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

Comparação do perfil de mutagenicidade e da composição
química do material particulado atmosférico de Limeira,
Estocolmo e Quioto

Bianca de Souza Maselli

Tese para obtenção do Título de DOUTOR

Orientador: Prof. Dr. Fábio Kummrow

São Paulo
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*“O desejo profundo da humanidade pelo conhecimento é justificativa
suficiente para nossa busca contínua.”*

Stephen Hawking

RESUMO

MASELLI, B. S. **Comparação do perfil de mutagenicidade e da composição química do material particulado atmosférico de Limeira, Estocolmo e Quioto.** 2018. 134f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2018.

O material particulado (MP) atmosférico é associado a vários agravos e doenças, sendo recentemente classificado como carcinogênico para humanos (Grupo 1) pela Agência Internacional de Pesquisa em Câncer (IARC). Embora sua toxicidade, incluindo a genotoxicidade, esteja reconhecidamente ligada ao tamanho das partículas, a contribuição da sua composição química para esses efeitos ainda não está completamente elucidada. O tamanho e a composição das partículas são influenciados por características meteorológicas e climáticas, com destaque para a temperatura e o período de radiação solar. O teste *Salmonella*/microsoma é o mais utilizado para avaliação da mutagenicidade de amostras de MP, contudo um número reduzido de linhagens é usado. Assim, o objetivo desse estudo foi investigar e comparar a influência das diferentes condições atmosféricas e climáticas das cidades de Limeira (Brasil), Estocolmo (Suécia) e Quioto (Japão) nos perfis de mutagenicidade, utilizando 11 linhagens com diferentes seletividades, e nos perfis de composição química de amostras compostas de partículas totais em suspensão (PTS) coletadas durante o inverno dessas cidades. Para que os resultados pudessem ser diretamente comparados foi adotada a mesma metodologia, incluindo o procedimento de amostragem, o método de preparo de amostra, o protocolo do teste de mutagenicidade e as técnicas de análises químicas para identificação de hidrocarbonetos policíclicos aromáticos (HPA) e de seus derivados alquilados. Limeira apresentou a maior concentração de PTS ($99,0 \mu\text{g}/\text{m}^3$), seguida por Quioto ($28,0 \mu\text{g}/\text{m}^3$) e Estocolmo ($6,2 \mu\text{g}/\text{m}^3$). Apesar da concentração de PTS em Limeira ser 16 vezes maior que em Estocolmo e 3,5 vezes maior que em Quioto, as porcentagens de material orgânico extraído (MOE) obtidas foram 9, 15 e 5%, respectivamente. Os extratos das amostras de PTS coletadas nas três cidades apresentaram atividade mutagênica para todas as linhagens, tanto na ausência quanto na presença de S9, com exceção da TA102 que não detectou a atividade mutagênica em nenhum dos extratos, e para YG7108 na presença de S9, apenas para a amostra de Limeira. Apesar das diferenças nas condições meteorológicas e climáticas dessas cidades, seus perfis de mutagenicidade foram semelhantes. A menor potência mutagênica para a YG7108 na ausência de S9 e a ausência de mutagenicidade na presença de S9 revela a menor contribuição de agentes alquilantes para a mutagenicidade da amostra de Limeira em relação as demais cidades. Considerando a seletividade das linhagens utilizadas, foi observado maior contribuição de compostos que causam danos oxidativo ao DNA e de agentes alquilantes para a mutagenicidade da amostra de Quioto. Porém, de acordo com as respostas das linhagens que possuem atividade aumentada das enzimas nitroredutase (NR) e/ou *O*-acetiltransferase (OAT), YG1021, YG1024 e YG1041, revelam a maior contribuição de nitroarenos e aminas aromáticas para a mutagenicidade das três cidades, com destaque para aqueles mutágenos que dependem da ativação via OAT. As potências mutagênicas expressadas em função da massa de MOE (rev./ μg MOE) foram similares para todas as amostras. Quando expressas por volume de ar (rev./ m^3), as potências mutagênicas foram proporcionais às concentrações de PTS das três cidades (Limeira > Quioto > Estocolmo). Quando aplicadas as razões diagnósticas foi possível verificar uma mistura de fontes de poluição em Limeira e Estocolmo. As razões diagnósticas dos HPA indicam que a

amostra de Limeira é composta por partículas frescas, com um leve predomínio de fontes de combustão. A amostra de Estocolmo apresenta partículas envelhecidas e com predomínio de fontes petrogênicas. Esse resultado foi inesperado, uma vez que as condições atmosféricas de Limeira são mais favoráveis ao envelhecimento das partículas que as de Estocolmo. O estudo do transporte das massas de ar explica, pelo menos em parte, a presença de partículas envelhecidas na amostra de Estocolmo. Sugere também que a redução das concentrações de HPA em Limeira depende do controle das fontes locais, enquanto em Estocolmo o controle das fontes locais não seria eficiente na diminuição desses poluentes. Em paralelo a avaliação das amostras de PTS, foi desenvolvido um novo protocolo miniaturizado do teste Salmonella/microsossoma em microssuspensão. O protocolo utilizando microplacas de 12 poços foi validado empregando treze compostos mutagênicos testados com três linhagens de Salmonella selecionadas baseadas em suas diferentes frequências de reversão espontânea (baixa, média e alta). Tanto o teste miniaturizado em microplacas de ágar (MPA) quanto o em microssuspensão apresentaram sensibilidades semelhantes, concluindo que o MPA é uma ferramenta promissora e pode ser particularmente adequado para estudos ambientais como Análise Dirigida pelo Efeito (EDA) ou programas de monitoramento.

Palavras-chaves: Partículas totais suspensas; Ensaio Salmonella/microsossoma em microssuspensão; Razões diagnósticas moleculares; Processo de envelhecimento do PTS, Miniaturização do teste Salmonella/microsossoma.

ABSTRACT

MASELLI, B. S. **Comparison of mutagenicity and chemical profile of the atmospheric particulate matter from Limeira, Stockholm and Kyoto.** 2018. 134f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2018.

Atmospheric particulate matter (PM) is associated with various diseases and has recently been classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC). Although the PM toxicity, including genotoxicity, is known to be related to particle size, the contribution of its chemical composition to the observed effects has not yet been fully elucidated. The size and composition of the particles are influenced by meteorological and climatic characteristics, especially the temperature and the period of solar radiation. The Salmonella/microsome assay is the most used for the evaluate of the mutagenicity of PM samples, however a small number of strains are used. The objective of this study was to investigate and compare the influence of the different atmospheric and climatic conditions of the cities of Limeira (Brazil), Stockholm (Sweden) and Kyoto (Japan) on mutagenicity profiles, using 11 strains with different selectivity, and chemical composition profiles of total particles suspended (TPS) composed samples collected during the winter of these cities. For the results to be directly compared, the same methodology was adopted, including the sampling procedure, the sample preparation protocol, the mutagenicity test protocol, and the chemical analysis techniques for the identification of polycyclic aromatic hydrocarbons (PAH) and their alkylated derivatives. Limeira presented the highest concentration of TPS ($99.0 \mu\text{g}/\text{m}^3$), followed by Kyoto ($28.0 \mu\text{g}/\text{m}^3$) and Stockholm ($6.2 \mu\text{g}/\text{m}^3$). Although the TPS concentration in Limeira was 16-fold higher than in Stockholm and 3.5-fold higher than in Kyoto, the percentage of extracted organic material (EOM) obtained was 9, 15 and 5%, respectively. The extracts from the TPS samples collected in the three cities presented mutagenic activity for all strains, both in the absence and presence of S9, except for TA102 that did not detect the mutagenic activity in any of the extracts, and for YG7108 in the presence of S9, only for the Limeira sample. Despite the differences in meteorological and climatic conditions of these cities, their mutagenicity profiles were similar. The lower mutagenic potency for YG7108 in the absence of S9 and the non-mutagenicity in the presence of S9 reveals the lower contribution of alkylating agents to the mutagenicity of the Limeira sample in relation to the other cities. Considering the selectivity of the strains used, we can also observe a greater contribution of compounds that cause DNA oxidative damage and of alkylating agents for the mutagenicity of the Kyoto sample. However, according to the responses of the strains that have increased activity of the nitroreductase (NR) and/or *O*-acetyltransferase (OAT) enzymes, YG1021, YG1024 and YG1041, show the highest contribution of nitroarenes and aromatic amines for the mutagenicity of the three cities, with emphasis on those mutagens that depend on OAT activation. The mutagenic potencies expressed as a function of EOM mass (rev./ μg EOM) were similar for all samples. When expressed by air volume (rev./ m^3), the mutagenic potencies were proportional to TPS concentrations of the three cities (Limeira > Kyoto > Stockholm). When applying the diagnostic ratios, it was possible to verify a mixture of sources of pollution in Limeira and Stockholm. The diagnostic ratios of the PAH indicate that Limeira sample is composed of fresh particles with a slight predominance of combustion sources. The Stockholm sample present aged particles with a predominance of petrogenic sources. This result was unexpected, since Limeira's

atmospheric conditions are more favorable to particle aging than Stockholm's. The study of the transport of air masses explains, at least in part, the presence of aged particles in the Stockholm sample. It also suggests that the reduction of PAH concentrations in Limeira depends on the control of local sources, while in Stockholm control of local sources would not be efficient in reducing these pollutants. In parallel to the evaluation of the TPS samples, a new miniaturized protocol of the Salmonella/microsome assay in microsuspension was also developed. The protocol using 12-well microplates was validated by employing thirteen mutagenic compounds tested with three selected Salmonella strains based on their different spontaneous reversion frequencies (low, medium and high). Both the miniaturized microplate agar (MPA) and the microsuspension assay presented similar sensitivities, concluding that MPA is a promising tool and can be particularly suitable for environmental studies such as effect-directed analysis (EDA) or monitoring programs.

Keywords: Total particles suspended; Salmonella/microsome assay in microsuspension; Molecular diagnostic reasons; Aging process of TPS, Miniaturization of the Salmonella/microsome assay.

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1. INTRODUÇÃO

A poluição atmosférica difere de todos os outros tipos, em que a exposição é evitável, por afetar todos os segmentos de uma população. Por exemplo, quando uma fonte de água está contaminada é possível buscar água de outra fonte ou ainda tratar a contaminação, porém no caso da poluição atmosférica isso não é viável (Oliveira e Kummrow, 2014). Assim, estudos sobre a qualidade do ar em ambientes urbanos são de grande importância, pois inúmeras atividades antrópicas podem levar a poluição atmosférica e acarretar prejuízos à saúde humana e ao meio ambiente (Callén et al., 2013). Entre as doenças e agravos à saúde humana associados à exposição à poluição atmosférica, e em especial ao material particulado (MP) do ar, estão o aumento do risco de mortalidade, agravamento de doenças respiratórias incluindo asma e bronquite, arteriosclerose, alterações na frequência cardíaca (Kok et al., 2006; Lewtas, 2007; Cohen et al., 2017), e recentemente, a Agência Internacional de Pesquisa em Câncer (IARC) concluiu que a exposição à poluição atmosférica e ao MP é carcinogênica para humanos (Grupo 1), causando preponderantemente câncer de pulmão (IARC, 2016). DeMarini (2012) considera que já existem evidências suficientes para que a poluição atmosférica seja declarada como mutagênica para células germinativas humanas. Além disso, o MP atmosférico interfere nos processos climáticos, diretamente por meio da absorção e espalhamento da irradiação solar, ou indiretamente, pela formação de núcleos de condensação das nuvens e pela alta concentração de partículas finas dificultando a visibilidade (Meire et al., 2007; Walgraeve et al., 2010). Assim, o MP é considerado um dos problemas prioritários, tanto do ponto de vista ambiental quanto de saúde pública (Burkart et al., 2013).

O MP atmosférico é constituído de uma mistura de partículas sólidas e líquidas de tamanhos e composições bastante variados, e a sua origem pode ser natural ou antropogênica, podendo ser emitido diretamente (partículas primárias) ou formado na própria atmosfera (partículas secundárias) (Yu et al., 2012; Oliveira e Kummrow, 2014; Collins, 2015). Suas principais fontes são: exaustão veicular (ex.: motores a diesel), emissões de processos industriais (ex.: queima de carvão em altos-fornos), combustão (ex.: queima de biomassa), ressuspensão de poeira do solo e de rodovias, sistemas de aquecimento doméstico, construção civil e demolição, pulverização de agrotóxicos, bioaerossóis, cinzas vulcânicas, incêndios florestais e erosão eólica (Rocha et al., 2005; Curtis et al., 2006; Samet e Krewski, 2007; Sarigiannis et al., 2014; Souza et al., 2017).

As partículas grandes ($> 10 \mu\text{m}$) são geralmente provenientes de fenômenos da natureza ou de processos físicos. Atividades humanas também podem dar origem a este tipo de partículas. Por outro lado, as partículas menores ($\leq 10 \mu\text{m}$), são provenientes principalmente de processos químicos ou da condensação de espécies ainda menores, como moléculas (Franco, 2006). Comprovadamente a manifestação dos efeitos tóxicos decorrentes da exposição ao MP, incluindo efeitos genotóxicos, está associada ao tamanho das partículas, principalmente àquelas com diâmetro aerodinâmico menor que $10 \mu\text{m}$ (MP_{10}) pois penetram mais profundamente no sistema respiratório (Kok et al., 2006; Lewtas, 2007). Muitas emissões dos processos de combustão contêm partículas com um diâmetro aerodinâmico menor, mas essas partículas tendem a apresentar elevados níveis de mutágenos em sua matéria orgânica extraída (MOE). Por outro lado, as partículas com alto diâmetro aerodinâmico geralmente contêm quantidades relativamente menores de MOE (Claxton et al., 2004; Sato et al., 1995). Além disso, as reações atmosféricas podem produzir mutágenos (Ex.: nitroarenos) que podem condensar em partículas pequenas ou combinar com outras moléculas gerando produtos de baixa volatilidade, que podem então formar partículas ultrafinas e depois partículas maiores (Claxton et al., 2004). Devido à sua elevada área superficial, as partículas menores tendem a adsorver diversos compostos orgânicos, como os Hidrocarbonetos Policíclicos Aromáticos (HPA), principalmente aqueles com maior peso molecular e menor volatilidade, e seus derivados (Ex.: nitro-HPA) (Rainho et al., 2013). Porém, ainda são necessários estudos para elucidar a relação entre a composição química do MP e os seus efeitos tóxicos sobre as populações expostas (Lewtas, 2007).

Nos centros urbanos, a combustão do diesel e da gasolina, utilizados no transporte, é considerada a principal fonte de poluição atmosférica (Lewtas, 2007; CETESB, 2016; Kumar et al., 2016), e consequentemente a intensidade do tráfego tem importante impacto tanto em aspectos qualitativos como quantitativos do MP emitido, incluindo suas características químicas, físicas e toxicológicas (Kok et al., 2006). Contudo, recentemente McDonald et al. (2018) demonstraram que a contribuição de compostos orgânicos voláteis (COV) presentes na composição de produtos de uso doméstico, comercial e industrial, como por exemplo os solventes de tintas e vernizes, em ambientes urbanos industrializados pode ser maior que aquela proveniente do uso de combustíveis fósseis em veículos. Alves et al. (2016) demonstraram que a contribuição de poluentes gerados na combustão de biomassa (Ex.: a queima da palha da cana de açúcar), misturada a poluição emitida nos centros urbanos aumenta a mutagenicidade de amostras de

partículas totais em suspensão (PTS). Assim, a análise da movimentação das massas de ar se torna uma importante ferramenta para o entendimento da distribuição dos poluentes em diferentes áreas, sua influência para a formação de poluentes secundários e finalmente a sua contribuição para a mutagenicidade observada em amostras de MP.

Amostras MP são quimicamente muito complexas, contendo de centenas a milhares de diferentes compostos orgânicos, muitos deles em concentrações extremamente baixas (Ringue et al., 2012; Ahmed et al., 2015). A fração orgânica do MP representa de 10 a 90% em massa (Alves et al., 2008; Ringue et al., 2012). Particularmente a mutagenicidade da fração orgânica do MP está relacionada com poluentes primários e/ou secundários, e a sua distribuição pode variar amplamente de acordo com as fontes de poluição, o transporte das massas de ar, e as condições meteorológicas, incluindo a temperatura, a incidência de luz solar e a umidade relativa do ar (Alves et al., 2012; Keyte et al., 2013; Jariyasopit et al., 2014; Shrivastava et al., 2017). De acordo com Claxton et al. (2004), a mutagenicidade observada nos extratos orgânicos de MP está associada a mais de 500 compostos químicos já identificados. Porém, esse montante representa apenas em torno de 10 a 20% da composição total da fração orgânica presente em amostras de MP (Walgraeve et al., 2010). O que torna a contribuição de inúmeros mutágenos presentes no MOE para a atividade mutagênica das amostras de MP pouco conhecida (Lewtas, 2007).

Dentre as classes químicas reconhecidamente ligadas a mutagenicidade dos extratos orgânicos do MP estão os HPA, porém a sua presença não explica grande parte do efeito mutagênico associado a este tipo de amostra (Claxton et al., 2004). Contudo, parte desta mutagenicidade está sendo atribuída a outras classes químicas, como os nitroaromáticos, as aminas e as cetonas aromáticas (Claxton et al., 2004; Umbuzeiro et al., 2008a, 2008b). Nesse contexto, Lewtas (2007) sugere que novas abordagens visando à identificação efetiva de compostos carcinogênicos presentes em misturas complexas, como o MP, empreguem testes com bactérias para avaliação de mutagenicidade, como por exemplo o teste *Salmonella*/microsoma, combinados com a estratégia analítica denominada *Análise Dirigida pelo Efeito* (EDA).

O teste *Salmonella*/microsoma é o mais empregado em todo o mundo para a detecção, caracterização e elucidação da mutagenicidade de amostras do MP (~ 50 % dos trabalhos publicados internacionalmente) (Claxton et al., 2004; Claxton e Woodall Jr., 2007; Claxton et al., 2010). Existem diferentes protocolos desse teste e, quando há restrições na quantidade de amostra disponível, ou quando se pretende utilizar amplo

conjunto de linhagens, o protocolo microssuspensão (teste de Kado) é o mais recomendado (De Martinis et al., 1999; Claxton et al., 2001; Alves et al., 2016; IARC, 2016). Entre as vantagens do teste *Salmonella*/microssoma estão as inúmeras linhagens de *Salmonella enterica* serovar Typhimurium (anteriormente denominada *Salmonella typhimurium*) (McClelland et al., 2001; DeVos et al., 2005; Gal-Mor et al., 2014), com características que as tornam sensíveis a classes específicas de mutágenos. E, em virtude da sua seletividade, essas linhagens estão sendo aplicadas com sucesso na elucidação da contribuição de determinadas classes químicas, presentes em amostras de MP, a sua atividade mutagênica (Azuma et al., 1997; De Martinis et al., 1999; Claxton et al., 2001; Vargas, 2003; Umbuzeiro et al., 2008a,b; Umbuzeiro et al., 2011). A seletividade destas linhagens está associada principalmente à produção de diferentes tipos e níveis de enzimas envolvidas nas reações de biotransformação e a expressão de diferentes sistemas de reparo no DNA.

As linhagens mais comumente utilizadas para avaliar amostras ambientais são a TA98 e TA100, que detectam mutações do tipo deslocamento no quadro de leitura e substituição dos pares de base, respectivamente (Reifferscheid e Buchinger, 2010; Umbuzeiro et al., 2016), pois foi observado que a combinação delas detecta a maior parte dos mutágenos, detectados pelo teste *Salmonella*/microssoma, neste tipo de amostra (Zeiger et al., 1985; Felton e Wu, 2003). A TA98, é mais sensível aos HPA (Lepers et al., 2014), aminas aromáticas e nitroarenos do que a TA100 (Chen e White, 2004). Considerando a importante contribuição de nitroarenos e aminas aromáticas para a mutagenicidade das amostras de MP (Nohmi, 2007; Umbuzeiro et al., 2014; Alves et al., 2016; IARC, 2016), linhagens com diferentes níveis das enzimas nitroredutase (NR) e *O*-acetiltransferase (OAT), ambas envolvidas na ativação dessas classes de mutágenos, foram incluídas em estudos de avaliação da mutagenicidade de amostras atmosféricas (Ducatti e Vargas, 2003; Umbuzeiro et al., 2008a; Franco et al., 2010; Pereira et al., 2013; Rainho et al., 2013). Entre as linhagens que apresentam produção aumentada dessas enzimas, as mais utilizadas são YG1021 (NR), YG1024 (OAT) e YG1041 (NR e OAT) (IARC, 2016). A enzima NR é vista como um fator limitante para a bioativação de nitroarenos mutagênicos para as linhagens de *S. typhimurium* (Watanabe et al., 1989), enquanto que a OAT é uma enzima chave para a ativação intracelular de nitroarenos e aminas aromáticas (Watanabe et al., 1990).

A linhagem YG1041 possui como diferencial a adição do plasmídeo pYG233 que acarreta em elevados níveis de ambas enzimas, NR e OAT, sendo a mais sensível na

detecção de nitroarenos e aminas aromáticas. O aumento na mutagenicidade de uma amostra para a linhagem YG1041 em relação a TA98 é uma resposta típica de compostos nitroaromáticos (Claxton et al., 2004; Umbuzeiro et al., 2011). A YG1021 é uma linhagem que possui níveis elevados de NR (Watanabe et al., 1989), especializada e altamente sensível a compostos nitroaromáticos, na ausência de (-S9). Enquanto que a YG1024, com elevada produção de OAT, é altamente sensível a aminas aromáticas, especialmente na presença de (+S9).

Entre as demais linhagens empregadas para a caracterização da mutagenicidade das amostras de PTS, encontra-se a TA1538, que detecta o mesmo tipo de mutágenos que a TA98 (Maron e Ames, 1983), porém, em geral, a TA98 é considerada mais sensível que a sua parental por possuir o plasmídeo pKM101 (Herbold, 1982; Maron e Ames, 1983). Esse plasmídeo carrega o gene *mucAB* que aumenta a capacidade de expressão do sistema de reparo no DNA suscetível ao erro, denominado “*error-prone*” (Maron e Ames, 1983). Herbold (1982) concluiu que a TA1538 pode ser substituída por outras linhagens, como por exemplo, a própria TA98, por apresentarem respostas semelhantes para inúmeros compostos químicos. Contudo, a TA1538 mostrou-se mais sensível para a mutagenicidade de extratos orgânicos de amostras de PTS coletadas anteriormente em Limeira (Alves et al., 2016) e para o benzo[*a*]pireno (B[*a*]P) (Skopek et al., 1978; Umbuzeiro et al., 2014). Alves et al. (2016), sugeriram que o aumento da mutagenicidade observada para a TA1538 com relação a TA98 pode estar relacionada a presença de mutágenos que causam adutos no DNA e, portanto, lesões dependentes do sistema de reparo recombinacional presente na TA1538.

Embora a YG5185 nunca tenha sido empregada para avaliação da mutagenicidade de amostras de MP, Umbuzeiro et al. (2014) empregaram a linhagem YG5161, que apresenta sensibilidade similar aos HPA que a YG5185, buscando distinguir a contribuição desta classe química para a mutagenicidade de amostras de PTS coletadas anteriormente em Limeira e Estocolmo. A YG5161 além de detectar HPA, também apresenta níveis expressivos das enzimas NR e OAT, que favorecem a detecção de nitroarenos e aminas aromáticas. Os autores concluíram que a YG5161 não foi capaz de distinguir a resposta dos HPA que atuam como o B[*a*]P e outras classes químicas de mutágenos nas concentrações encontradas nos extratos das amostras estudadas, sendo sua mutagenicidade mascarada principalmente pela presença dos nitroarenos. Para evitar que a atividade mutagênica dos HPA fosse mascarada pelos nitroarenos e/ou aminas aromáticas, Yamada et al. (2006) desenvolveram a linhagem YG5185, que semelhante a

YG5161, possuem o plasmídeo pYG768, responsável pela maior expressão da DNA polimerase IV, o que a torna também especialmente sensível os HPA. Porém, a YG5185 é deficiente na produção das enzimas NR e OAT, o que torna essa linhagem menos sensível aos nitroarenos e as aminas aromáticas (Yamada et al., 2006). Uma resposta mutagênica em presença de S9 aumentada para essa linhagem em relação às demais, pode indicar a contribuição de compostos como o B[a]P (Yamada et al., 2006) para a mutagenicidade observada.

Linhagens com maior sensibilidade a outras classes de mutágenos, que induzem tipos específicos de mutações, podem ser úteis para um estudo abrangente de amostras de MP (Umbuzeiro et al., 2011). A TA102 que detecta a atividade de mutágenos capazes de causar ligações cruzadas no DNA (Mortelmans e Zeiger, 2000), como por exemplo os hidroperóxidos (Ames, 1984; Mortelmans e Zeiger, 2000), foi anteriormente utilizada para avaliar extratos orgânicos de MP (De Flora et al., 1989; Crebelli et al., 1991; Landkocz et al., 2017) e detectou atividade mutagênica apenas na presença de S9 (Crebelli et al., 1991). De Flora et al. (1989) utilizou a linhagem TA104, que detecta mutágenos que causam danos oxidativos ou ligações cruzadas no DNA, para avaliar extratos orgânicos de MP, porém, essa linhagem não foi capaz de detectar atividade mutagênica. Segundo Mortelmans e Zeiger, (2000), o uso da TA102 em paralelo com a TA104 é recomendado, principalmente quando são obtidos resultados negativos com a TA104 e há suspeita da presença de mutágenos que causam danos oxidativos ao DNA não detectados por esta linhagem. A TA102 carrega múltiplas cópias do plasmídeo pAQ1, tornando está mais sensível aos agentes oxidantes como a Mitomicina C e Bleomicina, e a TA104 que carrega a mutação *hisG428*, não detectando a Mitomicina C, mas aldeídos não detectados pela TA102 (Levin et al., 1982). A sensibilidade da linhagem TA97a, que foi desenvolvida a partir da TA1537, aos extratos de MP sugerem a presença de mutágenos que induzem ligações cruzadas no DNA (Maron e Ames, 1983; Mortelmans e Zeiger, 2000; Silva Júnior e Vargas, 2009). Crebelli et al. (1991) utilizando TA97a, observaram atividade mutagênica em ausência e presença de S9. Em um estudo realizado para avaliar a especificidade da TA97a com relação a TA1537, foram testados 21 compostos (dentre eles, B[a]P, 2-aminofluoreno e 2-nitrofluoranteno) reconhecidamente mutagênicos para estas linhagens. A TA97a foi consideravelmente mais sensível a estes compostos do que a TA1537 (Donovan, 1990).

A linhagem YG7108 tem capacidade de detectar mutágenos que causam substituição dos pares de bases, com maior sensibilidade ao grupo dos agentes alquilantes.

Característica dada a esta linhagem devido à deleção dos genes (*ada_{ST}* e *ogt_{ST}*) envolvidos no mecanismo responsável pelo reparo do DNA causados por esses compostos (Yamada et al., 1997). Alves (2011) utilizou a YG7108, para a caracterização da mutagenicidade de amostras de PTS coletadas anteriormente em Limeira, porém essa linhagem não foi capaz de detectar atividade mutagênica em nenhuma das amostras.

A distribuição dos HPA e a formação dos seus derivados (ex.: nitro-HPA) é fortemente influenciada pelas fontes de poluição e pelas características atmosféricas e climáticas (Burkart et al., 2013; Keyte et al., 2013). Os HPA podem ter origem através de processos naturais, porém em regiões altamente urbanizadas ou industrializadas, esses compostos são gerados por fontes antropogênicas (Tobiszewski e Namieśnik, 2012). As fontes de poluição dos HPA podem ser identificadas comparando-se a proporção da concentração entre pares selecionados de HPA (Zhang et al., 2005). Esta ferramenta é chamada de razões diagnósticas moleculares (Tobiszewski e Namieśnik, 2012; Huang et al., 2014; Singh et al., 2015). Os pares de HPA utilizados para determinar as razões diagnósticas, geralmente são de mesma massa molar e de propriedades físico-químicas semelhantes (Tobiszewski e Namieśnik, 2012). Devido a essas semelhanças, esses pares de HPA estão sujeitos a processos atmosféricos e destino ambiental semelhantes (Tobiszewski e Namieśnik, 2012; Katsoyiannis e Breivik, 2014). Assim, razões diagnósticas moleculares têm sido amplamente utilizadas como uma ferramenta adicional para a identificação ou distinção de fontes de poluição que contribuem para a composição do PTS (Alves, 2008; Tobiszewski e Namieśnik, 2012; Huang et al., 2014; Singh et al., 2015).

O movimento das massas de ar pode transportar tanto os poluentes antropogênicos quanto os naturais entre latitude em semanas, entre os trópicos e os polos em meses, e entre os hemisférios norte e sul dentro do período de um ano (Jacob, 2000). Em estudos da distribuição atmosférica do MP, o cálculo de trajetórias é uma das ferramentas mais utilizadas (Pérez et al., 2015). Diferenças na volatilidade, taxa de fotodegradação e taxa de biodegradação dos HPA e seus derivados podem levar a diferentes perfis destes compostos em amostras de MP envelhecidas antes da amostragem (Kim et al., 2009). Os fenômenos de poluição do ar, dispersão de compostos mutagênicos e o envelhecimento das partículas formadas por estes compostos, podem ser melhor compreendidos quando estudos sobre as trajetórias de massas de ar são incluídos (Pérez et al., 2015; Alves et al., 2016).

Em geral é difícil comparar resultados de estudos sobre a mutagenicidade de amostras de MP conduzidos em diferentes lugares, devido as diferenças nos procedimentos de amostragem, nos protocolos de preparo das amostras e dos testes de mutagenicidade. Assim, como todas as amostras avaliadas neste estudo foram coletadas com diferenças mínimas (número de filtros e duração do período de amostragem) e processadas e testadas usando os mesmos protocolos, torna-se possível realizar comparações diretas entre os resultados obtidos para essas amostras. Assim, espera-se que cidades com grandes variações, tanto nas fontes de poluição como nas características atmosféricas, possam ter perfis distintos de composição química e de mutagenicidade da matéria orgânica de PTS. O uso de diferentes linhagens selecionadas pode identificar padrões de resposta de certos tipos de mutágenos presentes nos extratos de PTS.

2. OBJETIVOS

O primeiro objetivo geral desse trabalho foi investigar e comparar os perfis de mutagenicidade e de composição química dos extratos orgânicos de amostras compostas de PTS provenientes de Limeira (Brasil), Estocolmo (Suécia) e Quioto (Japão), cidades com diferentes condições atmosféricas e climáticas, utilizando um conjunto abrangente de linhagens de *S. typhimurium* e técnicas cromatográficas. Como segundo objetivo geral, a influência da movimentação das massas de ar foi avaliada tanto na composição química quanto na atividade mutagênica de amostras coletadas, processadas e testadas empregando os mesmos protocolos.

Apresenta como objetivos específicos:

- Avaliar o perfil de mutagenicidade dos extratos orgânicos de amostras de PTS de Limeira, Estocolmo e Quioto frente às linhagens do teste Salmonella/microsoma TA97a, YG5185, TA98, TA1538, YG1021, YG1024, YG1041, TA102, TA104, YG7108 e TA100, na ausência e presença de ativação metabólica (S9).
- Verificar se o uso das linhagens selecionadas é capaz de discriminar a contribuição de diferentes tipos de mutágenos responsáveis pelo efeito observado em amostras de PTS das cidades selecionadas.
- Quantificar HPA e alguns dos seus derivados alquilados nos mesmos extratos orgânicos.
- Aplicar razões diagnósticas de HPA para investigar fontes predominantes desses poluentes e também o envelhecimento das partículas.
- Estudar se a movimentação das massas de ar pode influenciar sobre a composição química e para a atividade mutagênica das amostras de PTS das três cidades estudadas e assim ser utilizada como ferramenta para este tipo de estudo.

3. METODOLOGIA

Inicialmente foram coletadas amostras compostas de PTS durante o inverno das três cidades estudadas. Para essa etapa foi adotado o mesmo procedimento de amostragem e foram utilizados filtros pertencentes ao mesmo lote visando minimizar possíveis interferências nas amostras obtidas. Contudo, devido a diferenças nos níveis de poluição e nas condições atmosféricas, o número de filtros amostrados e a duração da amostragem, foi diferenciado em cada cidade, visando garantir as quantidades necessárias de PTS para o desenvolvimento de todas as etapas do estudo proposto. Os filtros amostrados foram enviados para Estocolmo onde os extratos orgânicos foram obtidos pela técnica de extração acelerada por solventes (ASE) empregando o tolueno como solvente extrator. Esse procedimento foi desenvolvido e validado para análise de HPA e seus derivados, e foi conduzido pela equipe do Dr. Roger Westerholm do Departamento de Ciências Ambientais e Química Analítica da Universidade de Estocolmo.

Os extratos obtidos foram aliquotados e encaminhados para os laboratórios envolvidos nas diferentes análises desse projeto. As alíquotas destinadas ao teste Salmonella/microsoma, realizado no Laboratório de Ecotoxicologia e Genotoxicidade (LAEG - Ames) da Faculdade de Tecnologia da Unicamp – *campus* I, foram enviadas a Limeira e recebidas no mês de outubro de 2015. Logo após sua chegada iniciou-se os testes com as linhagens TA97a, YG5185, TA98, TA1538, YG1021, YG1024, YG1041, TA102, TA104, YG7108 e TA100 na ausência e presença de sistema de ativação metabólica (S9), em que foram avaliadas as sensibilidades das linhagens propostas frente aos extratos dessas amostras. Em paralelo à avaliação mutagênica dos extratos, foi realizada, com as linhagens YG5185, TA98 e TA1538, testes com o benzo[*a*]pireno (B[*a*]P) para calcular a contribuição deste HPA para a mutagenicidade dos extratos de Limeira e Estocolmo.

Outras alíquotas dos mesmos extratos foram destinadas a determinação química dos HPA e seus derivados alquilados. Em Estocolmo, parte dessas alíquotas foram enriquecidas com padrões internos visando a determinação dos compostos de interesse empregando a técnica de cromatografia líquida de alta eficiência-cromatografia gasosa/espectrometria de massa (HPLC-GC/MS) (Sadiktsis et al., 2014). Com base em razões diagnósticas moleculares dos HPA, foram avaliadas as principais fontes desses poluentes e o envelhecimento das partículas de PTS (Tobiszewski e Namieśnik, 2012; Huang et al., 2014; Singh et al., 2015). E para a melhor compreensão sobre o

envelhecimento das partículas de PTS, foram realizadas ainda o estudo da influência da movimentação das massas de ar em colaboração com o Dr. Arnaldo A. Cardoso, do Departamento de Química Analítica da Universidade Estadual Paulista (UNESP). Em paralelo a este estudo foi realizado também o desenvolvimento de um novo protocolo miniaturizado do teste Salmonella/microsoma em micro suspensão utilizando microplacas de 12 poços ao invés das placas convencionais, reduzindo a complexidade do teste em micro suspensão (Teste de Kado) e a quantidade de amostras e reagentes consumida, reduzindo assim os custos.

3.1 Coleta das amostras de PTS

Foram coletadas amostras compostas de PTS, durante o inverno de Limeira, no Brasil, Estocolmo, na Suécia, e Quioto no Japão. Cidades sobre influência de diferentes fontes de emissão de MP e outros poluentes atmosféricos, e com grande variação das temperaturas e dos períodos de radiação solar (Figura 1), fatores que tem influência direta nas reações atmosféricas. Em Limeira (Latitude: -22,5645, Longitude: -47,4004, 22° 33' 52" Sul, 47° 24' 1" Oeste), a amostra foi coletada ao nível da rua, dentro das instalações da Faculdade de Tecnologia (Unicamp). O local de amostragem é impactado pelo tráfego pesado, incluindo veículos leves e pesados, emissões industriais e queima de biomassa associada à produção de etanol, atividade econômica predominante desta região (Umbuzeiro et al., 2008a; Carvalho et al., 2014; Alves et al., 2016). Em Estocolmo (Latitude: 59,3328, Longitude: 18,0645, 59° 19' 58" Norte, 18° 3' 52" Leste), a amostra foi coletada ao nível do telhado na Universidade de Estocolmo. Este local também é impactado pelo tráfego pesado, com as emissões de automóveis e caminhões como a principal fonte de poluição (Lim et al., 2015). Em Quioto (Latitude: 35,0210700, Longitude: 135,7538500, 35° 1' 16" Norte, 135° 45' 14" Oeste), as amostras também foram coletadas ao nível do telhado em um edifício de três andares na Universidade Farmacêutica de Quioto. O local está localizado em uma área residencial da cidade, e as principais fontes de poluição que afetam o local de amostragem são o tráfego (emissão de carros). Quioto está localizada na parte central da ilha de Honshu, no Japão, principalmente impactada pela produção tecnológica, têxteis e atividades de turismo (He et al., 2004).

Figura 1. Representação gráfica de algumas das principais características climáticas e atmosféricas das cidades de Limeira, Estocolmo e Quioto.

Para isso foram empregados amostradores de partículas suspensas totais de grande volume (Hi-vol PTS) (Figura 2) e filtros de fibra de vidro (254×203 mm, Pallflex, Pall Corporation, Putnam, CT, USA) pré-condicionados e pré-pesados (Umbuzeiro et al., 2014). Sendo que as concentrações de PTS foram determinadas por gravimetria. As características da amostragem de cada cidade estão apresentadas na Tabela 1.

Figura 2. Amostrador de grande volume para partículas totais em suspensão (PTS) empregado na coleta da amostra de Limeira.

Fonte: Faculdade de Tecnologia da Unicamp – *campus* Limeira. Rua Paschoal Marmo, 1888 – Jardim Nova Itália – Limeira-SP.

Tabela 1. Características do processo de amostragem do material particulado total (MP) realizado durante o inverno nas cidades de Limeira, Estocolmo e Quioto.

Cidades	Datas de amostragens	Tempo de amostragem (h)	Concentração de PTS por filtro ($\mu\text{g}/\text{m}^3$)	Parte dos filtros utilizados na extração	MOE (%)
Limeira	25/06/2014	24	126,0	Metade dos filtros utilizados	9,0
	01/07/2014	24	119,2		
	19/07/2014	24	37,5		
	06/08/2014	24	101,1		
	19/08/2014	24	113,3		
	29/08/2014	24	126,3		
	05/09/2014	24	74,2		
Concentração média de PTS ($\mu\text{g}/\text{m}^3$)				99,0	
Estocolmo	02-08/10/2013	140	6,1	Filtros inteiros utilizados	15,0
	08-15/10/2013	168	3,5		
	23-29/10/2013	142	4,1		
	29/10-05/11/2013	169	3,0		
	05-12/11/2013	167	2,8		
	12-19/11/2013	168	3,5		
	19-26/11/2013	167	3,2		
	26/11-03/12/2013	169	4,3		
	03-10/12/2013	168	3,0		
	21-28/01/2014	169	4,1		
	18-25/02/2014	168	6,6		
	25/02-04/03/2014	168	20,5		
	04-11/03/2014	167	16,2		
	11-19/03/2014	192	6,3		
Concentração média de PTS ($\mu\text{g}/\text{m}^3$)				6,2	
Quioto	21-28/02/2014	168	33,3	Filtros inteiros utilizados	5,0
	21-28/02/2014	168	32,0		
	28-07/02/2014	168	22,4		
	28-07/02/2014	168	25,0		
Concentração média de PTS ($\mu\text{g}/\text{m}^3$)				28,0	

3.2 Extração orgânica das amostras de PTS e determinação do material orgânico extraído (MOE)

Todas as amostras de PTS coletadas foram submetidas à extração acelerada com solventes utilizando um sistema extrator ASE 200 (Dionex Co., Sunnyvale, CA) empregando como fase extratora o tolueno. Os filtros de microfibras de vidro foram pesados e inseridos em células de extração de 5 mL. Filtros não amostrados e pertencentes ao mesmo lote foram usados como branco. Padrões internos deuterados (fenantreno- d_{10} , pireno- d_{10} , benz[*a*]antraceno- d_{12} , benzo[*a*]pireno- d_{12} e benzo[*g,h,i*]perileno- d_{12}) foram adicionados aos filtros amostrados e brancos. Foram realizados 5 ciclos estáticos de 30 minutos cada, com o instrumento operando na sua capacidade máxima (200 °C e 3000 psi). Esse procedimento de extração foi desenvolvido e validado para análise de HPA em amostras de MP atmosférico por Bergvall e Westerholm (2008) e adaptado por Sadiktsis et al. (2014). As quantidades de PTS submetidos a extração variaram entre 5 e 10 mg. Alíquotas dos extratos foram evaporadas até completa secagem sob fluxo brando de nitrogênio a 55 °C e posteriormente foram ressuspensas em dimetilssulfóxido (DMSO) para o teste Salmonella/microsoma e nos solventes adequados para a análise cromatográfica. Esse método tem a capacidade de extrair de forma exaustiva os HPA e assim apresentar concentrações comparáveis ou superiores aqueles determinados pelo Instituto Nacional de Padrões e Tecnologia (NIST) para o material de referência - padrão urbano AIR SRM 1649a (Bergvall e Westerholm, 2008).

A metade dos sete filtros de PTS amostrados em Limeira, foram submetidos ao procedimento de extração, enquanto que para Estocolmo e Quioto, foram utilizados quatorze e quatro filtros inteiros, respectivamente. Os filtros brancos também foram submetidos ao processo de extração e o extrato obtido foi testado usando o teste Salmonella/microsoma com apenas quatro (TA98, TA1538, YG1041 e YG5185) das onze linhagens utilizadas neste estudo, porém os dados não serão apresentados.

Para a determinação do MOE foram transferidos para um frasco de vidro pré-pesado 3-4 mL dos extratos orgânicos. Estes frascos foram submetidos à evaporação até a total secagem. Após a total evaporação do solvente dos extratos, os frascos foram pesados e o MOE foi determinado pelas diferenças das massas iniciais e finais dos frascos de vidro.

3.3 Teste Salmonella/microsossoma com as diferentes linhagens

Os extratos de PTS de Limeira, Estocolmo e Quioto foram submetidos ao teste Salmonella/microsossoma em microssuspensão (Kado et al., 1983) em experimentos dose-resposta empregando as linhagens *Salmonella enterica* serovar Typhimurium (nesse trabalho será empregada a nomenclatura *S. typhimurium*) TA97a, YG5185, TA98, TA1538, YG1021, YG1024, YG1041, TA102, TA104, YG7108 e TA100 na ausência e presença do sistema de metabolização exógena (mistura S9), através do uso da fração S9, induzida com Aroclor 1254 (Moltox, USA), preparada de acordo com Mortelmans e Zeiger (2000). Este protocolo é recomendado quando se dispõe de pouca quantidade de amostra ou quando se pretende usar maior número de linhagens (Kado et al., 1983; Alves et al., 2016). Todos os testes foram conduzidos em duplicata e adicionados dos controles positivos e negativos (5 replicas). Os testes para avaliar a contribuição do B[a]P com as linhagens YG5185, TA98 e TA1538, foram realizados sob mesma condição. A Tabela 2 apresenta as principais características genéticas das linhagens diagnósticas empregadas nesse estudo.

Para a realização dos testes foram utilizadas culturas “overnight” concentradas 5 vezes, por centrifugação a 10.000 rpm, por 10 minutos, a 4 °C. Todos os extratos de PTS e os controles positivos foram diluídos e ressuspensos em DMSO (CAS 67-68-5, Sigma, pureza 99,5%). Foram realizados testes preliminares para determinar as doses finais testadas de cada amostra de acordo com cada linhagem empregada, e os testes de finais foram realizados, em geral, com doses máximas de 500 µg de EOM por placa.

Foram utilizadas placas de ágar mínimo com concentrações de glicose a 10% nos testes com todas as linhagens, exceto para a TA97a. Para esta linhagem, a concentração de glicose deve estar entre 0,4 e 2%, pois as colônias desta bactéria crescem muito lentamente causando o ressecamento das placas. Neste caso, foi utilizado para avaliar os extratos a concentração de 0,4% (Donovan, 1990). Esse crescimento lento é uma consequência do aumento da concentração da glicose à medida que a água contida no ágar é perdida e a sensibilidade a altas concentrações de glicose é característica da TA97a, que carrega a mutação *hisO242* (Donovan, 1990).

Tabela 2. Principais características das linhagens de *Salmonella enterica* serovar Typhimurium propostas para avaliação dos extratos das amostras de PTS de Limeira, Estocolmo e Quioto.

Linhagens	Descrição	Plasmídeo	Tipo de mutação	Referências	Razão pela inclusão da linhagem
TA97a	<i>hisD6610</i> , <i>hisO1242</i> Δ (<i>uvrB</i> , <i>bio</i>), <i>rfa</i> , <i>Ap^r</i> .	pKM101	Deslocamento do quadro de leitura	Donovan (1990)	Maior sensibilidade a mutágenos que induzem a ligação cruzada no DNA.
YG5185	<i>hisD3052</i> , Δ (<i>uvrB</i> , <i>bio</i>), <i>rfa</i> , <i>Ap^r</i> , <i>Cm^r</i> , <i>Km^r</i> , produção aumentada da DNA Pol. IV, e produção reduzida de NR e OAT.	pYG768	Deslocamento do quadro de leitura	Yamada et al., (2006)	Mais sensível aos HPA e sensibilidade reduzida aos nitroarenos (-S9) e as aminas aromáticas (+S9), respectivamente.
TA98	<i>hisD3052</i> , Δ (<i>uvrB</i> , <i>bio</i>), <i>rfa</i> , <i>Ap^r</i> .	pKM101	Deslocamento do quadro de leitura	Maron e Ames (1983)	TA98 e TA100 incluídas por detectar a maioria dos mutágenos ambientais (Zeiger et al., 1985).
TA1538	<i>hisD3052</i> , Δ (<i>uvrB</i> , <i>bio</i>), <i>rfa</i> .	Sem plasmídeo	Deslocamento do quadro de leitura	Maron e Ames (1983)	Incluída pela maior sensibilidade em relação a TA98 às amostras de MP coletados em Limeira e Estocolmo (Alves et al., 2016).
YG1021	<i>hisD3052</i> , Δ (<i>uvrB</i> , <i>bio</i>), <i>rfa</i> , <i>Ap^r</i> , produção aumentada de NR.	pKM101; pYG216	Deslocamento do quadro de leitura	Watanabe et al., (1989)	Altamente sensível aos nitroarenos (-S9) em amostras de MP.
YG1024	<i>hisD3052</i> , Δ (<i>uvrB</i> , <i>bio</i>), <i>rfa</i> , <i>Ap^r</i> , produção aumentada de OAT.	pKM101; pYG219	Deslocamento do quadro de leitura	Watanabe et al., (1990)	Altamente sensível às aminas aromáticas (+S9) em amostras de MP.
YG1041	<i>hisD3052</i> , Δ (<i>uvrB</i> , <i>bio</i>), <i>rfa</i> , <i>Ap^r</i> e <i>Cm^r</i> , produção aumentada de NR e OAT.	pKM101; pYG233	Deslocamento do quadro de leitura	Hagiwara et al., (1993)	Produção aumentada das NR e OAT, mais sensíveis a nitroarenos (-S9) e aminas aromáticas (+S9), respectivamente.
TA102	<i>hisG428</i> , <i>rfa</i> , <i>Ap^r</i> , <i>Tt^r</i> .	pKM101; pAQ1	Substituição dos pares de base	Maron e Ames (1983)	Mais sensível a agentes oxidantes e intercalantes, como Mitomicina C e Bleomicina.
TA104	<i>hisG428</i> , <i>rfa</i> , <i>Ap^r</i> .	pKM101	Substituição dos pares de base	Levin et al., (1982)	Mais sensível a mutágenos oxidativos e a alguns dos aldeídos.
YG7108	<i>hisG46</i> , Δ (<i>uvrB</i> , <i>bio</i>), <i>rfa</i> , <i>Cm^r</i> e <i>Cn^r</i> , Δ (<i>ogt_{ST}</i> and <i>ada_{ST}</i>).	Sem plasmídeo	Substituição dos pares de base	Yamada et al., (1995, 1997)	É altamente sensível aos agentes alquilantes.
TA100	<i>hisG46</i> , Δ (<i>uvrB</i> , <i>bio</i>), <i>rfa</i> , <i>Ap^r</i> .	pKM101	Substituição dos pares de base	Mortelmans e Zeiger (2000)	Idem TA98.

his – mutação responsável pela síntese da histidina; Δ *uvrB* – deleção do gene *uvrB*; Δ *bio* – deleção do gene da biotina; *rfa* – mutação que causa perda parcial da camada lipopolissacarídica; *Ap^r* – ampicilina resistente; NR – nitroredutases; OAT – *O*-acetiltransferases; *Km^r* – kanamicina resistente; *Cn^r* – cloranfenicol resistente.

Durante o procedimento, foi adicionado 2 µL de cada dose aos tubos de ensaio contendo 50 µL de cada linhagem e 50 µL da solução tampão fosfato diluído 1:13 v/v (em testes com – S9) ou da mistura S9 (testes + S9). Essa mistura foi incubada a 37 °C por 90 minutos, e depois desse período, foi adicionado 2 mL de ágar de superfície (acrescido de biotina/histidina), e fez-se o plaqueamento em 20 mL de ágar mínimo. As placas foram incubadas invertidas, durante 66 h a 37 °C. Após o tempo de incubação foi realizada contagem do número de colônias revertentes em todas as placas empregando contador automático de colônias, Interscience – Modelo Scan 1200. O controle negativo utilizado para todas as linhagens foi DMSO. Os controles positivos e suas doses estão descritos na Tabela 3.

Tabela 3. Controles positivos utilizados na ausência e presença de S9 para as linhagens empregadas.

Linhagens	Controles Positivos			
	- S9	Dose (µg/placa)	+ S9	Dose (µg/placa)
TA98	4NQO ^a	0,125	2AA ^c	0,625
TA100				
TA104				
YG5185				
YG1021	4NQO	1,0	2AA	1,0
YG1024				
TA1538	4NOP ^b	2,5	2AA	0,625
YG1041				
TA97a	Mitomicina C	0,5	2AA	2,5
TA102	9-Aminoacridina	50,0		
YG7108	Azida sódica	2,5	2AA	5,0

^a 4-nitroquinolina-1-óxido; ^b 4-nitro-o-diamino-fenilina; ^c 2-aminoantraceno

Os resultados da mutagenicidade foram analisados utilizando o software Salanal (Integrated Laboratory Systems, Research Triangle Park, NC). Foi realizada uma ANOVA que incluía um teste post hoc para comparar cada concentração testada com o respectivo controle negativo. Em seguida, uma regressão linear foi realizada utilizando o modelo de Bernstein (Bernstein et al. 1982). As potências mutagênicas foram expressas em função do MOE (revertentes/µg de MOE), e posteriormente, esses resultados foram transformados em função do volume de ar (revertentes/m³). A equação linear foi utilizada para calcular a Dose Mínima Efetiva (MED₂) que forneceu o dobro do número de revertentes do controle negativo expressa em µg de MOE /placa. Os resultados foram

expressos usando 1/MED₂. O extrato foi considerado positivo quando a ANOVA e a regressão linear forneceram respostas significativas ($p < 0,05$).

3.4 Análises químicas para determinação de HPA

Para determinação dos HPA, alíquotas dos extratos totais foram enriquecidas com soluções de padrão interno (PI) deuterados (fenantreno-*d10*, pireno-*d10*, benz[*a*]antraceno-*d12*, benzo[*a*]pireno-*d12* e benzo[*ghi*]perileno-*d12*) (Christensen et al., 2005; Bergvall e Westerholm, 2006). As alíquotas dos extratos brutos foram submetidas em etapa de *clen-up* utilizando cartuchos de extração em fase sólida (SPE) de sílica (100 mg, IST Isolute, Biotage, Cardiff, Reino Unido) com eluição de 3 mL de hexano (Christensen et al., 2005; Bergvall e Westerholm, 2006). Os extratos foram reduzidos até 100 µL sob um fluxo suave de nitrogênio e transferido para frascos tipo vial antes da injeção. A análise para a determinação de 27 congêneres de HPA e 15 alquil-HPA foi realizada por cromatografia líquida de alta eficiência-cromatografia gasosa/espectrometria de massa (HPLC-GC/MS) (Christensen et al., 2005; Bergvall e Westerholm, 2006; Sadiktsis et al., 2014). O sistema acoplado de HPLC-MS-GC utilizado para análise dos compostos foi constituído por um sistema de fornecimento de solvente, Varian 9012 (Varian Inc., Palo Alto, CA, EUA), com um micro-amostrador CMA/200 (CMA Microdialysis AB, Suécia) acoplado a um cromatógrafo a gás Agilent 6890 N (Agilent Technologies) com o sistema Agilent 5973 N MSD (Agilent Technologies) (Sadiktsis et al., 2014). As concentrações de HPA foram expressas em função do volume de ar (pg/m³).

3.5 Avaliação das fontes de emissão dos HPA e o envelhecimento do PTS

A fonte de emissão dos HPA e o envelhecimento do PTS foram avaliados utilizando as razões diagnósticas moleculares de HPA quantificados nas amostras de PTS coletadas nas três cidades. As fontes de emissão dos HPA foram investigadas a partir da proporção do Antraceno (Ant)/Ant + Fenantreno (Ant + Fe) para distinguir fontes petrogênicas ($< 0,1$) e pirogênicas ($> 0,1$). Para distinguir emissões não relacionadas ao tráfego ($< 0,6$) e as emissões relacionadas ao tráfego ($> 0,6$), a proporção B[*a*]P/Benzo[*ghi*]perileno (B[*ghi*]P) foi utilizada (Tobiszewski e Namieśnik, 2012; Singh et al., 2015). Para avaliar o envelhecimento das amostras de PTS foi empregada a relação

entre B[a]P e Benzo[e]pireno (B[e]P) (Panther et al., 1999; Okuda et al., 2002; Vasconcellos et al., 2010 Keyte et al., 2013). Proporções próximas de 1 indicam partículas frescas e grande impacto das fontes de emissão locais, enquanto proporções menores que 1, indicam partículas envelhecidas (Oliveira et al., 2011; Vasconcellos et al., 2011; Wu et al., 2012).

Afim de complementar a investigação sobre o envelhecimento do PTS das cidades de Limeira e Estocolmo, foram determinadas as trajetórias das massas de ar durante o respectivo período de amostragem utilizando o modelo HYSPLIT 4 da Administração Oceânica e Atmosférica dos Estados Unidos (NOAA) (Hybrid Single-Particle Lagrangian Integrated Trajectory 4) (Draxler e Rolph, 2015). As trajetórias das 48 h anteriores ao final de cada amostragem foram calculadas a 100 e 500 metros acima do solo sobre o local de amostragem.

3.6 Teste miniaturizado em Microplaca de Ágar (MPA)

Em paralelo ao estudo realizado para avaliar os perfis de mutagenicidade e composição química das amostras de PTS das cidades de Limeira, Estocolmo e Quioto, houve participação no desenvolvimento de um novo protocolo miniaturizado do procedimento em micro suspensão do teste *Salmonella*/microsoma. A estratégia utilizada para este novo protocolo foi avaliar diferentes compostos mutagênicos em paralelo empregando os protocolos em micro suspensão, desenvolvido por Kado et al. (1983), e a sua miniaturização, chamado Microplaca de Ágar (MPA). Foram empregados os mesmos mutágenos de referência, linhagens (TA1538, TA98 e YG1041) com diferentes frequências de reversão espontânea, concentração da mistura S9 e condições de pré-incubação em ambos os testes. A única diferença entre os testes, foi o tipo de placa de ágar utilizada; Placas de Petri de (90 x 15 mm) para micro suspensão e microplacas de 12 poços (21,4x17,5 mm) para o MPA.

Os dados de ambos os protocolos de ensaio foram analisados utilizando o software Salanal (Integrated Laboratory Systems, Research Triangle Park, NC). Uma ANOVA foi realizada para comparar as concentrações testadas com o respectivo controle negativo, e em seguida, uma regressão linear aplicando o modelo de Bernstein (Bernstein et al. 1982). O composto químico foi considerado positivo quando a ANOVA e a regressão linear forneceram respostas significativas ($p < 0,05$).

4. RESULTADOS

Os resultados obtidos neste trabalho foram organizados em três capítulos, apresentados a seguir, e que correspondem aos três primeiros artigos que resultaram deste trabalho. O Capítulo I - *“A worldwide comparison of the mutagenicity of atmospheric particulate matter using a comprehensive set of Salmonella strains”* apresenta a avaliação dos perfis de mutagenicidade obtidos e discute a sensibilidade das diferentes linhagens frente aos extratos orgânicos obtidos das amostras de PTS das cidades de Limeira, Estocolmo e Quioto.

Na sequência, o Capítulo II - *“Polycyclic aromatic hydrocarbons (PAHs) in the atmosphere of cities from opposite hemispheres: determination of particle sources, aging and its contribution to the mutagenic activity”* compreende a investigação da composição dos HPA presentes nas amostras de PTS coletadas em Limeira e Estocolmo visando determinar as fontes das partículas e o seu envelhecimento. Foi avaliada também a contribuição de B[a]P para a atividade mutagênica desses extratos.

E por fim, o Capítulo III - *“Miniaturization of the microsuspension salmonella/microsome assay in agar microplates – MPA”* apresenta o desenvolvimento de uma versão miniaturizada do teste Salmonella/microsoma em microssuspensão usando microplacas, onde podem ser empregadas em linhagens com frequências de mutações espontâneas baixas, médias e altas.

Capítulo I

A WORLDWIDE COMPARISON OF THE MUTAGENICITY OF ATMOSPHERIC PARTICULATE MATTER USING A COMPREHENSIVE SET OF SALMONELLA STRAINS

Running title: Worldwide TPS mutagenicity comparison

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Abstract

Atmospheric particulate matter (PM) organic fraction collected in urban centers are frequently mutagenic for the Salmonella/microsome assay. This mutagenic activity is related to both primary and secondary pollutants. Meteorological conditions such as temperature and period of solar radiation have great influence in the degradation of contaminants and the formation of secondary pollutants. The objective of this study was to compare the mutagenicity of atmospheric total suspended particulates (TPS) from Limeira (Brazil), Stockholm (Sweden), and Kyoto (Japan), cities with marked different meteorological conditions using a comprehensive set of Salmonella strains applying the same methodological conditions. The TPS concentration from Limeira was $99.0 \mu\text{g}/\text{m}^3$, followed by Kyoto ($28.0 \mu\text{g}/\text{m}^3$), and Stockholm ($6.2 \mu\text{g}/\text{m}^3$), and the percentages of extracted organic material (EOM) obtained were approximately 9, 15 and 5%, respectively. The samples from the three cities were mutagenic for all strains, except for TA102. Based on the selectivity of these strains, we could verify that the mutagens present in the samples are able to induce DNA intercalation, DNA adducts, DNA cross-linking, DNA alkylation and oxidizing DNA damage. Unexpected, the mutagenicity profiles obtained for Limeira, Stockholm and Kyoto were similar, despite the differences in average temperatures and solar radiation periods recorded during the sampling. The mutagenic potencies expressed as a function of EOM mass (rev./ μg EOM), were quite similar for the all samples. By air volume (rev./ m^3) the mutagenic potencies are proportional to the TPS concentrations from the three cities.

Key words: Total suspended particles; accelerated solvent extraction; Salmonella/microsome microsuspension assay; mutagenic profiles.

INTRODUCTION

There are several studies on the mutagenicity of atmospheric particulate matter (PM) around world using the Salmonella/microsome assay [Claxton et al., 2004; Claxton and Woodall Jr., 2007; Claxton et al., 2010], and the mutagenic potencies are positively related to the PM levels, and consequently correlated with the decline of air quality [IARC, 2016]. Several different protocols of the Salmonella/microsome assay have been used, such as plate incorporation [Sato et al., 1995; Claxton et al., 2001; Villalobos-Pietrini et al., 2007], pre-incubation [Azuma et al., 1997; Matsumoto et al., 1998; Kawanaka et al., 2004] and in microsuspension [Azuma et al., 1997; De Martinis et al., 1999; Claxton et al., 2001; Ducatti and Vargas, 2003; Umbuzeiro et al., 2008a,b]. The microsuspension protocol is 5 to 10 times more sensitive than the plate incorporation protocol and is widely used when there is limited amount of sample available or when the aim of the study is to use several strains and/or test conditions [Kado et al., 1983; DeMarini et al., 1989; Alves et al., 2016; IARC, 2016].

In general studies on PM mutagenicity employ a specific set of Salmonella strains. The most commonly pair of strains used to evaluate mutagenicity of environmental samples includes TA98 and TA100, which detects respectively frameshift and base-pair substitutions mutations [Reifferscheid and Buchinger, 2010; Umbuzeiro et al., 2016]. These strains were found to detect the majority of environmental mutagens [Zeiger et al., 1985; Felton and Wu, 2003]. Claxton et al. [2004] considered that the use of this pair of strains is the most appropriate for urban environments monitoring because it produces more reproducible and comparable results. Considering the important contribution of nitro-compounds and aromatic amines to the PM samples mutagenicity [Umbuzeiro et al., 2014; Alves et al., 2016; IARC, 2016], strains with different levels of nitroreductase (NR) and *O*-

acetyltransferase (OAT) enzymes, both involved in the activation of these chemical classes, have often been included in studies of PM mutagenicity [Ducatti and Vargas, 2003; Umbuzeiro et al., 2008a ; Franco et al., 2010; Pereira et al., 2013; Rainho et al., 2013]. Among the most frequently deficient NR and OAT strains used are TA98NR and TA98/1,8-DNP₆ respectively, and among the strains that overproduce these enzymes are YG1021 (NR), YG1024 (OAT) and YG1041 (both NR and OAT) [IARC, 2016]. However, strains with higher sensitivity to other classes of mutagens, which induce specific types of mutations, may be useful for a comprehensive study of PM samples [Umbuzeiro et al., 2011].

Among the other strains used for the characterization of the mutagenicity of PM samples we can cite TA1538 which detects the same type of mutagens that the TA98 but is supposed to be less sensitive [Maron and Ames, 1983], however this strain showed higher sensitivity for organic fraction obtained from PM samples [Alves et al., 2016]. Although TA102, which detects cross-linking agents (transitions and transversions), was used previously to evaluate organic extracts from PM [De Flora et al., 1989; Crebelli et al., 1991; Landkocz et al., 2017], this strain detected mutagenic activity only in the presence of S9 [Crebelli et al., 1991]. Crebelli et al. [1991] using TA97a, which also detects mutagens that induce cross-linking, observed mutagenic activity both in absence and presence of S9. De Flora et al. [1989] used TA104, which detects mutagens that cause oxidative damage and cross-linking damage, but this strain was not able to detect mutagenic activity in PM organic extracts. Umbuzeiro et al. [2014] used the YG5161, which detects polycyclic aromatic hydrocarbons (PAHs) with higher sensitivity, however due to the NR and OAT activities present in this strain, it was not possible to discriminate the mutagenic activity of mutagens such as benzo[*a*]pyrene (B[*a*]P), present in organic extracts of PM samples, from nitroarenes

induced mutagenicity. To avoid that the mutagenic activity of PAHs was masked by nitro-compounds and aromatic amines, Yamada et al. [2006] developed the YG5185 which has characteristics and sensitivity to PAHs similar to YG5161, since both strains have the plasmid pYG768, responsible for the higher expression of DNA polymerase IV. However, this strain is deficient in the production of NR and OAT enzymes, which makes YG5185 much less sensitive to nitroarenes and aromatic amines. Thus, an increased mutagenic response for this strain in the presence of S9, in relation to the others may distinguish the contribution of PAHs [Yamada et al., 2006]. So far, we have not found articles in the literature that used this strain to evaluation of PM sample. According to Yamada et al. [2006], YG5185 is the best choice when the objective of the study is to monitor the mutagenic activity of PAHs. The YG7108, which detects alkylating agents, was used by Alves [2011] but none of the organic extracts from PM samples evaluated presented mutagenic activity.

PM samples are chemically very complex, containing hundreds of organic compounds, many of them in concentrations extremely low. PM mutagenicity is related to both primary and secondary pollutants, and varies according to pollution sources, the transport of air mass and meteorological conditions, such as temperature, sunlight incidence and relative air humidity [Alves et al., 2012; Keyte et al., 2013; Jariyasopit et al., 2014; Shrivastava et al., 2017]. In this context cities with differences in intensity and type of pollution sources, and differences in atmospheric and meteorological conditions may present different PM mutagenicity profiles. However, it is important to note that the mutagenicity of organic extracts obtained from PM can vary according to protocols used for sample extraction, especially due to the type of solvents and method of extraction, the Salmonella/microsome assay protocols applied (i.e. plate incorporation x microsupension), the selected strains and the metabolic activation system used [Claxton

et al., 2004; Marvin and Hewitt, 2007; Mutlu et al., 2015]. Thus, for comparison PM mutagenicity harmonized sampling and analytical protocols are crucial [Sillanpää et al., 2006].

The objective of this study was to compare the mutagenicity of atmospheric total suspended particulates (TPS) from three cities with marked different meteorological conditions using a comprehensive set of *Salmonella* strains applying the same methodological conditions.

MATERIAL AND METHODS

Selection of sites and sampling

Knowing that atmospheric conditions can have a great influence on the composition of TPS organic fraction [Alves et al., 2012; Jariyasopit et al., 2014], we selected three cities, Limeira in Brazil, Stockholm in Sweden, and Kyoto in Japan that present differences in average temperatures and periods of solar radiation, especially during the Winter. This may lead to each of these cities presenting distinct profiles of mutagenic activity, strongly influenced by climatic and meteorological conditions that exert great influence on atmospheric chemical reactions [Li et al., 2015]. TPS composed samples were collected during the winters of the selected cities. The average temperatures and the average solar radiation periods at this season in Limeira, Stockholm and Kyoto are presented in Figure 1.

Fig. 1. Average temperatures and periods of solar radiation during the Winter in Limeira, Stockholm and Kyoto.

In Limeira, the samples were collected at the street level, on the School of Technology (University of Campinas - Unicamp). This sampling site is impacted by heavy traffic, including light and heavy vehicles, industrial emissions and biomass burning associated to ethanol production, the predominant economic activity of this region [Umbuzeiro et al., 2008a; Carvalho et al., 2014; Alves et al., 2016]. In Stockholm, the samples were collected at the rooftop level on the University of Stockholm. This site is also impacted by heavy traffic, with car and truck emissions as the main pollution source [Lim et al., 2015]. In Kyoto, the samples were collected also at rooftop of a three-storied building in Kyoto Pharmaceutical University. This site is located in a residential area of Kyoto, and main sources of pollution that direct impact this site is traffic (cars emission). Moreover, Kyoto is located in the central part of the island of Honshu, mainly impacted by technological production, textiles and also tourism activities [He et al., 2004].

In the TPS samplings, high-volume (Hi-vol TPS) samplers, and glass fiber filters (254 × 203 mm, Pallflex, Pall Corporation, Putnam, CT, USA) pre-conditioned and pre-weighed were used [Umbuzeiro et al., 2014]. Considering the importance of using harmonized methodology to provide reliable data for comparative studies on

atmospheric samples [Sillanpää et al., 2006], the TPS samples from all cities were collected using same filters batch and submitted to the same procedure of organic extraction and mutagenicity tests.

TPS samples organic extraction and determination of extracted organic material (EOM)

Pooled TPS samples were submitted to accelerated solvent extraction (ASE) employing toluene as extraction phase [Bergvall and Westerholm, 2008] in the Unit of Analytical and Toxicological Chemistry, Department of Environmental Sciences and Analytical Chemistry, Stockholm University, Sweden. The sampled filters were weighed and inserted into 5 mL extraction cells. Five static cycles of 30 minutes each were performed, with the instrument operating at its maximum capacity (200 °C and 3000 psi). According Bergvall and Westerholm [2008], toluene showed improved extraction for benzo[*a*]pyrene-d12 (internal standard) in ultrasonically assisted extraction (USE) compared to acetone and dichloromethane (DCM). Based on this result, the ASE method was further developed and validated to obtain higher extraction recovery. The current ASE method was developed to extract as much PAHs as possible from atmospheric PM, though the PAHs concentrations of standard reference materials were not in good agreement with those from National Institute of Standards and Technology (NIST) using DCM due to higher extraction efficiency [Bergvall and Westerholm 2008]. Aliquots of the extracts were evaporated to dryness under a gentle nitrogen flow at 55 °C and then resuspended in dimethylsulfoxide (DMSO) for the Salmonella/microsome assay.

Seven half filters were submitted to the extraction procedure for Limeira, fourteen and four whole filters were used for Stockholm and Kyoto, respectively. The

detailed description of date of the sampling, portion of the filters used for extraction, concentrations of TPS per filter and EOM percentage are presented in Table II. The blank filters were also submitted to the extraction process and the obtained extract was tested using the Salmonella/microsome assay with only four (TA98, TA1538, YG1041, and YG5185) of the eleven strains used in this study (data not shown).

For determination of the EOM, 3-4 mL of each organic extracts were transferred to a pre-weighed glass vial. These vials were evaporated to dryness, and then weighed again. The EOM was determined by differences between the initial and final masses of the vials and expressed in μg of EOM.

Salmonella/microsome microsuspension assay

The TPS extracts were submitted to the Salmonella/microsome microsuspension assay [Kado et al., 1983] in dose-response experiments employing the diagnostic strains of *Salmonella enterica* serovar Typhimurium TA97a, YG5185, TA98, TA1538, YG1021, YG1024, YG1041, TA102, TA104, YG7108 and TA100 (Table I) in the absence and presence of the exogenous metabolization system (S9 mixture), prepared according to Mortelmans and Zeiger [2000]. All tests were performed in duplicate and added to positive and negative controls (5 replicates).

TABLE I. Main characteristics of *Salmonella enterica* serovar Typhimurium strains used in this work.

Strains	Description	Plasmids	Reversion event	Reference	Reason for inclusion of strain
TA97a	<i>hisD6610</i> , <i>hisO1242</i> $\Delta(uvrB, bio)$, <i>rfa</i> , <i>Ap^r</i> .	pKM101	Frameshift	Donovan (1990)	Higher sensitive to mutagens that induce cross-linking in DNA.
YG5185	<i>hisD3052</i> , $\Delta(uvrB, bio)$, <i>rfa</i> , <i>Ap^r</i> , <i>Cm^r</i> , <i>Km^r</i> , DNA Pol. IV overproducing strain, NR and OAT reduced production.	pYG768	Frameshift	Yamada et al., (2006)	It's higher sensitive to PAHs and reduce sensitive to nitro (-S9) and aromatic amines (+ S9) respectively.
TA98	<i>hisD3052</i> , $\Delta(uvrB, bio)$, <i>rfa</i> , <i>Ap^r</i> .	pKM101	Frameshift	Maron and Ames (1983)	TA98 and TA100 strains were found to detect most environmental mutagens (Zeiger et al., 1985).
TA1538	<i>hisD3052</i> , $\Delta(uvrB, bio)$, <i>rfa</i> .	No plasmids	Frameshift	Maron and Ames (1983)	This strain was included because it was shown to be more sensitive than TA98 to mutagenicity from PM samples collected in Limeira and Stockholm (Alves et al., 2016).
YG1021	<i>hisD3052</i> , $\Delta(uvrB, bio)$, <i>rfa</i> , <i>Ap^r</i> , NR overproducing.	pKM101; pYG216	Frameshift	Watanabe et al., (1989)	It is highly sensitive to nitroarenes (- S9) in the PM samples.
YG1024	<i>hisD3052</i> , $\Delta(uvrB, bio)$, <i>rfa</i> , <i>Ap^r</i> , OAT overproducing.	pKM101; pYG219	Frameshift	Watanabe et al., (1990)	It is highly sensitive to aromatic amines (+ S9) in the PM samples.
YG1041	<i>hisD3052</i> , $\Delta(uvrB, bio)$, <i>rfa</i> , <i>Ap^r</i> and <i>Cm^r</i> , NR and OAT overproducing.	pKM101; pYG233	Frameshift	Hagiwara et al., (1993)	The YG1041 overproducing NR and OAT enzymes, more sensitive to nitroarenes (- S9) and aromatic amines (+ S9), respectively.
TA102	<i>hisG428</i> , <i>rfa</i> , <i>Ap^r</i> , <i>Tt^r</i> .	pKM101; pAQ1	Base-pair substitutions	Maron and Ames (1983)	Is higher sensitive to oxidizing and intercalating agents as Mitomycin C and Bleomycin.
TA104	<i>hisG428</i> , <i>rfa</i> , <i>Ap^r</i> .	pKM101	Base-pair substitutions	Levin et al., (1982)	Is higher sensitive to oxidative mutagens and some of the
YG7108	<i>hisG46</i> , $\Delta(uvrB, bio)$, <i>rfa</i> , <i>Cm^r</i> e <i>Cn^r</i> , $\Delta(ogt_{ST}$ and $ada_{ST})$.	No plasmids	Base-pair substitutions	Yamada et al., (1995, 1997)	It is highly sensitive to alkylating agents.
TA100	<i>hisG46</i> , $\Delta(uvrB, bio)$, <i>rfa</i> , <i>Ap^r</i> .	pKM101	Base-pair substitutions	Mortelmans and Zeiger (2000)	See TA98.

Note: *his* – mutation in genes in the histidine operon; $\Delta uvrB$ – deletion of *uvrB* gene; Δbio – deletion of biotin gene; *rfa* – mutation that cause partial loss of the lipopolysaccharide cell wall barrier; *Ap^r* – resistant to ampicillin; NR – nitroreductase; OAT – *O*-acetyltransferase; DNA Pol IV – DNA Polymerase IV; *Km^r* – resistant to Kanamycin; *Cm^r* – resistant to chloramphenicol; *Tt^r* – resistant to tetracycline; Δogt_{ST} e Δada_{ST} – deletion of genes responsible for the production of *O*₆-methylguanine DNA methyltransferase enzymes.

Overnight cultures concentrated 5-fold were used. All TPS extracts and positive controls were diluted and/or resuspended in DMSO (CAS 67-68-5, Sigma, purity 99.5%). Preliminary tests were performed to determine the final tested doses of each sample according to each strain employed, and then the final mutagenicity tests were performed using, in general, as maximum dose 500 µg of EOM per plate. Minimum agar plates containing 10% glucose were used in the assays with all strains, except TA97a. For TA97a, a glucose concentration should be reduced from 0.4 to 2% because revertants grow very slowly on plates which have dried out [Donovan, 1990], in this case, the 0.4% concentration was used. Slow growth is a consequence of increased glucose concentration as water is lost from the agar, and sensitivity to high concentrations of glucose is characteristic of strains such as TA97a which carry the *hisOI242* mutation [Donovan, 1990].

The test plates were incubated for 66 h at 37 °C and the number of revertant colonies was counted using automatic colony counter (Interscience - Model Scan 1200). The negative control used for all strains was DMSO, and the number of revertants in the negative control is the spontaneous reversion rate obtained for each strain under the test conditions. Positive controls and their doses are presented in the supplementary material (Table SI).

The mutagenicity results were analyzed using the Salanal computer software (Integrated Laboratory Systems, Research Triangle Park, NC). An ANOVA including a post hoc test was performed to compare each tested concentration with the respective negative control. Then a linear regression was performed using the Bernstein model [Bernstein et al. 1982]. The mutagenic potencies were expressed as a function of EOM (revertants/µg of EOM), and later, these results were transformed in function of air volume (revertants/m³). The linear equation was used to calculate the Minimal Effective

Dose (MED₂) that provided twice the number of revertants of the negative control and expressed in µg of EOM/plate. The lower MED₂ indicate higher mutagenicity, and in the Figure 3A and 3B the results were expressed using 1/MED₂. The extract was considered positive when both the ANOVA and linear regression provided significant responses ($p < 0.05$).

RESULTS AND DISCUSSION

During the sampling periods Limeira presented the highest TPS concentration (99.0 µg/m³) followed by Kyoto (28.0 µg/m³) and Stockholm (6.2 µg/m³). Umbuzeiro et al. [2014] observed a similar difference in TPS concentrations for samples previously collected in Limeira (95.8 µg/m³) and Stockholm (7.2 µg/m³). Although the TPS concentration (µg/m³) in Limeira was approximately 16-fold higher than in Stockholm and 3.5-fold higher than in Kyoto, the percentages of EOM obtained were approximately 9, 15 and 5%, respectively (Table II).

TABLE II: Information on the TPS samplings including the dates, amount of particles, concentrations of TPS per sampled filter, and EOM percentages.

Cities	Date of the sampling	Time of sampling (h)	TPS concentration per filter ($\mu\text{g}/\text{m}^3$)	Part of the filters used for extraction	EOM (%)
Limeira	2014/06/25	24	126.0	Used half filters	9.0
	2014/07/01	24	119.2		
	2014/07/19	24	37.5		
	2014/08/06	24	101.1		
	2014/08/19	24	113.3		
	2014/08/29	24	126.3		
	2014/09/05	24	74.2		
Average TPS concentration ($\mu\text{g}/\text{m}^3$)				99	
Stockholm	2013/10/02-08	140	6.1	Used whole filters	15.0
	2013/10/08-15	168	3.5		
	2013/10/23-29	142	4.1		
	2013/10/29-11/05	169	3.0		
	2013/11/05-12	167	2.8		
	2013/11/12-19	168	3.5		
	2013/11/19-26	167	3.2		
	2013/11/26-12/03	169	4.3		
	2013/12/03-10	168	3.0		
	2014/01/21-28	169	4.1		
	2014/02/18-25	168	6.6		
	2014/02/25-03/04	168	20.5		
	2014/03/04-11	167	16.2		
	2014/03/11-19	192	6.3		
Average TPS concentration ($\mu\text{g}/\text{m}^3$)				6.2	
Kyoto	2014/02/21-28	168	33.3	Used whole filters	5.0
	2014/02/21-28	168	32.0		
	2014/02/28-07	168	22.4		
	2014/02/28-07	168	25.0		
Average TPS concentration ($\mu\text{g}/\text{m}^3$)				28.0	

In atmospheric PM, the percentage of the organic fraction is highly variable and generally represents 10-90% by mass. This huge variation in the proportion represented by organic chemicals is related to several factors, among them the PM origin and the particle size distribution [Alves, 2008; Ringuet et al., 2012]. Many emissions from combustion processes contain particles with a smaller aerodynamic diameter, but these particles tend to present high levels of mutagens in the EOM. On the other hand, particles with high aerodynamic diameter usually contain relatively smaller amounts of EOM [Sato et al., 1995; Claxton et al., 2004]. Thus, although we have not determined the particle size distribution in the TPS collected samples, based on their EOM contents, the Stockholm sample appears to be predominantly composed by fine particles while the Kyoto and Limeira samples predominantly by coarse particles. In addition, atmospheric reactions may produce mutagens (e.g., nitroarenes) that may condense into small particles or combine with other molecules to produce liquid aerosols, which may then melt to form ultrafine particles and then larger particles [Claxton et al., 2004]. Because of their high specific surface area, the smaller particles tend to adsorb various organic compounds, such as PAHs (mainly those with higher molecular weight and lower volatility), nitro-PAHs and oxy-PAHs [Rainho et al., 2013]. These chemical classes are often related to the mutagenic activity of PM samples detected by the Salmonella/microsome assay [Claxton et al., 2004; Umbuzeiro et al., 2014; Alves et al., 2016; IARC, 2016].

The TPS samples extracts from the three cities presented mutagenic activity for all strains used, both in absence and presence of S9, except for the TA102 that was not able to detect mutagenic activity in all extracts (maximum tested dose - 50 µg of EOM/plate), and for YG7108 in the presence of S9, only for the Limeira sample extract (Figures 3A and 3B; Table III; and supplementary material Tables SII to SXII). Our

results indicate the presence of direct and indirect mutagens able to cause both frameshift and base-pair substitution mutations. In general, the highest mutagenic potencies were obtained in the absence of S9 (- S9) for all strains (Table III). Umbuzeiro et al. [2014] also observed the highest mutagenic potencies - S9 for TPS samples previously collected in Limeira and Stockholm. Furthermore, based on the selectivity of the used strains, it is possible to verify that the mutagenic compounds present in the organic fraction of TPS samples can induce DNA intercalation, DNA adducts, DNA cross-linking, DNA alkylation and oxidizing DNA damage (see Table I).

In general, the mutagenicity profiles of the three cities are quite similar (Figure 2), but some small differences can be observed. Based on the response of YG7108 - S9 and in the absence of mutagenicity in the presence of S9 (+ S9), it is possible to observe the lower contribution of alkylating agents to the mutagenic activity of the TPS organic extract of the sample from Limeira in relation to the other two cities. Considering selectivity of the strains used, we can also observe a higher contribution of alkylating agents and compounds that cause oxidative DNA damage for the mutagenic activity of the TPS sample from Kyoto. Despite of the recognized influence of meteorological conditions, especially the average temperatures and the solar radiation periods, under atmospheric reactions [Claxton et al., 2004; Bukart et al., 2013; Li et al., 2015], these factors appear not to have led to important differences in the mutagenicity profiles observed for Limeira, Stockholm, and Kyoto (Figure 2).

Fig. 2. Mutagenicity profile, expressed by revertants/ μg of EOM of Limeira, Stockholm and Kyoto TPS samples extracts with strains (-/+ S9) that detected mutagenic activity.

Due to the different spontaneous reversion rates of the used strains, the ratio $1/\text{MED}_2$, was employed to evaluate the sensitivities of the strains (Figures 3A and 3B). In general, the sensitivity of the strains that detect frameshift mutations - S9 decreases in the following order $\text{YG1041} > \text{YG1024} > \text{YG1021} \approx \text{TA1538} > \text{TA98} > \text{TA97a} \approx \text{YG5185}$ (Figure 3A). However, some exceptions were observed. YG1024 showed a slightly higher sensitivity than the YG1041 for the Limeira sample extract. For the samples collected in Stockholm, the sensitivity of TA1538 was similar to YG1041 and higher than the YG1021 and YG1024. The TA97a was slightly more sensitive to the extract from the sample collected in Kyoto than for the samples from both other cities. In the presence of S9 the sensitivity of the same strains decreased in the following order $\text{YG1041} > \text{TA1538} > \text{YG1024} \approx \text{YG1021} > \text{TA98} \approx \text{TA97a} \approx \text{YG5185}$ (Figure 3A). In this test condition (+ S9) the YG1024 response for the sample extract from Stockholm was relatively higher than for sample extracts from the other two cities.

*- not mutagenic at the maximum dose tested (500 µg of EOM/plate).

Fig. 3. Comparison of sensitivity according to the 1/MED₂ of the strains used in this study, separated to the reversion event without and with metabolic activation (A- frameshift strains; B- base-pair substitution strains).

In general, strains that detect frameshift mutations were more sensitive than those detecting base-pair substitution mutations (Figures 3A and 3B). The highest sensitivity of the strains that overproducing NR and/or OAT enzymes, YG1041, YG1024 and YG1021, in relation to the parental strain TA98 (with background levels of both enzymes), indicates a major contribution of nitroarenes and aromatic amines [Watanabe et al., 1989; Watanabe et al., 1990; Hagiwara et al., 1993] to the mutagenicity observed in all samples extracts. Nitroarenes and aromatic amines are recognized as two important classes of environmental mutagens ubiquitously present in atmospheric PM samples [Claxton et al., 2004; Nohmi, 2007; Umbuzeiro et al., 2011; IARC, 2016]. The NR enzyme has been shown to be a limiting factor for the metabolic activation of nitroarenes in the *Salmonella* strains [Watanabe et al., 1989], while OAT is a key enzyme for the activation of both nitroarenes and aromatic amines [Watanabe et al., 1990]. Thus, the YG1021 has high sensitive to nitroarenes but not to aromatic amines, on the other hand the YG1024 are sensitive to both nitroarenes and mainly to aromatic amines [Watanabe et al., 1989]. Hagiwara et al. [1993] state that YG1041 (overproducing both NR and OAT) is extremely sensitive to the mutagenicity of nitroarenes which require both NR and OAT activation, and that the activity of the OAT enzyme in YG1041 is 3 to 5-fold higher than in YG1024.

Aromatic amines usually require exogenous metabolic activation (+ S9) to be mutagenic in the *Salmonella*/microsome assay [Nohmi, 2007; Umbuzeiro et al., 2011]. The International Agency for Research on Cancer [IARC, 2016] observed the same behavior of our samples in relation to the YG1021 and YG1024, where in the absence of S9, the mutagenicity usually was higher for YG1024, highlighting the important contribution of mutagens that depend on OAT activation. Increases in the mutagenic potencies, when using strains that overproducing OAT in the presence of S9, are related

to the presence of aromatic amines [IARC, 2016]. Thus, considering the specificity of YG1024 + S9, the Stockholm TPS extract seems to present a higher contribution of mutagens that require OAT activation, including aromatic amines, in comparison to the sample extracts from both other cities.

Despite the TA98 was generally considered to be more sensitive than TA1538, due to the presence of the plasmid pKM101 [Herbold, 1982; Maron and Ames, 1983], the TA1538 was more sensitive to mutagenicity of the TPS samples extracts from all cities. TA1538 was also more sensitive than its derivative TA98 to TPS sample extracts previously collected in Limeira [Alves et al., 2016], and to B[a]P [Skopek et al., 1978; Umbuzeiro et al., 2014]. We included the TA98 because Claxton et al. [2004] consider, based on the data retrieved from the articles considered in their extensive review of the literature, that is the initial strain of choice for the evaluation of PM organic extracts due to its low spontaneous reversion background and its sensitivity to PAHs in the presence of S9. Nevertheless, both TA98 and TA1538 are sensitive to nitroaromatics and aromatic amines because of their endogenous production of the NR and OAT enzymes [Claxton et al., 2004]. Alves et al. [2016], suggested that the increase in the mutagenicity observed using TA1538 in relation to TA98 may be related to mutagens that cause gaps in one strand of DNA, and therefore depend on the recombinational repair system present in TA1538.

The TA97a is particularly sensitive to mutagens able to induce cross-linking damage in DNA [Donovan, 1990; Mortelmans and Zeiger, 2000]. Thus, the relatively higher sensitivity of this strain, especially in the absence of S9, for the extract from Kyoto sample, in relation to the other two cities, suggests a major contribution of cross-linking mutagens to this sample mutagenic activity.

YG5185 presented the lower sensitivity among the frameshift strains. This strain is highly sensitive to PAHs and has disrupted activity of NR and OAT enzymes [Yamada et al., 2006; Nohmi, 2007]. Considering that this strain presented similar sensitivity for the samples from all three cities, in the presence of S9, we can suggest that the contribution of mutagens that act as PAHs to the observed mutagenicity was similar, and lower than that of nitroarenes and aromatic amines, corroborating the results obtained with the NR and/or OAT overproducing strains.

For base-pair substitution strains, both in the absence and in the presence of S9, in the general the sensitivity of the strains decreased in the following order TA100 slightly > YG7108 > TA104 (Figures 3B). The exceptions that deserve mention are the lack of mutagenicity of the extract from the Limeira sample for YG7108 + S9, and the relatively higher sensitivity of this strain to the extract of the sample from Kyoto, mainly - S9. YG7108 is highly sensitive to alkylating agents [Yamada et al., 1997]. Alves [2011] evaluated the mutagenicity of TPS samples previously collected in Limeira and did not detect mutagenic activity using YG7108, indicating that the alkylating agents, if present, are not in sufficient amounts to be detected under the test conditions. According to Alves [2011] alkylating agents do not necessarily require S9 to induce mutagenicity. Considering the response obtained with YG7108, Kyoto was the city with higher contribution of alkylating agents to the TPS mutagenicity, mainly in the absence of S9. The TA100 - S9 presented elevated sensitivity for the Kyoto sample extract, in relation to the other cities, but in the presence of S9 this sensitivity was greatly reduced. Thus, it seems that reactive base-pair substitution mutagens that causing DNA adducts [Benigni and Bossa, 2008] are present in higher amounts in TPS from Kyoto.

The lack of mutagenicity for TA102 of all TPS samples, suggests that compounds able to cause oxidative DNA damage, such as hydroperoxides [Ames, 1984;

Mortelmans and Zeiger, 2000], if present, are not in sufficient amounts to induce mutagenic activity. Because TA104 is more sensitive to aldehydes, not detected by TA102 [Levin et al., 1982], this type of mutagens seems to contribute to the mutagenic activity detected in extracts from Limeira, Stockholm, and Kyoto.

Mutagenicity potencies were expressed both as function of the EOM mass (rev./ μg of EOM) and air volume (rev./ m^3) (Table III). When the mutagenic potencies were expressed as a function of EOM mass, considering strain by strain, all sample extracts presented quite similar potencies (Figure 2; Table III). According to IARC [2016], based on 250 articles containing results of the Salmonella/microsome assay from studies performed in different parts of the world, the mutagenic potency of PM extracts, expressed per mass of EOM, in general show less than 10-fold variation, despite of the test protocol and the strain used, locations and site conditions (i.e. source contributions, weather, and season). By air volume (rev./ m^3) the mutagenic potencies are directly related to the concentration of TPS recorded in the cities during the sampling period (Limeira > Kyoto > Stockholm), and consequently the amount of EOM present in each cubic meter. IARC [2016] also observed that, regardless of the particle size selected, the mutagenic potency expressed per air volume (rev./ m^3), ranged over 5 orders of magnitude.

The lower variation observed among the mutagenic potencies of the Limeira, Stockholm and Kyoto TPS samples, mainly when expressed as a function of air volume (rev./ m^3), in relation to the variation described by the IARC [2016], seems to be associated with the chosen particle size (TPS) and the organic extraction protocol used. The extraction procedure and the extraction phase (pure solvent or solvents mixture) used can significantly affect the mutagenicity potencies of particulate matter samples [Mutlu et al., 2015; Palacio et al., 2016]. According Marvin and Hewitt [2007]

approximately 55-95% of the inter-laboratory variability in the PM samples mutagenic potencies is due to differences in the methodological approach rather than real differences in the samples mutagenicity. Typically, it is difficult to compare results on the mutagenicity of PM from studies performed in different countries, due to differences in sampling procedures, sample preparation protocols, and mutagenicity tests conditions [Claxton et al., 2004; Marvin and Hewitt, 2007]. Furthermore, it is important to note that the relative contribution of the different classes of mutagenic compounds present in PM is directly related to the organic extraction procedures used and to the choice of Salmonella/microsome assay strains [Claxton et al., 2004].

We compare the results from Limeira, Stockholm and Kyoto with the data obtained by Umbuzeiro et al. [2014] for TPS samples previously collected in Limeira and Stockholm, that contain similar particles concentration to our samples, and used the same organic extraction procedure (ASE with toluene). In general, the mutagenic potencies (rev./m³) obtained by Umbuzeiro et al. [2014] using TA1538, TA98, and YG1041 (-/+ S9) were similar to the potencies obtained for Limeira and Stockholm samples. The Kyoto sample presented slightly lower mutagenic potencies for all the three strains in relation to the data presented by Umbuzeiro et al. [2014]. In general, the samples evaluated by Umbuzeiro et al. [2014] and the samples from Limeira, Stockholm, and Kyoto presented higher mutagenic potencies (rev./μg of EOM and rev./m³) than the TPS samples submitted to ultrasonication-based organic extraction with DCM and also were evaluated using the Salmonella/microsome assay in microsuspension with the same strains [Azuma et al., 1997; Alves, 2011; Lemos et al., 2011; Umbuzeiro et al., 2014; Alves et al., 2016].

TABLE III. Limeira, Stockholm and Kyoto TPS sample mutagenic potencies, expressed in EOM (revertants/ μg of EOM) and in air volume (revertants/ m^3), for the strains TA97a, YG5185, TA98, TA1538, YG1021, YG1024, YG1041, TA102, TA104, YG7108 and TA100 (-/+ S9).

Strains		Mutagenic potency (rev./ μg of EOM)			Mutagenic potency (rev./ m^3)		
		Limeira	Stockholm	Kyoto	Limeira	Stockholm	Kyoto
TA97a	-S9	15 (14-16)*	22 (18-26)	38 (33-42)	130 (120-150)	20 (17-24)	53 (47-59)
	+S9	8 (7-9)	11 (10-13)	15 (13-16)	71 (63-80)	10 (9-12)	21 (19-22)
YG5185	-S9	3 (2-4)	4 (3-5)	3 (2-4)	27 (23-35)	4 (3-4)	4 (3-6)
	+S9	1 (1-2)	2 (1-2)	2 (1-2)	9 (8-15)	2 (1-2)	3 (2-3)
TA98	-S9	18 (14-22)	26 (24-28)	9 (8-10)	160 (120-190)	24 (23-26)	13 (11-14)
	+S9	2 (10-2)	2 (1-2)	2 (2-3)	18 (15-22)	2 (1-2)	3 (3-4)
TA1538	-S9	17 (15-18)	30 (26-35)	17 (16-18)	150 (130-160)	28 (24-33)	24 (22-25)
	+S9	10 (9-12)	11 (9-12)	10 (9-11)	89 (77-100)	10 (8.6-11.5)	14 (13-16)
YG1021	-S9	28 (22-33)	31 (26-36)	18 (14-22)	250 (190-300)	29 (24-34)	25 (19-31)
	+S9	11 (7-14)	13 (10-16)	7 (6-9)	98 (66-130)	12 (10-15)	10 (8-12)
YG1024	-S9	100 (86-121)	50 (43-59)	37 (28-45)	920 (760-1100)	47 (40-55)	52 (39-53)
	+S9	11 (8-14)	22 (15-28)	9 (8-10)	98 (69-130)	20 (14-26)	13 (11-14)
YG1041	-S9	270 (250-290)	200 (180-220)	300 (280-320)	2400 (2200-2600)	190 (170-210)	420 (390-450)
	+S9	45 (42-48)	60 (57-63)	90 (86-96)	400 (370-420)	56 (53-59)	127 (120-130)
TA102	-S9	not detected	not detected	not detected	not detected	not detected	not detected
	+S9	not detected	not detected	not detected	not detected	not detected	not detected
TA104	-S9	3 (2-4)	4 (3-5)	3 (2-4)	27 (20-31)	4 (2-5)	4 (3-6)
	+S9	0.6 (0.5-0.7)	1 (0.8-2)	1 (0.9-1.4)	5 (4-6)	1 (0.7-2)	1 (1-2)
YG7108	-S9	0.6 (0.5-0.7)	3 (2-3)	4 (3-5)	5 (4-6)	3 (2-3)	6 (5-7)
	+S9	not detected	2 (1-2)	2 (1-2)	not detected	2 (1-2)	3 (2-3)
TA100	-S9	11 (10-13)	12 (11-13)	19 (17-20)	98 (89-110)	11 (10-13)	27 (24-29)
	+S9	8 (6-8)	14 (11-17)	7 (6-8)	71 (59-75)	13 (11-16)	10 (9-11)

*95% confidence interval.

At least in part, the explanation for this observation must be related to the performance of the extraction procedure used previously by Umbuzeiro et al. [2014] and in this study, along with the solvent used, toluene. Bergvall and Westerholm [2008] validated this procedure in order to extract much PAHs as possible, and demonstrated that the ASE extraction protocol is more efficient for extracting this chemical class from urban atmospheric PM. This ASE extraction procedure has been shown to exhaustively extract PAHs and to produce comparable or higher PAHs concentrations than those assigned by the National Institute for Standards and Technology (NIST) for the Urban

Air standard reference material SRM 1649a [Bergvall and Westerholm, 2008]. The concentration obtained for B[a]P, for example, was more than 15-fold higher than the NIST certified value, which is of considerable importance since B[a]P is traditionally used as an indicator of carcinogenic PAHs. Based on the comparison of our data with the data retrieved from the literature, we can suggest that direct comparisons are impracticable. Thus, to make possible direct comparisons of mutagenic potencies of TPS samples from different studies, it's important that the sampling procedure, the extraction protocol, and the mutagenicity assessment method were similar [Claxton et al., 2001, Vargas 2003, Sillanpää et al., 2006; Umbuzeiro et al., 2016; Alves et al., 2016].

CONCLUSION

The use of a broad set of the Salmonella/microsome assay strains allowed a comprehensive evaluation of the mutagenicity of the TPS samples from the studied cities. All TPS samples organic fractions were able to cause all types of DNA lesions detected by the used strains. The strains that detect frameshift mutations, mainly the NR and OAT overproducing strains, were more sensitive than those detecting other types of damage.

The mutagenicity profiles and potencies expressed by organic matter (rev./μg EOM) obtained for Limeira, Stockholm and Kyoto were similar, despite the differences in temperature and solar radiation from three cities. Therefore, mutagenic potencies by air volume (rev./m³) were proportional to the TPS concentrations from the three cities during the sampling period (Limeira > Kyoto > Stockholm). The, it was possible to observe e that the same extraction procedure seems to be very important to allow proper mutagenicity comparisons among different locations. Considering that in general the

mutagenic potencies obtained for the three cities evaluated in relation to the data found in the literature, show that for comparisons between different studies to be made properly it is necessary that the same methodologies have been employed, especially the organic extraction procedure.

AUTHORS' CONTRIBUTION

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Development of experiments - Bianca S. Maselli, Macelle C. G. Giron, Hwanmi Lim, Christoffer Bergvall, Roger Westerholm

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Supplementary Material

**A WORLDWIDE COMPARISON OF THE MUTAGENICITY OF
ATMOSPHERIC PARTICULATE MATTER USING A COMPREHENSIVE
SET OF SALMONELLA STRAINS**

Running title: Worldwide TPS mutagenicity comparison

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TABLE SI. Positive controls used in the absence and presence of S9 for each Salmonella strain used.

Strains	Positive Controls			
	- S9	Dose (µg/plate)	+ S9	Dose (µg/plate)
TA98	4NQO ^a	0.125	2AA ^c	0.625
TA100				
TA104				
YG5185				
YG1021	4NQO	1.0	2AA	1.0
YG1024				
TA1538	4NOP ^b	2.5	2AA	0.625
YG1041				
TA97a	Mitomycin C	0.5	2AA	2.5
TA102	9-Aminoacridine	50.0		
YG7108	Sodium azide	2.5	2AA	5.0

Note: ^a4-nitroquinoline-1-oxide; ^b4-nitro-o-phenylenediamine; ^c2-aminoanthracene.

TABLE SII. Dose response experiments, ANOVA, potencies in revertants/μg of EOM and MED₂ in μg of EOM/plate for Limeira, Stockholm and Kyoto for the strain TA97a without and with (S9).

TA97a																																				
Number of revertants/plate and mean																																				
Doses (µg of EOM/plate)	Limeira											Stockholm										Kyoto														
	-S9						+S9					-S9						+S9				-S9					+S9									
	Plates					Plates					Plates					Plates					Plates					Plates										
	1	2	3	4	5	Mean	1	2	3	4	5	Mean	1	2	3	4	5	Mean	1	2	3	4	5	Mean	1	2	3	4	5	Mean	1	2	3	4	5	Mean
DMSO ^b	120	132	122	138	147	132	138	161	142	151	166	151	189	199	189	173	192	189	202	188	220	204	211	204	164	159	182	167	181	167	192	184	185	203	203	192
0.3	138	156				147	163	158				161	200	197				199	182	235					209	201	192				197	183	195		189	
1	165	138				152	148	168				158	236	213				225	226	197					212	210	241				226	216	201		209	
3	209	186				198	166	180				173	242	259				251	242	233					238	303	274				289	246	223		235	
10	301	329				315	254	212				233	453	448				451	323	317					320	502	595				549	367	362		365	
30	563	558				561	411	391				401	980	636				808	499	582					541	1073	1279				1176	582	650		616	
50	1037	961				999	594	499				547	1130	1255				1193	627	655					641	1375	1453				1414	725	829		777	
Positive control ^c	580	599	550			580	704					704	3825	5683				4754	2668	3607					3138	1124	1543				1334	2168	2100		2134	
ANOVA ^a	p<0.001						p<0.001					p<0.001						p<0.001				p<0.001					p<0.001									
Potency	15						8					22						11				38					15									
(rev./ µg of EOM)	13.8 - 16.4						7.1 - 9.0					17.7 - 25.6						9.9 - 12.5				33.3 - 42.2					13.4 - 15.7									
IC ^{***}	9.1						18.9					8.7						18.7				4.6					12.9									
MED ₂ ^c	8.3 - 9.9						16.4 - 21.4					7.4 - 10.8						16.4 - 20.7				4.2 - 5.3					12.4 - 14.5									
(µg of EOM/plate)																																				
IC																																				

Note: ^aExtracted organic material; ^bDimethylsulfoxide; ^cMinimal effect dose; *Positive controls: see details in material and methods, ** Analysis of Variance, p<0.05 indicates a positive response, *** Confidence interval.

TABLE SIII. Dose response experiments, ANOVA, potencies in revertants/ μg of EOM and MED_2 in μg of EOM/plate for Limeira, Stockholm and Kyoto for the strain YG5185 without and with (S9).

YG5185																																				
Number of revertants/plate and mean																																				
Doses (µg of EOM ² /plate)	Limeira												Stockholm												Kyoto											
	-S9						+S9						-S9						+S9						-S9						+S9					
	Plates					Mean	Plates					Mean	Plates					Mean	Plates					Mean	Plates					Mean	Plates					Mean
	1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5	
DMSO ^b	17	20	26	33	25	25	24	30	19	22	28	24	17	20	26	33	25	25	24	30	19	22	28	24	16	31	26	28	20	26	27	13	24	21	24	24
0.1	22	21				22							26	24				25							27	29				28						
0.3	16	29				23	40					40	26	29				28	27	31				29	34	29			32	23	18			21		
1	35	31				33	25	24				25	23	35				29	27	28				28	16	40			28	36	27			32		
3	34	37				36	29	32				31	33	34				34	30	41				36	52			52	34	29			32			
10	73	71				72	38	35				37	66	76				71	56	43				50	77	80			79	44	49			47		
30	106	116				111	62	74				68	139	140				85	85					85	114	102			108	76	77			77		
50							67	68				68						96	93					95						90	109			100		
Positive control ¹	589	560	521			560	1120	1403				1262	589	560	521			560	1120	1403				1262	332	357	383			357	926	975			951	
ANOVA ^{**}	p<0.001						p<0.001						p<0.001						p<0.001						p<0.002						p<0.001					
Potency	3						1						4						2						3						2					
(rev./ µg of EOM)	2.6 - 3.9						1.1 - 1.7						3.3 - 4.6						1.4 - 2.0						2.3 - 3.9						1.4 - 2.0					
IC ^{***}	8.0						22.0						6.1						13.4						8.9						11.6					
MED ₂ ^c	6.2 - 9.3						14.8 - 22.9						5.3 - 7.5						13.1 - 19.1						6.9 - 11.6						11.6 - 16.6					
(µg of EOM/plate)																																				
IC																																				

Note: ^aExtracted organic material; ^bDimethylsulfoxide; ^cMinimal effect dose; ^{*}Positive controls: see details in material and methods, ^{**} Analysis of Variance, $p<0.05$ indicates a positive response, ^{***} Confidence interval.

TABLE SIV. Dose response experiments, ANOVA, potencies in revertants/ μg of EOM and MED₂ in μg of EOM/plate for Limeira, Stockholm and Kyoto for the strain TA98 without and with (S9).

TA98																																				
Number of revertants/plate and mean																																				
Doses (μg of EOM ^a /plate)	Limeira												Stockholm												Kyoto											
	-S9						+S9						-S9						+S9						-S9						+S9					
	Plates					Mean	Plates					Mean	Plates					Mean	Plates					Mean	Plates					Mean						
	1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5							
DMSO ^b	50	50	49	41	52	50	45	48	50	44	42	45	50	50	49	41	52	50	45	48	50	44	42	45	21	21	26	23	23	23	24	22	24	25	24	24
0.1	57	72				65							53	42				48							20											
0.3	52	64				58	41	39				40	63	50				57	52	41				47	21	23				22	21	24			23	
1	74	79				77	51	67				59	72	73				73	52	51				52		35			35	17	20			19		
3	99	102				101	69					69	85	122				104	55	50				53		43			43	29	36			33		
10	368	373				371	77	70				74	293	302				298	60	60				60	120				120	47	53			50		
30	812	849				831	115	105				110	860	946				903	112	101				107	140	98			119	94	86			90		
50							115	130				123							146	140				143						108	111			110		
Positive control ^c	761	790	766			766	551	388	356			388	761	790	766			766	551	388	356			388	171	180	172			172	391	236			314	
ANOVA ^{**}	p<0.001						p<0.001						p<0.001						p<0.001						p<0.001						p<0.001					
Potency	18						2						26						2						9						2					
(rev./ μg of EOM)	13.6 - 21.5						1.7 - 2.5						24.2 - 28.1						1.7 - 2.1						7.9 - 10.3						1.9 - 2.5					
IC ^{***}	2.9						24.7						1.8						23.0						2.4						11.9					
MED ₂ ^c	2.4 - 3.8						19.8 - 29.1						1.6 - 1.9						21.9 - 27.1						2.1 - 2.7						9.5 - 12.5					
(μg of EOM/plate)																																				
IC																																				

Note: ^aExtracted organic material; ^bDimethylsulfoxide; ^cMinimal effect dose; ^{*}Positive controls: see details in material and methods, ^{**}Analysis of Variance, p<0.05 indicates a positive response, ^{***}Confidence interval.

TABLE SV. Dose response experiments, ANOVA, potencies in revertants/ μg of EOM and MED_2 in μg of EOM/plate for Limeira, Stockholm and Kyoto for the strain TA1538 without and with S9.

TA1538																																				
Number of revertants/plate and mean																																				
Doses (μg of EOM ^a /plate)	Limeira												Stockholm												Kyoto											
	-S9						+S9						-S9						+S9						-S9						+S9					
	Plates					Mean	Plates					Mean	Plates					Mean	Plates					Mean	Plates					Mean	Plates					Mean
	1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5	
DMSO ^b	9	27	16	20	17	17	16	15	13	12	18	15	9	27	16	20	17	17	16	15	13	12	18	15	20	22	18	19	15	19	22	15	17	17	15	17
0.1	23	22				23	19	19				19	19	27				23	14	24				19	22	20				21	14	17			16	
0.3	31	36				34	23	22				23	30	19				25	28	32				30	27	29				28	26	28			27	
1	35	34				35	49	47				48	54	58				56	62	57				60	70	57				64	49	58			54	
3	96	99				98	72	71				72	288	298				293	115	110				113	179	222				201	113	124			119	
10	196	231				214	217	199				208	551	536				544	236	254				245	555	520				538	345	282			314	
30	417	504				461	238	210				224							313	318				316							283	338			311	
50																																				
Positive control ^c	387	340	391			387	855	963				909	387	340	391			387	855	963				909	206	243	208			208	884	780			832	
ANOVA ^a	p<0.001						p<0.001						p<0.001						p<0.001						p<0.001						p<0.001					
Potency (rev./ μg of EOM)	17						10						30						11						17						10					
IC ^{***}	14.7 - 18.0						8.6 - 12.1						25.8 - 35.0						9.3 - 12.4						15.8 - 18.1						9.3 - 11.1					
MED ₂ ^c (μg of EOM/plate)	1.2						1.5						0.6						1.4						1.0						1.6					
IC	1.1 - .14						1.2 - 1.7						0.5 - 0.7						1.3 - 1.7						1.0 - 1.1						1.5 - 1.7					

Note: ^aExtracted organic material; ^bDimethylsulfoxide; ^cMinimal effect dose; ^{*}Positive controls: see details in material and methods, ^{**} Analysis of Variance, $p<0.05$ indicates a positive response, ^{***} Confidence interval.

TABLE SVI. Dose response experiments, ANOVA, potencies in revertants/μg of EOM and MED₂ in μg of EOM/plate for Limeira, Stockholm and Kyoto for the strain YG1021 without and with (S9).

YG1021																																				
Doses (µg of EOM ^b /plate)	Number of revertants/plate and mean																																			
	Limeira											Stockholm										Kyoto														
	-S9						+S9					-S9						+S9				-S9						+S9								
	Plates					Mean	Plates					Mean	Plates					Mean	Plates					Mean	Plates					Mean						
	1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5	
DMSO ^b	39	32	24	37	28	32	40	32	43	31	31	32	39	32	24	37	28	32	40	32	43	31	31	32	39	32	24	37	28	32	40	32	43	31	31	32
0.1	51	41				46	29	37				33	38	41				40	49	35				42	33	27				30	32	38			35	
0.3	42	36				39	37	37				37	48	48				48	34	51				43	36	32				34	37	38			38	
1	73	65				69	43	51				47	71	66				69	37	55				46	54	50				52	39	44			42	
3	116	107				112	55	47				51	118	123				121	52	63				58	87	82				85	58	45			52	
10	324	269				297	208	121				165	365	286				326	205	180				193	280	273				277	103	118			111	
30	368	427				398	513	674				594	688	608				648	491	457				474	673	642				658	582	215			399	
Positive control ^c	1421	1638	1537			1537	1211	1720				1466	1421	1638	1537			1537	1211	1720				1466	1421	1638	1537			1537	1211	1720			1466	
ANOVA ^{***}	p<0.001						p<0.002						p<0.001						p<0.001						p<0.001											
Potency	28						11						31						13						18						7					
(rev./ µg of EOM)	21.8 - 33.4						7.4 - 14.0						25.4 - 36.3						10.5 - 15.8						13.5 - 22.4						5.5 - 8.8					
IC ^{***}	1.2						3.1						1.1						2.6						1.7						4.9					
MED ₂ [‡]	1.0 - 1.6						2.4 - 4.5						0.9 - 1.3						2.1 - 3.2						1.4 - 2.3						3.9 - 6.3					
IC																																				

Note: ^aExtracted organic material; ^bDimethylsulfoxide; ^cMinimal effect dose; ^{*}Positive controls: see details in material and methods, ^{**} Analysis of Variance, p<0.05 indicates a positive response, ^{***} Confidence interval.

TABLE SVII. Dose response experiments, ANOVA, potencies in revertants/ μ g of EOM and MED₂ in μ g of EOM/plate for Limeira, Stockholm and Kyoto for the strain YG1024 without and with (S9).

YGI024																																				
Number of revertants/plate and mean																																				
Doses (μ g of EOM ^a /plate)	Limeira												Stockholm												Kyoto											
	-S9						+S9						-S9						+S9						-S9						+S9					
	Plates					Mean	Plates					Mean	Plates					Mean	Plates					Mean	Plates					Mean						
	1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5							
DMSO ^b	43	37	36	47	40	32	42	38	41	35	38	43	37	36	47	40	32	42	38	41	35	38	43	37	36	47	40	32	42	38	41	35	38			
0.1	47				47	39	28				34	65	30			48	36	52				44	41	45			43	39				39				
0.3	73	85			79	47	48				48	62	44			53	37	51				44	51	58			55	41	49			45				
1	133	147			140	49	43				46	98	96			97	39	68				54	93	66			80	48	46			47				
3	277	257			267	58	76				67	203	178			191	167	131				149	57	59			141	57	59			58				
10	739	760			750	193	110				152	485	456			471	261				526	571	480			526	141	120			131					
30	1011	929			970	622	333				478	856	744			800	454	399				427	1268	989			1129	397	330			364				
Positive control ^c	1829	1631	1195		1631	1359	1506	1129			1359	1829	1631	1195		1631	1359	1506	1129			1359	1829	1631	1195		1631	1359	1506	1129		1359				
ANOVA ^{**}	p<0.001						p<0.002						p<0.001						p<0.004						p<0.001						p<0.001					
Potency	103						11						51						22						37						9					
(rev./ μ g of EOM)	85.5 - 120.6						7.7 - 14.1						42.8 - 59.3						15.1 - 27.8						28.0 - 45.1						7.6 - 10.1					
IC ^{***}	0.4						3.6						0.8						1.7						1.1						4.1					
MED ₂ ^c	0.3 - 0.5						2.7 - 5.0						0.7 - 0.9						1.3 - 2.4						0.9 - 1.5						3.7 - 4.9					
(μ g of EOM/plate)																																				
IC																																				

Note: ^aExtracted organic material; ^bDimethylsulfoxide; ^cMinimal effect dose; *Positive controls: see details in material and methods, ** Analysis of Variance, p<0.05 indicates a positive response, *** Confidence interval.

TABLE SVIII. Dose response experiments, ANOVA, potencies in revertants/ μg of EOM and MED_2 in μg of EOM/plate for Limeira, Stockholm and Kyoto for the strain YG1041 without and with (S9).

YG1041																																				
Number of revertants/plate and mean																																				
Doses (µg of EOM ^a /plate)	Limeira											Stockholm											Kyoto													
	-S9					Mean	+S9					Mean	-S9					Mean	+S9					Mean	-S9					Mean	+S9					Mean
	Plates					Plates					Plates					Plates					Plates					Plates					Plates					
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	
DMSO ^b	115	119	124	105	114	115	92	103	95	90	111	95	115	119	124	105	114	115	92	103	95	90	111	95	115	119	124	105	114	115	92	103	95	90	111	95
0.03							116	103				110	132	120				126	90	86				88	121	110				116	116	102			109	
0.1	127	129				128	103	110				107	124	198				161	95	94				95	210	213				212	104	96			100	
0.3	203	228				216	114	117				116	169	213				191	115	122				119	234	259				247	113	101			107	
1	375	369				372	120	132				126	253	360				307	139	118				129	369	375				372	120	127			124	
3	806	827				817	230	235				233	842	864				853	229	300				265	995	1154				1075	362	323			343	
10	1977	1877				1927	539	580				560	2181	1887				2034	690	722				706	2291	2422				2357	1126	994			1060	
20	2642	2664				2653	1180	1119				1150	3032	2856				2944	1323	1322				1323	2828	2849				2839	2006	1797			1902	
Positive control ^c	1748	1703	1777			1748	1482	1380	1328			1380	1748	1703	1777			1748	1482	1380	1328			1380	1748	1703	1777			1748	1482	1380	1328			1380
ANOVA ^{**}				p<0.001																																
Potency																																				
(rev./ µg of EOM)						270						45						203						60						299			91			
IC ^{***}						247.2 - 285.9						41.6 - 47.5						185.2 - 221.5						57.0 - 63.4						278.2 - 318.7			85.6 - 96.1			
MED ₂ ^c																																				
(µg of EOM/plate)						0.4						2.2						0.6						1.5						0.4			1.0			
IC						0.4 - 0.5						2.1 - 2.4						0.5 - 0.7						1.4 - 1.6						0.3 - 0.4			1.0 - 1.1			

Note: ^aExtracted organic material; ^bDimethylsulfoxide; ^cMinimal effect dose; ^{*}Positive controls: see details in material and methods, ^{**}Analysis of Variance, $p < 0.05$ indicates a positive response, ^{***}Confidence interval.

TABLE SIX. Dose response experiments, ANOVA, potencies in revertants/ μg of EOM and MED₂ in μg of EOM/plate for Limeira, Stockholm and Kyoto for the strain TA102 without and with (S9).

TA102																																			
Number of revertants/plate and mean																																			
Doses (μg of EOM ^a /plate)	Limeira												Stockholm										Kyoto												
	-S9						+S9						-S9					+S9					-S9					+S9							
	Plates					Mean	Plates					Mean	Plates					Mean	Plates					Mean	Plates					Mean					
	1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5		1	2	3	4	5
DMSO ^b	192	174	181	177	183	181	151	183	179	162	151	162	171	167	190	182	177	159	182	185	165	173	173	172	191	200	181	172	181	205	211	206	197	197	205
0.3	192	179				186	201					201	174	179			177	160				160	178	151				165	197	195			196		
1	152	147				150	135					135	168	185			177	158	187			173	169	178				174	214	193			204		
3	149	174				162	206	173				190	180	198			189	169				169	186					186	199				199		
10	219	201				210	195	194				195	199	195			197	182	191			187	157	161				159	191	181			186		
30	187	188				188	187	165				176	208	174			191	191	181			186	190	197				194	185	193			189		
50	182	196				189	160	173				167	207				207	188	214			201	185	192				189	192				192		
Positive control ^c	1086					1086	1169					1169	1522	1488			1505	1252	1246			1249	1344	1273				1309	1211				1211		
ANOVA ^{**}	P=0.666						P=0.187						P=0.314					P=0.130					P=0.248					P=0.165							
Potency	0						0						0					0					0					0							
(rev./ μg of EOM)	-						-						-					-					-					-							
IC ^{***}	-						-						-					-					-					-							
MED ₂ ^c	>50						>50						>50					>50					>50					>50							
(μg of EOM/plate)	-						-						-					-					-					-							
IC	-						-						-					-					-					-							

Note: ^aExtracted organic material; ^bDimethylsulfoxide; ^cMinimal effect dose; *Positive controls: see details in material and methods, ** Analysis of Variance, p<0.05 indicates a positive response, *** Confidence interval.

TABLE SX. Dose response experiments, ANOVA, potencies in revertants/ μg of EOM and MED₂ in μg of EOM/plate for Limeira, Stockholm and Kyoto for the strainTA104 without and with (S9).

TA104																														
Number of revertants/plate and mean																														
Doses (μg of EOM ^b /plate)	Limeira										Stockholm										Kyoto									
	-S9					+S9					-S9					+S9					-S9					+S9				
	Plates					Plates					Plates					Plates					Plates					Plates				
	1	2	3	4	5	Mean	1	2	3	4	5	Mean	1	2	3	4	5	Mean	1	2	3	4	5	Mean	1	2	3	4	5	Mean
DMSO ^a	259	218	272		259	261	252	323		261	259	218	272		259	261	252	323		259	218	272		259	261	252	323		261	
10	294	270			282	262	296			279	245	260			253	241	217			229	282			282	225	266			246	
30	312	344			328	275				275	332	385			359	273	291			282	264	309			287	317			317	
50	331	385			358	271	302			287	405	499			452	321	328			325	447	403			425	378	411		395	
100	525	582			554	415	425			420	597				597	408	505			457	467	575			521	399	423		411	
300	659	690			675	441				441	777	812			795	637				637	660	705			683	608	630		619	
500	892				892	606	567			587	808	858			833	667				667	812				812	846	811		829	
Positive control ^c	1690	1343			1517	1216	1313			1265	1690	1343			1517	1216	1313			1265	1690	1343			1517	1216	1313		1265	
ANOVA ^{**}	p<0.002					p<0.002					p<0.002					p<0.002					p<0.001					p<0.001				
Potency (rev./ μg of EOM)	3					0.6					4					1					3					1				
IC ^{***}	2.2 - 3.5					0.5 - 0.7					2.6 - 4.8					0.8 - 1.8					2.1 - 4.0					0.9 - 1.4				
MED ₂ ^c	81.4					471.6					62.8					279.7					84.6					293.9				
(μg of EOM/plate)																														
IC	69.7 - 111					404.2 - 565.9					52.3 - 96.6					155.4 - 349.6					63.5 - 120.9					209.9 - 326.6				

Note: ^aExtracted organic material; ^bDimethylsulfoxide; ^cMinimal effect dose; ^{*}Positive controls: see details in material and methods, ^{**} Analysis of Variance, p<0.05 indicates a positive response, ^{***} Confidence interval.

TABLE SXI. Dose response experiments, ANOVA, potencies in revertants/ μg of EOM and MED_2 in μg of EOM/plate for Limeira, Stockholm and Kyoto for the strain YG7108 without and with (S9).

YG7108																																			
Number of revertants/plate and mean																																			
Doses (µg of EOM ^a /plate)	Limeira										Stockholm										Kyoto														
	-S9					+S9					-S9					+S9					-S9					+S9									
	Plates					Plates					Plates					Plates					Plates					Plates									
	1	2	3	4	5	Mean	1	2	3	4	5	Mean	1	2	3	4	5	Mean	1	2	3	4	5	Mean	1	2	3	4	5	Mean					
DMSO ^b	43	42	43	40		43	43	41	44			43	40	46	39	47	47	46	48	64	36	46	53	48	38	40	33	32	46	38	43	41	44		43
0.3													38					38	48	45				47	33	46				40					
1													36					36	42	40				41	43	45				44					
3													55	68				62	54	55				55	52	60				56					
10	50	55				53							82	65				74	51	56				54	89	77				83	54		54		
30	55					55	46	50				48	122	118				120	109	94				102	161	163				162	98	85		92	
50	76	74				75	44					44	138	112				125	122	133				128	195	179				187	140	136		138	
100	89					89																									146	182		164	
300	34					34	60	43				52																		180			180		
500							76	72				74																		200	166		183		
Positive control ^c	130	114				122	1313	1228	1345			1313	1603	1598			1601	1540	1495				1518	1486	1492				1489	1313	1228	1345		1313	
ANOVA ^{**}																																			
Potency																																			
(rev./ µg of EOM)																																			
IC ^{***}																																			
MED ₂ ^c																																			
(µg of EOM/plate)																																			
IC																																			

Note: ^aExtracted organic material; ^bDimethylsulfoxide; ^cMinimal effect dose; *Positive controls: see details in material and methods, ** Analysis of Variance, $p < 0.05$ indicates a positive response, *** Confidence interval

TABLE SXII. Dose response experiments, ANOVA, potencies in revertants/ μg of EOM and MED_2 in μg of EOM/plate for Limeira, Stockholm and Kyoto for the strain TA100 without and with (S9).

TA100																																			
Number of revertants/plate and mean																																			
Doses (μg of EOM/plate)	Limeira										Stockholm										Kyoto														
	-S9					+S9					-S9					+S9					-S9					+S9									
	Plates					Plates					Plates					Plates					Plates					Plates									
	1	2	3	4	5	Mean	1	2	3	4	5	Mean	1	2	3	4	5	Mean	1	2	3	4	5	Mean	1	2	3	4	5	Mean					
DMSO ^b	147	125	124		125	126	132	128	141	147	132	227	235	235	207	212	227	212	176	180	223	175	180	174	157	154	133		156	129	137	136	145	160	137
0.3	147	161			154	149	119				134	241	222				232	191	227				209	145	169				157	137	134			136	
1	152	123			138	141					141	238	222				230	221	186				204	178	177				178	135	142			139	
3	186	171			179	156	139				148	297	243				270	263	233				248	196	201				199	147	143			145	
10	254	325			290	227	215				221	350	339				345	310	395				353	343	351				347	203	236			220	
30	436				436	332	375				354	681	578				630	672	549				611	714	717				716	360	390			375	
50	678	724			701	365					365	815	806				811	641	694				668	771	739				755	517	485			501	
Positive control ^c	3643	3293			3468	2613	3295				2954	2530	2507				2519	3132	3034				3083	1506	1820				1663	3112				3112	
ANOVA ^{**}																																			
Potency																																			
(rev./ μg of EOM)																																			
IC ^{***}																																			
MED ₂ ^c																																			
(μg of EOM/plate)																																			
IC																																			

Note: ^aExtracted organic material; ^bDimethylsulfoxide; ^cMinimal effect dose; *Positive controls: see details in material and methods, ** Analysis of Variance, $p < 0.05$ indicates a positive response, *** Confidence interval

Capítulo II

Polycyclic aromatic hydrocarbons (PAHs) in the atmosphere of cities from opposite hemispheres: determination of particle sources, aging and its contribution to the mutagenic activity

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ABSTRACT

The distribution of polycyclic aromatic hydrocarbons (PAHs) in atmospheric particulate matter is related to both emission sources and atmospheric reactions, which are driven by temperature and the meteorological conditions. Thus, it is expected that cities with differences in average temperatures and solar radiation periods, such as Limeira (south hemisphere) and Stockholm (north hemisphere), will have different PAHs distributions on total suspended particulate (TPS). Our objective was to investigate the PAHs content in TPS from both cities to determine the TPS sources and aging. We also evaluated the benzo[*a*]pyrene (B[*a*]P) contribution to the TPS mutagenicity using strains with different sensitivities to this pro-mutagen. The PAHs diagnostic ratios indicated a mixture of pollution sources in both cities with a slight predominance of combustion in Limeira and vehicular in Stockholm. Stockholm presented older particles although Limeira presented climatic/meteorological conditions more favorable to TPS aging. Air masses from the European continent can explain, at least in part, the aged TPS in Stockholm. Therefore, changes in aerosol chemistry depend more on the TPS lifetime than the presence of oxidants, or temperature and sunlight. The strain YG5185 was more suitable for assessing the B[*a*]P contribution to the TPS mutagenicity in comparison to TA98 and TA1538 and seems to be a better choice to monitor sites where compounds that act similarly as B[*a*]P are suspected to be the major pollution type. The PAHs concentration reduction in Limeira depends on the local sources control, while in Stockholm the local sources control would not decrease these pollutants in the air.

Keywords: PAHs diagnostic ratios; TPS aging process; backward trajectories; TA1538; TA98; YG5185.

1. Introduction

The negative impact of atmospheric particulate matter (PM) on the urban environment has been scientifically well established. PM affects visibility, air quality, and impacts on human health and climate (Seinfeld and Pankow, 2003; Robinson et al., 2007). The PM composition includes inorganic elements, ions, carbon, organic compounds, and water, in varying proportions. The PM may be internally mixed (containing various chemical species within the same particle), or externally mixed (different species in separate particles). Products of atmospheric reactions secondary organic aerosols (SOAs) are another possible source of new PM. Once the vapor pressure of the SOA exceeds the saturation vapor pressure the compound condenses to form a particle (Holmes, 2007). Size and composition of the particles are information that can help in understanding the origin of the PM and also to predict possible behavior and impact on the environment (Souza et al., 2017). Furthermore, PM from urban centers around the world contains many classes of mutagens. The mutagenicity of the

PM organic fraction is related to more than 500 individual compounds already identified (Claxton et al., 2004). Among these compounds, polycyclic aromatic hydrocarbons (PAHs), such as benzo[*a*]pyrene (B[*a*]P), have received great attention because the recognized carcinogenicity and pro-mutagenicity of several of their congeners (Srogi, 2007).

Reactive gases such as O₃ and NO_x play a central role in tropospheric chemistry. These gases are involved in photochemical processes with formation of radical species, responsible for the oxidation reactions of the organic compounds in the atmosphere. In this way, atmospheric pollutants and environmental conditions may have a great influence on the composition of the PAH content of PM (Alves et al., 2012; Jariyasopit et al., 2014). Direct photochemical reactions are mainly influenced by the intensity spectrum of available light in the atmosphere, and dependent on location characteristics as the altitude and latitude, period of the day, particle density, and cloud cover (George et al., 2015). Shrivastava et al. (2017) showed that the organic fractions of the aerosol coating are more effective in shielding PAHs in the middle/high latitudes, avoiding photochemical degradation, compared with regions of high humidity and luminosity. Moreover, the phenomena of air pollution and dispersion of toxic compounds can be better understood when studies on the trajectories of air masses are included (Pérez et al., 2015; Alves et al., 2016). The movement of air masses can carry both the anthropogenic and natural pollutants around latitude bands within weeks, between the tropics and the poles in months, and between the northern and southern hemispheres within a year (Jacob, 2000).

In the atmosphere, the PAHs may be distributed in the gas phase or adsorbed to PM (Alves et al., 2012; Zhou et al., 2012). Most of atmospheric PAHs are associated with PM (~ 70-90%) (Piekarska, 2010). PAHs are released into the environment from incomplete combustion of transport fuels, biomass, coal and oil, as well as during processing of fossil fuels, metal smelting and coke production. Natural sources of PAHs include wildfires, coal, crude oil, and volcanoes (Finlayson-Pitts and Pitts Jr., 1999; Keyte et al., 2013). Although often considered as persistent organic pollutants (POPs), PAHs are reactive in the air (Keyte et al., 2013). Their atmospheric concentrations are strongly dependent on the meteorological conditions under which emissions occurs. The ambient temperature directly influences PAH volatilization from PM, whereas photodegradation process is governed by solar radiation (Alves et al., 2012). Differences in volatility, photodegradation rate and biodegradation rate of the parent PAHs can lead to different PAH profiles in PM samples aged prior to the sampling (Kim et al., 2009).

PAHs are emitted into the atmosphere as complex mixtures, and different sources of pollution present different distributions of the various congeners from this chemical class, making their identification a difficult task (Tobiszewski and Namieśnik, 2012; Katsoyiannis and Breivik, 2014). The pollution sources of PAHs can be identified by comparing the proportions of concentrations of selected pairs of PAHs (Zhang et al., 2005). This tool is called molecular diagnostic ratios (Tobiszewski and Namieśnik, 2012; Huang et al., 2014; Singh et al., 2015). Usually molecular

diagnostic ratios involve pairs of PAHs with the same molecular weight and similar physicochemical properties. Because of these similarities these PAH pairs are subject to the similar atmospheric processes and environmental fate (Tobiszewski and Namieśnik, 2012; Katsoyiannis and Breivik, 2014). Thus, molecular diagnostic ratios have been widely used as an additional tool for the identification or apportionment of pollution sources (Alves, 2008; Tobiszewski and Namieśnik, 2012; Huang et al., 2014; Singh et al., 2015).

Air pollution characterized by dispersion of toxic compounds of anthropogenic origin most often has mutagenic properties for living beings. The mutagenicity properties can be characterized using bioassays, which will provide a biological response that would correspond to the levels of mutagenic compounds present in the sample as well as their possible additive, synergistic, or antagonistic effects. The Salmonella/microsome assay is the most widely used method for the evaluation of PM mutagenicity (Claxton and Woodall, 2007). Numerous studies have demonstrated the association between the mutagenic PM activity and atmospheric PAHs levels (IARC, 2016), however, this class of pro-mutagens generally does not correspond to most of the organic extract mutagenicity of PM samples collected in different parts of the world (Claxton et al., 2004). According to Durant and Lafleur (2011), PAHs and some of their derivatives (alkyl-PAHs, nitro-PAHs, hydroxynitro-PAHs, nitro-PAHs lactones, and ketone derivatives of PAHs) are responsible for approximately 20-25% of the total mutagenicity of whole PM samples. However, it is important to highlight that the relative contribution of the different classes of mutagenic compounds, present in the PM, is directly related to the organic extraction procedures used and the choice of the strains of the Salmonella/microsome assay (Claxton et al., 2004).

Harmonized sampling and analytical protocols is a key factor to provide reliable data for comparative studies on PM (Sillanpää et al., 2006). The total suspended particulate (TPS) samples from Limeira (south hemisphere) and Stockholm (north hemisphere) were collected using same filter batch and submitted to the same procedures of organic extraction, chemical analysis, and mutagenicity tests. The objective of this study was to investigate the PAHs content of TPS collected in both cities to determine the particle sources and aging. We also evaluated the B[a]P contribution to the TPS mutagenic activity.

2. Material and methods

2.1. Selection of sites and sampling

TPS samples from Limeira, Brazil (south hemisphere) and Stockholm, Sweden (north hemisphere) were collected during winter time. In general, TPS samples collected during colder seasons (winter) are more mutagenic (per cubic meter) than TPS samples collected during warm seasons (summer) (IARC, 2016). The meteorology of the studies regions is characterized by very

different profiles. At Limeira, in winter the temperature has average 20.4 °C, precipitation is low (average of 35 mm), average sunlight periods of 11 hours per day and humidity decreases, especially during the daytime (FT-Unicamp, 2015). The dry winter conditions favor the accumulation of particles in the atmosphere. Stockholm, during winter presents average temperatures of -2.0 °C and average sunlight period of 2 hours per day (WW&CI, 2015). Limeira samples were collected from June to September (2014), at ~ 2.0 above ground level at the campus of the School of Technology - Unicamp. Limeira is a town with approximately 270 thousand inhabitants, where the economy is based on industrial and agriculture production. Two highways linking major cities of the region cross the city's urban boundaries. The sampling site is impacted by heavy traffic, industrial emissions, and sugar cane biomass burning. TPS samples collected at this site presented one of the highest mutagenic potencies found in the literature (Alves et al., 2016). Stockholm with approximately 935 thousand inhabitants, where the economy is based on business and finance. In Stockholm samples were collected from October (2013) to March (2014) at rooftop level at the campus of the Stockholm University. This site is mainly impacted by traffic (Lim et al., 2015), but its TPS is much less mutagenic than Limeira (Umbuzeiro et al., 2014).

TPS samples were collected using high-volume total suspended particulate (Hi-vol TPS) samplers with pre-conditioned and pre-weighed glass fiber filters (254 × 203 mm, Pallflex, Pall Corporation, Putnam, CT, USA). The PM samples were collected using filters from the same batch and under similar sampler operating conditions. Organic extracts were prepared with combined TPS samples. The number of filters varied between the two cities and their quantities were defined based on the amount of PM needed for mutagenicity tests (Table 1).

Table 1. Detailed information on the collection of TPS samples including the date, amount of particles and concentrations of TPS per filter.

Cities	Date of the sampling	Initial and final hour of the sampling	Duration (h)	Total amount of particles per filter (mg)	Total flow per filter (m ³)	TPS concentration per filter (µg/m ³)
South hemisphere (Limeira)	2014/06/25	0:00 – 24:59	24	346	2,745	126
	2014/07/01	0:00 – 24:59	24	325	2,722	119
	2014/07/19	0:00 – 24:59	24	111	2,954	37.5
	2014/08/06	0:00 – 24:59	24	273	2,699	101
	2014/08/19	0:00 – 24:59	24	313	2,763	113
	2014/08/29	0:00 – 24:59	24	348	2,756	126
	2014/09/05	0:00 – 24:59	24	207	2,789	74.2
North hemisphere (Stockholm)	2013/10/02-08	14:02 - 10:01	140	58.1	9,582	6.06
	2013/10/08-15	10:14 - 9:46	168	39.6	11,424	3.47
	2013/10/23-29	11:50 - 10:04	142	40.0	9,704	4.12
	2013/10/29-11/05	10:16 - 10:49	169	33.1	10,975	3.02
	2013/11/05-12	11:01 - 10:13	167	29.8	10,732	2.78
	2013/11/12-19	10:27 - 10:27	168	38.8	10,949	3.54
	2013/11/19-26	10:39 - 10:03	167	34.9	10,930	3.19
	2013/11/26-12/03	10:16 - 10:55	169	46.5	10,707	4.34
	2013/12/03-10	11:07 - 11:36	168	31.5	10,435	3.02
	2014/01/21-28	9:53 - 11:22	169	43.4	10,508	4.13
	2014/02/18-25	9:37 - 9:51	168	71.2	10,723	6.64
	2014/02/25-03/04	10:00 - 10:22	168	218	10,610	20.5
	2014/03/04-11	10:31 - 9:49	167	176	10,896	16.2
	2014/03/11-19	9:59 - 9:36	192	77.4	12,196	6.35

2.2. TPS sample organic extraction and PAH analysis

TPS samples were submitted to the accelerated solvent extraction using the ASE 200 extractor system (Dionex Co., Sunnyvale, CA, USA) with toluene (Bergvall and Westerholm, 2008). The sampled filters were weighed and inserted into 5 mL extraction cells. Five static cycles of 30 minutes each were performed, with the instrument operating at its maximum capacity (200 °C and 3000 psi). The method has been shown to exhaustively extract PAHs and to give comparable or higher PAHs concentrations than those assigned by the National Institute for Standards and Technology (NIST) for the Urban Air standard reference material SRM 1649a (Bergvall and Westerholm, 2008). For the determination of the extracted organic material (EOM), 3-4 mL of each organic extract was transferred to a pre-weighed glass vial. These vials were evaporated to dryness, and then the vials were weighed again. The EOM was determined by differences between the initial and final masses of the vials.

Aliquots of the extracts were spiked with solutions of internal standard (phenanthrene-*d*₁₀, pyrene-*d*₁₀, benz[*a*]anthracene-*d*₁₂, benzo[*a*]pyrene-*d*₁₂ and benzo[*ghi*]perylene-*d*₁₂), and then

submitted to a clean-up using silica solid phase extraction cartridges (100 mg, IST Isolute, Biotage, Cardiff, UK) with the elution of 3 mL hexane (Christensen et al., 2005; Bergvall and Westerholm, 2006). The eluate was reduced until 100 μ L under a gentle nitrogen flow and transferred to a micro vial prior to the injection. Analysis for determination of 27 PAHs congeners (Table 2) were performed by high performance liquid chromatography-gas chromatography/mass spectrometry (HPLC-GC/MS) (Christensen et al., 2005; Bergvall and Westerholm, 2006; Sadiktsis et al., 2014). The PAH concentrations were expressed as a function of air volume (pg/m^3) (Table 2).

2.3. Evaluation of PAH sources and TPS aging

The source of PAHs and TPS aging were evaluated using molecular diagnostic ratios. The PAH sources were investigated using the ratio of anthracene (Ant)/Ant + phenanthrene (Ant + Phe) to distinguish petrogenic (< 0.1) and pyrogenic (> 0.1) sources, and the ratio B[a]P/benzo[ghi]perylene (B[ghi]P) is used to distinguish non-traffic emissions (< 0.6) and traffic emissions (> 0.6) (Tobiszewski and Namieśnik, 2012; Singh et al., 2015). The ratio between B[a]P and benzo[e]pyrene (B[e]P) have been employed to evaluate the PM aging (Panther et al., 1999; Okuda et al., 2002; Vasconcellos et al., 2010; Keyte et al., 2013). When this ratio is close to 1 indicates negligible photochemical degradation and major impact from local emission sources (fresh particles), while lower ratios indicate aged particles (Oliveira et al., 2011; Vasconcellos et al., 2011; Wu et al., 2012). Although both isomers are emitted by most pollution sources in the same proportion, B[a]P is photodegraded more rapidly during the transportation, while the B[e]P is slowly photodegraded (Alves, 2008; Oliveira et al., 2011; Vasconcellos et al., 2011).

To complement the investigation on the aging of the TPS from both cities, the trajectories of air backtracking of both cities during the respective sampling period were calculated using the United States National Oceanic and Atmospheric Administration (NOAA) HYSPLIT 4 model (Hybrid Single-Particle Lagrangian Integrated Trajectory 4) (Draxler and Rolph, 2015). The 48 h back-trajectories were calculated 100 m and 500 m above the ground over the sampling site. In studies of the atmospheric distribution of PM, the calculation of backward trajectories is one of the most used tools; and the most common model used for this purpose is the HYSPLIT (Pérez et al., 2015).

2.4. Mutagenicity evaluation

To investigate the contribution of the B[a]P to the mutagenicity of the organic extracts obtained from the TPS samples collected in both cities, the *Salmonella enterica* serovar Typhimurium strains TA1538, TA98 and YG5185 were chosen. Both TPS sample organic extracts and B[a]P were tested and only in the presence of exogenous metabolism system (S9 mixture) because PAHs like B[a]P need enzymatic activation to be mutagenic (Umbuzeiro et al., 2014). To selectively evaluate the

contribution of B[a]P to the total PM mutagenicity we used the YG5185. Yamada et al. (2006) developed this strain to selectively detect the mutagenicity of PAHs, even though this class of pro-mutagens is present in complex mixtures. Briefly, YG5185 possesses a plasmid carrying *dinB* encoding DNA polymerase IV into *ΔnfsBΔoat* from a derivative of TA1538. This specific DNA polymerase can bypass a variety of DNA lesions such as those promoted by B[a]P making this strain more sensitive to PAHs, which acts similarly to B[a]P (Yamada et al., 2006; Nohmi, 2007). This new strain also is much less sensitive to nitroaromatics and aromatic amines because the genes *nfsB* and *oat* encoding nitroreductase and *O*-acetyltransferase activation enzymes were disrupted (Yamada et al., 2006). This is an important feature because the intention of using this strain is to highlight the presence of PAHs in the samples that would be confounded by the high mutagenicity of the nitroaromatics and aromatic amines also present in PM (Yamada et al., 2006; Umbuzeiro et al., 2014).

We included the TA98 because Claxton et al. (2004) consider, based on the data retrieved from the articles included in their extensive review of the literature, that TA98 is an initial strain of choice for the evaluation of PM organic extracts due to its low spontaneous reversion background and its sensitivity to PAHs in the presence of S9 mixture. The TA1538 was included because this strain was more sensitive than its derivative TA98 to B[a]P (Skopek et al., 1978; Umbuzeiro et al., 2014) and to TPS sample extracts previously collected in Limeira (Alves et al., 2016). Nevertheless, both TA98 and TA1538 are highly sensitive to nitroaromatics and aromatic amines because of their endogenous production of the enzymes, nitroreductase and *O*-acetyltransferases, both involved in the bioactivation of these chemical classes (Claxton et al., 2004). Thus, in many cases the mutagenicity of PAHs is masked when these strains were used (Yamada et al., 2006; Nohmi, 2007).

Aliquots from the same extracts used for PAH analysis were evaporated to dryness under a gentle nitrogen flow at 55 °C and then resuspended in dimethylsulfoxide (DMSO - CAS 67-68-5, Sigma, purity 99.5%). The extracts were submitted to the Salmonella/microsome assay using the microsuspension protocol (Kado et al., 1983) in dose-response experiments, only in the presence of S9 mixture (+S9), prepared according to Mortelmans and Zeiger (2000). TA1538 was kindly provided by Dr. Takehiko Nohmi; TA98 was kindly provided by Dr. Larry Claxton; and YG5185 was kindly provided by Dr. Masami Yamada. The tests were performed in duplicate and added to positive and negative controls. 2-aminoanthracene (CAS 613-13-8, Sigma, purity 96%) (0.625 µg/plate) was the positive controls used in the tests for all strains employed. The negative control used in all tests was DMSO. The tested doses ranged from 2.7 - 445.0 and 0.3 - 47.0 µg per plate for Limeira and Stockholm, respectively. The B[a]P (CAS 50-32-8, Sigma, purity 99%) was also tested with the three strains used (+S9) to verify their relative sensitivities and the contribution of this mutagen to the mutagenic activity detected in the organic extracts from the TPS samples collected in both cities.

The mutagenicity results were analyzed using the Salanal computer software (Integrated Laboratory Systems, Research Triangle Park, NC). An ANOVA including a post hoc test was performed to compare each tested concentration with the respective negative control. Then a linear

regression was performed using the Bernstein model (Bernstein et al. 1982). The extract was considered positive when both the ANOVA and linear regression provided significant responses ($p < 0.05$). The mutagenic potencies were expressed as a function of air volume (revertants/m³), and the contribution of B[a]P to the mutagenicity of extracts was calculated as a function of its concentrations in both PM samples. The sensitivity of all strains to B[a]P was evaluated using the minimum effective dose (MED). MED₂ was determined as a minimum dose that presented a 2-fold higher response than the negative control, based on non-slope of the curve calculated in linear regression by the Bernstein model (Bernstein et al., 1982).

3. Results and discussion

Limeira presented average TPS concentration of 99.0 µg/m³ ($n = 7$ sampled filters; standard deviation = 32.9), 16-fold higher than in Stockholm (6.2 µg/m³; $n = 14$ sampled filters; standard deviation = 5.4). This result agrees with those previously reported by Umbuzeiro et al. (2014) where Limeira presented higher TPS concentration (13-fold) than Stockholm. The total PAHs (sum of the 27 congeners) concentration (pg/m³) was 8.4-fold higher in Limeira in comparison to Stockholm (Table 2). The EOM percentages obtained from the samples were approximately 9 and 15 % for Limeira and Stockholm respectively, resulting in a difference of ~ 10-fold in the amount of organic compounds per cubic meter. The difference in the PAH levels between both cities agree with the EOM percentage of the samples, and can be explained, at least in part, by differences in pollution sources and the age of the particles.

Table 2. PAH concentrations determined in organic extracts from the TPS samples collected in Limeira and Stockholm.

Number of rings	PAHs	Concentration	
		Limeira (pg/m ³)	Stockholm (pg/m ³)
3	Anthracene	113	8.52
	Phenanthrene	696	91.0
	2-Phenylnaphthalene	71.7	15.5
Sub-total concentration		881	115
4	Benz[<i>a</i>]anthracene	435	61.9
	Fluoranthene	817	152
	Pyrene	921	133
	Benzo[<i>a</i>]fluorene	72.1	16.1
	Benzo[<i>b</i>]fluorene	46.5	8.94
	Benzo[<i>b</i>]naphto[1,2- <i>d</i>]thiophene	13.4	1.15
	Benzo[<i>c</i>]phenanthrene	90.6	17.7
	4 <i>H</i> -Cyclopenta[<i>def</i>]phenanthrene	63.9	13.5
Sub-total concentration		2,460	404
5	Benzo[<i>b</i>]fluoranthene	970	138
	Benzo[<i>k</i>]fluoranthene	424	58.7
	Benzo[<i>a</i>]pyrene	822	78.7
	Dibenz[<i>a,h</i>]anthracene	131	16.4
	Benzo[<i>ghi</i>]fluoranthene	408	47.1
	Benzo[<i>e</i>]pyrene	790	89.3
	Picene	121	16.2
	Perylene	140	10.7
6	Benzo[<i>ghi</i>]perylene	1,400	104
	Dibenzo[<i>a,e</i>]pyrene	130	14.4
	Dibenzo[<i>a,h</i>]pyrene	5.30	1.25
	Dibenzo[<i>a,i</i>]pyrene	15.0	3.13
	Dibenzo[<i>a,l</i>]pyrene	13.4	1.50
	Indeno[1,2,3- <i>cd</i>]fluoranthene	81.2	12.7
	Indeno[1,2,3- <i>cd</i>]pyrene	796	83.3
7	Coronene	875	57.2
Sub-total concentration		7,120	733
Total concentration		10,500	1,250

The percent composition of 3, 4 and ≥ 5 ring PAHs in the TPS from both cities are similar (Figure 1) despite differences in the mass of PAHs per m³ (Table 2). TPS collected in cities with higher temperatures as Limeira (average 20.4 °C) are expected to have less volatile congeners in comparison with TPS collected in cities with lower temperatures as Stockholm (average -2.0 °C) (Zhou et al., 2012; Keyte et al., 2013). The majority of the 3 ring-PAHs congeners are expected in the gas phase, whereas those with 6 or more rings predominantly in the TPS. PAHs with four or five rings are distributed among both phases (Zhou et al., 2012), and the gas-particle partitioning is influenced by both adsorption and absorption processes that is strongly linked to temperature (Keyte et al., 2013). Other important factors that influence the distribution of PAHs in the atmosphere are the vapor pressure of the PAHs, the amount of fine particles with available surface area for adsorption of PAHs,

and the affinity of individual PAHs for the organic matrix of the particles (Baek et al., 1991). Furthermore, PAHs present different rates of degradation during transport and deposition processes (Kim et al., 2009).

Figure 1. Polycyclic aromatic hydrocarbons (PAHs), grouped by the number of rings, present in the organic extracts of TPS samples from Limeira and Stockholm expressed in percentage. The size of the circles represents the difference in TPS concentration ($\mu\text{g}/\text{m}^3$) of both cities.

Limeira and Stockholm presented a variety of PAH sources but the Ant/(Ant + Phe) and B[a]P/B[ghi]P ratios suggest a slight predominance of biomass burning sources (pyrogenic, 0.14 and non-traffic, 0.59) in Limeira and vehicle emissions sources (petrogenic, 0.09 and traffic, 0.76) in Stockholm. Jarvis et al. (2014), suggested that for samples previously collected at the same sampling sites, PAHs at the Limeira site can mainly be attributed to heavy traffic, industrial emissions and biomass burning, whereas those at the Stockholm site can mainly be attributed to traffic emissions. However, the source profile also depends on the air mass trajectory.

Surprisingly, Limeira presented fresher particles than Stockholm with B[a]P/B[e]P ratios of 1 for Limeira and 0.9 for Stockholm. According Panther et al. (1999) and Tan et al. (2009), in locations with lower temperatures and sunlight hours the B[a]P/B[e]P ratio was significantly higher than in locations with higher temperatures and sunlight hours. Based on the atmospheric and climatic characteristics recorded in Limeira and Stockholm on average during the winter and considering their influence in the process of particle aging; aged particles were expected in the Limeira sample. The photochemical reactions involved in the aging process of TPS are directly influenced by the intensity

of solar radiation received. Another important factor is that liquid-like PM organic coatings, typical of warm/humid regions (tropics), favor heterogeneous reactions of PAHs degradation, while semisolid coatings, typical of cold/dry regions (middle/high latitudes), protect PAHs from degradation (Zhou et al., 2012; Jariyasopit et al., 2014; Shrivastava et al., 2017). However, in the Limeira region during the winter the relative humidity is low, which may hinder some heterogeneous reactions (Caetano-Silva et al., 2013). The lack of humidity in Limeira may be avoiding the reactions between the oxidizing agents and the PAHs, and thus making the aging process of the particles difficult (Reid and Sayer, 2003). Furthermore, the heterogeneous oxidation of particle-bound PAHs decreases due to accumulation SOA species over particles during their atmospheric transport and chemical aging (Jariyasopit et al., 2014). Highly viscous SOA coatings limit the diffusion of PAH molecules from particle bulk to particle surface, effectively shielding PAHs from heterogeneous reactions (Shrivastava et al., 2017). It is also important to note that other factors not related to temperature, sunlight hours and humidity could be influencing the PAH congeners' distribution and aging of particles in both studied locations, including atmospheric transport.

TPS collected at one sampling site is composed not only of particles emitted locally, but also by primary and secondary particles transported from other regions. During the sampling period, there was a large variation in TPS concentrations in Stockholm, but in Limeira this variation was considerably lower (Table 1). In general, when air masses that arrived at Stockholm had their origin in the North Sea, the observed particle concentrations were low (Figure 2A). However, when the masses of air had their origin in the Europe Continent, passing through Poland among other countries, the concentrations of TPS were approximately 3-fold higher than the average TPS concentration (Figure 2B). This is in agreement with a recent report from the European Environmental Agency (EEA, 2016). Studies on the trajectory of air masses revealed that in general continental air masses are more polluted, while those masses coming from the North Sea are among the cleanest of this hemisphere (Pérez et al., 2015). This important contribution of particles from European countries may explain, at least in part, the fact that we observed relatively aged particles in Stockholm than in Limeira.

In contrast, TPS concentrations in Limeira were high throughout the sample period and so air masses that passed through the metropolitan region of the city of São Paulo (MRSP) (Figure 3A), one of the largest in the world, as those coming from the interior of the State (Figure 3B) seem to contribute to the high TPS levels. While the particles coming from the MRSP are derived mainly from vehicular and industrial sources, those generated in the interior of the State are also related to the biomass burning. Also, based on the results of the PAHs diagnostic ratios presented above, we can conclude that, during the sampling period in Limeira, the contribution of sources related to biomass burning at local and regional scale seems to be the most important.

Figure 2. The 24-h back-trajectories arrived at a height of 100 m (blue line) and 500 m (red line) above the ground level (AGL), over the Stockholm sampling site. 2A. Sample collected: October 29, 2013 – total PM concentration $3.0 \mu\text{g}/\text{m}^3$. 2B. Sample collected: February 25, 2014 – total PM concentration $20.5 \mu\text{g}/\text{m}^3$.

Figure 3. The 24-h back-trajectories arrived at a height of 100 m (blue line) and 500 m (red line) above the ground level (AGL), over the Limeira sampling site. 2A. Sample collected: August 29, 2014 – total PM concentration $126.3 \mu\text{g}/\text{m}^3$. 2B. Sample collected: July 1, 2014 – total PM concentration $119.2 \mu\text{g}/\text{m}^3$.

In PM, the organic fraction is highly heterogeneous and generally represents 10-90% by mass (Alves, 2008; Ringuet et al., 2012), and this fraction is mostly concentrated in smaller particles ($< 2 \mu\text{m}$) (Holmes, 2007). The difference in EOM amounts of both samples was around 10-fold. The sample collected in Limeira contains $8.9 \mu\text{g}$ of EOM/ m^3 while the sample collected in Stockholm contains $0.9 \mu\text{g}$ of EOM/ m^3 . The observed difference in the EOM amount can be explained by the higher total concentration of particles in Limeira. However, the ratio EOM/mass of TPS shows that the particles collected in Stockholm have a larger composition of the nonpolar organic phase than the particles collected in Limeira. The differences may be related to the intensity particles sources directly emitted into the atmosphere or formed there by chemical reactions. The dry winter in the region of Limeira, favors the emission of soil particles that are poor in non-polar organic matter (Rocha et al., 2005).

The combustion processes release gas and vapor compounds to the atmosphere, which can generate new particles or favor the growth of previously nucleated particles resulting in the increase of atmospheric fine particle concentrations (Rocha et al., 2005; Holmes, 2007; Souza et al., 2017). Conversely, particles with high aerodynamic diameter usually contain relatively smaller amounts of EOM (Sato et al., 1995; Claxton et al., 2004). Thus, it seems that secondary particles formed into the atmosphere are the main sources of particles in Stockholm. These particles are mainly formed as due of the condensation of organic vapors favored by low local temperature in the winter (-2.0°C in average). In addition, lower temperatures, lower incidences of solar radiation, lower height of the atmosphere boundary layer, and more frequent stable winter weather conditions may favor the condensation of semi-volatile organic compounds on freshly nucleated particles (Li et al., 2015; Souza et al., 2017). Consequently, these particles tend to accumulate organic compounds, such as PAHs and their derivatives (Li et al., 2015). While the lower proportion of EOM obtained from the Limeira TPS sample indicates larger particles, a result of dry soil that favors its resuspension by the winds and as result of biomass burning (Rocha et al., 2005; Souza et al., 2017). Although we have not collected different particle sizes fractions, differences in the distribution of particles of different sizes may also influence TPS aging and the profile of PAH congeners present in the particulate phase. As a continuation of this study we intend to work with certain PM fractions, for example with $\text{PM}_{2.5}$.

Both TPS samples were mutagenic for all strains (+S9) used (Table 3). TA1538 presented 5 times higher mutagenic potencies than TA98 (Table 3). This has already been observed by Umbuzeiro et al. (2014) for TPS samples previously collected at the same locations.

Table 3. Mutagenic potencies of the Limeira and Stockholm TPS sample organic extracts using the YG5185, TA1538 and TA98 strains (+ S9).

Strains (+S9)	Mutagenic potency (revertants/m ³)	
	Limeira	Stockholm
YG5185	9 (8.8-15.1)	2 (1.3-2.0)
TA1538	89 (76.5-107.7)	10 (8.6-11.5)
TA98	18 (15.1-22.2)	2 (1.6-2.0)

Because of the higher sensitivity of YG5185 to B[a]P (Table 4) and its reduced sensitivity to nitroaromatics and aromatic amines (Yamada et al., 2006) the potencies obtained for this strain was lower than both TA1538 and TA98. To the best of our knowledge this strain had not yet been used for the selective detection of mutagenicity related to this class of pro-mutagens in atmospheric TPS samples. Only one study was found in the literature that used YG5185 strain to evaluate complex mixtures (Mladjenovic et al., 2013). The authors used this strain to assess the mutagenicity of cigarette smoke condensates (CSC), obtained from different types of cigarettes marketed in Canada, and observed that the mutagenicity detected by YG5185 was related with PAH levels of some CSC (Mladjenovic et al., 2013). Umbuzeiro et al. (2014) did not use the strain YG5185, however the authors used YG5161, which has similar sensitivity to PAHs (Yamada et al., 2006), but has activity of the nitroreductase and *O*-acetyltransferase enzymes similar to its parental strain (TA1538). The YG5161 failed to distinguish the mutagenicity related to the PAHs present in organic extracts from TPS samples previously collected at the same sampling sites (Umbuzeiro et al., 2014) because it also was relatively sensitive to nitroaromatics and aromatic amines (Nohmi, 2007; Umbuzeiro et al., 2014). Therefore, the mutagenic potencies for TA1538 and TA98 strains, although associated with the concentrations of PAHs clearly are not caused by B[a]P concentrations found in each sample (Table 4).

The contributions for the total mutagenicity that can be attributed to B[a]P are presented in the Table 4. The highest B[a]P contributions were observed for YG5185. Our results confirm that YG5185 is the best strain to evaluate the contribution of PAHs present in complex mixtures, as organic extracts from PM samples. For Limeira extract B[a]P explained more than 50% of the mutagenicity and for Stockholm more than 30%. The remaining mutagenic activity may be related to other PAHs, which act by the same mechanism of B[a]P.

Table 4. Contribution of the B[a]P to the mutagenicity of TPS sample organic extracts of the Limeira and Stockholm using the YG5185, TA1538 and TA98 strains with S9.

Sample site	Strains	Sample potency (rev./m ³)	B[a]P concentration (ng/m ³)	B[a]P potency (rev./ng)	Contribution	
					(rev./m ³)	(%)
Limeira	YG5185	9 (8.8-15.1)*	0.822	YG5185 6.000	4.770	53.00
	TA1538	89 (76.5-107.7)		(5.170-6.630)	0.134	0.15
	TA98	18 (15.1-22.2)		TA1538 0.164	0.020	0.09
Stockholm	YG5185	2 (1.3-2.0)	0.079	(0.145-0.183)	0.530	23.00
	TA1538	10 (8.6-11.5)		TA98 0.108	0.015	1.35
	TA98	2 (1.6-2.0)		(0.818-0.135)	0.010	6.75

* 95% confidence interval

4. Conclusion

The total concentrations of PAHs (pg/m³) in Limeira are approximately 8.4-fold higher than in Stockholm, and this difference agrees with the EOM levels obtained from both samples. The distribution of PAHs with 3, 4 and ≥ 5 rings in the TPS samples from both cities were similar, despite the relatively high average temperature and the favorable conditions for the photochemical reactions observed in Limeira.

The PAH diagnostic ratios indicated a mixture of pollution sources in both cities with a slight predominance of biomass combustion sources in Limeira and of vehicular sources in Stockholm. Stockholm presented older particles although Limeira presented solar radiation and photochemistry reaction apparently more favorable to TPS aging. Air masses from the Europe continent suggest that, at least in part, the aged TPS collected in Stockholm is imported from other parts of Europe. Therefore, our results indicate that changes in aerosol chemistry depend more on the TPS lifetime than only presence of oxidants, or temperature and sunlight. Environmental factors, such as relative humidity, need to be better understood in future PAHs reactivity studies.

The strain YG5185 was more suitable for assessing the B[a]P contribution to the TPS mutagenicity in comparison to TA98 and TA1538 and seems to be a better choice to monitor sites where compounds that act similarly as B[a]P, such as other non-substituted PAHs, are suspected to be the major pollution type.

The reduction of the PAHs concentration in Limeira depends on the control of local sources, since in Stockholm the control of local sources would not significantly decrease these aromatics in the atmosphere.

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Capítulo III

MINIATURIZATION OF THE MICROSUSPENSION

SALMONELLA/MICROSOME ASSAY IN AGAR MICROPLATES

Running title: MINIATURIZED SALMONELLA ASSAY

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ABSTRACT

The Salmonella/microsome assay (Ames Test) is the most widely used mutagenicity test for evaluation of pure chemicals and environmental samples. There are several versions of protocols available in the literature, including those that reduce the amount of sample needed for testing with liquid and agar media. The Microsuspension version of the Salmonella/microsome assay is more sensitive than the standard protocol. It is performed 5-times concentrated bacteria and less sample and S9 mixture, but still used conventional Petri dishes (90 x 15 mm). It has been extensively used for environmental samples testing, including in effect-directed Analysis (EDA). The objective of this study was to miniaturize the microsuspension assay using 12-well microplates instead of the conventional plates. For validation of this miniaturization, thirteen mutagenic compounds were tested using three Salmonella strains that were selected based on their different spontaneous reversion frequencies (low, medium and high). The conditions of the miniaturized procedure were made as similar as possible to the microsuspension protocol, using the same testing design, metabolic activation, and data interpretation, and the tests were conducted in parallel. The miniaturized plate assay (MPA) and microsuspension procedures provided similar sensitivities although MPA is less laborious and require less sample and reagents, thereby reducing overall costs. We conclude that the MPA is a promising tool and can be particularly suitable for environmental studies such as EDA or monitoring programs.

Keywords: Mutagenicity, Effect-directed analysis, Monitoring studies, Ames test, MPA, Validation.

INTRODUCTION

The Salmonella/microsome assay is the most widely used mutagenicity test both for evaluation of pure chemicals and environmental samples and is considered a reliable tool in the toxicology of the 21st century (Claxton et al., 2010). Some researchers have modified the test conditions and developed reduced volume protocols that are available in the literature (Kado et al., 1983; Brooks 1995; Flamand et al. 2001; Pant et al. 2016; Proudlock and Evans 2016). These modifications provide clear advantages, such as cost-effectiveness, reduced hands-on time and especially the reduction in S9 mix and sample volume needed.

A reduced version of the standard assay using liquid media, called the Microplate Fluctuation Protocol (MPF), was developed and provided higher sensitivity than the standard Ames for chemical substances (Flückiger-Isler and Kamber, 2012), environmental samples (Umbuzeiro et al. 2010), and in effect-directed analysis (EDA) (Muz et al., 2017). MPF showed good performance in interlaboratory studies (Reifferscheid et al. 2012).

Several other reduced protocols using agar media have been developed (Brooks 1995; Flamand et al. 2001) which used 2.5 times less sample and 5 times less S9 mix per plate in comparison with the standard protocol (Mortelmans and Zeiger, 2000). Pant et al. (2016) used 5 times less sample and Proudlock and Evans (2016) used 20 times less sample and S9 per plate. All agar-based reduced protocols provided good agreement with the standard protocol.

Another reduced version of the Ames test is the microsuspension assay (Kado et al., 1983). The authors showed that the assay was 10 to 13 times more sensitive than the standard protocol. Agurell and Stensman (1992) tested single chemicals and particulate matter extracts and showed that the microsuspension procedure was 3 to 37 times more sensitive than the standard protocol. Watanabe et al. (1995) observed a greater variation in the sensitivity of the microsuspension procedure (2 – 4400 times more sensitive) for a specific class of nitro

compounds. Černá et al. (1999) found the microsuspension procedure 4 to 8 times more sensitive when testing atmospheric particulate extracts. It is clear that the microsuspension protocol is more sensitive than the standard Ames test protocol but how much depends on the type/class of compound and the conditions/strains employed in the test. It uses 5 fold concentrated bacterial culture, 10 times less sample and S9 mixture, but is performed with conventional Petri dishes (90 x 15 mm). This protocol has been extensively used for environmental testing (Umbuzeiro et al. 2004; Crebelli et al. 2005; Di Giorgio et al. 2011; Alves et al. 2016), and in effect-directed analysis (Umbuzeiro et al., 2011; Brack et al., 2016).

The aim of this study was to develop a miniaturized version of the Salmonella/microsome microsuspension assay using agar microplates, which would work equally well with strains having low, medium and high spontaneous mutation frequencies.

MATERIALS AND METHODS

The strategy used was to evaluate a number of mutagenic compounds in parallel in the new protocol, called microplate agar (MPA) and in the microsuspension procedure developed by Kado et al. (1983). We used the same compounds, tester strains, S9 concentration and preincubation conditions in both assays, the difference being in the type of agar plates used; 90 x 15 mm plates for the microsuspension and 12-well microplates (21.4 x 17.5 mm) for the MPA.

Selection of bacterial strains, test compounds, controls

Three different strains of *Salmonella enterica* serovar Typhimurium were selected based on their spontaneous mutant frequencies. We wanted to develop a protocol that could be used with strains with low, medium and high spontaneous frequencies. Typically, in our laboratory TA1538 provides 10 - 20 colonies per plate, TA98, 20 – 40, and YG1041, 100 –

200 in the microsuspension protocol. TA1538 and TA98 were kindly supplied by Dr. Larry Claxton and, YG1041 by Dr. Takehiko Nohmi.

To compare the potency of both assays we selected thirteen variably mutagenic compounds. Six were tested only without metabolic activation (S9), because they provide higher potencies in this condition and the other seven were tested only with S9 because they require S9 to be mutagenic or provide higher responses under this condition (Table I). We also included some dyes that were expected to provide negative or weak responses with some of the strains used in this study (TA1538 and TA98) to verify the qualitative agreement of both protocols. All compounds were diluted in dimethyl sulfoxide (DMSO) and tested over a series of concentrations which were selected based on the literature and expressed in ng/μL of the pre-incubation mixture.

The negative control consisted of DMSO and positive controls were included (4-100 nitroquinoline-1-oxide at 1.25 ng/μL for TA98 and 4-nitro-o-phenylenediamine at 25 ng/μL for TA1538 and YG1041 without S9, and 2-aminoanthracene at 6.25 ng/μL for TA1538, TA98 and at 0.3125 ng/μL for YG1041 with S9).

TABLE I. Name, CAS number, supplier and purity of the compounds selected

	Compounds	CAS	Supplier	Purity
	1,6-dinitropyrene	42397-64-8	Sigma-Aldrich Co.	98%
	3,6-dinitrobenz(a)pyrene	847862-64-0	NARD Chemical Ltd.	99%
-S9	1-nitropyrene	5522-43-0	Sigma-Aldrich Co.	99%
	4-nitropyrene	57835-92-4	Tokyo Chemical Industry	97%
	3-nitrofluoranthene	892-21-7	Santa Cruz Biotechnology	94%
	4-nitroquinoline 1-oxide	56-57-5	Sigma-Aldrich Co.	98%
	2-aminoanthracene	613-13-8	Aldrich Chem Co.	96%
	Benzo(a)pyrene	50-32-8	Aldrich Chem Co.	97%
	Disperse Blue 373	51868-46-3	Shanghai Orgchem Co. Ltd.	95%
+S9	Disperse Orange 30	12223-23-3	Shanghai Orgchem Co. Ltd.	95%
	Disperse Orange 37	13301-61-6	Sigma-Aldrich Co.	96%
	Disperse Violet 93	52697-38-8	Shanghai Orgchem Co. Ltd.	95%
	Disperse Yellow 3	2832-40-8	Sigma-Aldrich Co.	96%

Microsuspension Assay

The microsuspension assay was performed using the protocol developed by Kado et al. (1983) using the buffer concentration recommended by DeMarini et al. (1989). Overnight cultures were concentrated 5-fold by centrifugation at 10,000 g at 4 °C for 10 min and resuspended in 0.015 M sodium phosphate buffer. Volumes of 50 µL of cell suspension (0.5 to 2x10¹⁰ cells per mL), 50 µL of S9 mix or 0.015 M sodium phosphate buffer, and 2 µL of the sample were added to a tube, mixed by vortexing, and incubated with shaking at 180 rpm at 37 °C for 90 min. After pre-incubation, 2 mL of molten agar was added, and the mixture was poured onto a minimal agar plate. Colonies were counted after 66 h of incubation at 37 °C, using an automatic counter. The S9 mix was prepared according to Mortelmans and Zeiger (2000) at a concentration of 5% v/v lyophilized Aroclor-1254-induced rat liver S9

fraction (Moltox Inc, NC, USA). Toxicity was evaluated by observing the background densities of the agar plates. Duplicate plates were used for each concentration tested.

Microplate Agar (MPA) assay

The microplate Salmonella/microsome assay was developed using 12-well microplates. A scheme with the key steps of the test, including a link to a video, is presented in Figure 1. Overnight cultures were concentrated 5-fold by centrifugation at 10,000 g at 4 °C for 10 min and resuspended in 0.015 M sodium phosphate buffer. Aliquots of 50 µL of cell suspension (0.5 to 2x10¹⁰ cells per mL) were added to a tube containing 2 µL of the sample then this volume was split into halves, by transferring 26 µL to a new tube. In one tube 25 µL of S9 mix was added and in the other 25 µL 0.015 M sodium phosphate buffer. Both tubes were incubated with shaking at 180 rpm at 37 °C for 90 min. After pre-incubation 1 mL of molten agar was added to each tube, and 250 µL of the mixture was distributed in 4 wells (21.4 x 17.5 mm) containing 2.8 mL of minimal agar. Colonies were counted by hand using a stereomicroscope after 66 h of incubation at 37 °C. The maximum number of colonies we could count per plate was 150 due to the small size of the wells. The negative control and the S9 mix used in the experiments were the same as described above. Toxicity was also evaluated using a stereomicroscope. The assay is performed in only one tube that later on is divided into four aliquots and poured into four wells. Therefore four counts are obtained for each concentration tested. We decided to use only one tube to reduce as much as possible the quantity of sample used in the assay. This test design was based on the miniaturized pre-incubation protocol described by Proudlock and Evans (2016) except that they used 3 wells derived from a single treatment and we used 4 wells.

Fig. 1. Diagram depicting the steps to Microplate Agar (MPA) assay. For details watch the video https://www.youtube.com/watch?v=nZFcP_ooqwY

Data Analysis

The data from both assays were analyzed using the Salanal computer program (Integrated Laboratory Systems, Research Triangle Park, NC). An ANOVA including a post hoc test was performed to compare each tested concentration with the respective negative control. Then a linear regression was performed using the Bernstein model (Bernstein et al. 1982). The linear equation was used to calculate the Minimal Effective Concentration (MEC₂) that provided twice the number of revertants of the negative control and expressed in ng/μL. The lower this value, the more mutagenic is the tested sample. A substance was considered positive when both the ANOVA and linear regression provided significant responses ($p < 0.05$).

RESULTS AND DISCUSSION

The protocol was refined to correct for the problem of using small amounts of sample (no less than 2 μ L) and the best result was obtained with 12-well microplates (Figure 2). 24-well microplates were also tested but they provided a high incidence of zero mutant frequency, especially in the strains with lower reversion frequencies (data not shown). In the 12-well microplates, although we obtained 3.4% wells for TA1538, 2.4% wells for TA98 and 0% for YG1041 with zero revertants, we have not observed more than one well with a zero count for any tested concentration. We also observed that the microplates get less microbial contamination in comparison with the regular Petri dishes, which is an advantage of the miniaturized method.

Fig. 2. Picture showing the revertant colonies in the 12-wells microplates after 66h incubation period.

The MPA and microsuspension protocols showed 100% agreement of the qualitative responses in the 39-paired tests (13 for each of the 3 strains tested). All substances were positive in strains TA98 and YG1041, and 5 were negative in TA1538 in both protocols (Tables II and III and Supplementary Material). The concentration response curves showed a similar behavior for each compound in both protocols (Figures 3 to 15).

Fig. 3. Response curve, in log-scale, for the 1,6-Dinitropyrene using the strains TA1538, TA98 and YG1041 without S9, for the protocols Microsuspension and MPA assay.

Fig. 4. Response curve, in log-scale, for the 3,6-Dinitrobenz(a)pyrene using the strains TA1538, TA98 and YG1041 without S9, for the protocols Microsuspension and MPA assay.

Fig. 5. Response curve, in log-scale, for the 1-Nitropyrene using the strains TA1538, TA98 and YG1041 without S9, for the protocols Microsuspension and MPA assay.

Fig. 6. Response curve, in log-scale, for the 4-Nitropyrene using the strains TA1538, TA98 and YG1041 without S9, for the protocols Microsuspension and MPA assay.

Fig. 7. Response curve, in log-scale, for the 3-Nitrofluoranthene using the strains TA1538, TA98 and YG1041 without S9, for the protocols Microsuspension and MPA assay.

Fig. 8. Response curve, in log-scale, for the 4-Nitroquinoline 1-oxide using the strains TA1538, TA98 and YG1041 without S9, for the protocols Microsuspension and MPA assay.

Fig. 9. Response curve, in log-scale, for the 2-Aminoanthracene using the strains TA1538, TA98 and YG1041 with S9, for the protocols Microsuspension and MPA assay.

Fig. 10. Response curve, in log-scale, for the Benzo(a)pyrene using the strains TA1538, TA98 and YG1041 with S9, for the protocols Microsuspension and MPA assay.

Fig. 11. Response curve, in log-scale, for the Disperse Blue 373 using the strains TA1538, TA98 and YG1041 with S9, for the protocols Microsuspension and MPA assay.

Fig. 12. Response curve, in log-scale, for the Disperse Orange 30 using the strains TA1538, TA98 and YG1041 with S9, for the protocols Microsuspension and MPA assay.

Fig. 13. Response curve, in log-scale, for the Disperse Orange 37 using the strains TA1538, TA98 and YG1041 with S9, for the protocols Microsuspension and MPA assay.

Fig. 14. Response curve, in log-scale, for the Disperse Violet 93 using the strains TA1538, TA98 and YG1041 with S9, for the protocols Microsuspension and MPA assay.

Fig. 15. Response curve, in log-scale, for the Disperse Yellow 3 using the strains TA1538, TA98 and YG1041 with S9, for the protocols Microsuspension and MPA assay.

MPA was more sensitive than the microsuspension assay in 12 of the 34 positive responses and less in 15. Identical values were observed in the 7 remaining comparisons, (Tables II and III). The differences between the MEC_2 values obtained in each protocol were in the range of the expected variation from intra- and inter-laboratory studies reported in the literature when the laboratories used the same protocol for the same substance (Claxton et al. 1991a, b). Myers et al. (1987) when analyzing a interlaboratory study found that only slopes that differ by at least one order of magnitude (>10) could be considered statistically different. Therefore we can't claim that MPA is more or less sensitive than the microsuspension protocol.

The MPA protocol uses 4 times less sample and 4 times less S9 than the microsuspension protocol because although the concentration tested in the pre-incubation

tube is the same in both methods, we use only one tube with half of volume and split it in two for the test with and without S9 in contrast with the microsuspension where 2 tubes with and 2 tubes without S9 are used per concentration. This was done to allow for the maximum reduction of sample quantity to be used in the assay. Because the quantity of sample available for test can be very small in environmental studies, Daiber et al. (2016) used single plates for each dose when testing disinfection byproducts. The reduction of sample volume allows analyzing more sampling sites and the use of different diagnostic strains with the same amount of resources. This is particularly important in monitoring studies, where we want to find hot spots or specific sources of mutagenic discharges (Alves et al. 2016; Umbuzeiro et al. 2016). Another advantage of the MPA is anticipated in EDA studies where several fractions need to be tested with the objective to find the most potent ones to perform chemical characterizations (Brack et al. 2016). The MPA protocol could also be applied in specific situations such as drug impurity testing when only few mg of sample are available, with the limitations already pointed out by Proudlock and Evans (2016). The MPA protocol allows for the application of more strains with the same amount of sample used in the microsuspension protocol, enhancing the ability of detection of a mutagenic response for chemicals like C.I. Disperse Yellow 3 (Table III), which are not detected in the standard Salmonella strains. The use of YG1041 in this case allowed the detection of a clear positive response in comparison with TA1538 (negative) and TA98 (weak response).

The negative control means observed for the MPA protocol were one order of magnitude lower than for the microsuspension because of the miniaturization procedure (Figure 16). In general, lower counts will produce a higher variance in the coefficient of the mean. Indeed, we observed increased coefficient of variation of the mean of the colony counts for the MPA when compared to the microsuspension protocol, especially for the strains with low and medium spontaneous mutation frequencies (Figure 17). However, this

did not influence the qualitative and quantitative responses obtained for the tested compounds (Tables II and III), although the MPA protocol might have a reduced ability to detect weak mutagens when strains with low and medium spontaneous mutant frequencies are used. This remains to be verified, but we assume that the limitation of MPA may be compensated by the reduction of sample quantity and costs provided by the miniaturization procedure. Therefore MPA can be considered a suitable tool especially with strains with higher spontaneous mutant frequencies, e.g. TA100, TA97a, TA102, TA104 and YG1041.

Fig. 16. Coefficients of variation for the negative controls observed for each strain in MPA and Microsuspension assays.

Fig. 17. Coefficients of variation for the negative controls observed for each strain in microsuspension and MPA protocols.

TABLE II - Minimal effect concentration (MEC₂) in ng/μL and ratio (higher MEC₂ / lower MEC₂) for the tested compounds in the absence of metabolic activation (-S9) for MPA and Microsuspension assay.

MEC ₂ ng/μL Compounds	Strains/Protocols								
	TA1538			TA98			YG1041		
	MPA	Microsuspension	Ratio	MPA	Microsuspension	Ratio	MPA	Microsuspension	Ratio
1,6-dinitropyrene	0.002	0.005	2.5	0.001	0.001	1.0	0.00003	0.00005	1.6
3,6-dinitrobenz(a)pyrene	0.008	0.007	1.1	0.002	0.004	2.0	0.00005	0.0001	2.0
1-nitropyrene	0.02	0.01	2.0	0.001	0.002	2.0	0.0002	0.0001	2.0
4-nitropyrene	0.005	0.002	2.5	0.004	0.002	2.0	0.0001	0.00009	1.1
3-nitrofluoranthene	0.009	0.002	4.5	0.001	0.001	1.0	0.00001	0.00004	4.0
4-nitroquinoline 1-oxide	0.003	0.003	1.0	0.003	0.003	1.0	0.007	0.006	1.1

TABLE III - Minimal effect concentration (MEC₂) in ng/μL and ratio (higher MEC₂ / lower MEC₂) for the tested compounds in the presence of metabolic activation (+S9) for MPA and Microsuspension assay.

MEC ₂ ng/μL Compounds	Strains/Protocols								
	TA1538			TA98			YG1041		
	MPA	Microsuspension	Ratio	MPA	Microsuspension	Ratio	MPA	Microsuspension	Ratio
2-aminoanthracene	0.4	0.4	1.0	0.9	1.2	1.3	0.1	0.3	3.0
Benzo(a)pyrene	2.0	1.6	1.2	3.0	4.0	1.3	0.5	0.1	5.0
Disperse Blue 373	Negative	Negative		0.20	0.16	1.2	0.16	0.12	1.3
Disperse Orange 30	Negative	Negative		1,100	1,100	1.0	150	180	1.2
Disperse Orange 37	Negative	Negative		10	17	1.7	2.70	2.00	1.3
Disperse Violet 93	Negative	Negative		0.4	1.0	2.5	0.70	0.20	3.5
Disperse Yellow 3	Negative	Negative		>10,000*	>10,000*		620	460	1.3

* Not possible to calculate MEC₂ because the maximum concentration tested did not provided twice the negative control, although ANOVA indicated a positive response.

CONCLUSIONS

This is the first study to examine a miniaturized version of the microsuspension *Salmonella*/microsome assay using agar microplates and demonstrates that the modified method exhibits a similar sensitivity to the microsuspension assay. The method requires less sample and less reagents in comparison to the microsuspension protocol, thereby reducing overall costs. It can be particularly suitable for environmental studies such as EDA or monitoring programs.

AUTHORS' CONTRIBUTION

Concept of the idea - Umbuzeiro and Brack.

Development of experiments - Zwarg, Morales and Maselli.

Data analysis and Interpretation of results – Zwarg, Morales, Maselli, Brack, Umbuzeiro.

Writing the manuscript – Zwarg, Morales, Umbuzeiro.

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5. CONSIDERAÇÕES FINAIS

- A amostra de Limeira apresentou a maior concentração de PTS ($99,0 \mu\text{g}/\text{m}^3$), seguida por Quioto ($28,0 \mu\text{g}/\text{m}^3$) e Estocolmo ($6,2 \mu\text{g}/\text{m}^3$).
- Embora a concentração de PTS/ m^3 em Limeira seja que em Estocolmo e em Quioto, as porcentagens de MOE obtidas foram aproximadamente de 9, 15 e 5% respectivamente. As diferenças na distribuição do tamanho das partículas e nas suas concentrações, parecem ter influenciado diretamente na quantidade de MOE obtida nos extratos de cada cidade.
- O uso de um amplo conjunto de linhagens de *Salmonella*/microsoma permitiu uma avaliação abrangente da mutagenicidade das amostras de PTS das cidades de Limeira, Estocolmo e Quioto.
- Os extratos orgânicos das amostras de PTS das três cidades foram capazes de causar todos os tipos de lesões no DNA detectadas pelas linhagens empregadas. Exceto, aqueles detectados pela TA102 na ausência e presença de S9 para os extratos das três cidades, e pela YG7108 na ausência de S9 para os extratos de Limeira.
- As linhagens que detectam mutações do tipo deslocamento do quadro de leitura, principalmente as que apresentam produção aumentada das enzimas NR e OAT, foram as mais sensíveis aos mutágenos presentes nos extratos das amostras em relação aquelas que detectam substituição de pares de bases.
- Os perfis de mutagenicidade e as potências mutagênicas expressas em função da massa de MOE (rev./ μg MOE) obtidas para Limeira, Estocolmo e Quioto, foram semelhantes, apesar das diferenças de temperatura e período de radiação solar observada entre as três cidades.
- As potências mutagênicas obtidas por volume de ar (rev./ m^3) foram proporcionais às concentrações de PTS das três cidades durante o período de amostragem (Limeira > Quioto > Estocolmo).
- Foi possível observar que, a aplicação do mesmo procedimento de extração entre as três cidades, parece ser muito importante para permitir comparações de mutagenicidade de amostras coletadas em diferentes locais. Considerando que em geral, as potências mutagênicas obtidas para as três cidades avaliadas em relação aos dados encontrados na literatura, mostraram que para comparações entre diferentes estudos, é necessário que as mesmas metodologias tenham sido empregadas, principalmente o procedimento de extração orgânica das amostras de MP.

- A concentração total dos HPA (pg/m^3) em Limeira foi aproximadamente 8,4 vezes maior do que em Estocolmo, e essa diferença é proporcional as quantidades de MOE obtidos nas amostras de ambas as cidades. Diferenças quantitativas podem ser explicadas, pela elevada concentração de PTS observada em Limeira e pelas fontes de poluição das duas cidades.
- A distribuição dos HPA com 3, 4 e ≥ 5 anéis nas amostras de PTS de Limeira e Estocolmo foram semelhantes, apesar da temperatura média relativamente alta e das condições favoráveis para as reações fotoquímicas observadas em Limeira.
- As razões diagnósticas moleculares entre os HPA indicaram uma mistura de fontes de poluição em Limeira e Estocolmo, com uma leve predominância de fontes de combustão de biomassa em Limeira e de fontes veiculares em Estocolmo.
- Estocolmo apresentou partículas envelhecidas, embora Limeira apresentasse radiação solar e reação fotoquímica aparentemente mais favorável ao envelhecimento do PTS.
- As massas de ar do continente europeu sugerem que, pelo menos em parte, o PTS envelhecido coletado em Estocolmo é proveniente de outras partes da Europa. Portanto, nossos resultados indicam que mudanças químicas nos aerossóis dependem mais do tempo de residência do PTS do que apenas a presença de oxidantes, temperatura e luz solar. Fatores ambientais, como a umidade relativa, precisam ser melhor compreendidos em estudos futuros de reatividade dos HPA.
- A linhagem YG5185 foi a mais adequada para avaliar a contribuição do B[a]P para a mutagenicidade do MP em comparação com as linhagens TA98 e TA1538, sendo a melhor escolha para monitorar locais onde compostos que agem de forma semelhante ao B[a]P, como outros HPA.
- A redução da concentração dos HPA em Limeira irá depender do controle das fontes locais, já que em Estocolmo o controle dessas fontes não diminuiria significativamente a concentração desses compostos na atmosfera.
- O primeiro estudo realizado para avaliar uma versão miniaturizada do protocolo em microssuspensão do teste Salmonella/microsoma utilizando microplacas de ágar, demonstrou que o método modificado possui sensibilidade semelhante ao teste em microssuspensão.
- Este novo método requer menos quantidades de amostras e reagentes em comparação ao protocolo em microssuspensão, reduzindo assim os custos globais do teste.
- O MPA pode ser particularmente adequado para aplicação em estudos ambientais, como EDA ou programas de monitoramento.

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ANEXO I

Ficha do Aluno

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

9141 - 8851071/1 - Bianca de Souza Maselli

Email: bsmaselli@usp.br
Data de Nascimento: 30/09/1984
Cédula de Identidade: RG - MG-13.007.094 - MG
Local de Nascimento: Estado de Minas Gerais
Nacionalidade: Brasileira
Graduação: Licenciado em Ciências Biológicas - Fundação Educacional de Lavras - Minas Gerais - Brasil - 2010
Mestrado: Mestra em Ecologia e Tecnologia Ambiental (1) - Universidade Federal de Alfenas - Minas Gerais - Brasil - 2013

Curso: Doutorado
Programa: Toxicologia e Análises Toxicológicas
Data de Matrícula: 05/12/2013
Início da Contagem de Prazo: 05/12/2013
Data Limite para o Depósito: 04/04/2018
Orientador: Prof(a). Dr(a). Gisela de Aragão Umbuzeiro - 05/12/2013 até 10/02/2015. Email: giselau@usp.br
Orientador: Prof(a). Dr(a). Fábio Kummrow - 11/02/2015 até o presente. Email: fkummrow@gmail.com
Proficiência em Línguas: Inglês, Aprovado em 05/12/2013
Prorrogação(ões): 120 dias
Período de 05/12/2017 até 04/04/2018
Data de Aprovação no Exame de Qualificação: Aprovado em 02/03/2016
Data do Depósito do Trabalho:
Título do Trabalho:
Data Máxima para Aprovação da Banca:
Data de Aprovação da Banca:
Data Máxima para Defesa:
Data da Defesa:
Resultado da Defesa:
Histórico de Ocorrências: Primeira Matrícula em 05/12/2013
Prorrogação em 22/11/2017

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 05/02/2018

Impresso em: 13/03/2018 16:19:36

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Faculdade de Ciências Farmacêuticas

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9141 - 8851071/1 - Bianca de Souza Maselli

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBC5803-3/3	Sistemas de Garantia da Qualidade em Laboratórios de Ensaio	11/03/2014	24/03/2014	30	2	100	A	N	Concluída
FBC5802-3/4	Tópicos Avançados em Toxicologia I	11/03/2014	23/06/2014	15	1	90	A	N	Concluída
CEN5772-1/3	Bioquímica e Biologia Molecular de Células (Centro de Energia Nuclear na Agricultura - Universidade de São Paulo)	12/03/2014	24/06/2014	225	0	-	-	N	Matrícula cancelada
FBC5784-3/5	Tópicos Avançados em Toxicologia II	05/08/2014	17/11/2014	15	1	75	A	N	Concluída
FBC5883-6/1	Avaliação da Toxicidade de Contaminantes em Amostras Ambientais	11/08/2014	07/09/2014	60	4	100	B	N	Concluída
FBA5728-3/10	Aprimoramento Didático	19/08/2014	15/09/2014	60	4	88	A	N	Concluída
FBC5758-2/2	Toxicologia dos Praguicidas	16/09/2014	27/10/2014	90	6	75	A	N	Concluída
CEN5780-1/1	Sustentabilidade Ambiental (Centro de Energia Nuclear na Agricultura - Universidade de São Paulo)	01/12/2014	21/12/2014	90	6	95	A	N	Concluída

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	10	20	24
Estágios:			
Total:	10	20	24

Créditos Atribuídos à Tese: 167

Observações:

1) Curso com validade nacional, de acordo com o disposto na Portaria MEC nº 1.225, de 05.10.2010..

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 05/02/2018

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Comissão julgadora da tese de doutorado:			
NUSP	Nome	Vínculo	Função
3082961	Fábio Kummrow	UNIFESP - Externo	Presidente

Última ocorrência: Matrícula de Acompanhamento em 05/02/2018

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