commercial formulation (Ferraz et al., 2011; Vacchi et al., 2013). More recently, it was shown that DR1 affects the regeneration and fecundity of the freshwater planarian *Girardia tigrina* (Ribeiro and Umbuzeiro, 2014). This dye also showed genotoxic potentials in the *Salmonella*/microsome assay, the comet assay using HepG2 cells, as well as the micronucleus assay involving human lymphocytes and HepG2 cells (Chequer et al., 2009; Ferraz et al., 2011; Oliveira et al., 2010). Another study showed that DR1 induces cytotoxic and genotoxic effects in mouse germ cells, indicating the harmful activity of this dye (Fernandes et al., 2015). Nevertheless, currently no regulatory thresholds exist for DR1 to ensure the protection of aquatic biota or human from this important group of compounds (Ribeiro and Umbuzeiro, 2014).

In this context, the derivation of Predicted No-Effect Concentrations (PNEC), i.e. the concentrations at which no adverse effects on the ecosystem are expected, is an important step in the risk assessment of these chemicals. PNECs are commonly derived from standard toxicity tests, using well-defined protocols for a limited number of reference species, as well as an assessment factor to account for the uncertainty related to laboratory-field extrapolations. To derive sound PNECs, it is advantageous to use various chronic tests from species covering different trophic levels, representing a wide variety of taxonomic groups and sensitive species, although acute tests can also be used (European Commission, 2011). Thereby, it is most crucial that the endpoints used to derive the respective PNECs are reliable (Moermond et al., 2015). To adequately test chemical substances, it is necessary to have the pure compound dissolved in the testing media. This can be challenging for pure disperse dyes because of their poor water solubility. Therefore testing the commercial formulation is easier, but this approach does generally provide less reliable information. Nevertheless, a PNEC for the main compound can be derived when this is considered.

The main objective of this study was therefore to derive short and long-term PNECs for the protection of freshwater biota towards Disperse Red 1, based on a set of relevant and reliable ecotoxicity tests on the commercial dye. Furthermore, we assessed the risk of Disperse Red 1 for aquatic life due to its occurrence in freshwaters of São Paulo State that are influenced by textile industries discharges.

2. Methods

2.1 Commercial Dye Disperse Red 1

The commercial dye used in this study was previously characterized and the main dye Disperse Red 1 was purified (>99%) (Vacchi et al., 2013). It contains 60% of Disperse Red 1 (i.e. *N*-Ethyl-*N*-(2-hydroxyethyl)-4-(4-nitrophenylazo) aniline; CAS number 2872-52-8), another six similar dye components (20%) and one unknown surfactant (20%). All ecotoxicity tests performed using the commercial dye was dissolved in the appropriate test medium for each organism without any solvent; except for tests with *Daphnia similis* performed with the purified Disperse Red 1, which was dissolved in water containing 1% of methanol for acute test and 0.01% of dimethyl sulfoxide (DMSO) for chronic test.

2.2 Ecotoxicity testing

Chronic toxicity was tested with the freshwater algae *Raphidocelis subcapitata* (former *Pseudokirchneriella subcapitata*) according to OECD guideline 201 (OECD, 2006). The inoculum was composed of algae cells harvested from a liquid stock algal culture that was 3 days old and in a logarithmic phase of growth. The initial cell density was 10,000 ± 1,000 cells/mL. The final volume was 45 mL (test sample, algal inoculum and enrichment medium). The test was performed under static conditions for 72 hours without media renewal, at 24 ± 2 °C under continuous fluorescent light (4,000 ± 400 lux). The effect measurement was the growth inhibition rate, for which the endpoint IC50 (median inhibition concentration) was determined.

Acute toxicity tests with *Daphnia similis*, *Daphnia magna*, *Ceriodaphnia silvestrii* and *Ceriodaphnia dubia* were performed according to OECD guideline 202 (OECD, 2004). Twenty neonates (<24 h old) from 2-3 week-old mothers were placed in 4 replicates for each concentration (5 organisms/replicate). Tests were performed at 21 ± 1 °C under a photoperiod of 16 hours light and 8 hours darkness. After 48 hours, the number of immobile daphnids was recorded. The results were statistically analyzed using the Trimmed Spearman–Karber method for estimating the endpoint EC50 (median effect concentration) according to Hamilton et al. (1977).

The chronic toxicity test was done with *Ceriodaphnia dubia* according to USEPA method 1002.0 (USEPA, 2002). This method measures the chronic toxicity, using ten neonates (less than 24h-old) for each concentration during seven days, in a static renewal test. The effect measurement was reproduction inhibition. Significant differences among concentrations were analyzed with the paired T-test to determine the NOEC.

The chronic toxicity test was done with *Daphnia similis* according to OECD guideline 211 (OECD, 2012) and the exposure time was modified to 14 days based on the study of Lameira (2008). This method measures the chronic toxicity, using ten neonates (less than 24h-old) for each concentration in a total renewal test. The effect measurement was reproduction inhibition. Significant differences among concentrations were analyzed with the paired T-test to determine the NOEC.

The acute toxicity test with *Hydra attenuata* was conducted in 12-well microplates, with 3 replicate wells for each concentration (Trottier et al., 1997). 5 mL of sample and 3 organisms were put in each well. Organisms were exposed to the test solution for 96 hours. The results were statistically analyzed using the Trimmed

Spearman–Karber method for estimating the median lethal concentration (Hamilton et al., 1977).

The chronic toxicity test using *H. attenuata* was performed to detect adverse effects on reproduction on cnidarians (Holdway, 2005). The test format involves immersing test animals over 7 days in a range of concentrations of the test solution with 100% daily solution renewal. Fifteen budding hydras were randomly assigned to each concentration and fed daily with alive brine shrimp nauplii (*Artemia salina*). After feeding, test solutions were changed and the number of organisms was observed. A one-way ANOVA followed by the Dunnet test was used to detect differences between the treatments and the control as well as to determine the NOEC.

The acute toxicity test using larvae of *Danio rerio* was performed according to OECD guideline 210 (OECD, 2013). 72h-old larvae were exposed to the dye test solutions for 96h. Thirty organisms were used per replicate, in duplicates per concentration. The acute toxicity was also tested with juveniles of *D. rerio* according to OECD guideline 203 (OECD, 1992). We exposed juveniles with 2.0 ± 1.0 cm of body length for 96 hours. We used 3 replicates per concentration, with 5 organisms each. The effect measurement was mortality in both tests and the endpoint of EC50 was calculated using the Trimmed Spearman–Karber method (Hamilton et al., 1977).

The acute toxicity test using larvae of *Chironomus xanthus* was performed according to Novelli et al. (2012). *C. xanthus* larvae (IV instar – 7/8 days age) were exposed to dye solutions at 23 °C ±2 temperature and photoperiod of 12:12 h light/dark. Six larvae were added in chambers containing 240 mL of test solution and 50 g of sediment composed of sterilized fine sand (550 °C for 2 h), in four replicates. After 96 h, the living organisms were counted and the EC50 was calculated using the Trimmed Spearman–Karber method (Hamilton et al., 1977).

2.3 Evaluating the quality of the ecotoxicity tests

To date, the evaluation of the reliability and relevance of the ecotoxicity test that is finally used for the derivation of quality criteria (i.e. often referred to as key study) is often based on the established Klimisch method (Klimisch et al., 1997). This method is commonly used in the regulatory context and favors highly standardized tests conducted from GLP-certified laboratories. It therefore penalizes test results that for example are produced in a scientific context with greater time and budget restraints. In order to address this bias, the “Criteria for Reporting and Evaluating ecotoxicity Data” (CRED)

method was recently introduced (Kase et al., 2016; Moermond et al., 2015), applying a set of 20 reliability and 13 relevance criteria (Supplementary Material I). CRED evaluates ecotoxicity tests according to transparent questions that allow the objective assessment of whether the test was conducted under appropriate conditions, as well as whether it is suitable for the given regulatory context (i.e. the derivation of Quality Criteria for surface waters). It aims at improving the reproducibility, transparency and consistency of aquatic ecotoxicity study evaluations amongst regulatory frameworks, countries, institutes and individual assessors. For this purpose, the assessor has to answers each question as to whether the criteria is i) fulfilled, ii) not fulfilled, iii) not applicable or iv) not reported. In general, a study should only be assigned to being "reliable without restrictions" when all important information is provided, and the study has no critical flaws in experimental design and results. Similarly, "reliable with restrictions" can be assigned to studies for which not all details are given, and/or raw data is not provided, and/or in which there are some minor flaws in experimental design, but for which it can still be assumed with reasonable certainty that the results are still reliable. A properly performed and reported peer-reviewed study (whether GLP or not) may be evaluated as "reliable without restrictions", just as a poorly designed and/or performed guideline and/or GLP study should be assigned as "not reliable", if relevant criteria are not fulfilled. "Not assignable" should be assigned to studies that lack the details necessary to evaluate their reliability. Please note that these studies cannot *per se* be regarded as "not reliable". In a similar way, the relevance of a study is assigned.

2.4 PNEC derivation and risk assessment

The PNECs values for Disperse Red 1 dye were derived according to European guidelines (European Commission, 2011) by using the deterministic approach. Essentially the deterministic approach takes the most sensitive reliable and relevant toxicity endpoint of the available set of test results and applies a respective assessment factor (AF) to extrapolate to an environmentally protective concentration. In order to cover both long- and short-term effects resulting from heterogeneous environmental exposures (i.e. average and peak concentrations), two PNECs are normally recommended by these guidelines: a long-term quality standard based on chronic toxicity data; and a short-term quality standard based on acute toxicity data.

In the first step of any chemical risk assessment for the aquatic environment, the risk quotient (RQ) compares the measured environmental concentration level (MEC, i.e. based on the maximum or average concentration) to the respective PNEC. In case the MEC/PNEC ratio exceeds 1, an ecological risk cannot be excluded (European Commission, 2003). This usually triggers further actions, such as extensive monitoring obligations or direct risk mitigation measures.

2.5 Chemical analysis of Disperse Red 1 in river water samples

A total of fourteen samples were collected at three river sites (i.e. one sample every 2-3 month during one year) located in São Paulo State, Brazil, that are influenced by textile discharges. Five samples were taken at the first site, which is located at Piracicaba river upstream of Quilombo river discharge (Figure 1, site A). Another five samples were taken at the second site, which lays downstream of this confluence (Figure 1, site B) while four samples were taken at the third site at the Quilombo river (Figure 1, site C), which is an affluent of Piracicaba river.

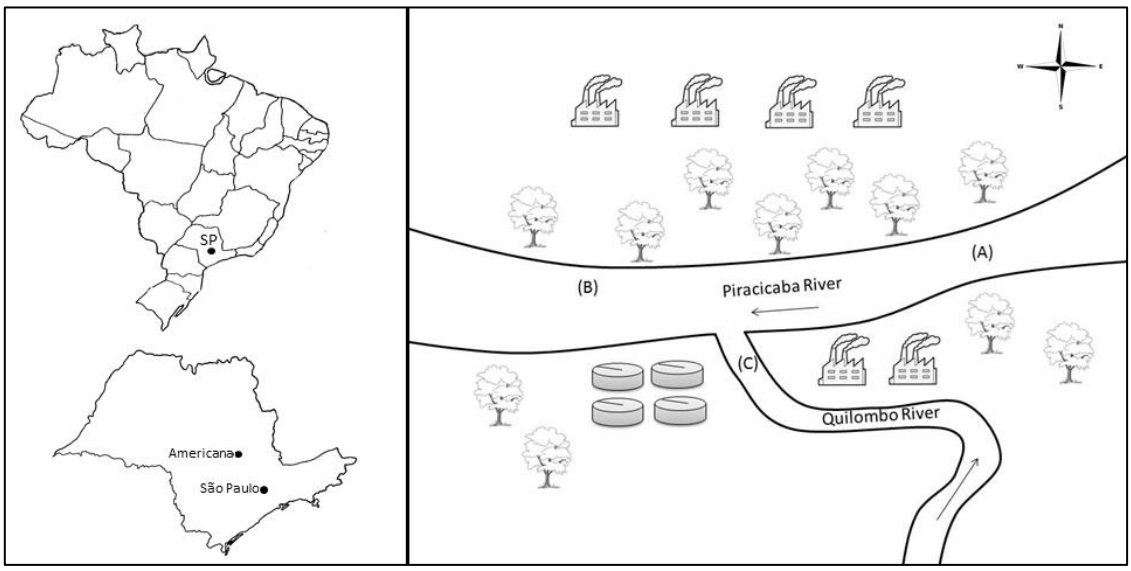

Figure 1. Sites of sampling at Piracicaba river upstream (A), Piracicaba river downstream (B), and Quilombo river (C) in Americana city, São Paulo State, Brazil.

The extraction of the environmental samples was done by liquid-liquid extraction, using dichloromethane and methanol (2.5:1, v/v) as organic phase. The extracts were dried under a gentle stream of nitrogen gas. Chemical analysis was performed in the extracts using a high performance liquid chromatography (HPLC) Agilent 1200 system (Waldbronn, Germany) coupled to an AB Sciex 3200 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (MS). The extracts were diluted with

methanol:water (50:50, v/v) containing 0.1% formic acid. Chromatographic separation was accomplished using a Kinetex PFP analytical column (150 mm x 4.6 mm; 5 µm, Phenomenex). The mobile phase constituted of water (A) and acetonitrile (B), both spiked with 0.1% formic acid, at flow rate of 1.5 mL min^{-1} with the following gradient program for water/acetonitrile: 0 - 1 min, 5% B; 1 - 5.5 min, 5 - 9% B; 5.5 - 6.5 min, 9 - 25% B; 6.5 – 9.5 min, 25 – 40% B; 9.5 – 11.5 min, 40 – 45.5% B; 11.5 – 16.5 min, 45.5 – 60.5% B; 16.5 – 18.5 min, 60.5 – 100% B, 18.5 - 23 min, 100% B and re-established by 5% B over 7 min, total run length was 30 min. Column temperature was set to 40 °C and injection volume was 20 µL. The chromatography system was coupled to the 3200 QTRAP via an electrospray ionization (ESI) source, operating in positive ion mode with the following ionization parameters: spray voltage, 5500 V; capillary temperature, 650 °C; the nebulizing gas (Nitrogen, 45 psi); the heating gas (Nitrogen, 45 psi) and the curtain gas, 15 psi. Identification of the compound was performed in the selected reaction monitoring (SRM) mode, where two SRM transitions were selected to eliminate any false result. The Limit of Detection (LOD) of the dye was 0.3 ng L^{-1} and the Limit of Quantification (LOQ) was 1 ng L^{-1}.

3. Results & Discussion

3.1 Ecotoxicity tests and their evaluation

Tests with eight aquatic organisms were carried out to evaluate the ecotoxicity of the commercial dye Disperse Red 1 (Table 1). Results of the acute toxicity of a commercial formulation containing Disperse Red 1 and the purified dye for *Daphnia similis* (Vacchi et al., 2013), as well as the results of commercial dye on the planarian *Girardia tigrina* (Ribeiro and Umbuzeiro, 2014), were previously published. The other tests have been conducted exclusively for this paper to allow for a more sound risk evaluation of DR1 (Table 1).

Since *D. similis* was by far the most sensitive species in the acute toxicity tests for the commercial product, we decided to test only *D. similis* with the purified dye. The respective acute and chronic endpoints for the purified dye were a NOEC of 3 µg L^{-1}, and an EC50 of 180 µg L^{-1}, respectively (Table 2). Both tests were also the most sensitive endpoints with regard to the commercial product data and were therefore selected for PNEC derivation.

The CRED evaluation method has been applied for acute and chronic studies used in the PNEC derivation (Supplementary Material II for CRED of chronic study;

Supplementary Material III for CRED of acute study; and Supplementary Material IV for raw data). Both studies fulfilled 18 of the 20 reliability criteria (i.e. being strictly no GLP study and using nominal concentrations) and 11 of the 13 relevance criteria (the other two not being applicable) and have been assigned to be reliable and relevant to derive a sound PNEC.

Table 1. Aquatic toxicity data for a commercial product containing Disperse Red 1.

Phylum	**Specie**	**Observed Effect**	**Exposure**	**Chronic NOEC (mg L^{-1})**	**Acute E(L)C50 (mg L^{-1})**
Chlorophyta	*Raphidocelis subcapitata*	Growth inhibition	72 hours	25	102
Crustacea	*Ceriodaphnia dubia*	Reproduction inhibition	7 days	0.1	-
		Acute lethality	48 hours	-	0.55
	Ceriodaphnia silvestrii	Acute lethality	48 hours	-	0.80
	Daphnia magna	Acute lethality	48 hours	-	0.58
	Daphnia similis	Reproduction inhibition	14 days	0.003	-
		Acute lethality[(1)]	48 hours	-	0.13
Cnidaria	*Hydra attenuata*	Reproduction inhibition	7 days	1	-
		Acute lethality	96 hours	-	48
Plathyhelminthes	*Girardia tigrina* [(2)]	Fecundity	5 weeks	0.1	-
		Newborn acute lethality	96 hours	-	79
		Adult acute lethality	96 hours	-	154
Chordata	*Danio rerio*	Larvae acute lethality	96 hours	-	>50
		Adult acute lethality	96 hours	-	>100
Arthropoda	*Chironomus xanthus*	Larvae acute lethality	96 hours	-	>100

(1) Vacchi et al (2013), (2) Ribeiro and Umbuzeiro (2014).

Table 2. Ecotoxicity data of purified dye in chronic and acute tests with *Daphnia similis.*

Concentration purified dye (µg L^{-1})	**Chronic data** (% of reproduction inhibition)	**Acute data** (% of organisms immobilised)
0	0	0
1	5	-
3	6	-
10	45	0
30	55	-
50	-	5
75	-	0
100	65	10
150	-	25
250	-	85
500	-	100
750	-	100
1000	-	100
Endpoint	NOEC 3 µg L^{-1}	EC50 180 µg L^{-1}

- not tested.

3.2 PNEC derivation

Using the deterministic approach, two PNECs were derived for the dye Disperse Red 1, a short term $PNEC_{acute}$ and a long term $PNEC_{chronic}$, both based on the toxicity of the purified dye. Nevertheless, the tests with the commercial formulation were also considered, due to the fact that Disperse Red 1 is responsible for most of its toxicity (Vacchi et al., 2013).

A long-term $PNEC_{chronic}$ of 60 ng L^{-1} was derived based on the NOEC for *D. similis* (3 µg L^{-1}) divided by an assessment factor of 50. This AF was selected because although four long-term NOECs (i.e. Algae, Cladocerans, Cnidarians and Platyhelminthes) are available, no valid NOEC for fish (secondary consumers) was available. The latter taxonomic group is requested for the base set of species (i.e. Algae, Daphnia and Fish), and its being missing justifies a higher AF.

A short-term $PNEC_{acute}$ of 1,800 ng L^{-1} was proposed for Disperse Red 1, based on the lowest LC50 (i.e. *D. similis* 180 µg L^{-1}) divided by an assessment factor of 100, considering the availability of at least one short-term L(E)C50 from each of the three trophic levels of the base set.

With information on the sensitivity of species representing four different trophic levels towards the commercial dye as well as data on the purified compound (for *D. similis* only), it was possible to derive short and long-term PNECs for the azo dye Disperse Red 1. We used the approach of combining all available effect data, because disregarding the data on the commercial formulation the PNEC would have become overestimated, due to a respectively higher AF. Another reason is that it would have been highly laborious to obtain reliable data for the purified compound with all test organisms, since it is very difficult to produce a high quantity of the purified dye, which is poorly water soluble (Vighi et al., 2003). Nevertheless, we recommend that more chronic data should be generated to complement the current data set, i.e. a chronic fish assay should be performed to allow for the reduction of the assessment factor to 10 and a more accurate PNEC.

3.3 Occurrence and risk of Disperse Red 1 in river waters

Samples of Piracicaba River upstream (A), Piracicaba River downstream (B) and Quilombo River (C) were analyzed after organic extraction. Disperse Red 1 dye was detected in six of the 14 samples, in a concentration range of about 50 - 500 ng L^{-1} (Figure 2).

Risk quotients (RQs) were calculated for both PNECs, considering the highest concentration of the dye in the river water samples for the $PNEC_{acute}$ and the average concentration for $PNEC_{chronic}$. A chronic RQ_1 of 1.7 was determined based on the average MEC (100 ng L^{-1}) and a long term PNEC of 60 ng L^{-1}, indicating potential adverse effects at all observed river sites. Please note that even a somewhat higher $PNEC_{chronic}$ of 300 ng L^{-1}, based on an AF of 10 instead of 50 would have been exceeded at least once, usually triggering a country-wide screening study in Europe. An acute RQ_2 of 0.3 was found for the maximum MEC (500 ng L^{-1}), suggesting rather chronic effects due to exposure to this dye. However, please note that the maximum concentration measured in this study is most likely underestimating the real exposure level; given the fact that only a few random grab samples were taken. A much higher variability is actually assumed for the dyeing process, since a batch process is the most

common method used for dyeing in textile industries (Hunger, 2003). So, the dye concentration in the effluent and, consequently, in the river may vary along with the stage of the dyeing process.

Moreover, the low number of sites and samples is not representative for Brazilian surface waters. Therefore, a monitoring study should be considered for this dye, considering its confirmed occurrence and potential risks, using a much larger number of sampling sites and a longer period of sampling with shorter frequency. The monitoring seems also indicated due to its genotoxicity potential for humans, which has been demonstrated not only *in vitro* but also by *in vivo* testing with mammals (Fernandes et al., 2015). Sediment samples should be analyzed as well, because Disperse Red 1 itself is poorly water soluble due to its relatively high log K_{ow} (4.2) and low water solubility (0.8 mg L^{-1}) and therefore, it is expected to easily adsorb to sediment.

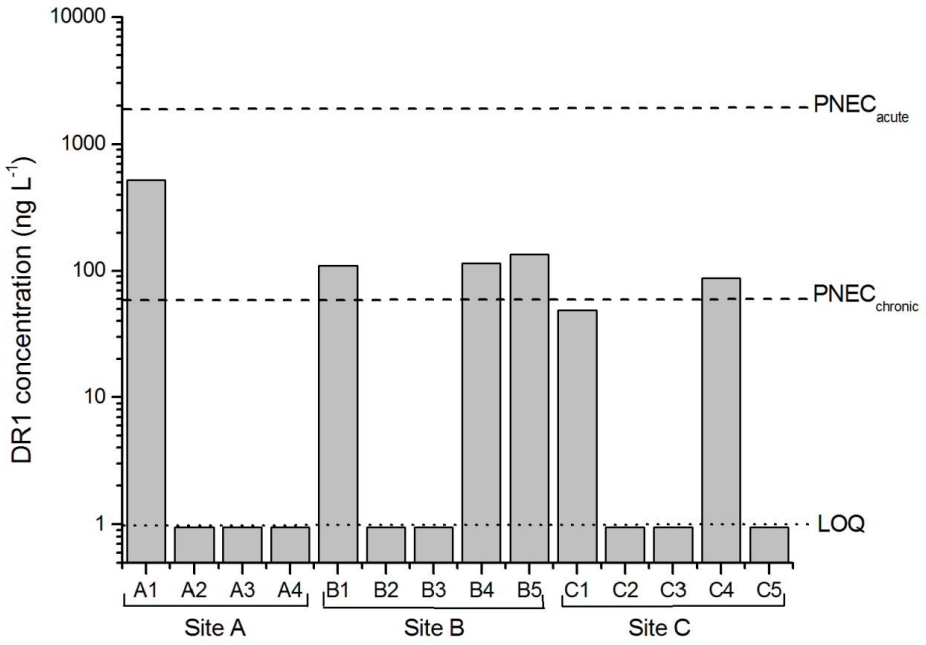

Figure 2. Occurrence of Disperse Red 1 (DR1) in river waters from four samples of site A (Piracicaba River upstream), five samples of site B (Piracicaba River downstream) and five samples of site C (Quilombo River).

4. Conclusions

This work presents a practical approach to derive PNECs for a disperse dye with a limited set of data on the purified compound. Testing of the commercial product helped to characterize the toxicity of the dye needed for risk assessment. The RQs obtained in this work suggest a potential risk to freshwater biota of Brazil. Therefore, a broader investigation study should be considered for this dye to improve the limited occurrence data. Moreover, additional chronic tests data (i.e. a fish partial or full lifecycle study) would be eligible to complement and improve PNEC derivation and preliminary risk assessment.

5. Acknowledgement

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6. Supplementary Material

Supplementary Material I

Criteria for Reporting and Evaluating ecotoxicity Data (CRED)

Explanatory guidance

Evaluated study (full reference):

Test substance:

Evaluated test:

Evaluated test species:

Evaluated test endpoint(s):

Evaluator (institution):

Please note that the questions and explanations / examples in the following are mainly based on the CRED system (Moermond et al. 2015). A weighting of criteria / questions has been included as suggested by Hobbs et al. (2005) and Breton et al. (2009). An overall score is derived for the evaluated data. Based on this score and, for reliability, the results of the evaluation of the data with regard to two cut-off criteria, a relevance and reliability class are proposed by the excel tool.

A. Relevance of the data

Remark: Relevance of a study mainly depends on the scope of the assessment / the regulatory framework, for which the study is evaluated. The following 12 questions should therefore be answered in the context of the overall assessment.

1 Is the tested species relevant for the compartment under evaluation?

Example: An aquatic species should be tested to evaluate risks for the aquatic environment.

2 Are the tested organisms relevant for the tested compound?

Example: In case of an ERA for an antibiotic, cyanobacteria should be used as test species instead of algae.

3 Are the reported endpoints appropriate for the regulatory purpose?

Example: Acute effects on aquatic organisms are not relevant for the environmental risk assessment of human pharmaceuticals.

4 Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?

Explanation: When a risk assessment is performed for a substance, for which information is available on a specific mode of action that is considered relevant for environmental organisms, studies including endpoints assessing this particular mode of action are most appropriate. For instance, if an API is known to affect reproduction of vertebrates, the endpoints of the fish early life stage test may not be appropriate. Instead, fish tests should include endpoints such as vitellgenin levels, secondary sex characteristics, sex ratio and reproduction depending on the specific mode of action of the substance (OECD 2012).

5 Is the effect relevant on a population level?

Explanation: Endpoints of the guideline studies, on which the ERA of human pharmaceuticals is based, are generally population relevant. For non-standard tests, population relevance has to be evaluated on a case by case basis.

6 Is the recorded effect statistically significant and biologically relevant?

Explanation: In the context of environmental risk assessment, a biologically relevant effect is an effect that is important and meaningful for environmental health (EFSA 2011). In a test system with relatively little control variation, minor changes may be statistically significant without necessarily being biologically relevant.To evaluate risks caused by chronic exposure, NOEC or EC_{10} values are used, while EC_{50} values are not appropriate. For the EC_{10}, it has to be evaluated on a case by case basis, if the effect is within biological variation of the control response. To evaluate risks caused by acute exposure (note that this is only relevant for some terrestrial tests with human pharmaceuticals), EC_{50} values are preferred.

7 Are appropriate life-stages studied?

Explanation/example: The tested life stage should be (a) appropriate for the selected test and test design and (b) relevant for the expected effect of the API. For instance, fish early life stages are not appropriate for studying effects on reproduction.

8 Are the test conditions appropriate for the tested species and relevant for the assessment?

Explanation/example: Test organisms should be tested under appropriate conditions. For instance, freshwater species should be tested in freshwater, and saltwater species in saltwater. If a test with freshwater or saltwater species is required depends on the scope of the assessment.

9 Is the timing and duration of exposure relevant and appropriate for the studied endpoints and species?

Explanation: The required exposure time should be appropriate for the test organism and the studied endpoint. Chronic studies should include sensitive life stages or cover the whole life cycle.

10 If recovery is studied, is this relevant for the framework for which the study is evaluated?

Explanation: In most regulatory frameworks (including the environmental risk assessment of human pharmaceuticals), recovery is not relevant (exception: authorisation of plant protection products).

11 Is the substance tested representative and relevant for the substance being assessed?

Explanation: Sufficient information should be provided to allow a clear identification of the test item. A substance may be tested as pure active substance or in a formulation. Tests performed with formulations are relevant for plant protection products, but less relevant within many other regulatory frameworks. Studies with mixtures of different substances are relevant for assessing toxicity of these mixtures, but not for assessing the individual substances contained in the mixture. For salts, the counter ion may influence toxicity. For pro-drugs, the active moiety and, if entering the environment in >10% of the administered does, the pro-drug need to be assessed (EMA/CHMP 2011). Depending on the regulatory framework, effects of transformation products may need to be considered. If the substance causing the effect is not the substance being assessed, expert judgement is needed to decide on how to deal with the results of the study and the resulting risk assessment.

12 Is the tested exposure route relevant for the assessment?

Explanation/example: The exposure route should be appropriate for the assessment. For instance, exposure by injection is generally not appropriate (Harris et al. 2014). For pharmaceuticals, exposure should be continuous. Intermittent exposure is generally not relevant. Exposure duration has to be sufficiently long. However, note that acute tests with some terrestrial organisms are also required in the environmental risk assessment of human pharmaceuticals.

B. Reliability of the data

General information

Remark: Before evaluating the test, please check the physico-chemical characteristics of the test substance (what is the solubility, log K_{OW}, pK_a, is the compound volatile, does it hydrolyse, photolyse etc.?)

1 Use of a standard (e.g. OECD, ISO, US EPA) or modified standard method:

Explanation: Please answer questions a-c with 'yes' or 'no'.

a Is an unmodified standard method used?

b Is a slightly modified standard method used?

c Is a substantially modified standard method used?

2 Is the test, including chemical analysis of the test substance where required, performed under GLP conditions?

3 Validity criteria:

a Are all validity criteria fulfilled if applicable?

Explanation: For standard tests, compliance with the validity criteria of the guideline is crucial for a study to be considered as reliable. Please check the corresponding test guideline where relevant. For non-guideline tests with standard species, validity criteria as described in a guideline for a similar test should be met if applicable.

b Are validity criteria clearly failed?

Explanation: If one or more validity criteria are clearly failed, a test is classified as '3' (not reliable).

4 Inclusion of appropriate controls:

Explanation: It depends on the test substance and test type which controls should be included; please check the corresponding test guideline where relevant. In addition to the negative control, a solvent control has to be included in all cases where a solvent is used. The concentration of solvent in the solvent control should correspond to the highest solvent concentration used in the test treatments. In some tests, a positive control with a reference substance is required. For standard tests, the corresponding guidelines provide information on how the controls should perform, e.g. with regard to survival, growth or reproduction. For non-standard tests and non-standard test organisms, expert judgement is needed to decide if performance of the controls is acceptable. Performance of the solvent control should preferably not differ significantly from performance of the negative control. If performance of one or several control(s) has already been covered in question 3a (validity criteria), question 4b, d and/or f should be answered with 'Not applicable' to avoid that this aspect is considered twice when deriving the overall quality score.

a Was a negative control included?

b Was performance of the negative control acceptable?

c Was a positive control included, if required?

d Was performance of the positive control acceptable?

e Was a solvent control included, if required?

f Was performance of the solvent control acceptable?

Test substance

5 a Is the test substance clearly identified with either name, CAS-number or SMILES code and, where relevant, information on stereochemistry?

Explanation/example: If the salt of an API was tested, information on the type of salt should be provided. If the test substance is not clearly identified, a test is classified as '3' (not reliable).

	b	**Is it specified if test concentrations relate to free acid / free base or salt where relevant?**
6	a	**Is the purity of the test substance reported and in an acceptable range (>95%)?**
	b	**Is the source of the test substance reported and trustworthy?**
7		**If a formulation is used or if impurities are present:**
	a	**Can it be excluded that other ingredients in the formulation or impurities exert an effect?**
	b	**Is the amount of test substance in the formulation indicated?**
		Test organism
8		**Description of the test organisms:**
	a	**Is the test species clearly identified?**
		Explanation: If the test species is not clearly identified, a test is classified as '3' (not reliable).
	b	**For algae: is mean cell density at the test start within an appropriate range? For other test organisms: Is mean body weight/length of the test organism in an appropriate range?**
		Explanation for 8 b-e: For standard tests, the corresponding guidelines provide information on required range of mean cell densities, age / life stage of the test organisms etc. at the test start.
	c	**Is age/life stage of the organisms at test start reported and in the required range, where appropriate (e.g. not for algae)?**
	d	**Is sex of the test organisms reported and is sex ratio appropriate, where relevant (e.g. when evaluating sexual-endocrine effects)?**
	e	**Is the species strain reported where required?**
9	a	**Are the test organisms from a reliable source? For field collected organisms: is the site of origin well-described?**
	b	**Have the organisms been acclimatized to test conditions (e.g. water type, temperature) before the start of exposure, where relevant? For tests with embryonic stages: have the parental organisms been held at appropriate conditions?**
	c	**Are the test organisms exempt from previous exposure or any other kind of stressor?**
		Experimental system and chemical analysis
10		**Appropriateness of the experimental system for the test substance:**
	a	**Is the type of exposure (e.g. static, semi-static, flow-through) appropriate for the test substance, taking its physico-chemical characteristics into account?**

Explanation: Static systems are in most cases only appropriate for short-term tests (exception: water/sediment tests). Where appropriate, guideline requirements should be followed.

b In case that the test substance is a difficult substance as defined in OECD (2000): is the selected test system appropriate for testing of this substance?

Explanation: Difficult test substances are substances which are e.g. poorly water soluble, volatile, photo-degradable, hydrolytically unstable, oxidizable, biodegradable, complexing or strongly adsorbing to surfaces of test vessels etc. In order to obtain reliable test results with such substances, test systems generally have to be adapted to take the difficult properties of the substance into account (e.g. by using a closed test system without headspace for volatile substances). For further details, please see OECD (2000). It has to be verified on a case-by-case basis, if the used test system is appropriate for the test substance.

c For ionisable substances: has the test been performed in an appropriate pH-range?

Explanation: Relatively small changes in pH can significantly alter the balance between dissociated and non-dissociated forms of some substances. An altered dissociation equilibrium may significantly affect the water solubility and the partition coefficient of the substance and hence, its bioavailability and toxicity.
For substances with a pKa that is in the normal pH range of the respective test, a preliminary test should be performed to evaluate if the two or more forms of the substance differ in toxicity. The definitive testshould be performed at a pH, within the pH range required for maintaining the health of the test organisms, at which the more toxic form of the test substance prevails. For further guidance, see OECD (2000), section 3.10.

11 Is the experimental system appropriate for the test organism (e.g. choice of medium / test water or soil, feeding, water or soil characteristics, temperature, light/dark conditions, pH, oxygen content)? Have conditions been stable during the test?

Explanation: The general requirements of the test species should be considered with regard to the characteristics of the selected test medium etc. Temperature, pH and oxygen content should be stable and within the appropriate range for the organism (where applicable, check the corresponding guideline). If control performance is not good (e.g. high mortality), this may indicate that test conditions were not appropriate. Where applicable, feeding should follow the guideline requirements, and all excess should be removed after feeding to avoid decreased bioavailability of the test substance.

12 a For aquatic tests: were exposure concentrations below the limit of water solubility?

b For aquatic tests: if a solvent was used, was solvent concentration within the appropriate range (i.e. not higher than 0.01%)?

13 Is a correct spacing between exposure concentrations applied?

Explanation: For standard tests, the corresponding guidelines provide information on the spacing factor. A factor of 3.2 is often recommended. As rule of thumb, the spacing factor should not be >10.

14 Is the exposure duration defined and appropriate?

15 Chemical analysis

a Are chemical analyses performed to a sufficient extent to verify test substance concentrations over the duration of the study where required, and is information on the results of these analyses presented?

Explanation: If required in the corresponding test guideline, nominal test substance concentrations should be verified by chemical analysis. Non-guideline test should be evaluated based on test guidelines for similar tests where appropriate. If it is stated that nominal substance concentrations were verified by chemical analysis, but no information is provided on the results of the chemical analysis, please answer with 'No' and add a comment. If question 15a is ans+B59wered with 'No', questions 15b-e should be answered with 'Not applicable' to avoid that a test with insufficient chemical analysis receives a lower score than a test without chemical analysis.

b Is an appropriate analytical method used to measure test substance concentrations?

c Are the measured test substance concentrations within the calibration range of the analytical method?

d Are samples analysed from a sufficient number of treatments and controls, and from a sufficient number of time intervals?

Explanation: The frequency of chemical analyses should be evaluated based on the requirements of the corresponding test guideline or, for non-guideline studies, on a guideline for a similar test if appropriate.

e Are test substance concentrations sufficiently stable during the course of the exposure ?

Explanation: Please evaluate according to the requirements of the corresponding test guideline or, for non-guideline studies, a test guideline for a similar test where appropriate.

16 Is the biomass loading of the organisms in the test system within an appropriate range?

Explanation: For standard tests, the corresponding guidelines provide information on maximum biomass loading. For non-standard tests / non-standard test species, expert knowledge is required to decide if the loading rate is appropriate.

Statistical design

17 a Is a sufficient number of replicates used for all controls and treatments?

b Is a sufficient number of organisms per replicate used for all controls and test concentrations?

Explanation for 17 a and b: For standard tests, the guideline requirements should be followed. When a non-guideline study is evaluated, expert judgement is needed to assess if the study design is appropriate to obtain statistically reliable results.

18 Are appropriate statistical methods used to derive the effect concentrations?

Explanation: Generally, a description of the statistical methods is needed to assess the reliability of the test results. For standard tests, the corresponding guideline requirements should be followed. Further guidance is e.g. provided by OECD (2006). When a non-guideline study is evaluated, expert judgment may be needed. EC_X values should not be extrapolated considerably beyond the range of tested concentrations.

19 a Is a concentration-response curve observed?

Explanation: The requirement for a concentration-response relationship depends on the objective of the study. If a limit test is performed at one (or two) concentration(s) to verify the lack of toxicity and no toxicity is recorded, a concentration-response relationship is obviously not needed to conclude that the LC_{50} or NOEC is above the highest tested concentration. However, if the intention of the study is to demonstrate an effect, reliability of the test results is higher, if (1) a sufficient number of concentrations have been tested and (2) the observed effect is regularly increasing (or regularly decreasing) with increasing test concentration (i.e. the concentration-response relationship is monotonous). Expert knowledge is needed, if an effect is only observed at the highest tested concentration. Expert knowledge is also needed in the case of non-monotonous concentration-response curves (e.g. U-, J- or inverted U-shaped curves). In

such cases, the underlying mechanisms of effects and the reproducibility of the results should be considered (Harris et al. 2014).

b Is the observed effect statistically significant?

Explanation: The significance level and the statistical method used to evaluate the specific effect should be indicated.

20 Are sufficient data available to check the calculation of endpoints and (if applicable) fulfilment of the validity criteria (e.g. control data, concentration-response curves)?

Explanation: If enough data are presented, additional endpoints may be calculated by the assessor if not reported by the author of the study.

Supplementary Material II

Chronic assay CRED evaluation

Compound name	Disperse Red 1
Organism group	Crustaceans
Species	*Daphnia similis*
Marine/Freshwater	Freshwater
Effect Measured	NOEC
Relevant endpoint	Reproduction
Endpoint concentration/dose	3
Endpoint measurement unit	µg L^{-1}
Test duration	15
Test duration unit	days

CRED evaluation method for reliability to be used together with the accompanying guidance

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Before evaluating the test, please check the physico-chemical characteristics of your compound (handbooks/general sources). What is the solubility, log K_{OW}, pKa, is the compound volatile, does it hydrolyse, photolyse, and others?	**If you have checked the physico-chemical parameters of the substance tested, please continue with the evaluation of reliability, using reliability criteria 1-20.**				
1	Is a standard method (e.g., OECD/ISO) or modified standard used?*	**(X)**				Yes, OECD guideline 211 was used with a slight modification on the exposure time (14 days), based on experiences from a diploma thesis with *Daphnia similis* (Lameira, 2009). The test species is not mentioned in the guideline but a local species.

2	Is the test performed under GLP conditions?*		**(X)**			No, but the lab quality management system follows the requirements of ISO/IEC 17025/2005.
3	If applicable, are validity criteria fulfilled (e.g. control survival, growth)?	**X**				Yes, all validity criteria are fulfilled. The average control reproduction was 72 neonates per mother and no mortality was observed in the controls.
4	Are appropriate controls performed (e.g. solvent control, negative and positive control)?	**X**				Yes, an appropriate negative and positive control were performed. NaCl was used as reference substance in the positive control. DMSO was used as solvent in a concentration that excert no effect (0.01%). Please see Supporting Information Figure S1.
	*These criteria are of minor importance for study reliability, but may support study evaluation					
	Test compound					
5	Is the test substance identified clearly with name or CAS-number? Are test results reported for the appropriate compound?	**X**				Yes, the substance name is Disperse Red 1 and the CAS number is 2872-52-8.
6	Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	**X**				Yes, the dye was purified in a previous study (Vacchi et al., 2013). The purity was determined to be bigger than 95%.
7	If a formulation is used or if impurities are present: Do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	**X**				No, the purified dye was used. No effect from impurities is expected.
	Test organism					

8	Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	X				Yes, the species scientific name is *Daphnia similis*, the strain is *Daphnia similis* Claus, 1876, the body lenght is 3.5 mm. The age/life stage used was less than 24h old neonates.
9	Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	X				Yes, the organisms were donated by Prof. Clarice Botta from University of São Paulo, Brazil. Test organisms were exempt from previous stressors. They are held according to recommended culture conditions.
	Exposure conditions					
10	Is the experimental system appropriate for the test substance, taking into account its physico-chemical characteristics?	X				Yes, the open test system was appropriate for a test substance that is not volatile. The commercial formulation used contained a surfactant to guarantee that the substance is really dissolved. Moreover, the compound is not ionizable, so pH does not have an effect.
11	Is the experimental system appropriate for the test organism (e.g., choice of medium or test water, feeding, water characteristics, temperature, light/dark conditions, pH, oxygen content)? Have conditions been stable during the test?	X				Yes, the experimental system was appropriate for the test organism, i.e. according to the OECD test guideline. Test conditions have been stable during the test.

12	Were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	**X**				Yes, exposure concentrations were below the limit of water solubility of 100 mg L^{-1}. DMSO was used in a concentration of 0.1 % to facilitate the solubility. However, a solvent control was included, where no effect was observed.
13	Is a correct spacing between exposure concentrations applied?	**X**				Yes, a spacing factor of about 3 was used. Concentrations were 1, 3, 10, 30, 100 µg L^{-1}.
14	Is the exposure duration defined?	**X**				Yes, the exposure duration was 15 days and test solutions were changed every two days.
15	Are chemical analyses adequate to verify substance concentrations over the duration of the study?		**(X)**			No. Nominal concentrations were used. However, it is assumed that actual concentrations are similar to the nominal concentration due to the physico-chemical characteristics and the solvent used.
16	Is the biomass loading of the organisms in the test system within the appropriate range (e.g. < 1 g/L)?	**X**				Yes, the biomass is 30 organisms in 1000 mL in the culture and 1 organism in 50 mL in the test.
	Statistical Design and Biological Response					
17	Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	**X**				Yes, the required minimum number of 10 replicates, with 1 organism per replicate (i.e. 10 mothers) were used for the control and each concentration.
18	Are appropriate statistical methods used?	**X**				Yes, ANOVA with paired T-test was used.

19	Is a dose-response curve observed? Is the response statistically significant?	X				Yes, a dose-response curve was observed and the response was statistically significant ($p < 0.01$). Please see Supporting Information Figure S3.
20	Is sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, dose-response curves)?	X				Yes, raw data is available. Please see Supporting Information Table S1.
	Final score (R1 = Reliable, R2 = Reliable with restrictions, R3 = Not reliable, R4 = Not assignable)	**R1**				

CRED evaluation method for relevance to be used together with the accompanying guidance

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Before evaluating the test for relevance, indicate why you are evaluating this study. The relevance of the study might be different for different purposes (e.g., EQC derivation, PBT assessment, dossier evaluation for marketing authorisation), also depending on the framework for which the evaluation is requested.	**If you have indicated the purpose of the evaluation, please continue with the relevance criteria 1-13**				
	Biological relevance					
1	Is the species tested relevant for the compartment under evaluation?	X				Yes, *Daphnia similis* is a primary consumer and a standard test organism in risk assessment.
2	Are the organisms tested relevant for the tested compound?	X				Yes, the species group could be exposed to the dye.
3	Are the reported endpoints appropriate for the regulatory purpose?	X				Yes, long term NOEC on reproduction success are

						commonly used to assess chronic effects.
4	Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	**X**				Yes, the endpoint used is representative for chronic toxicity. It is covering various mode of action in a single endpoint.
5	Is the effect relevant on a population level?	**X**				Yes, reproduction inhibition is directly relevant for the population level.
6	Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	**X**				Yes, the long term NOEC represents the concentration at which no effect on the community is expected.
7	Are appropriate life-stages studied?	**X**				Yes, neonates are expected to be the most sensitive life stage. Moreover, a 15 day test covers one generation for *D. similis*.
8	Are the experimental conditions relevant for the tested species?	**X**				Yes, static renewal exposure is a common exposure scenario in risk assessment, i.e. according to the OECD guideline.
9	Is the time of exposure relevant and appropriate for the studied endpoints and species?	**X**				Yes, this is the standard time of exposure for chronic tests with this species.
10	If recovery is studied, is this relevant for the framework for which the study is evaluated?			**X**		Not applicable.
	Exposure relevance					
11	In case of a formulation, other mixture, salts or transformation products: Is the substance tested representative and relevant for the substance being assessed?			**X**		Not applicable.

12	Is the tested exposure scenario relevant for the substance?	**X**				Yes, long term toxicity can be expected from the continue input of this compound.
13	Is the tested exposure scenario relevant for the species?	**X**				Yes, the standard exposure scenario for this species was used.
	Final score (C1 = Relevant, C2 = Relevant with restrictions, C3 = Not relevant, C4 = Not assignable)	**C1**				

Supplementary Material III

Acute assay CRED evaluation

CAS Number	2872-52-8
Compound name	Disperse Red 1
Organism group	Crustaceans
Species	*Daphnia similis*
Marine/Freshwater	Freshwater
Effect Measured	EC50
Relevant endpoint	Mortality
Endpoint concentration/dose	176
Endpoint measurement unit	µg L^{-1}
Test duration	48
Test duration unit	hours

CRED evaluation method for reliability to be used together with the accompanying guidance

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Before evaluating the test, please check the physico-chemical characteristics of your compound (handbooks/general sources). What is the solubility, log K_{OW}, pKa, is the compound volatile, does it hydrolyse, photolyse, and others?	**If you have checked the physico-chemical parameters of the substance tested, please continue with the evaluation of reliability, using reliability criteria 1-20.**				
1	Is a standard method (e.g., OECD/ISO) or modified standard used?*	**X**				Yes, the OECD guideline 202, with no deviations, was used.
2	Is the test performed under GLP conditions?*		**(X)**			No, although without GLP/ISO certification, the lab quality management system follows the requirements of ISO/IEC 17025/2005.

3	If applicable, are validity criteria fulfilled (e.g. control survival, growth)?	X				Yes, validity criteria are fulfilled. No mortallity was observed in the negative control and no more than 10 per cent in the solvent control.
4	Are appropriate controls performed (e.g. solvent control, negative and positive control)?	X				Yes, an appropriate negative and positive control were performed. NaCl was used as reference substance in the positive control. Methanol was used in the solvent control and no toxicity was observed until 1%. Please see Supporting Information Figure S2.
	*These criteria are of minor importance for study reliability, but may support study evaluation					
	Test compound					
5	Is the test substance identified clearly with name or CAS-number? Are test results reported for the appropriate compound?	X				Yes, the substance name is Disperse Red 1 and has the CAS number 2872-52-8.
6	Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	X				The dye was purified in a previous study (Vacchi et al., 2013).
7	If a formulation is used or if impurities are present: Do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	X				The purified dye was used.
	Test organism					
8	Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	X				Yes, the species scientific name is *Daphnia similis*, the strain is *Daphnia similis* Claus, 1876, the body lenght is 3.5 mm. The age/life stage used was less than 24h old neonates.
9	Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	X				Yes, the organisms were donated by Prof. Clarice Botta from University of São Paulo, Brazil. Test organisms were exempt from previous stressors. They are held in our lab according to recommended culture conditions.

	Exposure conditions					
10	Is the experimental system appropriate for the test substance, taking into account its physico-chemical characteristics?	**X**				Yes, the open test system was appropriate for a test substance that is not volatile. Moreover, a solvent was used to guarantee that the substance is really dissolved.
11	Is the experimental system appropriate for the test organism (e.g., choice of medium or test water, feeding, water characteristics, temperature, light/dark conditions, pH, oxygen content)? Have conditions been stable during the test?	**X**				Yes, the experimental system was appropriate for the test organism, i.e. according to the OECD test guidance.
12	Were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	**X**				Yes, exposure concentrations were below the limit of water solubility of 100 mg L^{-1}. Methanol at 1% was used to facilitate the solubility. A solvent control was included, which was positive (i.e. no mortality).
13	Is a correct spacing between exposure concentrations applied?	**X**				Yes, concentrations were 10, 50, 75, 100, 150, 250, 500, 750 and 1000 µg L^{-1}.
14	Is the exposure duration defined?	**X**				Yes, the exposure duration was 48 hours under static conditions.
15	Are chemical analyses adequate to verify substance concentrations over the duration of the study?		**(X)**			No. Nominal concentrations were used. However, it is assumed that actual concentrations are similar to the nominal concentration due to the physico-chemical characteristics and the solvent used.
16	Is the biomass loading of the organisms in the test system within the appropriate range (e.g. < 1 g/L)?	**X**				Yes, the biomass was 30 organisms in 1000 mL in the culture and 1 organism in 50 mL in the test, according to the OECD test guideline.
	Statistical Design and Biological Response					
17	Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	**X**				Yes, 4 replicates with 5 organism per replicate were used for the control and each concentration.

18	Are appropriate statistical methods used?	X				Yes, Trimmed Spearman-Karber method was used.
19	Is a dose-response curve observed? Is the response statistically significant?	X				Yes, a dose-response curve was observed and the response was statistically significant ($p < 0.01$). Please see Supporting Information Figure S4.
20	Is sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, dose-response curves)?	X				Yes, raw data is available. Please see Supporting Information Table S2.
	Final score (R1 = Reliable, R2 = Reliable with restrictions, R3 = Not reliable, R4 = Not assignable)	**R1**				

CRED evaluation method for relevance to be used together with the accompanying guidance

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Before evaluating the test for relevance, indicate why you are evaluating this study. The relevance of the study might be different for different purposes (e.g., EQC derivation, PBT assessment, dossier evaluation for marketing authorisation), also depending on the framework for which the evaluation is requested.	**If you have indicated the purpose of the evaluation, please continue with the relevance criteria 1-13**				
	Biological relevance					
1	Is the species tested relevant for the compartment under evaluation?	X				Yes, *Daphnia similis* is a primary consumer and a standard test organism in risk assessment.
2	Are the organisms tested relevant for the tested compound?	X				Yes, the species group could be exposed to the dye.
3	Are the reported endpoints appropriate for the regulatory purpose?	X				Yes, short term EC50 on mortality is commonly used to assess acute effects.

4	Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	**X**				Yes, the endpoint used is representative for acute toxicity.
5	Is the effect relevant on a population level?	**X**				Yes, mortality is directly relevant for the population level.
6	Is the magnitude of effect statistically significant and biologically relevant for the regulatory purpose (e.g. EC10, EC50)?	**X**				Yes, the short term EC50 represents the concentration at which effect at 50% of organisms is expected.
7	Are appropriate life-stages studied?	**X**				Yes, neonates are expected to be the most sensitive life stage.
8	Are the experimental conditions relevant for the tested species?	**X**				Yes, static exposure is a common exposure scenario in risk assessment.
9	Is the time of exposure relevant and appropriate for the studied endpoints and species?	**X**				Yes, this is the standard time of exposure for acute tests with this species.
10	If recovery is studied, is this relevant for the framework for which the study is evaluated?			**X**		Not applicable.
	Exposure relevance					
11	In case of a formulation, other mixture, salts or transformation products: Is the substance tested representative and relevant for the substance being assessed?			**X**		Not applicable.
12	Is the tested exposure scenario relevant for the substance?	**X**				Yes, acute toxicity can be expected from the continues input of this compound.
13	Is the tested exposure scenario relevant for the species?	**X**				Yes, the standard exposure scenario for this species was used.
	Final score (C1 = Relevant, C2 = Relevant with restrictions, C3 = Not relevant, C4 = Not assignable)	**C1**				

Supplementary Material IV

Raw Data

Table S1. Raw data of chronic test of Disperse Red 1 to *Daphnia similis*

Concentration ($\mu g\ L^{-1}$)	**Day**	**Number of neonates per replicates**										**Total**
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	
0	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	0	0	0	0	0	0	0	0	0	
	6	8	7	10	0	0	11	10	0	0	9	
	8	12	8	13	18	17	14	16	23	6	14	
	10	8	10	14	10	10	12	13	6	12	6	
	12	16	17	15	6	18	14	26	35	18	12	
	14	28	24	22	26	16	20	28	34	24	26	
	Total	72	66	74	60	61	71	93	98	60	67	722
1	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	0	0	0	0	0	0	0	0	0	
	6	10	0	10	10	0	9	0	10	0	0	
	8	12	19	18	10	16	11	15	13	10	11	
	10	16	12	0	14	16	10	X	8	23	22	
	12	16	16	22	17	18	17	-	14	28	24	
	14	28	20	28	22	18	17	-	28	21	26	
	Total	82	67	78	73	68	64	15	73	82	83	685
3	1	0	0	0	0	0	0	0	0	0	0	
	3	0	0	0	0	0	0	0	0	0	0	
	5	0	0	0	0	0	0	0	0	0	0	
	7	7	8	10	9	7	0	0	7	7	10	
	9	13	13	15	19	10	4	8	10	12	8	
	11	13	2	0	0	16	14	14	13	13	14	
	13	23	35	39	33	26	10	15	18	20	16	
	15	12	20	14	15	19	17	18	24	20	19	
	Total	68	78	78	66	78	45	55	72	72	67	679

Table S1. Raw data of chronic test of Disperse Red 1 to *Daphnia similis* (Cont.)

Concentration (µg L^{-1})	**Day**	**Number of neonates per replicates**										**Total**
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	
10	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	0	0	0	0	0	0	0	0	0	
	6	9	11	10	10	0	0	0	0	4	0	
	8	11	11	10	10	8	10	6	4	8	8	
	10	10	6	10	8	6	4	10	12	9	8	
	12	X	10	14	12	10	14	15	8	10	6	
	14	-	12	10	10	8	14	10	6	8	6	
	Total	30	50	54	50	32	42	41	30	39	28	396
30	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	0	0	0	
	4	0	0	0	0	0	0	0	0	X	0	
	6	8	11	9	10	X	0	0	0	-	X	
	8	6	10	11	12	-	6	18	3	-	-	
	10	12	8	11	12	-	9	0	6	-	-	
	12	16	10	18	15	-	12	18	14	-	-	
	14	8	13	6	10	-	6	10	4	-	-	
	Total	50	52	55	59	0	33	46	27	0	0	322
100	0	0	0	0	0	0	0	0	0	0	0	
	2	0	0	0	0	0	0	0	X	0	0	
	4	X	0	0	0	0	0	0	-	0	0	
	6	-	7	5	7	3	8	0	-	0	X	
	8	-	9	10	8	2	8	8	-	7	-	
	10	-	12	10	16	3	4	6	-	12	-	
	12	-	18	12	15	8	12	X	-	12	-	
	14	-	10	0	5	2	6	-	-	10	-	
	Total	0	56	37	51	18	38	14	0	41	0	255

Table S2 – Raw data for acute test of Disperse Red 1 to *Daphnia similis*

Concentration (μg L^{-1})	**Number of organisms immobilised**				**Total**
	1	**2**	**3**	**4**	
0	0/5	0/5	0/5	0/5	0/20
10	0/5	0/5	0/5	0/5	0/20
50	0/5	0/5	0/5	1/5	1/20
75	0/5	0/5	0/5	0/5	0/20
100	0/5	1/5	1/5	0/5	2/20
150	2/5	2/5	0/5	1/5	5/20
250	5/5	4/5	4/5	4/5	17/20
500	5/5	5/5	5/5	5/5	20/20
750	5/5	5/5	5/5	5/5	20/20
1000	5/5	5/5	5/5	5/5	20/20

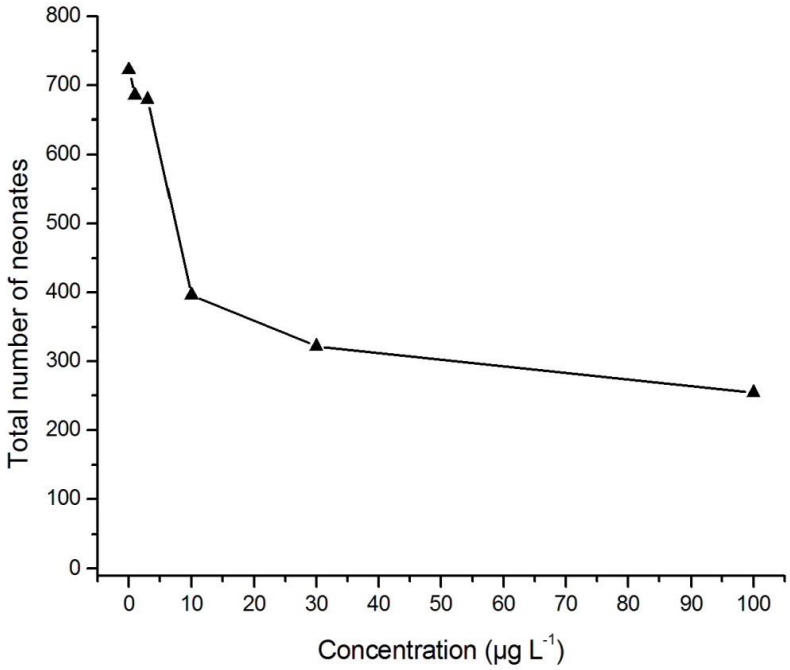

Figure S1. Dose-response curve chronic test of Disperse Red 1 to *Daphnia similis.*

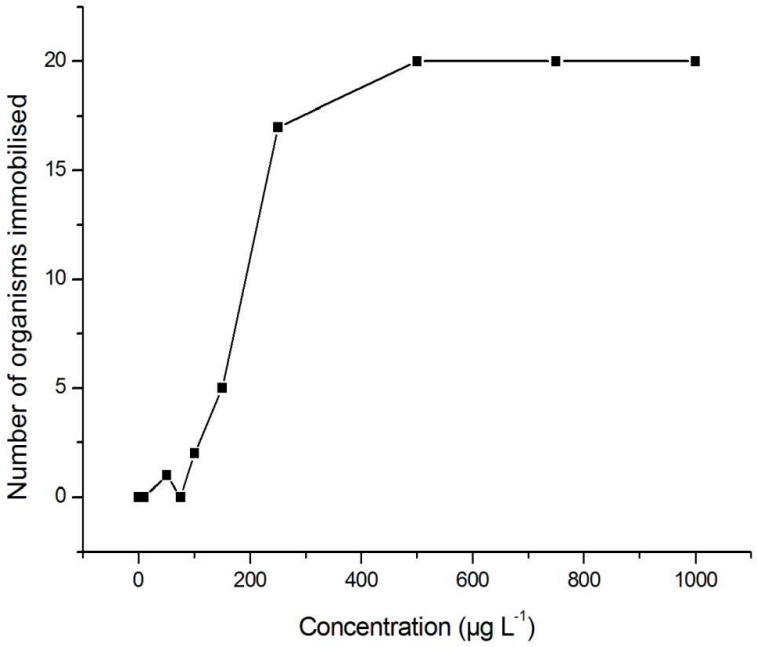

Figure S2. Dose-response curve of acute test of Disperse Red 1 to *Daphnia similis.*

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5. CONSIDERAÇÕES FINAIS

- Os corantes Disperse Blue 291, Disperse Blue 373, Disperse Orange 30, Disperse Red 1, Disperse Violet 93 e Disperse Yellow 3 foram detectados em amostras do Rio Piracicaba, Ribeirão Quilombo e efluente da ETE Carioba.
- As técnicas de extração líquido-líquido e cromatografia líquida acoplada a detector de massa (HPLC-MS/MS) se mostraram sensíveis para indicar a presença de corantes em amostras de efluentes e águas superficiais.
- A genotoxicidade do Rio Piracicaba, avaliada pelo teste *Salmonella*/microssoma e pelo ensaio do cometa, aumentou após o Ribeirão Quilombo e o efluente da ETE Carioba, mostrando uma possível contribuição destes lançamentos na genotoxicidade observada no Rio Piracicaba.
- O corante Disperse Red 1 foi o composto mais frequente, detectado em 8 das 16 amostras, porém sua contribuição para a mutagenicidade total foi baixa (de 0,004 a 2,7%); os corantes Disperse Blue 373 e Disperse Violet 93 foram os que mais contribuíram para o efeito observado.
- As concentrações do corante Disperse Red 1 nas amostras ambientais estão próximas ou acimas do critério para proteção da vida aquática derivado neste trabalho, mostrando que este corante apresenta risco ecotoxicológico e deve ser monitorado.
- Devido à alta hidrofobicidade dos corantes detectados nas amostras de efluente e água seria interessante analisar o sedimento da região para verificar a mutagenicidade, bem como a presença de corantes e outros produtos de transformação que poderiam persistir nessa matriz levando à exposição da biota por longos períodos.
- Seria importante ampliar a Estação de Tratamento de Efluentes Carioba para receber os efluentes lançados no Ribeirão Quilombo, e efetuar um tratamento que efetivamente remova esses grupos de compostos reduzindo os riscos a vida aquática. Outra pratica que deve ser evitada é a cloração dos efluentes, pois corantes podem gerar derivados reduzidos e clorados ainda mais mutagênicos que os produtos originais.
- Estudos utilizando marcadores de genotoxicidade em peixes ou organismos residentes da região poderiam ser realizados para uma avaliação mais ecologicamente relevante que ensaios *in vitro*, como os que foram realizados neste estudo.

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ANEXO I

Ficha do Aluno

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9141 - 8130215/1 - Francine Inforçato Vacchi

Email:	francinevacchi@usp.br
Data de Nascimento:	20/02/1986
Cédula de Identidade:	RG - 40.881.372-6 - SP
Local de Nascimento:	Estado de São Paulo
Nacionalidade:	Brasileira
Mestrado:	Mestre em Tecnologia (1) - Universidade Estadual de Campinas - São Paulo - Brasil - 2012

Curso:	Doutorado
Programa:	Toxicologia e Análises Toxicológicas
Data de Matrícula:	13/06/2012
Início da Contagem de Prazo:	13/06/2012
Data Limite para o Depósito:	13/06/2016
Orientador:	Prof(a). Dr(a). Silvia Berlanga de Moraes Barros - 13/06/2012 até 19/06/2012. Email: smbarros@usp.br
Orientador:	Prof(a). Dr(a). Gisela de Aragão Umbuzeiro - 20/06/2012 até o presente. Email: gisela@usp.br
Proficiência em Línguas:	Inglês, Aprovado em 13/06/2012
Data de Aprovação no Exame de Qualificação:	Aprovado em 01/09/2014
Data do Depósito do Trabalho:	31/05/2016
Título do Trabalho:	"Ocorrência, genotoxicidade e risco ecotoxicológico de corantes no ambiente aquático"
Data Máxima para Aprovação da Banca:	01/08/2016
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	
Histórico de Ocorrências:	Primeira Matrícula em 13/06/2012

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 5473 em vigor de 18/09/2008 até 19/04/2013).
Última ocorrência: Matrícula de Acompanhamento em 01/02/2016
Impresso em: 01/06/2016 11:52:38

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Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBC5803-2/2	Sistemas da Garantia da Qualidade em Laboratórios Analíticos	07/08/2012	20/08/2012	30	2	75	A	N	Concluída
FBC5883-5/1	Avaliação da Toxicidade de Contaminantes em Amostras Ambientais	09/08/2012	19/09/2012	60	4	100	A	N	Concluída
CEN5738-6/1	Ecotoxicologia (Centro de Energia Nuclear na Agricultura - Universidade de São Paulo)	20/08/2012	28/10/2012	150	10	90	A	N	Concluída
FBC5729-7/1	Fundamentos Básicos da Avaliação do Risco Oferecido por Substâncias Químicas	23/08/2012	19/09/2012	60	4	85	A	N	Concluída
EPP5766-1/9	Diálogos sobre o Ensino Superior (Escola Superior de Agricultura "Luiz de Queiroz" - Universidade de São Paulo)	18/03/2013	24/03/2013	30	0	-	-	N	Pré-matrícula indeferida
FBC5802-3/6	Tópicos Avançados em Toxicologia I (2)	03/03/2015	16/06/2015	15	1	90	A	N	Concluída
FBC5784-3/7	Tópicos Avançados em Toxicologia II (2)	04/08/2015	16/11/2015	15	1	90	A	N	Concluída

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	10	20	22
Estágios:			
Total:	10	20	22

Créditos Atribuídos à Tese: 167

Observações:

1) Curso com validade nacional, de acordo com o disposto na Portaria nº 590, de 18.06.2009..

2) Disciplina(s) cursada(s) voluntariamente pelo(a) candidato(a) após ter cumprido as exigências regulamentares.

Conceito a partir de 02/01/1997:

A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.

Última ocorrência: Matrícula de Acompanhamento em 01/02/2016

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FICHA DO ALUNO

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Comissão julgadora da tese de doutorado:

NUSP	Nome	Vínculo	Função
852540	Gisela de Aragão Umbuzeiro	UNICAMP - Externo	Presidente

Última ocorrência: Matrícula de Acompanhamento em 01/02/2016
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